THE EXPRESSION AND METABOLISM OF LOW DENSITY LIPOPROTEIN RECEPTORS IN FAMILIAL HYPERCHOLESTEROLAEMIA

Submitted in fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

in the

FACULTY OF MEDICINE (MEDICAL BIOCHEMISTRY)
UNIVERSITY OF CAPE TOWN

by

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February 1989

CERTIFICATE OF SUPERVISOR

In terms of Paragraph GP8 of "General rules for the degree of Ph.D.", I, as supervisor of the candidate, Anne Madeleine Fourie, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.
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ABSTRACT

The expression of two phenotypically-contrasting LDL receptor mutations was characterized in cultured fibroblasts from the genetically-homozygous Afrikaner subjects, FH1a and 1b, and FH3a and 3b, respectively. Surface receptor expression and functional activity were studied by ligand (^{125}I-LDL) and monoclonal antibody (^{125}I-IgG-C7) binding, and [^{35}S]-methionine pulse-chase experiments were used to analyze biosynthesis, processing and degradation of IgG-C7-immunoprecipitable mutant receptors.

Cells from the "receptor-negative" subjects, FH3a and 3b exhibited reduced, but significant (40-60% of normal) LDL receptor synthesis rates. Newly-synthesized precursors were processed slowly ($t_{½}$ 1.5 hours versus normal $t_{½}$ of approximately 15 minutes) to mature receptors which reached the cell-surface, but were rapidly degraded thereafter with a half-life of approximately 1.7 hours (normal value 12.6 hours) thus representing a new type of LDL receptor defect. Lysosomotropic weak bases such as ammonium chloride partially inhibited rapid degradation of the mutant receptors, suggesting the involvement of proteolysis in acidic compartments such as lysosomes or endosomes.

Fibroblasts from FH1a and 1b exhibited normal synthesis rates of LDL receptor precursors that were processed at a severely reduced rate ($t_{½}$ approximately 5 hours) to functionally heterogeneous mature surface receptors. One-third of the receptors (20% of normal levels) bound ^{125}I-LDL with normal affinity at 4°C and 37°C, whereas the majority were able to recognize only ^{125}I-IgG-C7, and apparently showed defective internalisation and subsequent degradation of the bound IgG-C7 at 37°C. The existence of the two receptor populations was further supported by selective intracellular trapping and degradation of only the active,

LDL-binding population, in the presence of ammonium chloride and LDL. The abnormal form predominated even in newly-synthesized receptors and reached a maximum of 50-70% of normal levels after 48 hours of upregulation. Upregulation kinetics and degradation rates ($t_{½}$ = 10-11 hours) of both functionally-active and abnormal receptor populations were similar to normal. A progressive increase in apparent molecular weight of the slowly-processed precursor receptors suggested a possible role for abnormal glycosylation in the formation of both "normal" and abnormal conformations of the same receptor molecule.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons:

Associate Professor Deneys van der Westhuyzen, my supervisor, for his intellectual guidance, insightful ideas and suggestions, and critical appraisal of the work.

Associate Professor Gerry Coetzee, for his enthusiastic and active involvement in the work, and much-appreciated practical advice and assistance.

Professor Wieland Gevers, for the privilege of working in a department that he has made into an active, dynamic centre of excellence in research.

Kathy Lyner and Rolene Liebenberg, for excellent and invaluable technical assistance.

Drs. M.S. Brown and J.L. Goldstein, for their gifts of polyclonal antibodies to the LDL receptor.

Drs. E. Leitersdorf, H.H. Hobbs and H.E. Henderson, for LDL receptor gene haplotype analyses of South African familial hypercholesterolaemic subjects.

Dr. I.S. Trowbridge, for his gift of the monoclonal antibody to the transferrin receptor.

The South African Medical Research Council, the University of Cape Town and the Loewenstein Research Scholarship for financial support.

Rose-Marie Estment, for typing of the thesis, and Valerie Myburgh, Mohammed Jaffer and Ingrid Cloete, for the artwork and photography.

Dr. Kevin Grant, for assistance with computer analysis of results.

Dr. Livia Casciola and Denver Hendricks, my fellow Ph.D. students, for their friendly support and unselfish help on many occasions.

Leon, my husband, for being undemanding in difficult times.

My family, for their continued support and interest.

ABBREVIATIONS

a.a.	amino acids
ACAT	acyl-CoA cholesterol acyltransferase
apo	apolipoprotein
BiP	immunoglobulin heavy-chain binding protein
BSA	bovine serum albumin
bp	base pairs
B_{max}	maximum binding capacity
β-VLDL	β-migrating very-low-density lipoprotein
(c)DNA	(complementary) deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane
CHD	coronary heart disease
CHO	Chinese hamster ovary
cpm	counts per minute
DMEM	Dulbecco's Modification of Eagle's Medium
dpm	disintegrations per minute
EDTA	(ethylenedinitrilo)-tetraacetic acid
EGF	epidermal growth factor
EMEM	Eagle's Minimum Essential Medium with Earle's Salts
EndoH	Endo-β-N-acetylglucosaminidase H
ER	endoplasmic reticulum
FH	familial hypercholesterolaemia
Gal	galactose
GalNAc	N-acetylgalactosamine
Glc	glucose
GlcNAc	N-acetylglucosamine
h	hours
HDL	high-density lipoprotein
HEPES	2-[4-(2-hydroxyethyl)-1-piperazine]ethane sulphonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl Coenzyme A
HSV-TK	Herpes Simplex Virus thymidine kinase
IDL	intermediate-density lipoprotein
IgG	immunoglobulin G
kb	kilobases

K_d	equilibrium dissociation constant
kDa	kilodaltons
LDL	low-density lipoprotein
LPDS	lipoprotein-deficient serum
Man	mannose
MEM	Eagle's Minimum Essential Medium
mRNA	messenger ribonucleic acid
M.W.	molecular weight
N.D.	not determined
n.d.	not detectable
N-linked	asparagine-linked
O-glycanase	Endo-α-N-acetylgalactosamine
O-linked	serine/threonine-linked
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PMSF	phenylmethylsulphonyl fluoride
Pristane	2,6,10,14-tetramethylpentadecane
RER	rough endoplasmic reticulum
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
rpm	revolutions per minute
S.D.	standard deviation
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
SSC	standard saline citrate
$t_{½}$	half-life of first-order degradation (or half-time for receptor maturation)
TCA	trichloroacetic acid
TE	Tris-EDTA (10mM Tris-HCl,1mM EDTA, pH 8)
TEMED	N,N,N'N'-tetramethylethylenediamine
Tris	Tris[hydroxymethyl]aminomethane
tRNA	transfer ribonucleic acid
Tween 20	polyoxyethylene sorbitan monolaurate
UDP	uridine-5'-diphosphate
VLDL	very-low-density lipoprotein
VSV	vesicular stomatitis virus
WHHL	Watanabe heritable hyperlipidaemic

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INTRODUCTION

Cholesterol is an essential component of the cell membrane and is the precursor for steroid hormone and bile acid synthesis (1). To provide for these functions, cholesterol is transported in the esterified form in plasma lipoproteins, and delivered to cells via lipoprotein receptors by the process of receptor-mediated endocytosis. If plasma cholesterol levels are elevated, this can lead to the formation of atherosclerotic plaques in the arteries, and eventually heart attacks and strokes. The concentration of cholesterol in the bloodstream must therefore be kept low. An important factor in regulating plasma cholesterol is the level of low-density lipoprotein (LDL) receptors.

The LDL receptor is a cell-surface protein which plays a central role in cholesterol homeostasis (1). This receptor binds LDL, the most abundant cholesterol transport lipoprotein, and carries it into the cell, thus clearing cholesterol from the plasma and initiating several regulatory processes within the cell (1). Study of the LDL receptor pathway has led to (i) the concept of receptor-mediated endocytosis (2,3,4), the process by which macromolecules enter cells after binding to receptors in coated pits, (ii) the elucidation of the mechanism by which the LDL receptor mediates feedback control of cholesterol synthesis (1,5), and how cholesterol levels in turn mediate transcriptional control of LDL receptor expression (6-9), and (iii) understanding of the genetic disease, familial hypercholesterolaemia, caused by mutations which disrupt the normal structure and function of the LDL receptor (1,3,10,11). For these, and other fundamental concepts emerging from their elegant studies of the LDL receptor and cholesterol homeostasis, Brown and Goldstein were awarded the Nobel Prize in Physiology or Medicine in 1985.

1.1 Familial hypercholesterolaemia and the low-density lipoprotein receptor - Historical perspective (1,10)

"The physiological, biochemical and genetic characterization of the LDL pathway in both normal and FH cells has had a profound impact on our understanding of sterol metabolism, hypercholesterolemia, atherosclerosis and endocytosis" (Krieger et al. ref. 12).

Familial hypercholesterolaemia (FH) was first described in 1938 by Carl Muller as an inborn error of metabolism, inherited as a dominant trait, which led to elevated blood cholesterol, xanthomata and myocardial infarctions in young people (10). Gofman et al. later showed that the hypercholesterolaemia resulted from a selective elevation of low density lipoprotein (LDL) particles (13). Kachadurian (14) clarified the clinical features of heterozygous and more severe homozygous FH. Fredrickson, Levy and co-workers demonstrated abnormally slow catabolism of both the apoprotein and cholesterol components of LDL in FH patients (15).

Brown and Goldstein began their classic studies in 1972 in an attempt to understand FH (1). They postulated that the elevated blood cholesterol observed in the disease was due to a failure of end-product repression of cholesterol synthesis. In their model system of cultured human skin fibroblasts, they showed that HMG-CoA reductase (a rate-limiting enzyme in cholesterol biosynthesis) was subject to negative regulation by LDL-cholesterol (16). The specificity of the regulation (only LDL- or VLDL-cholesterol) and the concentration of LDL required (less than 10^{-8} M) suggested the involvement of a high affinity receptor mechanism. In fibroblasts from homozygous FH subjects, no suppression of cholesterol synthesis by LDL was observed, and the cells exhibited HMG-CoA reductase activities 50 to 100 times greater than normal (16). These observations showed that the genetic defect was expressed in cell culture. However, when ethanolic cholesterol was

added directly to the FH cells, suppression of HMG-CoA reductase activity took place at the same rate and to the same extent as in normal cells. Thus, the defect in the cells appeared to be in the ability to extract cholesterol from lipoproteins such as LDL, possibly by high-affinity binding to a specific receptor. The existence of the LDL receptor was confirmed by ^{125}I-labelled LDL binding studies (16). Normal fibroblasts showed high-affinity binding of ^{125}I-LDL, followed by internalisation and degradation in the lysosomes, liberating free cholesterol from cholesterol esters, thus leading to suppression of HMG-CoA reductase activity. In contrast, fibroblasts from FH homozygotes were shown to lack high-affinity receptors. This deficiency in LDL receptors in these subjects would thus lead to reduced clearance of LDL from the plasma, explaining the elevation of LDL levels in FH. FH was thus shown to be caused by inherited defects in the LDL receptor.

The ability to measure LDL receptor activity in cultured cells was followed by methods to solubilize and purify the receptor in homogeneous form (17) and the development of antibodies directed against the protein (18). These advances led to a detailed understanding of the biosynthesis and intracellular itinerary of the LDL receptor, and of the disruption of these aspects in FH (18,19,20). In 1983, Russell et al. cloned a partial cDNA of the bovine LDL-receptor (6) and this was used by Yamamoto et al. in 1984 to isolate a full-length cDNA for the human LDL receptor (21). Subsequent work has led to the elucidation of the structure of the normal receptor gene (22) and the definition of mutations in the gene at the molecular level (10). In this way, the structural basis for the multiple biochemical classes of LDL receptor mutations in FH has been elucidated.

1.2 Structure-function relationships in the LDL receptor

1.2.1 LDL receptor protein

The extreme N-terminus of the LDL receptor consists of a hydrophobic signal sequence of 21 amino acids which

presumably mediates insertion of the nascent polypeptide into the endoplasmic reticulum (3). Excluding the signal sequence, the LDL receptor protein contains 839 amino acids and is composed of five functional domains (see Figure 1.1).

1.2.1.1 Ligand binding domain

The ligand binding domain was shown to be on the external surface of the membrane by anti-peptide antibody binding studies (3). This domain consists of 292 amino acids, made up of 7 repeats of approximately 40 amino acids each. Each repeat contains 6 cysteine residues, regularly spaced at intervals of 4 to 7 amino acids. All the cysteines are involved in disulphide bonds, creating a tightly cross-linked structure of extreme stability (3). At the carboxyl terminal of each repeat is a cluster of negatively-charged amino acids believed to be important for binding to positive charges on the ligands for the receptor. Since the centre of each repeat is relatively hydrophobic, it is likely that many of the disulphide-bonded cysteines are buried in hydrophobic pockets, flanked by polar residues, thus allowing the negatively-charged residues to be exposed at the surface of the binding domain (11) [See figure 1.1]. All of these features are highly conserved between the human and rabbit receptors, particularly the exact spacing of the cysteine residues and the groups of negatively-charged amino acids (23). Only the 16 N-terminal amino acids of this domain in the bovine receptor are known (3) and show strong sequence homology with the human and rabbit receptors, including the positions of the first two cysteine residues.

The protein ligands which bind to the LDL receptor are apolipoprotein(apo) E (found on particles of β-VLDL, HDL_C, VLDL, IDL) and apolipoprotein(apo) B-100 (found on β-VLDL, VLDL, IDL, and LDL particles). The LDL receptor binds lipoproteins containing multiple copies of apo E with higher affinity than LDL, which contains a single copy of apo B-100, but the receptor has a higher maximum binding capacity for LDL particles (24). The ligand apo E contains 299

amino acids, and has a cluster of positively-charged residues between amino acids 140 to 160, believed to occupy one face of a single α-helix. It has been shown with mutant and proteolyzed forms of apo E, and with monoclonal antibodies to different regions of the molecule, that this cluster represents the binding site of apo E for the LDL receptor (24). Apo B is produced in 2 forms: apo B-100 is produced by the liver, and apo B-48 by the intestine. Apo B-48 is produced from the same gene as apo B-100 by a post-transcriptional, tissue-specific modification to introduce a stop codon at residue 2153 (25), thus producing a shortened protein product representing the amino-terminal 2152 amino acids of apo B-100. Apo B-100 binds to the LDL receptor, whereas apo B-48 does not, suggesting that the binding site is situated in the carboxyl end of apo B-100. The cDNA for apo B-100 has been cloned (26,27) and the amino acid sequence (4536 amino acids) derived. Evidence for the carboxyl terminus as the site of the binding domain has been obtained from inhibition of binding by antibodies to this region, and striking homology between 2 basic amino acid sequences in this region (amino acids 3147 to 3157 and 3357 to 3367) and the receptor binding domain of apo E (24).

The numerous disulphide bonds in the binding domain of the LDL receptor create a very stable structure. This is presumably important in the acidic endosome, where the negatively-charged residues of the receptor become protonated, lose their charges and thus release the positively-charged ligand, accompanied by conformational changes in the receptor. The receptor does not become irreversibly denatured by this titration of the carboxyl groups because of structural stabilization of the binding domain by the disulphide cross-linking (3). Esser et al. (28) have used oligonucleotide-directed mutagenesis to alter the cysteine-rich repeats in the binding domain of expressible cDNA for the human LDL receptor. By expression of the mutated receptors in transfected monkey COS cells, the effect of the various mutations on the ability of the

receptor to bind LDL (apo B-100) or β-VLDL (apo B-100 plus multiple copies of apo E) could be measured. For example, the modification of the acidic binding sequence Ser-Asp-Glu to Ser-Tyr-Glu in repeat 5 caused a dramatic loss of binding to both β-VLDL and LDL. The same modification in repeat 6 decreased LDL binding by 50% but had no effect on β-VLDL binding, whereas this modification in the first repeat had no effect on binding of either LDL or β-VLDL (See Table 1.1). The results support the finding of van Driel et al. (29) that the first repeat binds monoclonal antibodies and Ca^{2+}, but is not required for ligand binding. Repeats 2 and 3 and repeats 6 and 7 were found to be required for maximal LDL, but not β-VLDL, binding. Repeat 5 was shown to be required for binding of both ligands. The results support a model where the various repeats play an additive role in ligand binding, but are not functionally equivalent.

Assuming that the first repeat has no functional role, and since a linker region of identical length (8 amino acids), but divergent sequence, has been found between repeats 4 and 5 in the human, rabbit and hamster receptor, the possibility of 2-fold symmetry of repeats 2 to 7 around the linker sequence has been proposed (28).

1.2.1.2 EGF-precursor homologous domain

The second domain of the LDL receptor consists of about 400 amino acids, of which 35% are homologous to a portion of the extracellular domain of the precursor for epidermal growth factor (EGF) (30). The sequence of this domain includes two sites used for N-linked glycosylation (21) (See figure 1.1). This region of the LDL receptor contains 3 cysteine-rich repeats, A, B and C, known as "growth factor repeats", that are present in many cell surface and secreted proteins, including clotting factors, complement proteins and membrane receptors (22). These cysteine-rich repeats have a different evolutionary origin from the 7 binding repeats (22), and, since they lack clusters of negative charges, are unlikely to participate directly in ligand binding.

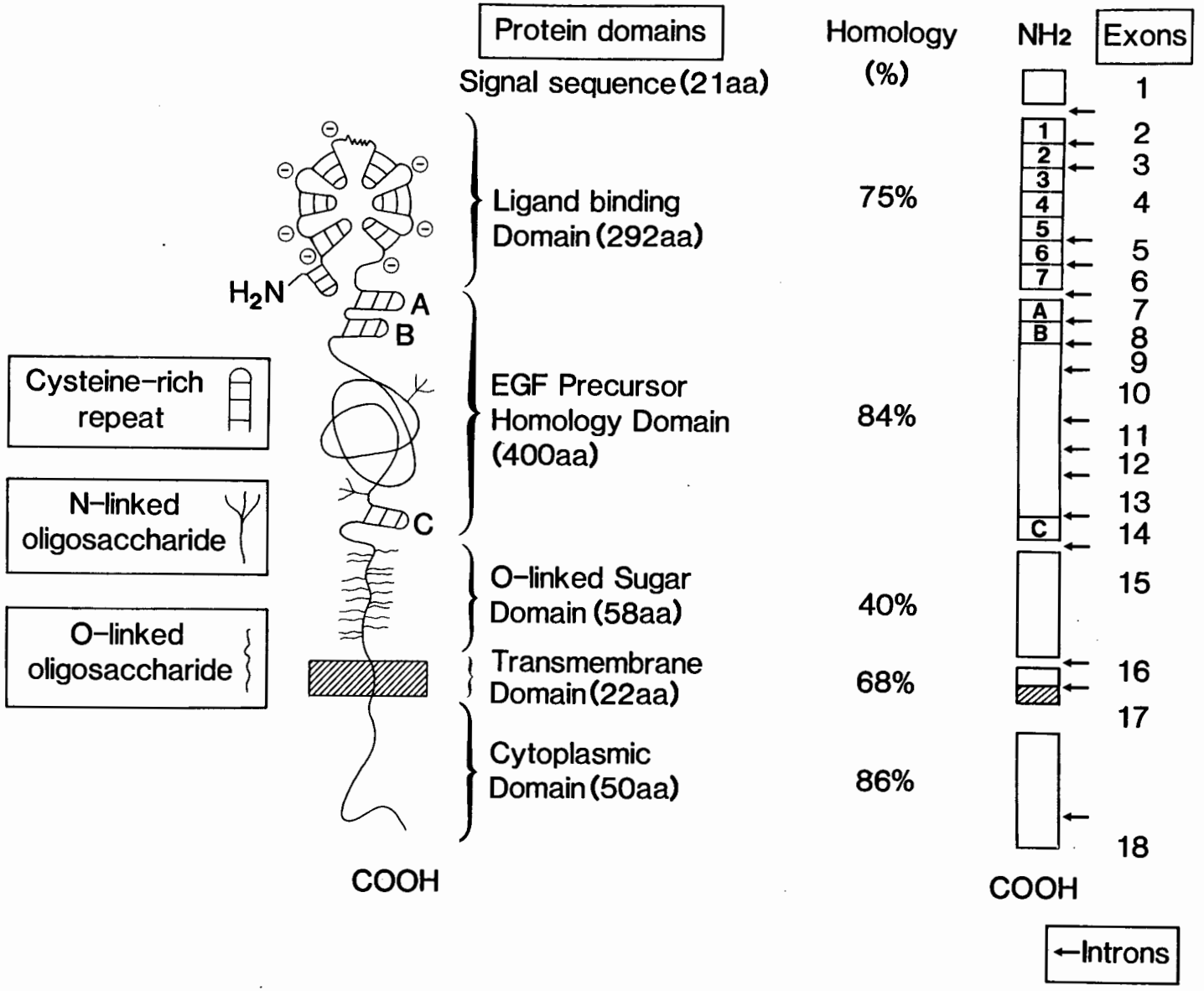

Figure 1.1 Model for protein domains and exon organization of the LDL receptor

Adapted from references 1, 22 and 28. See sections 1.2.1 and 1.2.3 for detailed description. The percentage homology between the human and rabbit LDL receptors in each domain (23) is indicated.

However, repeats A and B indirectly assist in the binding of LDL, since deletion of these two repeats, or the entire EGF-precursor homologous domain caused a dramatic loss in binding of LDL, but not β-VLDL at the cell surface (31). Esser et al. (28) showed that repeat A was required for LDL but not β-VLDL binding, but repeat B was not required for ligand binding (See Table 1.1). Since deletion of the repeats did not abolish the ability of the receptor to bind LDL on ligand blots (31) it was suggested that the repeats played a role in allowing LDL to gain access to the binding domain on the cell surface.

The growth factor repeats in the region of EGF-precursor homology appear to play an important role in release of bound ligand via acid-dependent conformational change in the endosome. When this entire domain, or only repeats A and B, were deleted from the protein, the receptor did not release its ligand at acid pH, did not recycle efficiently and was therefore rapidly degraded after ligand binding and internalisation (31). The importance of this region in the LDL receptor function is indicated by the high degree of conservation between the human, rabbit (23) and bovine (3) receptors.

1.2.1.3 O-linked sugar domain

Immediately external to the membrane-spanning domain of the LDL receptor is a threonine- and serine-rich region of 58 amino acids, encoded by a single exon (1). Various studies have shown that this region contains approximately 18 clustered O-linked oligosaccharide chains (30,32,33) attached to the serine and threonine residues. There is little sequence homology in this region between the bovine, human and rabbit receptors (23) except for the serine- and threonine-rich character. However, this region is glycosylated in all three species, suggesting that a primary sequence is not the signal for O-linked glycosylation of this region.

The function of this region is unknown, since it can be deleted without affecting normal function of the receptor in cultured hamster fibroblasts (33) [See Table 1.1]. It is possible that the clustered O-linked sugar domain is not important in non-hepatic cells, but may be crucial for the function of hepatic LDL receptors, which are major factors in determining serum cholesterol levels (see section 1.6). This possibility is supported by the fact that deletion of exon 15, which encodes the O-linked sugar domain, from one LDL receptor allele in 2 Japanese subjects, produces the clinical symptoms of heterozygous familial hypercholesterolaemia (34).

1.2.1.4 Membrane-spanning domain

A region of hydrophobic amino acids forms the membrane-spanning domain of the LDL receptor. Deletion of this domain causes the receptor to be secreted from the cell (35,36). The transmembrane domain serves only to anchor the receptor in the membrane and shows little sequence homology between the human, rabbit and bovine receptors, and variation in length (rabbit-25 amino acids, human-22 and bovine-27) (23).

1.2.1.5 Cytoplasmic domain

The cytoplasmic domain of the LDL receptor consists of 50 amino acids and shows a high degree of sequence conservation between the human (21), bovine (3) and rabbit (23) receptors. This region of the receptor is responsible for targeting the protein to coated pits, either through interaction with clathrin, or with some protein associated with clathrin on the cytoplasmic side of the membrane (1). The function of the cytoplasmic domain has been concluded from molecular analysis of 3 naturally-occurring internalisation-defective mutant LDL receptors (37,38), which all have defects in the cytoplasmic tail, and by the effects of site-directed mutagenesis of the cytoplasmic domain (39) [See Table 1.1]. Immediately internal to the membrane, the cytoplasmic tail has a cluster of positively-

TABLE 1.1 SITE-DIRECTED MUTATIONS IN THE LDL RECEPTOR

Affected Receptor Domain	Mutation	Phenotypic Result of Mutation	Reference
Ligand Binding Domain:			
Repeat 1	Deletion	No effect on ligand binding, loss of monoclonal antibody binding	29
	Cys^{6} → Ala $Cys^{6,18}$ → Ala Asp^{36} → Tyr	No effect on binding of LDL or β-VLDL	28
Repeat 5	Phe^{181} → Tyr	No effect	28
	Phe^{181} → Gly Ile^{189} → Asp Asp^{206} → Tyr Asp^{206} → Asn	LDL and β-VLDL binding markedly decreased	28
Repeat 6	Asp^{245} → Tyr	50% LDL binding, no effect on β-VLDL binding	28
Repeats 1-3	Deletion	30% LDL binding, 70% β-VLDL binding	28
Repeats 2-6	Deletion	No binding of lipoproteins, binding of monoclonal antibodies in Ca^{2+}-dependent fashion	29
Repeats 1-5	Deletion	No binding of lipoproteins	28
Repeats 6 and 7	Deletion	20% LDL binding, 80% β-VLDL binding	28
EGF Precursor Homologous Domain:	Deletion	Receptor binds β-VLDL, not LDL, unable to undergo acid-dependent dissociation from β-VLDL, not recycled, rapidly degraded	31
Repeat A	Cys^{297} → Ser $Cys^{297,308}$ → Ser	30% LDL binding, no effect on β-VLDL binding	28
	Asp^{310} → Ala	50% LDL binding, no effect on β-VLDL binding	28
	Deletion	30% LDL binding, no effect on β-VLDL binding	28
Repeat B	Asn^{349} → Ala Deletion	No significant effect on LDL or β-VLDL binding	28
Repeats A and B	Asp^{310}, Asn^{349} → Ala	20-25% LDL binding, no effect on β-VLDL binding	28
	Deletion	20-25% LDL binding, no effect on β-VLDL binding, defective acid-dependent dissociation from β-VLDL	28,31
O-linked Sugar Domain:	Deletion	Normal binding, internalization of LDL, normal recycling and $t_{\frac{1}{2}}$ for receptor, no apparent effect of deletion in cultured hamster fibroblasts	33
Cytoplasmic Domain:	Val^{806} → Cys	No effect	38,39
	Val^{806} → Pro	20% internalisation index	
	Tyr^{807} → Gly,Pro,Ala,Leu,Cys Ser,Thr,His,Glu,Lys	20% internalisation index	
	Tyr^{807} → Phe	No effect	
	Tyr^{807} → Trp	60% internalisation index	
	Gln^{808} → Cys	No effect	
	Gln^{808} → Pro	60% internalisation index	
Cytoplasmic Domain: (cont.)	Deletion Glu^{812} - Glu^{814} Glu^{835} - Asp^{837}	No effect	39
	Trp^{792} → Stop Tyr^{807} → Stop Glu^{808} → Stop	± 15% internalisation index	
	Glu^{812} → Stop	79% internalisation index	
	Arg^{829} → Stop	98% internalisation index	

charged amino acids, a common feature of plasma membrane proteins (3). Near the carboxyl terminal, a negatively-charged group of amino acids is found (3). Site-directed mutagenesis experiments (39) have shown that the first 22 amino acids of the cytoplasmic domain (residues 790-811) are sufficient for efficient receptor internalisation and that an aromatic residue at position 807 is especially critical. This was also shown by the fact that the molecular defect in the internalisation-defective mutant, FH 380, is the substitution of a cysteine residue for tyrosine-807 (38). Residues 812-839 do not seem to be required for internalisation or recycling (39), but are highly conserved, particularly the C-terminal 14 amino acids, residues 826-839, which are identical in the human (21), rabbit (23), bovine (3) and hamster (39) receptors. Possibly, these residues may perform specialized regulatory functions required in specific tissues such as the adrenal cortex (39). Although there seem to be strict structural requirements for efficient internalisation of the LDL receptor, no correlation is observed with cytoplasmic domains of 5 other coated pit receptors (3). The only structural feature shared between all these receptors is the presence of a single membrane-spanning region.

In addition to its role in clustering in coated pits, the cytoplasmic domain mediates self-association of the LDL receptor to form non-covalent dimers and higher order structures (40). However, the function of self-association is unknown and no causal connection between this process and LDL receptor-internalisation has been established, since a mutant receptor terminating at position 812 was internalised normally, but showed greatly reduced oligomer formation (40).

1.2.2 LDL receptor messenger RNA

The LDL receptor mRNA appears on Northern blots to be approximately 5.3 kilobases in length (3). Of this, 2.58 kb represents protein coding sequences, flanked by a 5'-

untranslated region of 0.09 kb, and an unusually-long 3'-untranslated region of 2.5 kb (21). This region terminates with a poly(A)$^+$ tract approximately 15 nucleotides downstream from a polyadenylation signal (3). The 3'-untranslated region of the mRNA contains two-and-a-half copies of Alu repetitive sequences, which occur once in every 5000 base pairs in the human genome (41). Each Alu repeat is approximately 300 bp in length, consisting of a tandem repeat of two monomeric units, a left arm of 130 bp and a right arm of 160 bp, owing to a 30 bp insertion (3). Although Alu repeats were originally believed to be confined to introns and intergenic regions (10), they are present in exon 18 of the human LDL receptor gene, in the region coding for the 3'-untranslated sequence in the mRNA. Since bovine LDL receptor mRNA contains no Alu sequences, and mRNA from gorilla and chimpanzee, but not from baboon, have Alu repeats at the same location as the human LDL receptor mRNA, it has been suggested that the insertion of Alu sequences into this genetic location occurred late in the evolution of primates (42).

1.2.3 LDL receptor gene

The haploid human genome contains one copy of the LDL receptor gene, which has been localized by *in situ* hybridization to bands p13.1 - 13.3 on chromosome 19 (43). The total human LDL receptor gene spans about 45.5 kilobases of DNA, and the primary sequence of about 25% of the gene has been determined, including all of the 18 exons and several of the 17 introns.

A striking correlation exists between the exons in the gene and the functional domains of the protein (22) (see Figure 1.1). The first exon codes for the short 5'-untranslated region plus the signal sequence, and exons 2 to 6 code for the seven 40 amino acid repeats of the binding domain. The seven repeats bear strong homology to a 40 amino acid, cysteine-rich sequence in complement component C9. Introns occur exactly at the ends of repeats 1, 2, 5, 6 and 7, with

exon 4 coding for repeats 3 to 5. The possibility thus exists that the 7 binding repeats arose by exon duplication. The region of the LDL receptor homologous to the EGF precursor is encoded by exons 7 to 14. A similar 8-exon cassette is found in the gene for the EGF precursor (44). The cysteine-rich "growth-factor" repeats, A, B and C in the LDL receptor, corresponding to repeats 2, 3 and 4 in the EGF precursor, are each encoded by a single exon. Similar repeat sequences are found in three proteins of the blood clotting system. In one of these clotting factors, a single exon also codes for the repeat structure (22). The O-linked sugar domain of the LDL receptor is encoded by exon 15, and the membrane-spanning domain by exon 16 and part of exon 17. Exon 17 and part of exon 18 encode the cytoplasmic tail, while the rest of exon 18 codes for the long 3'-untranslated region in the mRNA.

The correlation between exons and functional domains in the LDL receptor, the evidence for exon duplication of repeated structures, and the sharing of exons between the LDL receptor gene and other genes, support the exon-shuffling hypothesis of Gilbert, suggesting that proteins evolve as mosaic combinations of pre-existing functional units encoded by discrete exons (45). One possible mechanism for such exon-shuffling events is recombination between repetitive elements in the gene, such as Alu sequences. The LDL receptor gene contains Alu repeats in exon 18 (21) and in a number of introns (35). Although recombination between Alu sequences may have played a role in evolution of the LDL receptor protein, it may also render the gene susceptible to mutations. This possibility is supported by analysis of mutations in the LDL receptor and globin genes, showing that recombinations involving Alu sequences are causes of deletions in four LDL receptor mutations and four globin gene mutations (35). In addition, Alu-Alu recombination has been shown to result in duplication of seven exons in a mutant LDL receptor gene (46). Thus, the processes which permitted evolution of the LDL receptor may also account for

its frequent disruption by mutation in patients with familial hypercholesterolaemia (11).

The 5'-flanking region of the LDL receptor gene contains 2 TATA-like sequences of 7 base pairs each, 20 to 30 base pairs to the 5' side of the 2 major sites of transcription initiation between nucleotides -79 to -93 (22). This region also contains 3 imperfect direct repeat sequences of 16 nucleotides each (22). Sensitivity to sterol end-product repression depends on a 42 base pair element, containing two of the 16-base pair direct repeats, known as the sterol regulatory element (SRE42) (7). When SRE42 was inserted into the promoter of the herpes simplex virus thymidine kinase (HSV-TK) gene, this fusion gene promoted abundant mRNA production in transfected cells. When sterols were present, the mRNA was markedly reduced. Further studies have established that a 177 base pair fragment of the 5'-flanking region of the LDL receptor gene is sufficient for positive expression as well as negative regulation by sterols (8). This fragment contains the three 16-base pair repeats, each of which is required for mRNA expression, and the two TATA-like sequences, only one of which is required for expression. The three repeats contain a sequence homologous to the consensus eukaryotic transcription factor, Sp1, binding site. Insertion of a series of mutant SRE42 sequences, including 16-base pair repeats 2 and 3, into the HSV-TK promoter showed that repeat 3 is a constitutive positive transcriptional element which binds Sp1, while repeat 2 confers sterol-mediated repression upon repeat 3 (9). The results suggest that transcriptional regulation is mediated by a mechanism in which sterol-regulated binding of a protein to repeat 2 inhibits Sp1 binding activity in repeat 3.

A number of restriction fragment length polymorphisms (RFLP's) can be detected in the LDL receptor gene, as a result of the following polymorphic sites: Bsm I (47), Pvu II and Rsa I (48) in the 5' flanking region; Sph I in

intron 6 (47); Stu I in exon 8 (49); Ava II in exon 13 (50); Pvu II (42,51), Spe I (47) and Apa LI (52) in intron 15; Nco I in exon 19 (53); Apa LI (52) and Pst I (54) in the 3' flanking region; Bst EII (55) and Msp I (56) in the 3' half of the gene; Taq I in the 5' half of the gene (57); Hinc II in exon 12 (58). The development of a restriction fragment length polymorphism map for the LDL receptor gene is of great value in the study of mutations in familial hypercholesterolaemia, for the assignment of RFLP haplotypes to mutant genes. Haplotypes can be used to distinguish different mutations producing the same phenotype, or to show the presence of two different mutant alleles in a compound heterozygote (47). In addition, in a genetically homogeneous population group, linkage can sometimes be established between FH and a particular haplotype in that population (49,53,59,104) (see section 3.2.1).

1.3 Synthesis and post-translational processing of the LDL receptor

The N-terminal signal sequence presumably directs receptor-synthesizing ribosomes to the endoplasmic reticulum (ER) membrane (3) (see Figure 1.2), and is cleaved from the protein immediately after it is translated. The formation of the many intramolecular disulphide bonds, which stabilize the structure of the LDL receptor, is believed to take place co-translationally in the ER, as a result of the action of protein disulphide isomerase (60,61).

Glycosylation of the LDL receptor precursor protein begins in the ER and is completed in the Golgi apparatus to form the mature receptor (see Figure 1.2). Biosynthetic studies using [^{3}H]-glucosamine, and the migration behaviour of the LDL receptor on SDS-polyacrylamide gel electrophoresis, suggest that it contains two asparagine-linked (N-linked) oligosaccharide chains, and approximately 18 serine/threonine-linked (O-linked) chains (32). The N-linked oligosaccharides on the precursor are endo-β-N-acetylglucosaminidase H (EndoH)-sensitive and are believed

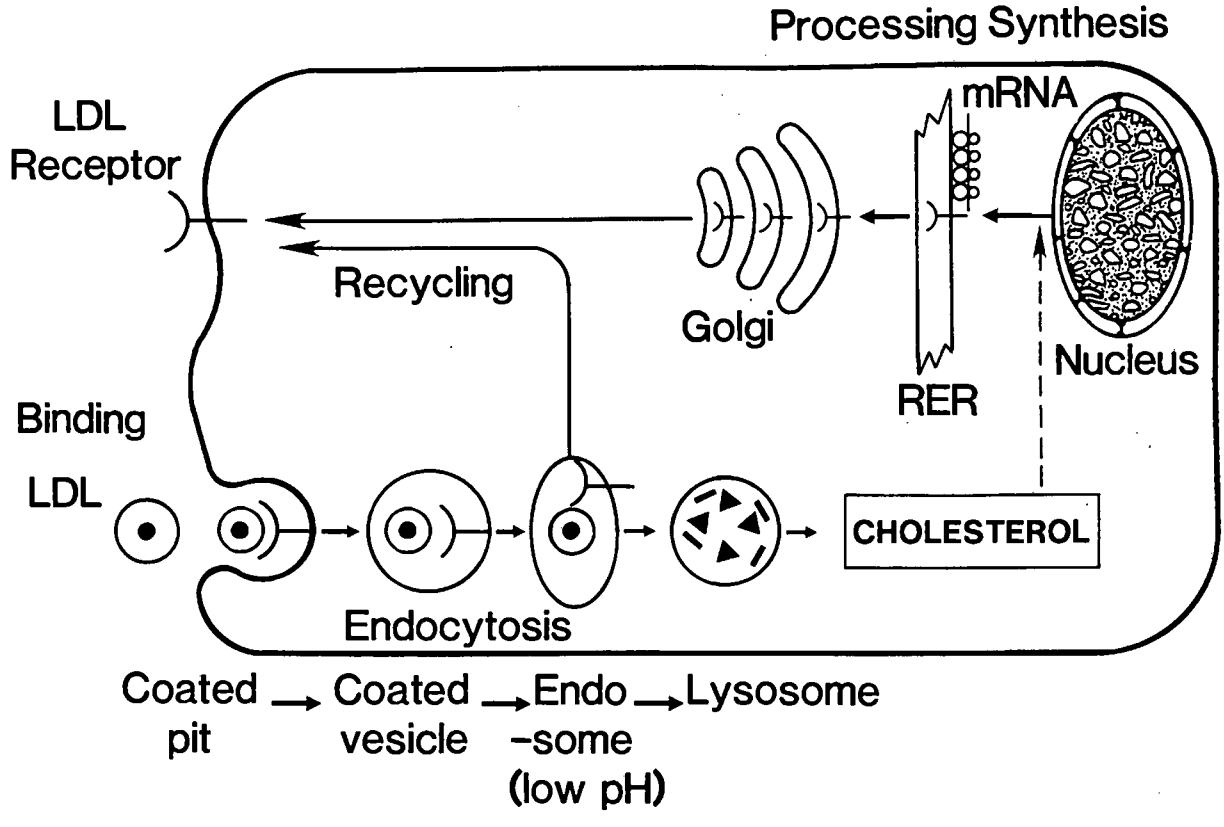

Figure 1.2 Intracellular pathway of the LDL receptor (Discussed in sections 1.3 and 1.4)

The LDL receptor is synthesized in the rough endoplasmic reticulum, transported to the Golgi complex for post-translational modifications and then to the cell-surface, where the receptors cluster in coated pits. The receptor binds LDL and the receptor-ligand complex is endocytosed. At the low pH in the endosome, the ligand dissociates from the receptor, which recycles to the cell-surface. The ligand is delivered to the lysosome and degraded. (Adapted from reference 12).

to have the composition $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$. The core O-linked oligosaccharides on the precursor in A431 cells consist of only N-acetylgalactosamine (GalNAc), whereas those on the precursor in fibroblasts contain GalNAc linked to another saccharide, postulated to be N-acetylglucosamine (GlcNAc). In fibroblasts, the N-linked oligosaccharides of the receptor precursor are processed to mature bi-antennary complex-type structures, and in A431 cells to tri- and tetra-antennary complex-type structures. The mature LDL receptor in A431 cells contains O-linked oligosaccharides consisting of galactose and N-acetylgalactosamine, plus one or two sialic acid residues (see Figure 1.3). In fibroblasts, the mature O-linked oligosaccharides are also sialylated, but are larger and have a different structure (32). Most of the O-linked chains can be isolated on a single glycopeptide fragment after Pronase digestion (32), and are thus clustered together in one domain of the receptor.

The LDL receptor is synthesized in the rough endoplasmic reticulum as a precursor which migrates on SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 120 kDa, although the protein component has a molecular weight of 93 kDa. Within 30 minutes, the receptor is converted to a mature form with an apparent molecular weight of 160 kDa. The calculated molecular weight of the receptor plus mature carbohydrates is approximately 115 kDa (3). This anomalous migration of the receptor on SDS-PAGE is largely due to the addition of sialic acid and galactose residues to the clustered O-linked N-acetylgalactosamine residues, leading to a conformational change (32,33). A small contribution to the increased apparent molecular weight of the receptor is made by the N-linked chains (32).

Biosynthesis of the N-linked oligosaccharides is a more complex process than that of O-linked oligosaccharides, but is better understood. The first step, catalyzed by

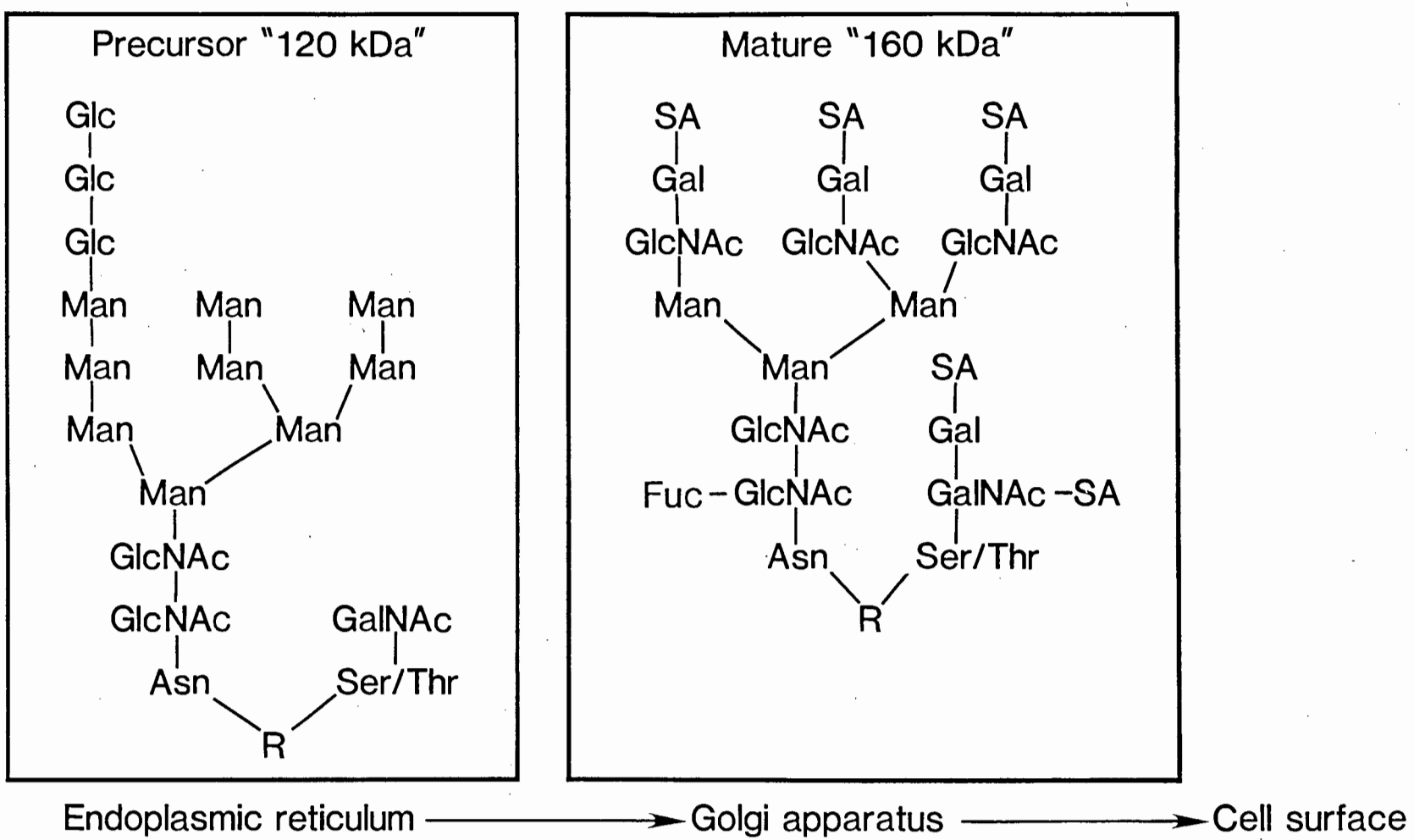

Figure 1.3 (legend overleaf)

Figure 1.3 Proposed structures of oligosaccharides attached to the LDL receptor during post-translational processing

High mannose asparagine (Asn)-linked (N-linked) oligosaccharides are added co-translationally to the LDL receptor (R) in the endoplasmic reticulum (ER). The core serine- or threonine-(Ser/Thr) linked (O-linked) sugars are added in the ER or in transitional elements between the ER and Golgi. The precursor receptor (120 kDa) is transported to the Golgi apparatus where carbohydrate trimming and additions produce the complex N-linked oligosaccharides and mature O-linked sugars. The mature form of the receptor (160 kDa) is then transported to the cell surface. The structures indicated are those proposed for the oligosaccharides on the LDL receptor in A431 cells (32). The N-linked oligosaccharides on the receptor from fibroblasts are processed to bi-antennary complex structures, and the O-linked oligosaccharides are larger than those for A431 cells, containing N-acetylglucosamine in addition to N-acetylgalactosamine, galactose and sialic acid. Abbreviations: Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; SA, sialic acid.

oligosaccharyl transferase, is the co-translational addition of $Glc_3Man_9GlcNAc_2$ from the lipid-linked oligosaccharide donor, dolichol pyrophosphate, to asparagine residues of the nascent polypeptide in the rough ER (32). The glucose residues are removed in the ER by glucosidases I and II before the protein is transported to the Golgi. Mannose residues are trimmed in the early Golgi region by α-mannosidase I, followed by addition of N-acetylglucosamine by GlcNAc transferase I and further mannose trimming by α-mannosidase II in the medial Golgi. At this stage the N-linked oligosaccharides become EndoH-resistant (62). Further modifications are carried out by other GlcNAc- and fucosyl transferases, before galactose and sialic acid residues are added in the trans Golgi to complete the maturation to complex-type N-linked oligosaccharides. There are five potential sites in the LDL receptor protein for N-linked glycosylation (Asn-X-Ser or Asn-X-Thr) (3). Apparently only the two sites situated in the region homologous to the EGF precursor are glycosylated, but not the three potential sites in the cysteine-rich N-terminal binding domain.

Addition of O-linked oligosaccharides to the LDL receptor is a post-translational event, initiated by the addition of N-acetylgalactosamine to serine and threonine residues (32). The subcellular localization of O-linked carbohydrate addition is unknown, and proposals range from the rough ER to the cis Golgi (63). The addition of N-acetylgalactosamine to the LDL receptor precedes the conversion of the N-linked oligosaccharides to the EndoH-resistant form in the medial Golgi (32). Therefore, the transfer of GalNAc must occur prior to entry of the protein into this region of the Golgi apparatus. Recently, immunocytochemical localization of mutant LDL receptors that fail to reach the Golgi, but do contain core O-linked sugars, has suggested that the enzymes which attach these residues must be located in the rough endoplasmic reticulum, or in a transitional zone of smooth membrane extensions from

the ER (64). The addition of GalNAc to the E1 glycoprotein of Mouse Hepatitis Virus-A59 has also been shown to occur in a budding compartment similar to transitional elements or vesicles (63). Approximately 75% of the O-linked oligosaccharides attached to the LDL receptor are clustered in a region immediately external to the cell membrane. The receptor also contains isolated O-linked carbohydrate chains in the NH_2-terminal portion of the protein (33).

The function of glycosylation of the LDL receptor is unknown. Davis et al. (33) have shown that deletion of the clustered O-linked sugar domain of the LDL receptor had no effect on receptor function in cultured hamster fibroblasts. However, Kingsley et al. (65) have shown that in a mutant Chinese hamster ovary cell line, O-linked carbohydrate chains, but not N-linked oligosaccharides, played a crucial role in stability of the LDL receptor. Considered together, these findings suggest that the isolated O-linked oligosaccharides in the N-terminal region of the receptor (33) are important for receptor stability, unless the clustered O-linked sugars protect a proteolytic site in that receptor domain. The latter possibility is supported by recent findings of Kozarsky et al. (66) showing that O-linked sugar-deficient receptors reached the cell-surface, but were abnormally unstable due to proteolytic cleavage within or near the domain usually containing clustered O-linked oligosaccharides, and release into the medium of most of the N-terminal extracellular domain. Chatterjee et al. (67) have shown that inhibition by tunicamycin of N-linked glycosylation of the LDL receptor affects the level, but not the functional capacity, of receptor expression.

Apart from glycosylation, the LDL receptor does not appear to undergo any other significant post-translational modification. Although the receptor in human fibroblasts can be labelled by ^{35}S-sulphate, which attaches to N-linked sugars (32), no functional significance for this modification has been demonstrated. Unlike the transferrin

receptor, the LDL receptor does not undergo acylation (3). There is no evidence for phosphorylation of the LDL receptor in vivo, although a kinase has been isolated which can phosphorylate the receptor in vitro (68). Recently, it has been shown that in bovine adrenal membranes, and in hamster fibroblasts transfected with human LDL receptors, a small proportion of the receptors undergoes noncovalent self-association, mediated by the cytoplasmic domain, to form dimers and higher order structures (40), but the functional significance of this process has not been established

1.4 LDL Receptor Function and Regulation

The mature LDL receptor is a cell-surface protein which binds plasma LDL particles and transports them into the cell by receptor-mediated endocytosis (1) (see Figure 1.3). The internalized LDL is delivered to the lysosomes, where the cholesterol ester core is hydrolyzed to release free cholesterol for plasma membrane, bile acid or steroid hormone synthesis, or conversion back to cholesterol ester for storage (1). The high affinity of the receptor for LDL, combined with the ability of each receptor to cycle many times between the plasma membrane and the cell interior, enables large amounts of cholesterol to be delivered to the tissues, while keeping plasma LDL-cholesterol at a low level. Disruption of the LDL receptor function by genetic defects or regulatory signals causes the plasma cholesterol level to increase and can lead to atherosclerosis (69).

Approximately 45 minutes after their synthesis, LDL receptors reach the cell surface, where they gather in clathrin-coated pits, which invaginate within 3 to 5 minutes to form coated endocytic vesicles (1). The receptors spontaneously move to coated pits and enter the cell continuously, even in the absence of ligand (3). The clathrin coat dissociates from the endocytic vesicles, which then fuse to form endosomes. The pH in the endosome is

decreased to below 6.5 by ATP-dependent proton pumps, causing the LDL to dissociate from the receptor, which then recycles to the surface to initiate another endocytic cycle. The LDL receptor recycles once every 10 minutes in the presence or absence of LDL (3). Recycling requires that the LDL receptors have a stable structure to pass repeatedly through the acidic endosomes. In the endosome, the receptor must undergo sufficient conformational change to release the ligand without becoming irreversibly denatured (3). The LDL released from the receptor is delivered to the lysosome, where the protein component is degraded to amino acids, and the cholesterol ester component is hydrolyzed by acid lipase to liberate free cholesterol (70).

This cholesterol (or oxygenated cholesterol derivatives (7-9,71) regulates three events in intracellular cholesterol metabolism. Firstly, cholesterol biosynthesis is inhibited by suppression of transcription of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), and acceleration of its degradation (72). Secondly, acyl CoA:cholesterol O-acyltransferase is activated to re-esterify and store excess cholesterol (70). The third regulatory response to the cholesterol is suppression of the transcription of LDL receptor messenger RNA, thereby reducing the LDL receptor level. Sterol repression of transcription is mediated by two 16-base pair imperfect direct repeat elements in the 5' flanking region of the LDL receptor gene (7-9). When cells are deprived of exogenous cholesterol, transcription of LDL receptor mRNA and expression of LDL receptors is increased (70). In contrast to HMG-CoA reductase, regulation of the degradation of the LDL receptor does not play a role in determining receptor levels (73). By means of the above regulatory mechanisms, cells can maintain a constant level of unesterified cholesterol, despite fluctuations in their cholesterol requirements and supply (70).

1.5 Classification and elucidation of LDL receptor mutations

1.5.1 Mutations in subjects with familial hypercholesterolaemia

The biochemical analysis of mutations in subjects with familial hypercholesterolaemia (FH) has revealed much information about structure-function relationships in the LDL receptor protein, and elucidated many of the steps in the complex cellular itinerary of the LDL receptor.

The earliest classification of FH mutations by clinical criteria could only differentiate between the heterozygous and homozygous forms of the disease. Heterozygotes, which occur at a frequency of one in 500 persons in most populations, have an LDL-cholesterol level (350-550 mg/dl) about 2.5-fold above normal and develop tendon xanthomata and coronary atherosclerosis after the age of 30 (74). Homozygotes constitute approximately one in a million persons in most populations, have severe hypercholesterolaemia (650-1000mg/dl), six to ten-fold higher than normal and develop planar cutaneous xanthomata in the first 4 years of life (74). Coronary heart disease develops in childhood and frequently causes death in the first or second decades from myocardial infarction.

The ability to measure LDL receptor activity in cultured cells on the basis of ^{125}I-LDL binding studies showed that FH heterozygotes produce approximately half the normal number of LDL receptors and homozygotes, virtually no functional receptors. Three types of LDL receptor defects in FH were identified: (i) receptor-negative, where less than 2% of the normal number of functional receptors are produced; (ii) receptor-defective, where markedly decreased receptor activities of 5 to 20% of normal are observed; (iii) receptor internalisation-defective, where no internalisation of receptors occurs and thus no internalisation and degradation of surface-bound ^{125}I-LDL (75).

The LDL receptor was purified to homogeneity (17) and antibodies to the receptor were developed (18). Using these antibodies as probes, at least four classes of LDL receptor mutations were distinguished (19,20), including defects in synthesis, intracellular transport, LDL binding ability or internalisation of the receptor. In this way, the receptor-negative and receptor-defective classes were shown to represent multiple defects on the basis of immunoprecipitable receptors (18), whereas variants of the internalisation-defective class, namely secreted receptors, could be detected (35,36). FH subjects considered "homozygous" from measurements of LDL receptor activity could be shown to be genetic compounds in cases where the two mutant alleles produced immunoprecipitable receptors of different molecular weights.

Class 1 mutations are those where no LDL receptors are synthesized. This is the most common class of mutations accounting for about half the defects analyzed to date (1). The designation of a class 1 mutation is based on the absence of LDL receptor protein as detected by reaction with polyclonal or monoclonal anti-receptor antibodies.

Class 2 mutations, the second most common class, lead to significant receptor synthesis, but slow transport from the endoplasmic reticulum to the Golgi apparatus, and therefore slow post-translational carbohydrate processing (see section 1.3). The mutant precursors contain high-mannose N-linked oligosaccharides and core O-linked N-acetylgalactosamine residues, and have molecular weights ranging from 100 kDa to 135 kDa (19), although most have normal molecular weights of 120 kDa. Biosynthetic immunoprecipitation studies show that the N-linked sugars are abnormally slowly converted to the complex EndoH-resistant form, and that the elongation of O-linked sugars is markedly retarded. The mutant receptors either do not appear on the cell surface, but remain in the ER, where they may eventually be degraded (76), or the

receptors appear on the surface at about one-tenth the normal rate (19,77). The reason for delayed transport in class 2 mutations is presumably abnormal folding of the mutant LDL receptor protein, since transport from the endoplasmic reticulum depends on a normal protein conformation (78-80). Some abnormal proteins retained in the ER have been shown to bind to a class of proteins including immunoglobulin heavy chain binding protein (BiP) (78,81). Binding to BiP has not yet been demonstrated for LDL receptor Class 2 mutants, although a putative "gatekeeper" protein has been proposed (23) and variable amounts of a protein migrating with the molecular weight of BiP have been detected in LDL receptor immunoprecipitation studies (82).

Class 3 mutant LDL receptors are processed and reach the cell surface, but fail to bind LDL normally although they bind antibodies to the receptor (18,19). The mature forms of these receptors have normal (160 kDa) or abnormal (140 or 210 kDa) apparent molecular weights (19), and are synthesized as precursors which are 40 kDa smaller than the mature receptors.

Class 4 mutations produce receptors which reach the cell surface and bind LDL, but fail to cluster in coated pits (3). The study of these mutants revealed the importance of coated pits in the process of receptor-mediated endocytosis. Class 4 variants produce LDL receptors which are secreted and can therefore be immunoprecipitated from the medium (35,36,83). Receptors are transported to the surface where some remain associated with the membrane, while the vast majority are secreted into the medium, possibly after initially being cell-associated (83). Those receptors associated with the membrane do not migrate to coated pits and are internalisation-defective.

Classification of genetic defects in the LDL receptor on the basis of binding activity and ^{35}S-biosynthetic immuno-

precipitation studies revealed a wide diversity of phenotypes associated with FH, suggesting that it was a heterogeneous disease at the molecular level. The isolation of full-length cDNA clones (21) to the LDL receptor mRNA and elucidation of the normal receptor gene structure (22) have provided the tools necessary to explore the molecular genetics of the LDL receptor in FH. The molecular basis of at least one of each of the different classes of mutations discussed above has been determined (summarized in Table 1.2). The mutations are distributed over the entire 45 kilobases of the gene, and affect all the different domains of the protein (10).

Molecular analysis of FH mutations has enabled further subdivision of class 1 mutations into null alleles that produce mRNA and those where no mRNA is detectable (47). Class 1 mutations are caused by promoter deletions (47,84) or deletions of exons coding for parts of the EGF precursor homology domain (47,85,86) and, in one case, also the O-linked sugar domain (86) [See Table 1.2].

Transport-deficient, or class 2, mutant LDL receptor alleles are the result of small in-frame deletions (10,23,87) or substitutions (82,88) in exons encoding both cysteine-rich and cysteine-poor (82) regions of the receptor. Mutations in cysteine-rich regions could be expected to distort intra-repeat disulphide bonding in some way, resulting in abnormal protein conformation and delayed transport from the ER to the Golgi (78-80). However, the mutation in one transport-deficient allele (FH429) is located in a part of the gene coding for a cysteine-poor region of the molecule (82). Presumably, this mutation alters the protein folding without involvement of abnormal disulphide bonding. In this regard, it is noteworthy that 4 different class 2 mutations elucidated at the molecular level (see Table 1.2) involve deletions or substitutions of glycine residues (10,23,82,88). Glycine, with its single hydrogen side chain, is often found at space-restricted regions in

proteins, such as β-turns. Thus deletion of a glycine residue, or substitution with a larger amino acid, could potentially disrupt protein conformation, leading to impaired intracellular transport (78-80).

Defective LDL binding in class 3 mutants appears to be caused by deletions (89) or insertions (46) involving exons 2 to 8, which code for cysteine-rich repeats in the binding and EGF precursor homology domains. It is noteworthy that these mutant receptors with deletions or insertions of entire repeats exhibit normal rates of intracellular transport, in contrast to those produced by small mutations within cysteine-rich repeats. This is consistent with the idea that disulphide bonds form within each repeat (90), so that small deletions or insertions disrupt correct disulphide bond formation, whereas deletion or insertion of whole repeats has no effect.

Internalisation-defective (Class 4) alleles all have mutations in exon 17 coding for the cytoplasmic tail (37,38), whereas the class 4 variants, the secreted receptors, have deletions which produce receptors lacking the membrane-spanning and cytoplasmic domains (35,36,91,92).

The analysis of FH mutations on a molecular level has revealed a frequent involvement of Alu repeats in large deletions (35,36,85,86,89) and insertions (46). Four of these mutations, FH274 (36), FH626 (89), FH781 (35) and TD (85), are deletions caused by homologous recombinations between two Alu sequences. The deletion in FH381 (86) is the result of recombination between an Alu sequence in intron 15 and a sequence in exon 13 homologous to the Alu sequence. Although the mutations in FH781 (35) and FH274 (36) both result from recombination between Alu repeats in intron 15 and exon 18, different individual Alu repeats are involved in the two deletions (35). In the recombinations involved in FH274 and FH381, the Alu (or Alu-like) sequences were oriented in opposite directions, and formation of a

TABLE 1.2 NATURAL MUTATIONS AT THE LDL RECEPTOR LOCUS IN FAMILIAL HYPERCHOLESTEROLAEMIA (Adapted from (19))

Biochemical Class of Mutation	FH Subject	Ethnic Origin	Mutation	mRNA	Protein	Ref.
Class 1: No detectable protein	FH 49, 549, 808, 859	French-Canadian	> 10 kb deletion of promoter and exon 1	None	None	84
	FH 26	American	Compound heterozygote - one allele: ± 6 kb deletion of promoter and exon 1, other allele: no visible deletion	None	None	47
	FH 132	Italian-American	Compound heterozygote - complex rearrangement of one allele, possible complete deletion of other allele	6.2 kb	None	47
	FH 651	Italian	4 kb deletion of exons 13 and 14	5 kb	None	47
	T.D.	British	Unequal crossing-over between Alu sequences in introns 12 and 14 - 4 kb deletion of exons 13 and 14 (possibly same mutation as FH651)	N.D.	None	85
	FH 381 (heterozygote)	Italian-American	5 kb deletion of exons 13 to 15 as a result of recombination between exon 13 and Alu sequence in intron 15	5 kb	None	86
	FH 431 FH 485 FH 664 FH 842 FH 61 FH 790 FH 573	American Colombian American Italian Italian-American Chinese Italian	No deletion rearrangement detected by Southern blotting	Normal 5.3 kb	None	47
Class 1 (cont.)	FH 250	Greek-Cypriot	No deletion/rearrangement detectable by Southern blotting	N.D.	None	47
	FH 551	Japanese	No deletion/rearrangement detectable by Southern blotting	5.3/8.4 kb	None	47
Class 2: Delayed transport to cell surface	WHHL rabbit	-	12 bp deletion in exon 4	Normal	Loss of 4 amino acids (Asp^{115}.Gly^{116}.Ser^{117}.Asp^{118}) in repeat 3 of binding domain	23
	FH 563	American	3 bp deletion in exon 4	Normal	Loss of one Glycine residue in 5th binding repeat	10,23
	FH 264,550, 786,793	Lebanese-Arab	Nonsense mutation in exon 14	Normal	Truncated protein lacking O-linked sugar, membrane-spanning and cytoplasmic domains	86
	FH 429	Italian	Missense mutation in exon 11	Normal	Substitution of Val for Gly^{544}	10,82
	TT	Xhosa (South African)	In-frame deletion of 6 bp in exon 2	N.D.	Removal of $Asp^{26}Gly^{27}$ from repeat 1, loss of IgG-C7 recognition	87
Class 3: Defective LDL binding	FH 359,454	Dutch	4 kb deletion of exons 7 and 8	5.1 kb	Loss of Cys-rich repeats A and B in EGF precursor-like region, binds β-VLDL, but not LDL	35
	FH 626	French	0.8 kb deletion of exon 5 as a result of unequal crossing over between Alu sequences in introns 4 & 5	5.2 kb	Loss of 6th Cys-rich binding repeat, binds β-VLDL, but not LDL	89

Biochemical Class of Mutation	FH Subject	Ethnic Origin	Mutation	mRNA	Protein	Ref.
Class 3 (cont.)	FH 295	American	14 kb duplication of exons 2 to 8 caused by Alu-Alu recombination between introns 1 and 8	6.5 kb	Duplication of binding repeats 1 to 7 and repeats A and B from EGF precursor-like region	46
Class 4: Defective internalisation	FH 682,683	Saudi-Arabian	Nonsense mutation in exon 17 - stop codon 2 residues distal to membrane-spanning region	Normal	Only 2 cytoplasmic amino acids	37
	FH 763	French	4 kb duplication in exon 17 after codon for sixth amino acid of cytoplasmic tail → altered reading frame	Normal	6 normal amino acids, 8 random amino acids, then stop codon	37
	FH 380 (J.D.)	Italian-American	Nonsense mutation in exon 17	Normal	Tyr^{807} replaced by Cys	38
Class 4 variants: Secreted receptors	FH 274	American	5.5 kb deletion of exons 16 to 18 by intrastrand recombination between 2 Alu sequences in intron 15 and exon 18	8.5 kb	Truncated receptor lacking membrane-spanning and cytoplasmic domains, small amount of internalisation-defective cell-associated receptors	10,36
	FH 781	Japanese	7.8 kb deletion of exons 16 to 18 by recombination between Alu sequences in intron 15 and exon 18, splice acceptor sites deleted	6.2 kb	Truncated receptor, no membrane-spanning or cytoplasmic domain, novel 55 amino acids from transcription of intron 15, cell-associated receptors internalisation defective	35

Biochemical Class of Mutation	FH Subject	Ethnic Origin	Mutation	mRNA	Protein	Ref.
Class Unknown (No phenotypic studies)	KY, SO (Heterozygotes)	Japanese	Deletion of exon 15 encoding O-linked sugar domain	N.D.	Loss of O-linked sugar domain	34
	30% of 52 FH heterozygotes	Finnish	8 kb deletion of exons 16 and 17 and part of exon 18	4.2 kb	Loss of membrane-spanning and cytoplasmic domain (presumably secreted)	91,92

N.D. = Not Determined

double stem-loop structure was proposed as the mechanism for the deletions (36,86). Unequal crossing-over between Alu sequences in the same orientation is the simplest mechanism that could give rise to the mutations in FH626 (89), FH781 (35), FH295 (46) and TD (85). Nine different Alu sequences have been shown to be involved in mutations in the LDL receptor gene, while three Alu sequences have been reported to be involved in mutations in globin genes (35). Eight of the deletion points are clustered within a 36-base pair sequence of the left arm of the Alu repeat, suggesting that this region may adopt an unusual configuration during transcription that renders it prone to recombination (35). In general, the frequent involvement of Alu sequences in mutations in the LDL receptor suggests that the tendency for recombination between these elements may account in part for the prevalence of FH.

1.5.2 Experimentally-induced and site-directed mutations in the LDL receptor

Although invaluable information about structure and function of the LDL receptor has been obtained through the study of naturally-occurring mutations in FH (summarized in Table 1.2), the variety of these mutations is limited, and only mutations compatible with the growth and survival of the organism (or cultured cells) can be studied. The scope of these studies has been extended by analysis of experimentally-induced mutations in the LDL receptor (12) and by *in vitro* mutagenesis to create specific mutations to characterize particular regions of the LDL receptor protein (28,29,31,33) [summarized in Table 1.1].

Krieger and co-workers have treated Chinese hamster ovary cells with mutagens, and then screened for mutants exhibiting defects in LDL endocytosis (93). The LDL receptor-deficient mutants fall into four complementation groups, ldlA, ldlB, ldlC and ldlD. Analysis of ldlA mutants identified the ldlA locus as the structural gene for the LDL receptor. Three classes of mutant alleles were

distinguished at the ldlA locus (93): null alleles (class 1), class 2 mutations causing defective receptor processing, and alleles that code for normally-processed receptors which cannot bind LDL (class 3). The abnormally-processed (class 2) receptors were continually converted to novel unstable intracellular intermediates. The mutations in ldlB, ldlC and ldlD cells appeared to be general glycosylation defects (94). The mutation in ldlD cells was shown to be a deficiency in the enzyme UDP-galactose/UDP-N-acetylgalactosamine 4-epimerase (65). The epimerase deficiency leads to reversible defects in the synthesis of both N-linked and O-linked oligosaccharides on glycoproteins, including the LDL receptor. The ldlD mutants have been particularly valuable in showing that O-linked, but not N-linked, oligosaccharides play a crucial role in stability of the LDL receptor (65) by preventing the proteolytic removal of the N-terminal region as observed for O-linked sugar-deficient receptors (66).

The general approach of using FH mutations in the LDL receptor to broadly define the importance of a region of the protein, followed by the in vitro construction of additional mutations for further characterization by transfection studies, has been applied to all domains of the LDL receptor (summarized in Table 1.1). Particularly rigorous analysis of the structural requirements for internalisation, mediated by the cytoplasmic domain (39), and the contribution of the various cysteine-rich repeats in the ligand-binding and EGF precursor homologous domains to binding of the ligands, LDL and β-VLDL (28), has been carried out (discussed in sections 1.2.1.5, 1.2.1.1 and 1.2.1.2, respectively). Site-directed mutagenesis of the LDL receptor has also established that the first cysteine-rich binding repeat contains the epitope for IgG-C7 (29), that the EGF precursor homologous domain facilitates acid-dependent conformational changes to release bound ligand in the endosome (31), and that the clustered O-linked oligosaccharides have no important function in cultured hamster fibroblasts (33). In addition, site-

directed mutagenesis of the 5'-flanking region of the LDL receptor gene has identified the elements required for regulated expression (7-9).

1.6 Effect of genetic defects in the LDL receptor *in vivo* - therapeutic implications

When Goldstein and Brown began their studies of FH in 1972 (1) it was generally believed that all important events in cholesterol metabolism occurred in the liver and intestine. Since it was not feasible to study the LDL receptor in livers of human FH patients, cultured skin fibroblasts from the patients were used. Thus, the elucidation of LDL receptor structure, function and regulation, in both normal and FH subjects, was conducted in the artificial environment of tissue culture, using non-hepatic cells. The question arises as to how relevant these findings are to the situation *in vivo*, particularly as the LDL receptors in the peripheral tissues would be continually down-regulated at the levels of LDL encountered in the plasma (75). However, the LDL receptor obviously has an important role in the body, as shown by the serious problems caused by LDL receptor deficiency in FH homozygotes, and the proportionately less severe abnormalities in heterozygotes (1).

The first demonstration of LDL receptor activity *in vivo* was in lymphocytes isolated from the bloodstream (95). LDL receptor activity was very low in freshly-isolated lymphocytes, but when the lymphocytes were incubated for 72 hours in the absence of lipoproteins to "de-repress" LDL receptor activity, a progressive increase in high-affinity uptake and degradation of ^{125}I-LDL was observed. Consistent with results from cultured fibroblasts, lymphocytes from FH homozygotes developed less than 5% of normal receptor activity after 72 hours in the absence of lipoproteins, and FH heterozygotes exhibited an intermediate level of expression (95).

The function of the LDL receptor *in vivo* has been studied by determining the rate of disappearance from the plasma of intravenously injected ^{125}I-LDL (69). The rate in FH heterozygotes (mean life-span of ^{125}I-LDL in the plasma = 4.5 days) is slower than in normal cells (mean life-span 2.5 days), and even slower in FH homozygotes (mean life-span 6 days) (1). This decreased rate of LDL catabolism *in vivo* correlates with the relative deficiency of LDL receptors detected in lymphocytes and fibroblasts from FH subjects.

The fraction of total LDL clearance which represents the receptor-dependent fraction can be estimated by comparing the rate of ^{125}I-LDL catabolism in normal individuals and FH homozygotes. The fractional catabolic rate in normal subjects is 3 times higher than in FH homozygotes, suggesting that approximately two-thirds of LDL clearance is normally mediated through the LDL receptor (1). The same conclusion is obtained from the degradation rates of native LDL versus lysine- or arginine-modified LDL, which cannot bind to the LDL receptor.

The existence and importance of the hepatic LDL receptors has been established in a number of studies (reviewed in (1)). The bovine adrenal gland and corpus luteum showed the highest LDL receptor activity per gram of tissue, but, taking the weight into account, the liver expressed the most LDL receptors of all the tissues. Similar results were obtained with human foetal tissues. Perfused rat livers exhibited high-affinity, receptor-mediated uptake of ^{125}I-LDL that could be increased by 17α-ethinyl estradiol administration. Liver membranes from dogs were also shown to contain functional LDL receptors that were susceptible to metabolic regulation (96). Steinberg and co-workers, and Dietschy and co-workers have shown that approximately 70% of total-body uptake of radio-labelled LDL is mediated through hepatic LDL receptors (1), thus emphasizing the importance of the liver receptors in the regulation of plasma LDL levels.

An additional mechanism for determination of LDL levels by hepatic LDL receptors has been elucidated through kinetic studies in the WHHL rabbit, an animal model for FH (97). In addition to degrading LDL more slowly, FH homozygotes and heterozygotes appear to over-produce LDL. LDL is not secreted directly by the liver, but is produced from VLDL (very-low-density lipoprotein) by triglyceride hydrolysis by lipoprotein lipase to form IDL (intermediate-density lipoprotein), which can be taken up by the liver LDL receptors via high-affinity binding of multiple apoprotein E molecules on the IDL particle. IDL can alternatively undergo further triglyceride hydrolysis to form LDL, containing a single molecule of apoprotein B-100. Injection of ^{125}I-labelled VLDL into the WHHL rabbit showed that the apparent overproduction of LDL observed in FH is due to the failure of IDL to be removed from the plasma because of the deficiency in hepatic LDL receptors. The IDL remaining in the circulation is converted in increased amounts to LDL. Thus hepatic LDL receptors play a dual role in LDL metabolism by (i) limiting LDL production by enhancing removal of precursor IDL from the circulation and, (ii) enhancing LDL degradation by mediating receptor-mediated cellular uptake. The LDL receptors of the liver are thus crucial in modulating plasma LDL levels.

As a result of the central role of the liver in the control of plasma cholesterol, therapeutic approaches to lower plasma cholesterol are aimed at increasing the production of LDL receptors in the liver. FH heterozygotes have one normal LDL receptor allele which can be stimulated to produce an increased number of receptors, to overcome the genetic deficiency (1). Since the liver is the major site for LDL receptor expression, this stimulation can be achieved by increasing the hepatic demands for cholesterol by: (i) inhibition of intestinal re-absorption and re-use by the liver of bile acids by the administration of bile acid-binding resins such as cholestyramine, (ii) inhibition of

cholesterol synthesis by HMG-CoA reductase inhibitors, such as compactin or mevinolin. The combination of these two measures causes an increase in the amount of HMG-CoA reductase and LDL receptors synthesized by the liver. Total body cholesterol synthesis is slightly reduced, while plasma LDL levels fall by 50 to 60%, as a result of the increased number of hepatic LDL receptors. The decrease in LDL is balanced by the increase in receptors so that the absolute amount of cholesterol taken up by the liver is the same, but is occurring at a lower level of plasma LDL (1).

FH homozygotes do not respond to measures that stimulate LDL receptor production, since they do not have a normal allele which can synthesize functional LDL receptors. Removal of LDL from the plasma of FH homozygous subjects can be performed extracorporeally by repeated plasmapheresis. An alternative, more direct approach of liver transplantation, to provide a source of LDL receptors, was recently applied in the case of FH homozygote, SJ. The result was a decrease in plasma cholesterol from 1100 mg/dl to 200-300 mg/dl and treatment with the HMG-CoA reductase inhibitor, mevinolin, further decreased the level to 150-200 mg/dl. This dramatic response to liver transplantation emphasizes the importance of hepatic LDL receptors <u>in vivo</u> in regulating plasma LDL cholesterol levels.

An alternative to therapy for homozygous FH subjects is prevention of the incidence of homozygous FH by genetic counselling to heterozygous FH subjects. However, unequivocal assignment of the condition of heterozygous FH is not always possible. Clinical symptoms similar to those in FH may be observed in other lipid disorders, and assays of receptor activity are too complicated and time-consuming for routine screening. In addition, overlap in the ranges of LDL receptor activity for normal and heterozygous cells can occur (98). The most reliable diagnostic test for heterozygous FH would be at the genetic level. However, the heterogeneity of the disease at the molecular level

hampers approaches for genetic diagnosis. The situation may be different in populations with a relatively homogeneous genetic background, where founder gene effects can occur. In such populations, diagnostic Southern blotting or haplotype analysis may be possible, facilitating simple and definitive screening of potential FH heterozygotes. Founder gene mutations in the LDL receptor in populations exhibiting a high incidence of FH are discussed in section 1.7.

1.7 Founder-gene mutations in the LDL receptor

Founder effects are produced by "the establishment of a new population by a few original founders which carry only a small fraction of the total genetic variation of the parental population" resulting in a new, genetically unique, population (99) in which certain genetic disorders are unusually prevalent. FH is a heterogeneous disease, even within the same ethnic groups (10). However, exceptions to this heterogeneity are observed in genetically-isolated populations, where founder gene effects are present.

1.7.1 French-Canadian FH

Four unrelated French-Canadian homozygous FH subjects (FH49, 549, 808 and 859) have been shown to have the same LDL receptor mutation, a large deletion eliminating the promoter and exon 1 of the gene, giving rise to a "null" (class 1) allele. This deletion accounted for 63% of all FH mutations in this ethnic group (84). The present-day French-Canadian population is descended from a small group of 8000 French immigrants to Canada in the 17th and 18th centuries. Since this population has remained relatively isolated genetically, it seems likely that the high frequency of the promoter deletion is due to a founder gene effect. Since the mutation is readily detected by Southern blotting (84), rapid and accurate diagnosis of FH in the majority of French-Canadian hypercholesterolaemic patients should be possible.

1.7.2 Lebanese FH

The prevalence of heterozygous FH is approximately 1 in 500 in most populations in the world. However, in Lebanon, the heterozygote frequency is approximately 1 in 100, and, coupled with a high incidence of consanguinity, results in a 10-fold higher prevalence of the homozygous disease (88). The occurrence of a single Class 2 mutation in the homozygous form in four unrelated Arab patients with FH (three from Lebanon, one from Syria) may be attributable to a founder gene effect. This "Lebanese allele" produces an abnormally small receptor as a result of a single base substitution in exon 14 producing a premature termination codon. The truncated receptor lacks the O-linked sugar, membrane-spanning and cytoplasmic domains, and is not transported to the cell surface, but retained in the ER, probably as a result of abnormal folding. The mutation creates a novel HinfI site in exon 14, permitting diagnosis by Southern blotting. The postulate that the mutation represents a founder gene mutation in this population can thus be readily tested in future.

1.7.3 Finnish FH

Recent studies (91,92) have identified an 8 kilobase deletion from the 3'-end of the LDL receptor gene, in about one-third of Finnish patients with heterozygous FH (91). The mutation eliminates exon 16, 17 and part of 18 (92). Thus, the receptor would be expected to lack the membrane-spanning and cytoplasmic domains, and be secreted from the cell (35,36). The deletion can be easily diagnosed by Southern blotting (91).

The Finns are descended from relatively few ancestors, and no significant immigration from Sweden or Russia has occurred. The barrier of the Finnish language has further contributed to the genetic isolation of this population, which has a high coronary heart disease mortality rate. The possibility thus exists that the prevalence of the

deletion mutation is the result of a founder gene effect in the Finnish population.

1.7.4 South African Afrikaner FH

FH is particularly prevalent in the South African Afrikaner population (100-102), with an estimated heterozygote frequency of 1 in 100 (101), similar to that in the Lebanese population. The incidence of the homozygous form of FH, which is rarely found in most countries (frequency of 1 in 1 million), is as high as 1 in 30000 in the Afrikaner population (101). Homozygotes from this group exhibit variable and somewhat milder clinical features than observed for FH in other populations (102).

The reason for the prevalence of FH in this group is that the Afrikaners have remained genetically relatively homogeneous because of a history of geographical, religious and cultural isolation (103) and a great expansion of an originally small settler population (102). A founder gene effect has thus been proposed to account for the high incidence of FH in the Afrikaner population (101,102). It has been calculated that one or two defective genes among founder members of the Afrikaners would be sufficient to explain the high FH prevalence (101).

Support for the founder gene hypothesis has been obtained in studies of LDL receptor activity (98,103) and from haplotype studies (59,104). LDL receptor activity was determined in cultured cells from 21 clinically-defined Afrikaner FH homozygotes from two geographical areas, the Cape Province (98) and the Transvaal (103). The results showed a "receptor-defective" (75) type of mutation in all but one pair of siblings, which were apparently "receptor-negative". Using PvuII (42,51) and StuI (49) polymorphisms in the LDL receptor gene, Brink et al. (59) and Henderson et al. (104) showed that 16 out of 17 unrelated FH homozygotes from the groups described above were homozygous for one haplotype. Preliminary biosynthetic studies (103) suggested the

predominance of a mutation coding for receptors which undergo retarded post-translational processing. All the above findings are consistent with the proposal of a founder gene(s) to account for the high incidence of FH in the South African Afrikaner.

The unusually high frequency of FH in the Afrikaner population affords a unique opportunity to study LDL receptor mutations, in many cases, in the truly homozygous form, without interference from a normal gene (in FH heterozygotes) or from a different mutant gene (in compound heterozygotes). In addition, the development of a comprehensive restriction map of the LDL receptor gene, and the subsequent determination of haplotypes or markers associated with FH, particularly in the founder gene situation, should enable population screening and thus prenatal diagnosis of homozygous FH, or diagnosis of heterozygous FH and genetic counselling to reduce the incidence of the severe homozygous form. This is particularly relevant in the South African population where deaths from coronary heart disease are the highest in the world (102). The high incidence of FH must make a significant contribution to these cases, and therefore the diagnosis, understanding and, hopefully, prevention of this disease is medically very relevant to this country.

1.8 Research objectives

Although studies of LDL receptor activity in cells from Afrikaner FH homozygotes indicated the predominance of a receptor-defective phenotype, believed to be the result of one or more founder mutations, considerable heterogeneity in the level of functional activity was observed (98,103).Analysis of receptor synthesis, processing and degradation rates, and ligand binding affinity could potentially elucidate the mechanism(s) underlying the heterogeneity, and thus provide valuable information, in a broader context, on the defects responsible for Afrikaner

FH, and, more specifically, on interesting cellular processes affecting the level of receptor expression.

The biochemical analysis of FH mutations has contributed significantly to the understanding of structure-function relationships and the cellular events in the LDL receptor pathway (discussed in section 1.5). The elucidation of Class 4 mutations, for example, has provided valuable insights into the role of the cytoplasmic domain of the receptor in the interaction with coated pits required for receptor-mediated endocytosis (37,38). The Class 2 mutations, which exhibit defects in receptor transport from the endoplasmic reticulum to the Golgi apparatus, have the potential to provide fundamental information on the complex regulation of intracellular transport, a topic of great current interest in cell biology (78-80). It was therefore noteworthy that preliminary biosynthetic studies of selected Afrikaner subjects (103) showed retarded post-translational transport and processing of the mutant receptors (Class 2 mutations).

The aim of this research project was to characterize fully the phenotypic expression of mutant LDL receptors in selected homozygous Afrikaner FH subjects (and FH homozygotes from other South African ethnic groups), because of (i) their potential medical and genetic significance (section 1.7.4) and (ii) the fundamental importance of the cell biological mechanisms underlying the wide range of LDL receptor activity produced by transport-deficient alleles (discussed above).

CHAPTER 2
EXPERIMENTAL PROCEDURES

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2.1 Materials

2.1.1 Culture media and supplements

Eagle's Minimum Essential Medium (MEM), Dulbecco's Modification of Eagle's Medium (DMEM), RPMI medium and methionine- and glutamine-free Eagle's Minimum Essential Medium with Earle's salts (EMEM) were obtained from Flow Laboratories (Ayrshire, Scotland). Trypsin and tryptose phosphate broth were from Difco Laboratories (Detroit, MI, USA). Heat-inactivated foetal calf serum was prepared by heating foetal calf serum (State Vaccine Institute, Cape Town, South Africa) to 56°C for 30 minutes. Human lipoprotein-deficient serum was prepared as described below in section 2.2.1. Penicillin G and streptomycin sulphate were from Hoechst Pharmaceuticals (Johannesburg, South Africa).

2.1.2 Radio-isotopes

Sodium [^{125}I] iodide was obtained from Amersham (Buckinghamshire, England). Tran^{35}S label^{TM} (ICN Radiochemicals, Irvine, CA, USA), was the source of [^{35}S]-methionine, and is the hydrolysate of *E. coli* grown in the presence of $H_2{}^{35}SO_4$, consisting of 70% [^{35}S]-methionine, 20% [^{35}S]-cysteine, 7% [^{35}S]-methionine sulphoxide, 2% [^{35}S]-cysteic acid and 1% other ^{35}S-labelled compounds.

2.1.3 Reagents for SDS-polyacrylamide gel electrophoresis and fluorography

BDH Chemicals Ltd. (Poole, England) was the source for sodium salicylate and acrylamide, while N,N'-methylene-bisacrylamide and TEMED (N,N,N',N'-tetramethylethylene-diamine) were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Ammonium persulphate, 2-mercaptoethanol, dithiothreitol and Coomassie Brilliant Blue R250 were purchased from E. Merck A.G. (Darmstadt, West Germany).

2.1.4 Detergents

Sigma Chemical Co. (St. Louis, MO, USA) was the source

for CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate), Nonidet P-40 and Triton X-100. Tween 20 was obtained from Bio-Rad Laboratories (Richmond, CA, USA) and sodium dodecyl (lauryl) sulphate (SDS) from Saarchem Pty Ltd. (Krugersdorp, South Africa).

2.1.5 Enzymes

"Trypsin-EDTA", for dispersion of cultured cell monolayers, contained Trypsin (0.05% (w/v), Difco Laboratories, Detroit, MI, USA) and EDTA (0.02% (w/v), Merck) in phosphate-buffered saline (PBS). Neuraminidase was obtained from Calbiochem-Behring Diagnostics (San Diego, CA, USA) or from Sigma Chemical Co. Pronase and Endo-α-N-acetylgalactosaminidase (O-glycanase) were from Boehringer Mannheim (Mannheim, West Germany) and Endo-β-N-acetylglucosaminidase H (Endoglycosidase H) was a product of Miles Scientific (Naperville, IL, USA). Proteinase K was obtained from E. Merck A.G. (Darmstadt, West Germany).

2.1.6 Inhibitors

Aprotonin (Midran^{TM}) was obtained from Novo Industries (Pharmaceuticals) (Pty) Ltd. (Johannesburg, South Africa). Phenylmethylsulphonyl fluoride (PMSF) was from Merck and leupeptin from the Peptide Institute Inc. (Osaka, Japan). Chloroquine was purchased from Sigma Chemical Co., and ammonium chloride from BDH Chemicals, Ltd. Tunicamycin, obtained from Calbiochem-Behring Diagnostics, was dissolved in dimethyl sulphoxide at a concentration of 2 mg/ml (32).

2.1.7 Miscellaneous reagents

Pristane (2,6,10,14-tetramethylpentadecane) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Iodo-Gen and Immobilized Protein A were supplied by Pierce Chemical Company (Rockford, IL, USA). Sigma Chemical Co. was the source for HEPES (2-[4-(2-hydroxyethyl)-1-piperazine]ethane sulphonic acid), heparin and cholesterol. Suramin (Germanin^{TM}) was purchased from Bayer-Miles (Pty) Ltd. (Isando, South Africa) and 25-hydroxycholesterol from

Research Plus, Inc. All other reagents used were analytical grade from Merck.

2.1.8 Antibodies

IgG-C7, a mouse monoclonal antibody to the LDL receptor, was prepared as described below in section 2.2.2. Polyclonal anti-LDL receptor antibodies, 698-7 and 498-1, raised in rabbits against purified bovine LDL receptor (105) and non-immune IgG from rabbits were gifts from Drs. M.S. Brown and J.L. Goldstein (Departments of Molecular Genetics and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA). B3/25, a mouse monoclonal antibody to the human transferrin receptor, was provided by Dr I.S. Trowbridge (The Salk Institute for Biological Studies, San Diego, CA, USA). Goat anti-mouse IgG (heavy and light chain specific) and goat anti-rabbit IgG (heavy and light chain specific) were obtained from Cappel Laboratories (Malvern, PA, USA).

Goat anti-mouse IgG was radio-iodinated as described later for IgG-C7 (section 2.2.2.3) after prior dialysis against several changes of 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7) to remove sodium azide from the preparation.

2.1.9 Lipoprotein ligands

Human low density lipoprotein (LDL), which binds to the LDL receptor via apolipoprotein B-100, and rabbit β-migrating very-low-density lipoprotein (β-VLDL), which binds via the interaction of apolipoprotein E with the LDL receptor (discussed in section 1.2.1.1) were prepared as described below in section 2.2.1.

2.2. Methods

2.2.1 Preparation of low-density lipoprotein (LDL), lipoprotein-deficient serum (LPDS) and β-migrating very-low-density lipoprotein (β-VLDL)

Human low density lipoprotein (LDL) (density 1.019 - 1.063 g/ml) and human lipoprotein-deficient serum (LPDS)

(density > 1.25 g/ml) were isolated from the plasma of normolipidaemic donors by discontinuous gradient centrifugation (106) in a Beckman L8-70 ultracentrifuge as follows. Plasma (containing 0.01% (w/v) EDTA and protease inhibitors, aprotonin (0.4% (v/v) of a 10000 U/ml solution) and phenylmethylsulphonyl fluoride (PMSF) (0.2 mM)) was adjusted to a density of 1.3 g/ml with potassium bromide and 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7.0) (density 1.006 g/ml) was layered over the adjusted plasma. The various lipoproteins were separated according to their densities by centrifugation at 50000 rpm (206000 g) for 2.5 hours at 10°C in a vertical VTi50 rotor. The LDL fractions (density 1.019 to 1.063 g/ml) were pooled and washed at density 1.063 g/ml by centrifugation for 16 hours at 50000 rpm (170000 g) in a Ti60 rotor. After the initial 2.5 hour centrifugation, the lower portion of the discontinuous gradient was adjusted with potassium bromide to a density of 1.25 g/ml and high-density lipoprotein (HDL) was separated from the denser lipoprotein-deficient fraction by centrifugation at 50000 rpm (206000 g) for 6 to 8 hours in a VTi50 rotor. The LDL and lipoprotein-deficient fractions were dialyzed exhaustively against 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7.0). After dialysis, the lipoprotein-deficient plasma was converted to lipoprotein-deficient serum by incubation at 4°C for 24 hours with thrombin (2 units/ml). The resulting clot was removed by centrifugation for 10 minutes at 20000 rpm (30000 g) for 10 minutes in a JA20 rotor.

In some cases, an alternative, shorter method for the preparation of LPDS only was used. Plasma (containing 0.25 M EDTA) was adjusted to a density of 1.215 g/ml with potassium bromide and centrifuged at 59000 rpm (250000g) for 36 hours at 4°C in a Ti60 rotor. The VLDL, LDL and HDL fractions in the upper portion of each tube were discarded, and the yellow, lipoprotein-deficient layers in the lower portion of the tubes were pooled, dialyzed against 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7.0) and treated with thrombin as described above.

The LDL and LPDS were sterilized by filtration through a Millipore filter (0.45 μM pore diameter). The LPDS was heated to 56°C for 30 minutes to inactivate complement activity. Protein concentrations of the LPDS and LDL preparations were determined by the method of Lowry et al. (107) using bovine serum albumin (Fraction V, Boehringer Mannheim) as standard. The protein concentrations usually ranged from 8 to 20 mg protein/ml for LDL and 50 to 80 mg/ml for the LPDS. The LPDS was stored frozen at -10°C and the LDL was kept at 4°C. The LDL preparation was shown by lipoprotein electrophoresis (Beckman Paragon Lipoprotein Reagent Kit) to be essentially free of other human lipoproteins, and by SDS-polyacrylamide gel electrophoresis (5-12% polyacrylamide gradient) according to the method of Laemmli et al. (108), to contain mainly apolipoprotein B-100 (apparent molecular weight 340000 daltons) and thus to be essentially free (<1%) of contaminating plasma proteins.

β-migrating very low density lipoprotein (β-VLDL) was prepared from the plasma of cholesterol-fed rabbits, essentially as described by Kovanen et al. (109). The rabbit is extremely sensitive to dietary cholesterol. Within days after the initiation of a high cholesterol diet (2% cholesterol - 10% corn oil), the plasma cholesterol concentration increases by more than 10-fold. A large proportion of this cholesterol is contained in β-VLDL particles in which cholesteryl esters are the predominant lipids, and apoprotein E the major protein (109).

β-VLDL was isolated from rabbits fed the high cholesterol diet for 2 to 5 weeks. The hyperlipidaemic (density < 1.006) fraction was isolated by centrifugation at 40000 rpm in a SW40 rotor for 16 hours at 10°C. This fraction was washed twice by adjusting the sample to 0.22 M sodium chloride and then applying the adjusted solution under a layer of phosphate-buffered saline followed by centrifugation under the same conditions as above. SDS-polyacrylamide gel

electrophoresis of the β-VLDL preparation showed that 80-90% of the protein content was apoprotein E. The protein concentration was determined by the method of Lowry et al. (107). Because of the lipid-rich nature of the β-VLDL, it was necessary to extract the turbid samples (and bovine serum albumin standards) three times with hexane, before the level of colour development in the protein assay could be determined spectrophotometrically.

2.2.1.2 Radio-iodination of lipoproteins

^{125}I-labelled LDL was routinely prepared using the iodine monochloride method of MacFarlane (110), as modified by Bilheimer et al. (111). An amount of glycine-NaOH buffer (2M, pH 10) was added to the LDL, such that the final glycine concentration in the total iodination mixture would be 0.4 M. Carrier-free sodium [^{125}I] iodide (1 mCi/mg LDL protein or 0.5 mCi/mg protein) was then added and 40 µl of diluted iodine monochloride solution (35 µl 33 mM iodine monochloride in 2 ml 2M sodium chloride) per mg LDL protein was forcefully injected into the reaction mixture. The radiolabelled LDL was then dialyzed against several changes of 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7.0) to remove free ^{125}iodine. The specific radioactivity of ^{125}I-labelled LDL prepared in this way usually ranged from 300 to 700 cpm per nanogram of protein (150-350 cpm/fmol) and 98% of the ^{125}I-radioactivity was protein-bound as determined by precipitation with 5% (w/v) trichloroacetic acid. Less than 5% of the ^{125}I-label was extractable into chloroform-methanol (2:1), representing lipid labelling. The protein concentration of ^{125}I-labelled LDL was approximately 3 mg/ml. Radio-iodinated LDL was used within 2 weeks of iodination and was always centrifuged briefly (11000 g for 2 minutes) before use, to remove any precipitated or particulate matter.

β-VLDL was radiolabelled as described above for LDL except that a 20-fold higher concentration of iodine monochloride was used. Approximately 90% of the label was precipitable

by 5% trichloroacetic acid, and 15-30% of the label could be extracted into chloroform:methanol (2:1), representing lipid labelling.

2.2.2 Preparation of monoclonal anti-LDL receptor antibody IgG-C7

IgG-C7, a monoclonal antibody directed against an epitope in the first cysteine-rich repeat of the LDL receptor binding domain (29), was prepared from a commercially available hybridoma cell line (American Type Culture Collection, CRL1691). This cell line was originally established by Beisiegel et al. (18) by immunizing mice with a partially purified receptor from bovine adrenal cortex. Spleen cells from the mice were then fused with mouse myeloma cells using polyethylene glycol. Aliquots of medium from the hybridoma cultures were screened for antibodies to the LDL receptor and positive hybridomas were cloned by limiting dilution.

2.2.2.1 Production of ascites fluid

Female BALB/C mice (6-8 weeks old) were primed for ascites tumour formation by injection with pristane (2,6,10,14-tetramethyl pentadecane) at least ten days before injection of hybridomas. Hybridoma cells were grown in suspension at approximately $3x10^5$ cells per ml in 90 mm diameter Petri dishes containing 10 ml of either DMEM, supplemented with 4.5 g glucose per litre and 10% (v/v) foetal calf serum, or RPMI, supplemented with 300 mg L-glutamine per litre, 500 µM mercaptoethanol, 10% (v/v) heat-inactivated foetal calf serum, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Medium was changed every alternate day. For long-term storage, cells were collected by centrifugation, resuspended at $2x10^6$ cells per ml of 90% (v/v) heat-inactivated foetal calf serum, 10% (v/v) dimethylsulphoxide, and stored frozen under liquid nitrogen.

Hybridoma cells were harvested by aspiration when in the actively growing, logarithmic phase, washed once with serum-free DMEM and resuspended to a final density of $2x10^6$ to

$5x10^6$ hybridomas per 0.5 ml DMEM. Each primed mouse was injected intra-peritoneally with 0.5 ml of hybridoma cell suspension. Within 14 to 21 days, ascites tumours developed in most of the mice. The mice were sacrificed by cervical dislocation, the peritoneal cavities opened and the ascites fluid aspirated. Each mouse yielded 2 to 4 ml of ascites fluid which was centrifuged at 2000 rpm in a Beckman Model TJ-6 centrifuge for 15 minutes at room temperature to remove cells, and then stored at 4°C in the presence of 0.02% sodium azide. In some cases, the ascites fluid proteins were precipitated with an equal volume of saturated ammonium sulphate, and stored as a wet pellet at -20°C.

2.2.2.2 Purification of IgG-C7 from ascites fluid

The ascites fluid was applied to a column of immobilized Protein A (Pierce) that was equilibrated with 0.1 M sodium phosphate (pH 7.0). After elution of unbound material at pH 7, the IgG fraction was eluted with a solution of 1 M acetic acid and 0.1 M glycine (pH 3). The pH of the IgG fraction was immediately raised to pH 7.4 by the addition of the required volume of a 2 M Tris solution, and the IgG-containing solution was then dialyzed against 10 mM sodium phosphate, 50 mM NaCl (pH 7.4) (18) for 24 hours at 4°C. The dialyzed solution was concentrated using an Amicon 8200 ultrafiltration system (YM10 membrane) to a protein concentration (determined by the method of Lowry et al. (107)) of 1 to 2 mg/ml. The IgG-C7-containing solution was stored frozen under liquid nitrogen in aliquots which were never freeze-thawed more than once before use.

2.2.2.3 Radio-iodination of IgG-C7

The monoclonal antibody IgG-C7 was radio-labelled with ^{125}I using Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycol-uril) a solid-phase iodinating reagent (112) essentially as described by Beisiegel et al. (18). A typical iodination reaction contained 200 µg of Iodo-Gen adsorbed to a glass vial, to which was added 0.6 mg IgG-C7 and 0.25 - 0.5 mCi of carrier-free sodium [^{125}I] iodide in a total volume of 0.4

ml. After incubation for 5 minutes (112) or 20 minutes (18) at 4°C, the iodination mixture was removed from the reaction vial and dialyzed against several changes of 0.9% (w/v) NaCl, 0.01% (w/v) EDTA (pH 7.4) to remove free ^{125}iodine. The specific radioactivity of the ^{125}I-labelled IgG-C7 ranged from 400 to 2000 cpm per nanogram of protein (60-300 cpm/fmol), depending on the amount of sodium [^{125}I]iodide used and the incubation time. Approximately 98% of the ^{125}I-radioactivity was protein-bound as determined by precipitation with 5% (w/v) trichloroacetic acid.

2.2.2.4 Characterization of isolated IgG-C7

The purity of the isolated IgG-C7 was assessed by SDS-polyacrylamide gel electrophoresis and biological activity of the antibody was determined by the ability of ^{125}I-labelled IgG-C7 to bind to cell-surface LDL receptors (18), and by the ability of an immune complex containing IgG-C7 to specifically immunoprecipitate LDL receptors (section 2.2.7.6) from a total cell lysate (20).

The efficiency of IgG-C7 purification by Protein A affinity chromatography was routinely assessed by SDS-PAGE of the ascites fluid, the unbound material at pH 7, and the putative IgG fraction eluted at pH 3. The samples were subjected to electrophoresis in the presence of SDS, under both reducing and non-reducing conditions, in a 5% to 20% polyacrylamide gradient gel (108). The ascites fluid contained IgG (molecular weight 150 kDa (non-reduced) or 50 kDa and 25 kDa (reduced)) and various other proteins, particularly albumin (molecular weight 67 kDa). The unbound material at pH 7 contained mostly contaminating proteins, but also a small amount of IgG. However, the peak which eluted at pH 3 was essentially pure IgG, thus confirming the efficiency of the Protein A affinity chromatography.

The monoclonal antibody to the LDL receptor, IgG-C7, was shown by Beisiegel et al. (18) to bind with high affinity to

human skin fibroblasts, reaching levels equimolar to the amount of LDL bound at saturating concentrations. Typical concentration curves for the binding of ^{125}I-labelled LDL and ^{125}I-labelled IgG-C7 to normal human skin fibroblasts are shown in Figures 5.1(a) and (b), p.151, respectively. For either antibody or ligand, the curves showed two components: a high affinity component which approached saturation at an LDL concentration of 5 μg protein/ml (10 nM) (Fig. 5.1(a)) or at an IgG-C7 concentration of 0.5 μg protein/ml (3 nM) (Fig. 5.1(b)), and a non-specific component which increased linearly above these concentrations. High-affinity binding values, calculated by non-linear regression analysis (section 2.2.8), showed that half-maximal binding for ^{125}I-LDL to the receptor in normal cells occurred at 2.4 μg protein/ml (4.8 nM), and for ^{125}I-IgG-C7 at 0.08 μg/ml (0.5 nM). Maximum high affinity binding of ^{125}I-labelled LDL was calculated to be 237 ng/mg cell protein (474 fmol/mg), and for ^{125}I-labelled IgG-C7 to be 41 ng/mg cell protein (273 fmol/mg). The maximal ^{125}I-IgG-C7 binding was 60% of the ^{125}I-LDL binding level, and not exactly equimolar as expected. This molar ratio was found to be variable between 0.6 and 1.0 for different batches of radio-iodinated IgG-C7 and LDL. The variability could be minimized by performing iodination of the IgG-C7 in the presence of Iodo-Gen for only 5 minutes at 4°C, as originally suggested by Fraker and Speck (112) instead of 20 minutes, as described by Beisiegel *et al.* (18). Using this procedure, approximately equimolar ratios of ^{125}I-LDL to ^{125}I-IgG-C7 binding to normal fibroblasts were consistently obtained. To correct for any variability in the molar ratio of binding of different batches of radio-iodinated LDL and IgG-C7, the ratio of LDL to IgG-C7 binding at saturation in LDL receptor-normal cells was assumed to be unity, and binding values for cells from FH subjects were always compared by expression as percentages of LDL- and IgG-C7 binding to normal cells in the same experiment (as shown in Figure 5.1 for FH1a cells). Besides binding with high-affinity to cells in approximately equimolar amounts to LDL

binding, the IgG-C7 was able to inhibit binding of ^{125}I-LDL binding to the LDL receptor. When cell monolayers were pre-incubated for 1 hour at 4°C with IgG-C7 (1 µg/ml), subsequent binding of ^{125}I-LDL (2 hours at 4°C) was inhibited by approximately 50% (data not shown). In contrast, pre-incubation with LDL did not decrease subsequent binding of ^{125}I-IgG-C7. These results are consistent with those obtained by Beisiegel et al (18).

2.2.3 Cell culture

All media used for cell culture were routinely tested to ensure that they were pyrogen-free. Cell cultures were periodically checked for mycoplasma contamination using the BRL Mycotect Mycoplasma Detection system (Bethesda Research Laboratories, Maryland, USA). Culture media usually contained antibiotics (penicillin G and streptomycin sulphate) to prevent bacterial contamination.

2.2.3.1 Human skin fibroblasts

Skin fibroblast cultures were established from skin biopsies obtained from the ventral surface of the forearms of subjects (98,103). In addition, LDL-receptor-normal fibroblast strains GM3348 and GM0203 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ, USA). The cells were maintained in monolayer cultures in 75 cm^2 flasks in a humidified incubator (95% relative humidity, 5% CO_2) at 37°C. Fibroblasts were grown in approximately 12 ml of Dulbecco's Modification of Eagle's Minimum Essential Medium (DMEM), buffered with 43mM or 25mM bicarbonate, and supplemented with 10% (v/v) heat-inactivated foetal calf serum, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulphate. Cells were not used beyond the twentieth passage in culture. Confluent monolayers were dissociated by incubation with 5 ml 0.05% (w/v) trypsin/0.02% (w/v) EDTA for approximately 5 minutes at 37°C and then seeded at 0.1 x 10^6 to 0.15 x 10^6 cells per 60 mm Petri dish and used for experiments within 2 to 5 days as described in sections 2.2.5, 2.2.6. and 2.2.7. For long-term storage, trypsin-

EDTA dissociated fibroblasts were resuspended at 10^6 cells per ml of medium containing 10% (v/v) dimethyl sulphoxide, and stored frozen under liquid nitrogen.

2.2.4 Induction of maximal LDL receptor expression in cultured fibroblasts

The expression of maximal LDL receptor activity requires that: (i) the cells be actively growing, since confluent cells no longer require cholesterol for new membrane synthesis and the number of LDL receptors thus declines, and (ii) the cells be deprived of exogenous cholesterol (70). Active growth of cultured cells was established by seeding them initially in medium containing 10% (v/v) foetal calf serum. After a few days of growth, the cells were deprived of exogenous cholesterol by washing with phosphate-buffered saline and then incubating them for 48 hours in lipoprotein-deficient medium (DMEM, supplemented with 5 mg LPDS protein/ml). In some cases, the medium was supplemented with LPDS at 2.5 mg protein/ml instead of 5 mg/ml. This lower concentration of LPDS produced the same upregulated LDL receptor level in normal cells after 48 hours as was obtained for 5 mg/ml LPDS (results not shown). Although fibroblasts cannot grow indefinitely in lipoprotein-deficient medium, they continue dividing for 48 hours, and maximal surface LDL receptor expression is reached after this period (70). Therefore, unless stated otherwise, LDL receptor activity was upregulated in lipoprotein-deficient medium for 48 hours prior to each experiment.

Although 48 hours in LPDS were required to achieve a new, maximal steady state receptor level, receptor synthesis rates increased more rapidly, reaching a maximum after 20 to 30 hours in LPDS (see Fig. 5.13, p. 190). Therefore, upregulation periods of only 16 to 24 hours were required to achieve sufficient [^{35}S]-methionine incorporation into LDL receptors for biosynthetic studies (19,77).

2.2.5 Surface binding of ^{125}I-labelled lipoproteins and ^{125}I-labelled IgG-C7 at 4°C

Normal human skin fibroblasts were seeded at a density of 0.1 x 10^6 cells per 60 mm diameter Petri dish and grown for 5 days and then induced for maximal LDL receptor expression by incubation for 48 hours in lipoprotein-deficient medium. The cells then received 1.5 ml of ice-cold lipoprotein-deficient medium (MEM buffered with 10 mM HEPES (pH 7.4), supplemented with 2.5 or 5 mg protein/ml LPDS) containing the indicated concentrations of ^{125}I-labelled LDL, ^{125}I-labelled β-VLDL (section 2.2.1.2) or ^{125}I-labelled IgG-C7 (section 2.2.2.3). After incubation for 2 hours at 4°C, cells were washed four times at 4°C with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin, followed by three washes at 4°C with PBS. The cells were solubilized in 1 M NaOH and total radioactivity bound to the cells was measured. Cell protein was determined by the method of Lowry et al. (107) using bovine serum albumin as standard.

High-affinity binding values for ^{125}I-LDL and ^{125}I-IgG-C7 were calculated from concentration curves of total binding by non-linear regression analysis, assuming ligand binding to a single class of high-affinity sites plus non-specific binding (See section 2.2.8). Alternatively, surface binding levels of ^{125}I-labelled IgG-C7 (1 μg/ml, 6.7 nM) and ^{125}I-labelled-LDL (7 μg/ml, 14 nM) at 4°C were determined as described above, and high-affinity values were calculated from total binding by subtraction of non-specific values obtained in the presence of excess unlabelled IgG-C7 (50 μg/ml, 330 nM) or LDL (300 μg/ml, 600 nM), respectively.

Surface-bound ^{125}I-β-VLDL (1 μg/ml) was determined, in the absence or presence of excess unlabelled β-VLDL (90 μg/ml), as the fraction of total binding released by the anionic compound, suramin. After binding at 4°C for 2 hours, and washing as described above, each monolayer was incubated for 1 hour at 4°C with 1.5 ml of buffer containing 10 mM

suramin, 50 mM NaCl, 3 mM $CaCl_2$, 10 mM HEPES (pH 7.4) (31). After the incubation period, the medium was collected and the suramin-releasable fraction of ^{125}I-β-VLDL bound was determined. This procedure was necessary because of the high, variable (25-60% of total) background of ^{125}I-β-VLDL binding obtained after solubilization in 1 M NaOH.

LDL receptor activity in fibroblasts from subjects with homozygous FH was always expressed as a percentage of the high-affinity binding to a normal cell line in the same experiment.

2.2.6 Binding and metabolism of ^{125}I-labelled lipoproteins and ^{125}I-labelled IgG-C7 at 37°C

Fibroblasts were grown for 5 days as described for 4°C surface binding assays, and then LDL receptor activity was either upregulated for 48 hours in lipoprotein-deficient medium (2.5 or 5 mg/ml LPDS protein) or down-regulated in medium containing 1 μg/ml 25-hydroxycholesterol plus 12 μg/ml cholesterol to suppress LDL receptor synthesis (6,21,75). On day 7, cells received 2 ml of lipoprotein-deficient medium (DMEM/LPDS) containing the indicated concentrations of either ^{125}I-labelled LDL, ^{125}I-labelled β-VLDL or ^{125}I-labelled IgG-C7 in the absence or presence of excess unlabelled LDL, β-VLDL or IgG-C7, respectively. After incubation for 4 hours at 37°C, the cells were chilled to 4°C and the medium was collected for determination of ^{125}I-LDL or ^{125}I-IgG-C7 degradation. The cells were washed as described in section 2.2.5 and solubilized in 1 M NaOH for the determination of cell-associated (surface-bound and intracellular) ^{125}I-LDL or -IgG-C7, and cell protein by the method of Lowry *et al.* (107). Degradation of ^{125}I-labelled LDL or IgG-C7 was determined from non-iodine trichloroacetic acid-soluble radioactivity in the medium, as described previously (98,103). Briefly, protein was precipitated from the medium by the addition of 0.6 ml 50% (w/v) trichloroacetic acid and incubation on ice for at least 30 minutes, and then sedimented by centrifugation at 2000 rpm

(Beckman TJ-6 centrifuge) for 30 minutes at 4°C. An aliquot of the supernatant (1 ml) was removed, 20 µl 40% potassium iodide was added as a carrier, and 50 µl 30% hydrogen peroxide was then added to convert the free iodide to iodine. The iodine was extracted with 2 volumes of chloroform and the ^{125}I-tyrosine in the aqueous phase was measured to determine the extent of degradation of ^{125}I-labelled LDL, β-VLDL or IgG-C7. Background degradation values, obtained from ^{125}I-LDL, ^{125}I-β-VLDL or ^{125}I-IgG-C7 incubation in cell-free dishes, were subtracted from values obtained for degradation by fibroblasts.

High-affinity uptake and degradation values for ^{125}I-labelled LDL, β-VLDL and IgG-C7 were calculated by subtraction of non-specific values obtained in the presence of an unlabelled excess of each ligand or antibody, respectively. In some cases, high affinity values for ^{125}I-LDL or ^{125}I-IgG-C7 uptake and degradation were determined by subtraction of the values obtained for LDL receptor down-regulated cells, or by non-linear regression (section 2.2.8) of a concentration curve of total binding, assuming ligand or antibody binding to a single class of high-affinity sites .

2.2.7 Immunoprecipitation of ^{35}S-labelled LDL receptors from cultured fibroblasts

2.2.7.1 Preformed immune complexes

Preformed immune complexes of (i) mouse monoclonal antibodies, IgG-C7 or anti-transferrin receptor B3/25, and goat anti-mouse IgG second antibody, or (ii) rabbit polyclonal anti-LDL receptor antibodies, 698-7 or 498-1, and goat anti-rabbit IgG, were prepared essentially as described by Tolleshaug *et al*. (20), with some modifications. Monoclonal (or polyclonal) antibody (0.3 mg in approximately 150 µl 10 mM sodium phosphate, 50 mM NaCl (pH 7.4)) was added to goat anti-mouse (or anti-rabbit) IgG (3 mg in 300 µl 20 mM phosphate-buffered saline (pH 7.3)) and 300µl of immune complex buffer containing 50 mM Tris-HCl (pH 8), 200

mM NaCl, 1 mM EDTA and 0.5% (v/v) Nonidet P-40. After incubation at room temperature for 30 minutes, the complex was placed at 4°C for an additional 24 to 48 hours. The complex was then washed once by mild centrifugation (5 minutes at 2000 rpm at 4°C) and resuspended in 1 ml of immune complex buffer. Protein concentrations of immune complex suspensions were typically 2-3 mg/ml, representing precipitation of approximately 70% to 80% of the total protein. Increasing the time of precipitation to longer than 24 hours, or increasing the ratio of second to first antibody to greater than 10:1, did not result in more efficient precipitation (Casciola, L.A.F., unpublished observations).

2.2.7.2 Labelling of cell proteins with [^{35}S]-methionine

Human skin fibroblasts were seeded at 1.5 x 10^6 cells per 60 mm dish, and after 2 days' growth, the cells were incubated for 16 hours in lipoprotein-deficient medium (DMEM, supplemented with LPDS at 2.5 or 5 mg protein/ml) to induce LDL receptor synthesis (19,77). The cells were washed twice with 2 ml PBS, and then pulse-labelled at 37°C in 1.3 ml methionine-free Eagle's Minimum Essential Medium buffered with Earle's salts (EMEM) (supplemented with 5 mg/ml LPDS) with 30-130 µCi/ml [^{35}S]-methionine (Tran^{35}S-label^{TM}, described in Materials section 2.1.2).

After pulse-labelling (usually 0.5 to 2 hours as indicated in figure legends), the cells were either immediately washed and solubilized, or the medium was changed to complete DMEM/LPDS (containing 200 µM unlabelled methionine) and incubation at 37°C was continued for various chase times. The cells were then washed twice at 4°C with 2 ml of buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl and 2 mM $CaCl_2$ and lysed and scraped from the dishes in 200 µl of ice-cold buffer containing 10 mM HEPES (pH 7.4), 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (dissolved in dimethylsulphoxide, final concentration 0.25% (v/v)), 0.1 mM leupeptin and 1% (v/v) Triton X-100. Each

dish was rinsed with a second 200 µl of this buffer, which was pooled with the first 200 µl and centrifuged at 4°C for 10 minutes at 11000 g in a microfuge to remove insoluble material. An aliquot of 320 µl was taken from each supernatant and the pellet fractions were discarded. The 320 µl aliquots of the lysates were either used immediately for immunoprecipitation or stored frozen at -70°C.

2.2.7.3 Immunoprecipitation of ^{35}S-labelled receptors

The method for immunoprecipitation was based on a modification of the procedure of Terhorst et al. (113) as described by Tolleshaug et al. (19). ^{35}S-labelled receptors were precipitated by the addition of 80 µl of preformed immune complex (section 2.2.7.1) to 320 µl of cell lysate and incubation at 4°C for 1 hour on a horizontally-shaken platform. Immunoprecipitates were collected by centrifugation at 2000 rpm at 4°C for 5 minutes. The supernatant was removed and either discarded or, in some cases, used for a second immunoprecipitation reaction with an immune complex containing either the same monoclonal or polyclonal antibody (to assess the efficiency of the first precipitation) or a different antibody to immunoprecipitate another specific protein. The immunoprecipitates were then resuspended in lysis buffer and washed by centrifugation through a 4-step sucrose gradient as described by Tolleshaug et al. (19). The sucrose gradient contained 10 mM Tris-HCl (pH 8) and 1mM phenylmethylsulphonyl fluoride throughout, and in addition, the first layer contained 10% sucrose, 0.5% (w/v) CHAPS; the second layer, 20% sucrose, 0.5 M NaCl, 0.2% (v/v) Nonidet P-40; the third layer, 30% sucrose, 0.1% (w/v) SDS, 0.2% (v/v) Nonidet P-40; and the fourth (bottom) layer, 40% sucrose. After centrifugation at 4°C for 30 minutes at 2000 rpm in a Beckman TJ-6 centrifuge, the supernatant (gradient solutions) was carefully decanted, and the tube left upside down to drain. The immunoprecipitate pellet in each tube was resuspended in 200 µl of a buffer containing 50 mM Tris-HCl (pH 8), 2 mM $CaCl_2$, transferred to a microfuge tube and pelleted by centrifugation at 12000 rpm

(11000 g) for 20 seconds. This final immunoprecipitate was dissolved in 35 µl of a solution containing 75 mM Tris-HCl (pH 6.8), 4 M urea, 100 mM dithiothreitol, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 2.45% (w/v) SDS.

Fig. 2.1D shows the different stages in the isolation of the LDL receptor by immunoprecipitation with IgG-C7, from the initial cell lysate to the final, washed, immunoprecipitate. The transferrin receptor was isolated simultaneously by including the monoclonal antibody, B3/25, in the preformed immune complex. The results show that although the immunoprecipitation causes an enrichment of the receptor band, the most important step in isolating the receptor from contaminating proteins is the washing of the immunoprecipitate by centrifugation through the stepwise sucrose gradient, containing different combinations of detergents and ionic strengths. A second immunoprecipitation carried out on the supernatant of the first showed that the first immunoprecipitation precipitated 80% of the ^{35}S-labelled LDL receptors and transferrin receptors. It was previously shown by Casciola et al. (73) that the antibody would precipitate a constant proportion of the antigen (LDL receptor) over an appropriate range of antigen concentration. Their results showed a linear response of immunoprecipitated ^{35}S-labelled LDL receptor band to LDL receptor content.

2.2.7.4 Immunoprecipitation from culture medium

In order to investigate the possibility of LDL receptor secretion from fibroblasts of FH subjects 3a and 3b (section 4.2.4) immunoprecipitation was carried out on the culture medium after a long pulse period, as described by Lehrman et al. (36) or after a 2-hour pulse period, followed by a 4-hour chase, similar to the protocols used by Miyake et al. (83) and Kozarsky et al. (66) for the detection of secreted LDL receptors. For the immunoprecipitation following a long pulse period, cells were labelled with [^{35}S]-methionine (50 µCi/ml) in 1.3 ml EMEM supplemented with 5 µM unlabelled

methionine for 18 hours at 37°C. The medium was then collected, centrifuged at 11000 g for 10 minutes to remove any cellular material and immunoprecipitation was then performed for 1 hour at 4°C on half the total volume (0.75 ml) using 150 µl IgG-C7- and B3/25(anti-transferrin receptor)-containing immune complex suspension. The cells were washed and solubilized and immunoprecipitation performed as usual on 320 µl cell lysate using 80µl immune complex suspension. The second protocol for immuno-precipitation from the medium was as follows: cells were pulse-labelled in methionine-free EMEM with [^{35}S]-methionine (80 µCi/ml) for 2 hours at 37°C, and then chased in 2 ml medium containing 200 µM unlabelled methionine for 4 hours at 37°C. The medium was then removed and the cells were washed and solubilized and centrifugation performed on the cell lysate to remove insoluble material (see section 2.2.7.2). The medium was similarly centrifuged at 11000 g for 10 minutes, to remove any cellular material, and then a 1.8 ml aliquot was immunoprecipitated with 140 µl of immune complex suspension, containing either monoclonal IgG-C7 or polyclonal 489-1. The cell lysates were simultaneously subjected to immunoprecipitation with 80 µl of either immune complex. All immunoprecipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis and fluorography as described below (section 2.2.7.5).

2.2.7.5 SDS-polyacrylamide gel electrophoresis and fluorography

SDS-PAGE was performed according to Laemmli (108), using a resolving gel containing 5-20% 10-20%, 5-12% or 7% acrylamide (as indicated in the figure legends) and 0.1% SDS, with a 5% acrylamide stacking gel. Samples were heated to 90°C for 3 minutes and then applied to the gel. Electrophoresis was performed at 30-35 mA per slab gel (Hoefer Scientific Instruments DC or Pharmacia Fine Chemicals Electrophoresis power supply, run at constant current). The mobilities of molecular weight standards (myosin (200 kDa), phosphorylase b (94kDa), bovine serum

albumin (67 kDa), ovalbumin (43 kDa)), separated on the same gel, were used to calculate apparent molecular weights of labelled receptor bands. After electrophoresis, gels were stained for 30 minutes in 40% methanol, 20% acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R250, and then destained in 10% methanol, 10% acetic acid. Destained gels were first soaked for 30 minutes in water, followed by 30 minutes at room temperature in the water-soluble fluor, sodium salicylate (1 M), as described by Chamberlain (114). The gel was then dried under vacuum for 2 hours at 60°C and 30 minutes at room temperature (Hoefer Dual Temperature Slab Gel Dryer), and then exposed to X-ray film (Kodak XAR-5 X-OMat) at -70°C for 16 hours to 5 days, depending on the amount of radioactivity in the receptor bands. After exposure, films were warmed to room temperature and developed in an automated processor.

For quantitative determinations, the enhanced, dried gel was exposed to the X-ray film for a sufficient period at -70°C to allow the emission from low intensity bands to reach a level that was within the linear response of the X-ray film, i.e. such that the darkening of the film was proportional to the amount of radioactivity. The amount of ^{35}S-radioactivity in the receptor bands on the fluorograms was quantified by densitometric scanning using either a Beckman DU-8 spectrophotometer (with a Slab Gel program) or a Cliniscan densitometer (Helena Laboratories, Texas). The response of the film was shown to be linear at band intensities between 1.5 and 8 arbitrary integration units (Beckman DU-8 spectrophotometer, gel scanning slit 0.1x4 mm)

Although the integration values are proportional to the amount of ^{35}S-radioactivity in receptor bands within an experiment, they are referred to as "arbitrary" because they cannot be considered as absolute values which can be compared directly between experiments. The number of integration "units" in a band, representing ^{35}S-labelled receptors immunoprecipitated from one Petri dish of cells,

will depend on the amount of [^{35}S]-methionine in the original pulse-labelling period, the length of the pulse, the number of cells per dish and their level of metabolic activity, and finally on the time and conditions of exposure of the dried gel to X-ray film. Although these parameters vary between experiments and cell lines, they are constant within an experiment for a particular cell line and in this case the integration values for receptor bands can be directly compared.

2.2.7.6 Specificity of LDL receptor immunoprecipitation by IgG-C7

When normal fibroblasts were pulse-labelled with [^{35}S]-methionine for 30 minutes at 37°C, and the label then chased by incubation for 30 minutes at 37°C in complete DMEM/LPDS (containing 200 μM unlabelled methionine), two major ^{35}S-labelled bands were immunoprecipitated by the IgG-C7-containing immune complex (Fig. 2.1A). These bands had apparent molecular weights of approximately 120 kDa and 160 kDa, as reported by Tolleshaug et al. (20) for the precursor and mature forms of the LDL receptor, respectively. These two bands were undetectable in immunoprecipitates from cells which had been incubated for 16 hours in lipoprotein-deficient medium containing 1 μg/ml 25-hydroxycholesterol, in order to suppress LDL receptor synthesis (7-9), thus confirming their identity as LDL receptor bands. Immune complex containing a rabbit polyclonal antibody to the LDL receptor precipitated the same precursor and mature bands as IgG-C7 (Figure 2.1B), whereas non-immune rabbit IgG did not show any precipitation of these two bands, indicating that they are specifically LDL receptor bands. Cells from homozygous FH subject, TT (discussed in detail in section 3.2.2) express receptors which are able to bind LDL, but do not recognize IgG-C7. Thus, no receptor bands were immunoprecipitated by IgG-C7 from TT cells (Fig. 2.1B), whereas the polyclonal antibody was able to recognize and immunoprecipitate precursor and mature LDL receptor bands. In the experiment shown in Figure 2.1C, normal cells and TT

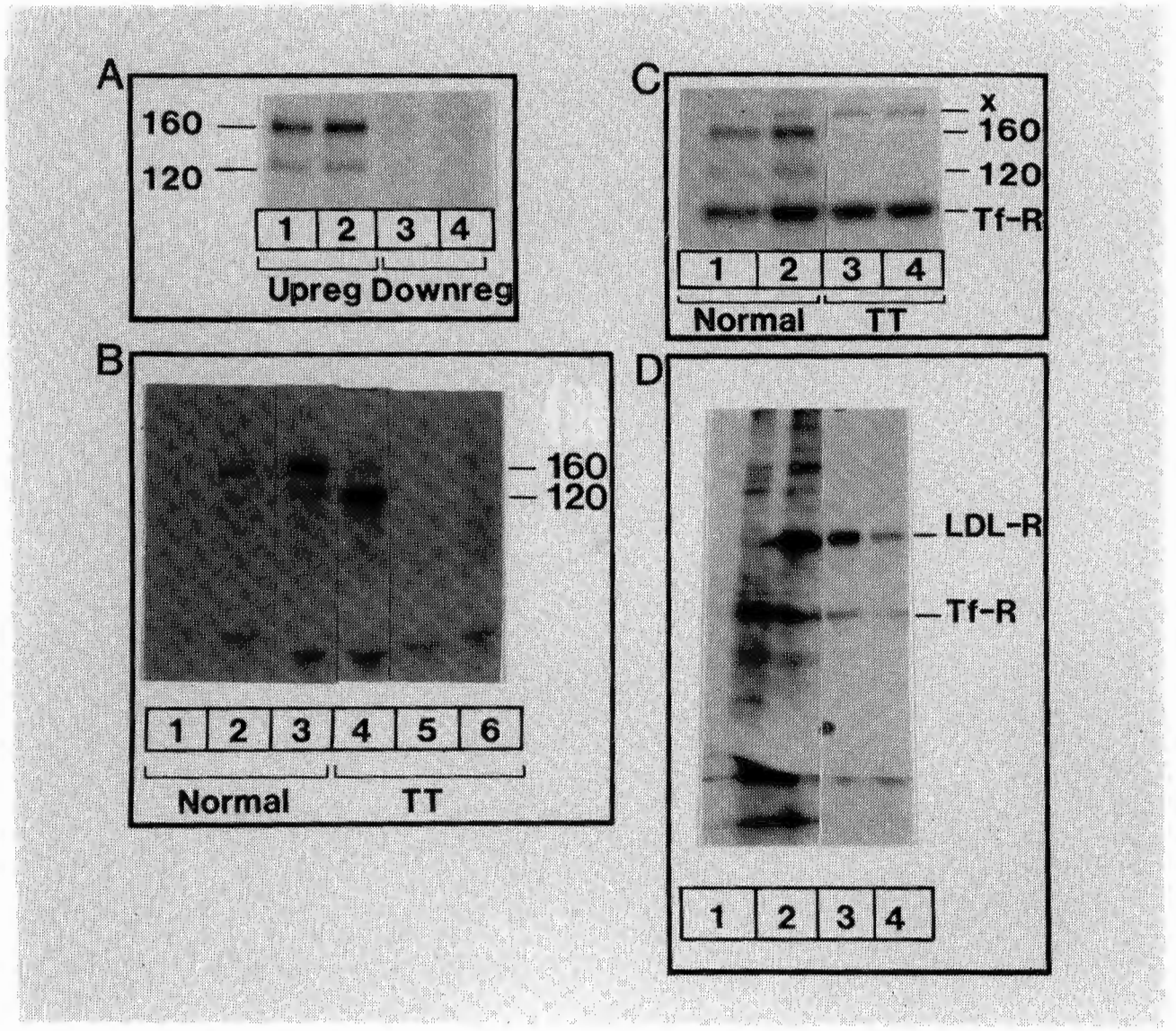

Figure 2.1 (legend overleaf)

Figure 2.1 Specificity of LDL receptor immunoprecipitation by IgG-C7

Fibroblasts from a normal subject or FH homozygote TT were incubated for 16 hours in lipoprotein deficient-medium to induce LDL receptor synthesis, or downregulated in lipoprotein-deficient medium containing 1 μg/ml 25-hydroxy-cholesterol (A, lanes 3 and 4). The cells were then pulse-labelled with [^{35}S]-methionine (A - 60 μCi/ml, B - Normal 120 μCi/ml, TT - 60 μCi/ml, C - 70 μCi/ml,D - 30 μCi/ml) in methionine-free EMEM for 30 minutes (D - 2 hours) at 37°C, followed by a 30 minute chase (D - 10 hour chase) in DMEM/LPDS containing 200 μM unlabelled methionine. The cells were then washed and solubilized, and ^{35}S-labelled receptors immunoprecipitated with preformed immune complexes containing either IgG-C7, plus (C and D) or minus B3/25 anti-transferrin receptor antibody (A; B - lanes 2 & 5), or polyclonal anti-LDL receptor IgG 698-7 (B - lanes 3 & 4) or non-immune rabbit IgG (B - lanes 1 + 6). The immunoprecipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis (5 - 20% acrylamide) and fluorography. The positions of the 160 kDa (mature), 120 kDa (precursor) LDL receptors, and of the 90 kDa transferrin receptor, are indicated. The band above the 160 kDa band is a non-specific contaminant. Various stages in the isolation of ^{35}S-labelled LDL receptors by immuno-precipitation with IgG-C7 (and transferrin receptor immunoprecipitation by B3/25) are shown in D. Samples: ^{35}S-labelled cell lysate after centrifugation to remove cell debris (lane 1), immunoprecipitate before (lane 2) and after (lane 3) the sucrose gradient wash, and a second immuno-precipitate from the supernatant of the first (lane 4).

cells were subjected to immunoprecipitation with an immune complex containing both IgG-C7 and B3/25, the monoclonal antibody to the transferrin receptor. In normal cells, three bands were precipitated, the 90 kDa transferrin receptor, the 120 kDa LDL receptor precursor and 160 kDa mature form. However, in TT cells, only the 90 kDa transferrin receptor was immunoprecipitated because the LDL receptors in these cells have a mutation in the epitope for IgG-C7 (see section 3.2.2).

Further evidence that the ^{35}S-labelled bands precipitated by IgG-C7 represented LDL receptors is shown in Figure 3.1A, p. 87. When normal fibroblasts were pulse-labelled for 1 hour with [^{35}S]-methionine, IgG-C7 immunoprecipitated two bands corresponding to the molecular weights of the precursor and mature forms of the LDL receptor. When the cells were then incubated in DMEM/LPDS containing 200 μM unlabelled methionine, the ^{35}S-label was chased from the 120 kDa band into the 160 kDa position, as conversion of precursor to mature receptors occurred. Within 15 minutes, 50% conversion to the mature form had taken place, and processing was complete within 60 minutes. This time course of processing corresponds to that originally described by Tolleshaug *et al*. (20) for the normal LDL receptor.

2.2.8 Data analysis using the computer program "Enzfitter"

Data for the concentration dependence of binding and degradation of ^{125}I-labelled ligands or antibody, or for LDL receptor half-life determinations from loss of ^{35}S-label in fluorographic bands, were analyzed by non-linear regression, using the computer program "Enzfitter" (Elsevier/BIOSOFT, Cambridge, U.K.). The advantage of non-linear regression is that it allows data to be fitted to the original non-linear form of the equation, avoiding transformation to the linear form which leads to an unequal error distribution throughout the data and therefore inaccurate estimation of parameters from the data. Using non-linear regression,

"Enzfitter" calculates the best-fit curve to one of several different equations provided. As well as producing best-fit values, the program also estimates the standard error of each of the parameters, thus showing how accurate their estimations are.

For half-life determinations, the rate of degradation (k) was calculated by fitting the data to a single exponential decay equation and the $t_{½}$ was calculated from the relationship, $t_{½} = 0.693/k$. Ligand and antibody binding and degradation data were fitted assuming binding to a single class of high-affinity sites, plus non-specific binding in the case of total binding data. In all the analyses, "simple" weighting of the data was used, assuming a constant error for all data points, together with the option of "robust" weighting to apply negligible weight to data points in extreme error, so that such points did not disproportionately affect calculations of parameters.

2.2.9 Pre-incubation of fibroblasts in the presence of ammonium chloride and LDL

In some experiments, prior to surface receptor binding assays or immunoprecipitation of ^{35}S-labelled LDL receptors, fibroblasts were pre-incubated in the presence of 10 mM ammonium chloride and 40 µg/ml LDL. In normal cells, this treatment led to a rapid decline in surface receptor activity, as measured by ^{125}I-LDL binding (see Figure 2.2A). A 70% decrease was observed within one hour of incubation with LDL and ammonium chloride (Casciola, L.A.F. *et al.*, manuscript in preparation). The loss in surface receptor activity was LDL-dependent, and therefore believed to be due to intracellular trapping of receptor through inhibition by the ammonium chloride of acid-dependent ligand dissociation in the endosome. Casciola *et al.* showed that the trapped receptors were degraded with a half-life of approximately 2 hours (see figure 2.2B). To assess the effects of this treatment on mutant LDL receptors, fibroblasts from normal and FH subjects were pre-incubated with 40 µg/ml LDL for 30

minutes at 37°C, followed by a one-hour incubation at the same temperature in the presence of both 40 μg/ml LDL and 10mM ammonium chloride. After releasing bound unlabelled LDL by incubation with 0.3% (w/v) heparin in phosphate-buffered saline for 40 minutes at 4°C, surface receptor activity was determined from ^{125}I-LDL or -IgG-C7 binding at 4°C (section 2.2.5). Alternatively, ^{35}S-labelled cells from normal and FH subjects were pre-incubated for 3- and 6-hour periods in the presence of LDL and ammonium chloride, followed by immunoprecipitation of cellular LDL receptors (section 2.2.7.3).

2.2.10 Determination of [^{35}S]-methionine incorporation into total trichloroacetic acid-precipitable protein

The amount of ^{35}S-radioactivity incorporated into total trichloroacetic acid-precipitable cell protein during a pulse-labelling period (as a measure of total protein synthesis) was determined by a filter-disc method (115) as modified by Bester (116). A small aliquot of the ^{35}S-labelled cell lysate (after 10 minutes centrifugation at 11000 g) was diluted with water (1/10) and then applied to a Whatman GF/C filter. The filter was subsequently consecutively immersed in 10% trichloroacetic acid (TCA) at 4°C for 30 minutes, 5% TCA for 15 minutes at room temperature, 5% TCA at 90°C for 20 minutes (to release [^{35}S]-methionine from amino acyl tRNA), 5% TCA at room temperature for 5 minutes, ethanol:diethyl ether (1:1) for 5 minutes, and finally, diethyl ether for 5 minutes. The ^{35}S-labelled trichloroacetic acid-precipitable protein was then measured by scintillation counting (Beckman Ready-Solv^{TM} EP scintillation cocktail) of the filter disc in a Packard TriCarb 4690 scintillation counter.

2.2.11 Pronase treatment of fibroblast monolayers

Pronase digestion of intact cells was carried out as a means of differentiating surface LDL receptors, which are accessible to a short Pronase digestion, from intracellular, Pronase-resistant receptors. Cells were pulse-labelled

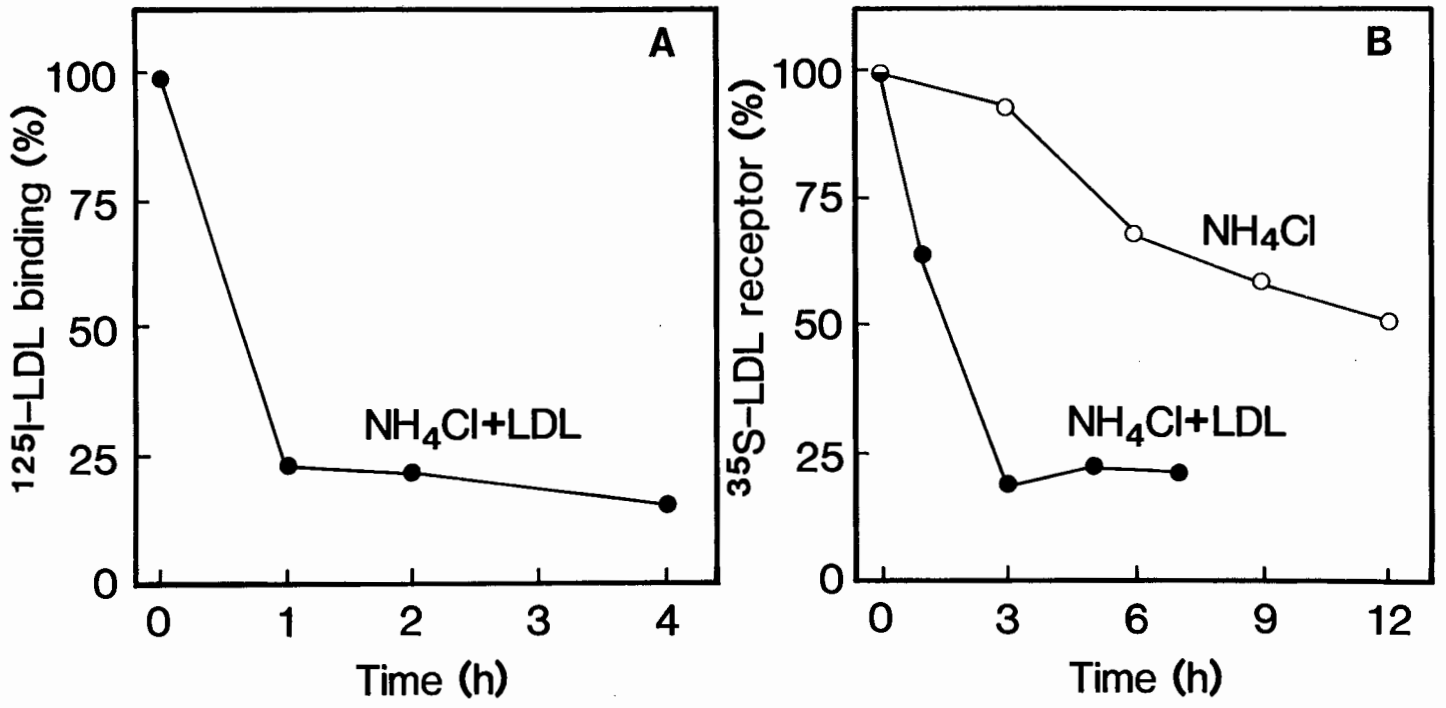

Figure 2.2 (legend overleaf)

Figure 2.2 Effect of incubation of fibroblasts with ammonium chloride and LDL in ^{125}I-LDL surface binding activity (A) and on IgG-C7-immuno-precipitable ^{35}S-labelled LDL receptors (B)

A. Upregulated normal fibroblasts were preincubated for 30 minutes at 37°C with 40 µg/ml LDL, followed by incubation with 40 µg/ml LDL plus 10 mM ammonium chloride for the indicated times at 37°C. At each time point, release of bound, unlabelled LDL by incubation at 4°C for 40 minutes with 0.3% (w/v) heparin, surface receptor activity was determined from the level of ^{125}I-LDL (7 µg/ml, 559 cpm/ng) binding at 4°C.

B. Normal fibroblasts were pulse-labelled with [^{35}S]-methionine (25 µCi/ml) for 15 hours at 37°C, and then chased for 2 hours at 37°C. The medium was then changed to one containing 10 mM NH_4Cl in the presence (●) or absence (o) of 40 µg/ml LDL and the cells were those for the indicated times at 37°C. At each time point, ^{35}S-labelled LDL receptors were immunoprecipitated, subjected to SDS-polyacrylamide gel electrophoresis and fluorography, and quantitated spectrophotometrically after NaOH extraction of the fluorographic bands (127).

with [^{35}S]-methionine, and chased for a period (indicated in figure legends) long enough to allow significant conversion of the precursor to the mature form of the LDL receptor which presumably reaches the cell-surface. The chase medium was removed and the cells were washed and incubated at 37°C for 20 minutes in 1.3 ml of buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl and 2 mM $CaCl_2$ in the presence or absence of 20 µg/ml Pronase (19). The incubation was stopped by chilling the cells to 4°C and adding 1 ml of ice-cold PBS containing 25% (v/v) LPDS, 25 mM EDTA and 2 mM phenylmethylsulphonyl fluoride (19). The cells were scraped from the dish and washed twice with PBS by centrifugation to remove any traces of Pronase, followed by lysis in buffer containing 1% (v/v) Triton X-100. LDL receptors were then immunoprecipitated from the cell lysates, washed and subjected to SDS-polyacrylamide gel electrophoresis and fluorography as described previously (section 2.2.7).

2.2.12 Analysis of LDL receptor glycosylation and the effects thereof on receptor activity

Although the function(s) of protein glycosylation has not been clearly elucidated (see section 5.3.4, p.207 for discussion), various glycosylation inhibitors and glycosidase enzymes are available as tools for the study of glycosylation and its role in protein function and/or structure. Some of these agents were used as described below to study glycosylation of the LDL receptor in normal and FH cells.

2.2.12.1 Neuraminidase and O-glycanase treatment of intact cells

Neuraminidase (sialidase) removes terminal sialic acid residues from N-linked and O-linked oligosaccharides (117). Endo-α-N-acetylgalactosaminidase (O-glycanase) liberates the disaccharide, Gal(β1→3)GalNAc from O-linked oligosaccharides bound to serine or threonine (118), provided the disaccharide is not substituted by other sugars (e.g. sialic

acid, fucose or N-acetylglucosamine). Susceptibility of the oligosaccharide residues on a protein to neuraminidase treatment can be used as an indication that the protein is exposed at the cell surface.

Cells were pulse-labelled with [^{35}S]-methionine and chased for 1 hour at 37°C. The cells were then washed and incubated for 30 minutes at 37°C in 1.3 ml of buffer (50 mM sodium acetate (pH 6), 100 mM NaCl, 2 mM $CaCl_2$) in the absence or presence of neuraminidase (Calbiochem-Behring, 0.05 units/ml). The cells were then chilled, washed and solubilized as usual. LDL receptors were immunoprecipitated from the cell lysates and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

To examine the role of sialic acid residues and O-linked oligosaccharides in LDL receptor function, upregulated fibroblasts were washed once with PBS and then incubated for 40 minutes at 37°C in 1.3 ml of the buffer described above containing (i) no additions, (ii) 0.1 units of neuraminidase (Sigma), (iii) 0.1 units of neuraminidase for 20 minutes, followed by the addition of 2.5 milliunits of O-glycanase for the last 20 minutes. The medium was removed and the cells were incubated for 1 hour at 37°C in lipoprotein-deficient medium to allow restoration of normal fibroblast morphology to cells which had "rounded-up" during incubation under acidic conditions. The cells were then chilled to 4°C and ^{125}I-LDL and ^{125}I-IgG-C7 surface binding activity determined.

2.2.12.2 Neuraminidase and O-glycanase treatment of immunoprecipitated LDL receptors

^{35}S-labelled, immunoprecipitated LDL receptors from normal and FH cells were resuspended in 50 µl of buffer containing 20 mM sodium citrate, 20 mM Tris-maleate (pH 6) and incubated for 18 hours at room temperature, in the absence or presence of 50 milliunits of neuraminidase (33). Some of the neuraminidase-treated samples were then treated with

4 milliunits of Endo-α-N-acetylgalactosaminidase (O-glycanase) for 24 hours at room temperature. After O-glycanase and/or neuraminidase treatment, 50 µl of buffer containing 20% glycerol, 125 mM Tris-HCl (pH 6.8), 4.6% SDS and 0.2 M dithiothreitol were added to each sample, followed by heating to 90°C for 3 minutes, SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography.

2.2.12.3 Tunicamycin treatment of fibroblast cultures

Tunicamycin inhibits the first step in the synthesis of the lipid-linked oligosaccharide donor for N-linked glycosylation (119). To determine the effect of N-linked glycosylation on surface LDL receptor activity, fibroblasts were incubated at 37°C for 24 hours in lipoprotein-deficient medium, in the absence or presence of 1 µg/ml tunicamycin. Under these conditions, Chatterjee et al. (67) showed that glycoprotein synthesis (as measured by [^{3}H]-glucosamine incorporation into trichloroacetic acid-precipitable protein) was inhibited by approximately 50%, while minimal inhibition of protein and nucleic acid synthesis ([^{3}H]-leucine and [^{3}H]-thymidine incorporation, respectively) was observed.

The effect of tunicamycin on the biosynthesis and processing of LDL receptors in normal and FH cells was determined by incubation for 24 hours in lipoprotein-deficient medium, followed by 3 hours in the absence or presence of 10 µg/ml tunicamycin. A dose-response study showed that, for the particular batch of tunicamycin used, this concentration was required for complete inhibition of N-linked LDL receptor glycosylation (Figure 2.3) within the 3-hour pre-incubation period. This treatment caused 60% inhibition of receptor synthesis as measured by incorporation of [^{35}S]-methionine into receptor bands. Subsequent pulse-labelling with [^{35}S]-methionine and chase incubations were also performed in the presence of 10 µg/ml tunicamycin. ^{35}S-labelled LDL receptors were then immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Figure 2.3 Concentration-dependence of inhibition of N-linked glycosylation of the LDL receptor by tunicamycin.

After induction of LDL receptor synthesis by pre-incubation in lipoprotein-deficient medium for 24 hours, normal fibroblasts were pre-incubated for 3 hours in the presence of tunicamycin at the indicated concentrations, after which the cells were pre-incubated for 30 minutes, and then pulse-labelled with [^{35}S]-methionine (30 µCi/ml) for 2 hours, at 37°C in methionine-free medium containing the same concentration of tunicamycin as in the pre-incubation. ^{35}S-labelled LDL receptors were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography.

2.2.12.4 Endoglycosidase H treatment of immunoprecipitated LDL receptors

Endo-β-N-acetylglucosaminidase H (Endoglycosidase H) removes "high mannose" N-linked oligosaccharides from asparagine residues (120). LDL receptors from normal and FH fibroblasts were labelled with [^{35}S]-methionine and immunoprecipitated with IgG-C7. The immunoprecipitates were resuspended in 50 µl of buffer containing 30 mM sodium citrate (pH 5.5), 0.75% SDS, 1 mM phenylmethylsulphonyl fluoride and 1 mM dithiothreitol, and incubated for 18 hours at room temperature in the absence or presence of 10 milliunits of Endoglycosidase H (88). After the incubation, 50 µl of buffer containing 20% glycerol, 125 mM Tris-HCl (pH 6.8), 4.6% SDS and 0.2 M dithiothreitol were added to each sample. The samples were then heated to 90°C for 3 minutes and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography.

2.2.13 Immunoblotting of solubilized LDL receptors

Immunoblotting was performed, with some modifications, according to the method of Van Driel *et al*. (40). Fibroblasts were seeded at approximately 1 x 10^6 cells per 145 mm diameter Petri dish in medium containing 10% heat-inactivated foetal calf serum (Day 0). On day 2 of cell growth, medium was changed to lipoprotein-deficient medium in the absence (upregulation) or presence (downregulation) of 2 µg/ml 25-hydroxycholesterol, and the cells were incubated in this medium for 48 hours. Upregulation medium was changed after 24 hours. The fibroblast monolayers were then washed twice at 4°C with buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl and 2 mM $CaCl_2$, and then scraped from the dishes in the same buffer, supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5 mM leupeptin. The cells were collected by centrifugation and solubilized in 200 µl of buffer containing 10 mM HEPES (pH 7.4), 200 mM NaCl, 2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM PMSF, 0.1 mM leupeptin and 1% (v/v) Triton X-100. Insoluble material was removed

by centrifugation at 4°C for 10 minutes at 11000 g. The samples were then adjusted to final concentrations of 4 M urea, 10% glycerol and 1% SDS and subjected to SDS-polyacrylamide gel electrophoresis (7% acrylamide) in the absence of reducing agents. Proteins and molecular weight standards were transferred electrophoretically to Hybond-C nitrocellulose membranes (Amersham) at 100 mA for 16 hours at 4°C in a buffer containing 20 mM Tris base, 150 mM glycine and 20% (v/v) methanol. The lane containing molecular weight standards was cut off and stained with Amido Black (0.1% (w/v) in 45% methanol, 10% acetic acid) while the rest of the membrane was incubated as follows: 1 hour at room temperature in Buffer D (50 mM Tris-HCl (pH 8), 80 mM NaCl, 2 mM $CaCl_2$, 0.05% (v/v) Tween 20) plus 3% bovine serum albumin (to block non-specific binding sites on the membrane); 1 hour in buffer D plus 5 µg/ml IgG-C7; three 10-minute washes in buffer D; 1 hour at room temperature in buffer D containing ^{125}I-labelled goat anti-mouse IgG (1700 cpm/ng, 3 x 10^6 cpm/ml), prepared as described in section 2.1.8. After three 15-minute washes in buffer D, the membrane was dried and subjected to autoradiography for 3 days at -70°C with intensifying screens.

2.2.14 Preparation of DNA for haplotype analysis

2.2.14.1 DNA isolation from blood

DNA was isolated from blood samples by a modification of the method of Kunkel (121). Blood (20 ml) from patients was collected in tubes containing heparin. Cell disruption was achieved by homogenization at 4°C in a glass vessel with a Teflon pestle in 60 ml lysis buffer (0.32 M sucrose, 1% (v/v) Triton X-100, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.6)). The nuclear pellets were sedimented by centrifugation for 20 minutes at 4°C at 2500 g (JA10 rotor, 4800 rpm) in 40 ml plastic tubes, and then resuspended in 4 ml 75 mM NaCl, 25mM EDTA (pH 8) and 1% SDS. (In some cases, samples were frozen at -70°C at this stage, and the DNA isolation continued at a later stage). After vortexing, proteinase K was added to a final concentration of 0.1 mg/ml, and samples were incubated

overnight at 50°C. The samples were then transferred to Corex tubes and 0.5 ml 5 M sodium perchlorate and 4 ml TE-(10 mM Tris-HCl, 1 mM EDTA, pH 8)-saturated phenol were added. The mixture was shaken gently until homogeneous and then the phases were separated by centrifugation at 10000 g for 10 minutes at 10°C. The aqueous phase was removed and extracted with an equal volume of chloroform:isoamylalcohol (24:1) followed by centrifugation at 10000 g for 10 minutes at 10°C. DNA was precipitated from the aqueous phase by the addition of 10 volumes of ice-cold 96% ethanol. The DNA was sedimented by brief centrifugation in a microfuge and washed twice with ice-cold 70% ethanol. Traces of ethanol were removed under vacuum and the final pellet was dissolved at 4°C in 600 µl TE. The DNA solution was re-extracted twice with an equal volume of TE-saturated phenol, and once with chloroform:isoamylalcohol (24:1). DNA was precipitated by the addition of a one-tenth volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold 96% ethanol. The DNA was washed twice with ice-cold 70% ethanol, dried under vacuum and dissolved at 4°C in approximately 0.6 ml TE.

2.2.14.2 DNA isolation from cultured cells

DNA was isolated from cultured human skin fibroblasts (ten 75 cm^2 flasks) or from Epstein-Barr virus-transformed lymphoblastoid cells (103)(100 x 10^6 to 200 x 10^6 cells) by a modification of the method described by Davis et al. (122). Cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS) by centrifugation. (In some cases, the cells were resuspended in 1 ml PBS and stored frozen at -70°C until later use). The cell pellet (after removal of PBS) was resuspended in 1% SDS, 1 mM EDTA, 1xSSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) (approximately 1 ml per 150 cm^2 flask of cells or per two 75 cm^2 flasks of cells) and proteinase K was added to a final concentration of 0.1 mg/ml. The samples were incubated at 50°C overnight or longer if the DNA was not yet in solution. The DNA solution was then extracted with an equal volume of

TE-saturated phenol, while shaking, for 1 hour. DNA was then precipitated by the addition of a one-tenth volume of 3M sodium acetate (pH 5.5) and ten volumes of ice-cold 96% ethanol. The DNA was sedimented by brief centrifugation in a microfuge and washed twice with ice-cold 70% ethanol. Traces of ethanol were removed under vacuum and the DNA was dissolved overnight or longer in TE (approximately 0.1 ml per 75 cm^2 flask) at 4°C. The DNA solution was re-extracted, re-precipitated and washed as described above for the DNA preparation from blood, and dissolved at 4°C in approximately 0.1 ml TE per 75 cm^2 flask of cells.

The concentration of DNA in samples isolated from blood or from cultured cells was determined by the optical density measured at 260 nm, and the level of protein contamination by the ratio of the absorbances at 260 nm and 280 nm. The A_{260}/A_{280} ratio for all samples was between 1.8 and 2.0. The DNA samples were subjected to electrophoresis in 1% agarose gels, after incubation in the absence or presence of EcoRI (Boehringer Mannheim) for 2 hours at 37°C, to ensure that the DNA was intact, essentially free of RNA and sufficiently free of protein and salts to be digested by restriction enzymes.

2.2.14.3 Haplotype analysis

Haplotype analysis of DNA from the various FH subjects was performed by H.E. Henderson (manuscript in preparation) or by E. Leitersdorf, J.L. Goldstein, M.S. Brown and H.H. Hobbs (manuscript in preparation). DNA samples were digested with each of the restriction enzymes known to result in polymorphic patterns (47,55-57) and analyzed by Southern blotting with the appropriate probes.

Haplotype analysis of homozygous FH subjects from the Cape region (those described in ref. 98 plus RI and TD) was carried out by Henderson (manuscript in preparation), using 10 different polymorphic restriction sites in the LDL receptor gene (Taq I (57), Stu I (49), Bst EII (55), Ava II

(50), Apa LI (5' and 3' sites) (52), Pvu II (42,51), Nco I (53), Msp I (56), Pst I (54)

The assignment of haplotypes in the large group of Afrikaner FH homozygotes from the Transvaal region (103) was done by Leitersdorf, Hobbs *et al*. (manuscript in preparation). In addition to Stu I, Ava II, Apa LI (5' and 3' sites), Pvu II, Nco I and Pst I (also used by Henderson), Leitersdorf, Hobbs et al. made use of DNA polymorphisms for the enzymes Bsm I, Sph I and Spe I (47) in the haplotype determinations.

CHAPTER 3

PRELIMINARY CHARACTERIZATION OF MUTANT LDL RECEPTORS IN SOUTH AFRICAN SUBJECTS WITH HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLAEMIA

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3.1 Introduction

The condition of homozygous familial hypercholesterolaemia (FH) was originally clinically defined by severe elevation of plasma cholesterol, xanthomata and coronary heart disease in childhood (74). It was later established that FH is caused by mutations in the gene for the low-density lipoprotein (LDL) receptor (74). On the basis of LDL-binding studies in cultured cells, three types of defects were defined by Goldstein and Brown: receptor-negative, receptor-defective and receptor internalisation-defective (75). The LDL receptor was later purified to homogeneity (17) and the monoclonal anti-LDL receptor antibody, IgG-C7 was produced (18). Using this antibody as a probe, at least four classes of LDL receptor mutations were distinguished (19,20), including defects in synthesis, intracellular transport, LDL binding and internalisation of the LDL receptor (3). More recently, the isolation of complementary DNA (cDNA) clones corresponding to the LDL receptor messenger RNA (21) and gene (22) has facilitated genetic haplotype assignments, by analysis of polymorphic restriction sites in LDL receptor genes (47), and elucidation of the molecular defects underlying the different classes of mutations (10). These studies have revealed the genetic heterogeneity of homozygous FH.

FH is particularly prevalent in the South African Afrikaner population, presumably because of founder-gene effects (section 1.7.4). Previous studies of LDL receptor activity in 23 clinically homozygous Afrikaner patients (98,103) and two FH homozygotes from other South African ethnic groups (98) indicated a predominance of a receptor-defective type of mutation, while one pair of siblings were apparently receptor-negative (103). The wide range of receptor activity in the Afrikaner group suggested the presence of more than one type of mutant allele. Haplotype analysis of 2 polymorphic restriction sites (59,104) suggested that FH homozygotes in all except one Afrikaner family shared a

common haplotype, thus supporting the idea of a founder gene mutation. Preliminary LDL receptor immunoprecipitation studies in 3 Afrikaner subjects suggested defects in intracellular receptor transport (103).

In the study reported in this chapter, the basis for the heterogeneity of receptor activity in South African FH homozygotes was investigated by studying receptor synthesis and processing, using [^{35}S]-methionine pulse-chase studies and immunoprecipitation by the monoclonal anti-LDL receptor antibody, IgG-C7. The results are interpreted on the basis of haplotype assignments through analysis of 10 polymorphic restriction sites in the LDL receptor genes of the FH subjects.

3.2 Results

The results of [^{35}S]-methionine pulse-chase experiments (section 2.2.7), assays of surface LDL receptor activity (section 2.2.5) and haplotype analysis (section 2.2.14.3) are summarized in Table 3.1, p. 86 .

3.2.1 Phenotypic expression of mutant LDL receptors in the Afrikaner population

Post-translational processing of the LDL receptor was studied in 10 unrelated Afrikaner kindreds, including 11 homozygous FH subjects. As shown in Figure 3.1A, the precursor form of the normal LDL receptor (apparent molecular weight 120 kDa) was rapidly and quantitatively processed ($t_{½}$ approximately 15 minutes) to the 160 kDa mature form by the addition of carbohydrate residues (see section 1.3). The newly-synthesized precursor in cells from all Afrikaner FH subjects studied had an apparently normal molecular weight (120 kDa), indicating that the mutation(s) caused no large deletions or insertions in the protein (see Fig. 3.1, B to F). However, the conversion of precursor into mature 160 kDa receptor, which accompanies transport from the endoplasmic reticulum to the Golgi complex and maturation of core sugars (32) was retarded in

TABLE 3.1. LDL RECEPTOR GENE HAPLOTYPES, RECEPTOR ACTIVITY AND POST-TRANSLATIONAL PROCESSING IN SOUTH AFRICAN HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLAEMIA

Ethnic Group	Subject	Haplotype[a]	LDL Receptor Activity (% Normal)			$t_{\frac{1}{2}}$ LDL receptor processing[c] (h)	LDL receptor phenotype
			37°C	4°C			
			^{125}I-LDL degradation[b]	^{125}I-LDL Bound	^{125}I-IgG-C7 Bound		
-	Normal	N.D.	100%	100%	100%	<0.5*	Normal transport/ processing; equimolar LDL and IgG-C7 binding
Afrikaner	JG	I/I	41%	20%+	72%+	6*	Impaired transport/ processing; defective LDL binding
	ES	I/I	28%	22%+	46%+	>2	Impaired transport/ processing; defective LDL binding
	¶ FH1a	3/3	25%	12%	39% (Ref. 103)	5.5*	Impaired transport/ processing; defective LDL binding
	FH7a	3/3	19%	N.D.	N.D.	5.5	Impaired transport/ processing
	FH2a	3/16	18%	N.D.	N.D.	>2	Impaired transport/ processing
	FH2b	3/16	15%	N.D.	N.D.	>2	Impaired transport/ processing
	GM	I/I	11%	3%+	27%+	>2	Impaired transport/ processing; defective LDL binding
	FH12b	3/3	11%	N.D.	N.D.	5	Impaired transport/ processing
	FH12a	3/3	10%	N.D.	N.D.	3.5*	Impaired transport/ processing
	FH6a	3/15	6%	7%	19%(ref.103)	5*	Impaired transport/ processing; defective LDL binding
	¶ FH3b	15/15	2%	n.d.	3%	1.5*	Impaired transport/ processing; receptor degradation
"Coloured"	CF	I/III	8%	10%+	28%+	4.5	Impaired transport/ processing; defective LDL binding
	RI	V/V	<1%‡	18%	42%	0.5‡	Normal transport/ processing; abnormal size; defective LDL binding
	TD	V/V	<1%‡	7%	15%	0.5‡	Normal transport/ processing; abnormal size; defective LDL binding
Xhosa	TT	IV/IV	30% (76)	30%	<1% (ref.87)	2	Impaired transport/ processing; LDL receptor not recognized by IgG-C7

N.D. = not determined

n.d. = not detectable

a - haplotypes were assigned by H.E. Henderson or E. Leitersdorf, H.H. Hobbs et al. as described in section 2.2.14.3.

b - ^{125}I-LDL degradation was determined from the noniodide trichloroacetic acid-soluble radioactivity in the medium after 4 hours at 37°C (98,103)

c - $t_{\frac{1}{2}}$ for LDL receptor processing is the chase time required to reach an equal distribution of ^{35}S-label between the precursor and mature LDL receptor bands, assuming no label in the mature receptor band at zero time.

* see Figure 3.1

+ Van der Westhuyzen and Coetzee, unpublished results

‡ Van der Westhuyzen, D.R., Stein, M., Henderson, H.E., Coetzee, G.A., manuscript in preparation.

¶ Homozygous siblings FH3a and FH1b described later in Chapters 4 and 5, respectively.

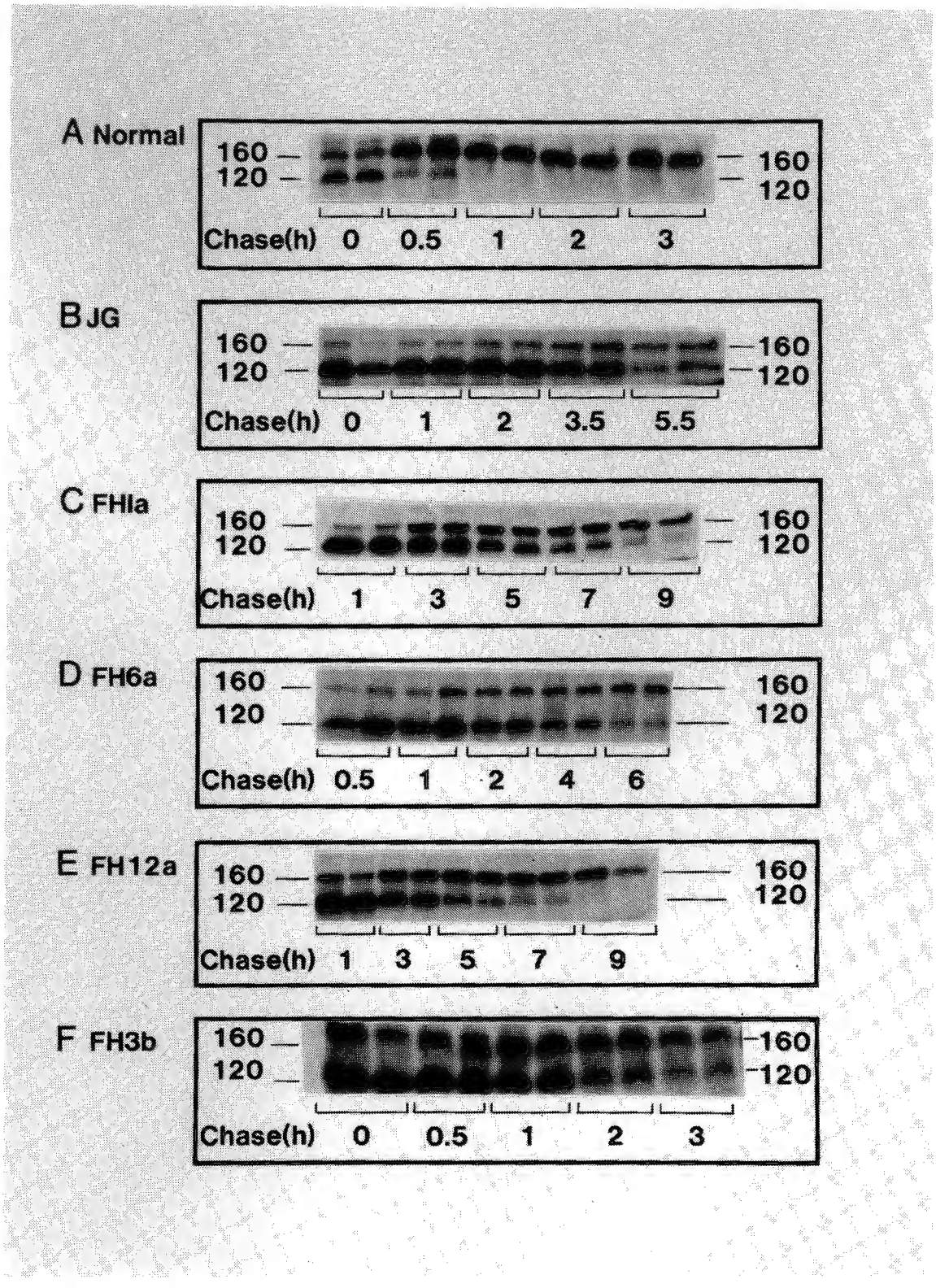

Figure 3.1 (legend overleaf)

Figure 3.1 Biosynthesis and processing of LDL receptors from normal cells and selected homozygous Afrikaner FH subjects

Fibroblasts from normal (A) and homozygous FH subjects were pre-incubated for 16 hours in lipoprotein-deficient medium to induce LDL receptor synthesis. The cells were then pulse-labelled with [^{35}S] methionine (50 µCi/ml) at 37°C,for 1 hour (Normal and FH3b), 2 hours (FH1a, 6a and 12a) or 2.3 hours (JG). The medium was then changed to DMEM/LPDS containing 200 µM unlabelled methionine and the cells were incubated at 37°C for various chase times. The cells were then washed and solubilized and ^{35}S-labelled LDL receptors immunoprecipitated with a preformed immune complex containing IgG-C7. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography. The positions of the 160 kDa (mature) and 120kDa (precursor) LDL receptors are indicated. The band above the 160 kDa band in some cases is a non-specific contaminant.

all cases, but to different degrees. Although all these mutations can thus be classified as Class 2 defects (3), the severity of the delay in transport and maturation of the receptor varied considerably, ranging from a half-time for processing (defined in footnotes to Table 3.1) of 1.5 hours to 6 hours, compared to the normal time of less than 30 minutes (see Table 3.1).

All the Afrikaner FH homozygotes analyzed by Henderson were homozygous for a haplotype designated "I" (see Table 3.1). By analysis of additional polymorphic sites, Leitersdorf, Hobbs et al. were able to sub-divide haplotype I into haplotypes "3" and "15", and detected an additional haplotype in kindred 2, designated "16". The heterogeneity in post-translational processing rates can thus be partly explained by the association of at least two different alleles (haplotypes 3 and 15), and possibly another (haplotype 16), with the slow processing phenotype. However, variations in receptor transport rates and levels of activity were observed even between individuals homozygous for the same haplotype, suggesting that genetic factors other than the LDL receptor locus may influence phenotypic expression of the mutations. Selected examples of post-translational processing in FH subjects, defined previously as high receptor-defective (FH1a, JG), low receptor-defective (FH6a, FH12a), and receptor-negative (FH3b), are shown in Figure 3.1 (B to F).

Cells from FH3b synthesized significant amounts of LDL receptor precursor and mature forms and are thus not truly receptor-negative. The virtual lack of surface receptor activity in FH3b may be related to the loss of more than half the original incorporation of ^{35}S-label from the receptor bands during the 3 hour chase period, indicating accelerated receptor degradation. (This aspect is discussed in detail in Chapter 4).

Although both haplotypes 3 and 15 are associated with slow processing phenotypes, haplotype 3 appears to represent the mutation giving rise to the most severe impairment of transport, but significant levels of surface receptor expression. In addition, the receptor protein associated with haplotype 3 may be defective in LDL binding, as shown by the higher proportion of surface antibody binding compared to LDL binding in FH1a. On the other hand, haplotype 15 is associated with a mutant receptor molecule processed at a rate only slightly slower than normal, but rapidly degraded at some stage, resulting in virtually no surface receptor activity (as seen for FH3b). It is interesting to note that FH6a, a compound heterozygote of haplotypes 3 and 15, expressed surface receptor activity intermediate between homozygotes of the two haplotypes (Table 3.1), and showed a higher proportion of antibody versus LDL binding, and the severely retarded processing phenotype associated with haplotype 3.

3.2.2 Immunoprecipitation of LDL receptors from homozygous FH Xhosa patient, TT

The LDL receptors in cells from a black (Xhosa) South African FH homozygote, TT, were able to bind LDL with high affinity, but not IgG-C7 (87, Fig. 2.1B, p. 86). Therefore, a polyclonal antibody, 698-7, to the bovine LDL receptor, was used to immunoprecipitate the LDL receptor produced by TT and normal cells. In normal fibroblasts (Fig. 3.2), essentially the same result was obtained as for normal cells using monoclonal IgG-C7 (Fig. 3.1A). However, the rate of processing in TT cells of the 120 kDa precursor to the mature form was markedly delayed (Fig. 3.2). Even after a two-hour chase, approximately half of the receptor protein was still in the precursor form.

Biosynthesis and processing of LDL receptors in cells from the heterozygous mother of TT were also analyzed using a sequential immunoprecipitation technique (Fig. 3.3). First, IgG-C7 was used to selectively precipitate the

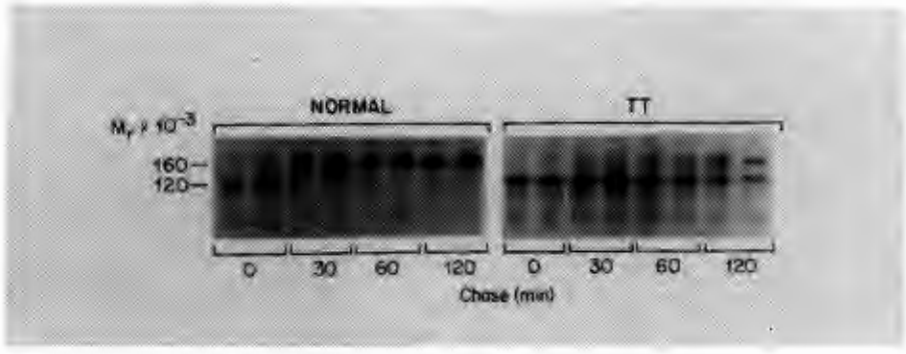

Figure 3.2 Biosynthesis and processing of LDL receptors in fibroblasts from TT and a normal subject

After 16 hours incubation in lipoprotein-deficient medium, cells were pulse-labelled for 30 minutes with [^{35}S]-methionine (normal cells - 120 µCi/ml, cells from TT - 60 µCi/ml) in methionine-free EMEM, followed by a chase for the indicated times in medium containing 200 µM unlabelled methionine. Cells were washed, solubilized and subjected to immunoprecipitation with polyclonal anti-LDL receptor antibody 698-7, after which SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) of immunoprecipitates and fluorography were performed. The positions of the mature (160 kDa) and precursor (120 kDa) LDL receptor bands are indicated.

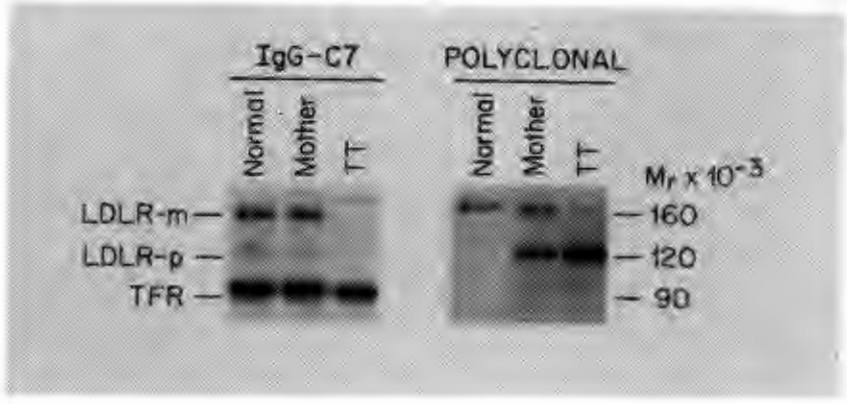

Figure 3.3 Biosynthesis and processing of LDL receptors in fibroblasts from FH homozygote, TT, his heterozygous mother and a normal subject

After 16 hours incubation in lipoprotein-deficient medium, cells were pulse-labelled for 30 minutes with [^{35}S]-methionine (70 μCi/ml) in methionine-free EMEM, followed by a chase for 30 minutes in medium containing 200 μM unlabelled methionine. The cells were washed, solubilized and subjected to immunoprecipitation with an immune complex containing monoclonal anti-LDL receptor antibody, IgG-C7, and an anti-transferrin receptor monoclonal antibody. After collection of the immunoprecipitates, the supernatants were subjected to a second immunoprecipitation with an immune complex containing the polyclonal anti-LDL receptor antibody, 698-7. Precipitates from the first and second immunoprecipitations were analyzed by SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography. The positions of the mature (160 kDa) and precursor (120 kDa) LDL receptors, and the 90 kDa transferrin receptor are indicated.

receptor produced from the normal allele, and then, polyclonal antibody 698-7 was used to precipitate the receptors produced by the mutant allele. As a control, the transferrin receptor was simultaneously immunoprecipitated by including B3/25, an antibody to the transferrin receptor, in the IgG-C7 containing immune complex. In cells from a normal subject and from the mother of TT, the first immunoprecipitation yielded 120 kDa, 160 kDa and 90 kDa bands, corresponding to the precursor and mature LDL, and transferrin receptors, respectively. Only the 90 kDa transferrin receptor was immunoprecipitated from TT cells. When the polyclonal anti-LDL receptor antibody, 698-7, was used to subsequently immunoprecipitate receptor protein not recognized by IgG-C7, a large amount of the 120 kDa precursor LDL receptor protein was precipitated from TT and his mother's cells, but not from normal cells. Some mature 160 kDa receptor was also seen in normal and TT's mother's cells, due to incomplete prior immunoprecipitation with IgG-C7. Only a very small amount of mature receptor was seen in TT cells, consistent with the slow processing of this mutant protein. This experiment showed that the presence of the abnormal protein produced by the mutant allele did not interfere with processing of the normal gene product in the heterozygous cells.

Haplotype analysis by Henderson and by Leitersdorf, Hobbs et al. (see section 2.2.14.3) of the LDL receptor gene in the Xhosa patient, TT, showed homozygosity for a single, unique haplotype. Since the first cysteine-rich binding repeat in the LDL receptor, coded for by exon 2, contains the epitope for monoclonal antibody IgG-C7 (29), and since the receptor from TT cells did not recognize IgG-C7, exon 2 was predicted as the site of the mutation (87). Amplification of this exon by the polymerase chain reaction technique and subsequent sequence analysis revealed a deletion of 6 base pairs, resulting in removal of two amino acids, aspartic acid and glycine, from the first cysteine-rich repeat (87). This mutation is thus also responsible for the slow

maturation of the LDL receptor precursor observed in these cells (Fig. 3.2). The small deletion in the receptor shortens the distance between two cysteine residues, and may thus interfere with correct disulphide bonding and protein folding (87), therefore causing slow transport of the protein from the endoplasmic reticulum to the Golgi apparatus (78-80). Abnormal conformation may also be responsible for loss of the epitope for IgG-C7 (87).

3.2.3 Mutant LDL receptors in FH homozygotes from the so-called "Coloured" population

Phenotypic expression of LDL receptors from three clinically-defined FH homozygotes from the "Coloured" group in South Africa was studied. Patient CF was previously shown to express approximately 10% of the normal level of LDL receptor activity at 37°C (98) and 4°C (unpublished results). Processing of the precursor receptor (120 kDa) to the mature form (160 kDa) in cells from subject CF was much slower than normal (Fig. 3.4). The half-time for conversion to the mature receptor was approximately 4 hours, compared to less than half an hour in normal cells (Fig. 3.1A). The mature surface receptors in CF appeared to be defective in LDL binding, as indicated by the higher proportion of IgG-C7 versus LDL binding. Haplotype analysis by H.E. Henderson (manuscript in preparation) showed that CF was a compound heterozygote for haplotype I (also found in Afrikaner FH subjects) and another haplotype, "III". Although slow processing and defective binding appear to be associated with haplotype I, it is not clear what contribution the gene product from the allele represented by haplotype III makes to the LDL receptor phenotype in CF.

Subjects RI and TD appeared to be receptor-negative when LDL receptor activity was assessed by a 4-hour incubation at 37°C with ^{125}I-LDL (Van der Westhuyzen D.R., Stein M., Henderson H.E., Coetzee G.A., manuscript in preparation), but showed approximately 20% of normal LDL receptor levels when assayed at 4°C (Table 3.1). After a 2-hour pulse-

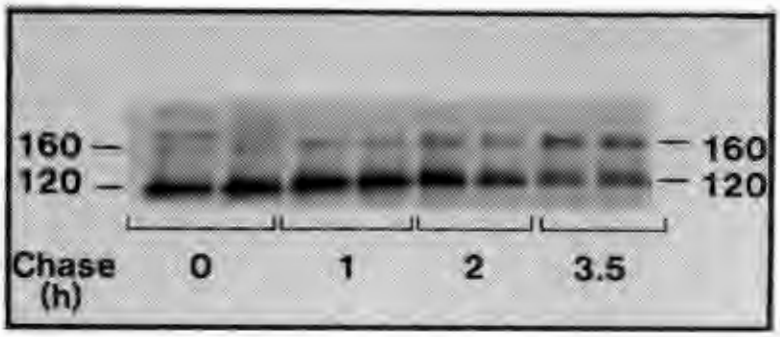

Figure 3.4 Biosynthesis and processing of LDL receptors in fibroblasts from subject, CF

Fibroblasts from FH homozygous patient, CF, were incubated for 16 hours in lipoprotein-deficient medium and then pulse-labelled with [^{35}S]-methionine (50 μCi/ml) for 2.3 hours at 37°C, followed by various chase periods in medium containing 200 μM unlabelled methionine. ^{35}S-labelled LDL receptors were then immunoprecipitated and subjected to SDS-PAGE (5-20% acrylamide) and fluorography. The positions of the 160 kDa (mature) and 120 kDa (precursor) LDL receptors are indicated. The band above the 160 kDa receptor band at zero time is a non-specific contaminant.

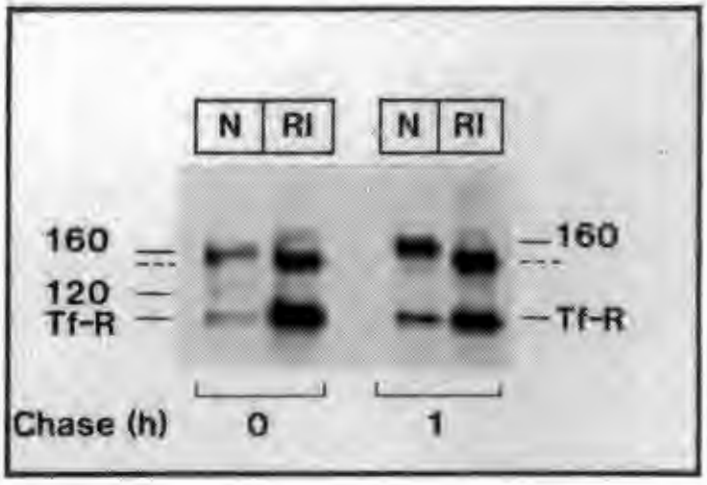

Figure 3.5 Biosynthesis and processing of LDL receptors in normal fibroblasts and in cells from homozygous FH subject, RI

Fibroblasts from a normal subject and FH homozygote, RI, were pre-incubated for 16 hours in lipoprotein-deficient medium and then pulse-labelled in methionine-free EMEM with [^{35}S]-methionine (80 μCi/ml) for 2 hours at 37°C, followed by a chase period of 1 hour in DMEM/LPDS containing 200 μM unlabelled methionine. ^{35}S-labelled LDL receptors and transferrin receptors were immunoprecipitated and subjected to SDS-PAGE (5-20% acrylamide) and fluorography. The positions of the normal LDL receptor precursor (120 kDa) and mature (160 kDa) forms and transferrin (90 kDa) receptors are shown by solid lines. The position of the RI mature (145 kDa) LDL receptor is indicated by the dotted line.

labelling period, LDL- and transferrin receptors were immunoprecipitated from normal cells and RI cells. In the normal cells, mainly the 90 kDa transferrin receptor and 160 kDa mature LDL receptor bands were seen, as well as a small amount of 120 kDa LDL receptor precursor (Fig. 3.5). In contrast, in RI, although a normal 90 kDa transferrin receptor band was observed, the mature LDL receptor band had a significantly lower apparent molecular weight than normal (145 kDa). The correspondingly smaller precursor band (105 kDa) was presumably of a similar molecular weight to the transferrin receptor and thus obscured. After the cells were chased for one hour at 37°C, only 160 kDa LDL receptor was seen in normal cells, and a smaller mature receptor in RI. The transferrin receptor band had the same molecular weight in both normal and RI cells. Similar results were obtained for TD cells (results not shown). The results suggest that the mutations in RI and TD cause similar deletions in the LDL receptor protein, but that post-translational processing of the mutant receptors is not retarded. This has been confirmed by Southern blotting (123) and by further pulse-chase experiments (Van der Westhuyzen, D.R., Stein, M., Henderson, H.E., Coetzee, G.A., manuscript in preparation), which also showed that the abnormally-small mature receptor was rapidly degraded. In addition, the mature LDL receptors in RI and TD were defective in LDL binding (see Table 3.1). TD and RI, were both true homozygotes for haplotype V, which was shown, by more detailed mapping by Southern blotting (123), to represent a 2.5 kb deletion in the LDL receptor gene. This deletion appears to result in the removal of exons 7 and 8, which code for the first two cysteine-rich repeats in the EGF precursor homologous domain, thus giving rise to the smaller protein observed in ^{35}S-methionine pulse-chase experiments.

3.3 Discussion

The biosynthesis and processing of mutant LDL receptors was studied in cultured fibroblasts from 15 clinically homozygous South African FH subjects, including 11 from the Afrikaner population. Previous cellular studies of these patients showed heterogeneity of LDL receptor activities, ranging from essentially receptor-negative up to 40% of normal receptor activity (98,103).

Subjects which have been designated FH homozygotes on the basis of clinical criteria and/or LDL receptor activity levels, may be true genetic homozygotes (i.e. possess two identical mutant alleles) or may be genetic compounds, having two different defective alleles. Compound heterozygotes can be detected by immunoprecipitation of ^{35}S-labelled receptors when the proteins produced by the defective alleles have different molecular weights (19,20). However, compound heterozygosity cannot be detected when the two mutant proteins have the same molecular weight, or, if one allele is a "null" allele, producing no detectable protein (38). In such cases, the assignment of haplotypes, based on combinations of the presence or absence of a series of polymorphic restriction enzyme sites in the LDL receptor gene, can differentiate between true homozygotes and genetically compound heterozygotes. The conclusion that mutant genes with different haplotypes represent different mutations is based on previous studies showing that, in general, RFLP haplotypes of chromosomes antedate mutations in the genes (124-126).

On the basis of 10 polymorphic restriction sites in the LDL receptor gene, H.E. Henderson (manuscript in preparation) detected 4 different haplotypes (I,III,IV,V) in the homozygous FH subjects examined. All the Afrikaner FH subjects (JG, ES, GM) were homozygous for the haplotype designated "I". The so-called "Coloured" patient, CF, was a compound heterozygote for haplotype I and another haplotype, "III". The Xhosa patient, TT, was homozygous

for a different haplotype, designated "IV". The other "Coloured" patients, TD and RI, were both true homozygotes for haplotype V. By analysis of the additional polymorphic sites for BsmI, SphI and SpeI, E. Leitersdorf, H.H. Hobbs et al. were able to further differentiate haplotype I (Henderson) into two haplotypes, designated "3" and "15". All the Afrikaner FH subjects analyzed (those reported in Table 3.1 and FH 5a, 8a, 9a, 10a, 11a, 14a (103)), except those from kindred 2, were homozygous for either haplotype 3 or 15, or were compound heterozygotes of these two haplotypes . The FH "homozygotes" in kindred 2 were compounds of haplotype 3 and an additional haplotype, "16". This haplotype has also been detected in a large Western Cape family of Afrikaner descent (Brink P.A., manuscript in preparation) and in an English-speaking subject and another Afrikaner from the Cape region (Henderson, haplotype "II", manuscript in preparation). On the basis of their phenotypes (surface LDL receptor activity and rate of post-translational processing), it can be predicted that JG and ES are probably homozygous for haplotype 3 and that GM may either also be homozygous for this haplotype or may be a compound heterozygote of haplotypes 3 and 15.

The only FH subjects which showed normal rates of receptor processing were RI and TD. They were also the only examples where abnormal receptor size was observed, as a result of deletion of exons 7 and 8, encoding cysteine-rich repeats A and B in the EGF precursor homology domain of the receptor (Van der Westhuyzen et al., manuscript in preparation). Defective LDL binding by the receptors in RI and TD is consistent with the finding of Esser et al. (28) that repeat A plays a permissive role in LDL binding. The rapid degradation of these mutant receptors is discussed further in section 4.3. A similar mutation has been detected in two Dutch FH subjects, FH 359 and 454 (11). In these subjects, a 4 kb deletion, also including exons 7 and 8, similarly gives rise to a receptor protein lacking the first two growth factor-like repeats.

All the other mutant alleles produced slowly-processed receptors of normal apparent molecular weight, probably resulting from small deletions, insertions or point mutations. This would be consistent with the findings for other mutant LDL receptors. The deletion of 4 amino acids from the third cysteine-rich binding repeat in the receptor from the WHHL rabbit (23) or the deletion of a single glycine residue from repeat 5 in FH563 (10,23), both resulted in delayed receptor transport, presumably as a result of abnormal folding (78-80) because of altered disulphide bonding (23). In contrast, loss of the entire sixth binding repeat in FH626 (89) or duplication of binding repeats 1 to 7, and growth-factor-like repeats A and B, in FH295 (46) produced receptor proteins of abnormal size, which were transported at a normal rate, and thus apparently were able to achieve a normal conformation. These results support the idea that disulphide bonds form within each repeat (90), so that small deletions or insertions interfere with the correct bonding pattern, whereas deletions or insertions of whole repeats have no effect. The mutation in subject TT (87) is a case in point, since deletion of two amino acids from the first repeat impaired transport of the TT receptor, whereas Van Driel et al. (29) have shown that deletion of the entire first repeat by site-directed mutagenesis produced a receptor that was transported and processed at a normal rate.

The fact that the LDL receptors in all the Afrikaner subjects studied showed slow post-translational processing supports the concept of a founder gene mutation. All the Afrikaner FH homozygotes analyzed by Henderson were homozygous for haplotype I. The "Coloured" subject, CF, was a genetic compound of a unique haplotype (III) and haplotype I, suggesting some Afrikaner ancestry. Leitersdorf, Hobbs et al. were able to show that haplotype I included two different haplotypes, 3 and 15, and that subjects from 12 different, unrelated Afrikaner families

were either homozygous for haplotype 3 or 15, or were genetic compounds of these two haplotypes. The high occurrence of these haplotypes in unrelated subjects strongly suggests that they may represent founder gene mutations in the Afrikaner population. Haplotype 16 has been found in 3 unrelated families and may represent a minor founder mutation. Founder gene mutations have also been identified in the French-Canadian (84), Lebanese (88) and possibly, the Finnish (91,92) populations. These founder mutations are readily detected by Southern blotting, allowing rapid and accurate diagnosis of FH in these populations. The high incidence of specific haplotypes associated with FH in the Afrikaner population suggests that similar diagnostic Southern blotting may be developed to detect FH mutations within this group.

FH1a and 3b have been shown to be respectively homozygous for 2 distinct LDL receptor gene haplotypes, 3 and 15. Each of these subjects has a sibling homozygous for the same haplotype. The LDL receptor mutations represented by haplotype 3 (FH1a and 1b) and haplotype 15 (FH3a and 3b) produce contrasting phenotypes in terms of levels of surface receptor activity and rates of receptor transport and processing (see Table 3.1). These sibling pairs thus afford a unique opportunity to study two contrasting LDL receptor mutations, probably representing founder mutations in the Afrikaner population, in true genetic homozygotes. Therefore, the phenotypic expression of the mutant LDL receptors in FH3a and 3b, and in FH1a and 1b, was characterized in detail, and the results are presented and discussed in Chapters 4 and 5, respectively.

CHAPTER 4

PHENOTYPIC EXPRESSION OF THE LDL "RECEPTOR-NEGATIVE" MUTATION IN AFRIKANER FH KINDRED 3

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4.1 Introduction

Previous studies of LDL receptor activity in 23 homozygous FH subjects from 17 Afrikaner kindreds (98,103) indicated receptor-defective mutations in all but two homozygotes, the sibling pair, FH3a and 3b, which were apparently receptor-negative on the basis of ^{125}I-LDL binding and metabolism. Consistent with this finding, the clinical features of the disease in FH3a and 3b were more severe than in the receptor-defective subjects. None of the receptor-defective homozygotes under the age of 16 showed signs of coronary heart disease (CHD), whereas even the younger (aged 10 at the time of study) of the receptor-negative siblings suffered from CHD (103).

On the basis of 10 polymorphic restriction sites in the LDL receptor gene, FH3a and 3b have been shown to be true genetic homozygotes for haplotype 15 (section 3.2.1). The phenotypic expression of the mutation represented by haplotype 15 could thus be characterized fully in cells from FH3a and 3b, without interference from another normal or mutant allele.

Preliminary biosynthetic studies (reported in section 3.2.1) showed significant synthesis and processing, albeit slower than normal, of ^{35}S-labelled, immunoprecipitable receptors in FH3b. Similar preliminary results had previously been obtained for FH3a (103). The mutation in kindred 3 is thus not a "null" allele, although it produces a receptor-negative phenotype. It was therefore of interest to elucidate the mechanism(s) responsible for the apparent lack of receptor expression, despite significant receptor synthesis. This was done mainly on the basis of [^{35}S]-methionine pulse-chase experiments, carried out to determine rates of synthesis, post-translational processing and degradation of immunoprecipitable LDL receptors.

4.2 Results

4.2.1 LDL receptor activity

FH3a and 3b were designated "receptor-negative" on the basis of ^{125}I-LDL binding and metabolism at 37°C (103). However, as discussed previously, biosynthetic studies using immunoprecipitation by the monoclonal anti-LDL receptor antibody, IgG-C7, indicated significant synthesis and processing of LDL receptor protein to the mature form, which in normal cells is expressed on the cell surface . The possibility thus existed that FH3a and 3b cells expressed abnormal surface receptor protein, recognized by IgG-C7, but not LDL. Previous studies of ^{125}I-LDL and ^{125}I-IgG-C7 surface binding at 4°C (Table 3.1, ref. 103, unpublished results) suggested the possibility of a low level (less than 10% of normal) of surface receptor expression in these cells. Therefore, a number of assays at 4°C for surface binding of both ^{125}I-labelled LDL and ^{125}I-labelled IgG-C7 to FH3b cells were performed, to ascertain whether or not surface expression of LDL receptor protein could be detected. Binding and metabolism of ^{125}I-LDL and ^{125}I-IgG-C7 at 37°C were also measured.

As shown in Table 4.1 (a), in 3 separate experiments, surface binding at 4°C of ^{125}I-LDL or -IgG-C7 to FH 3b cells ranged from below the detection limits of the assay to a small significant percentage of normal receptor activity. Similar results were previously obtained for FH3a (see Table 4.1). In another experiment, shown in Table 4.1 (b), binding levels at 4°C for ^{125}I-LDL, ^{125}I-IgG-C7 and another lipoprotein ligand, ^{125}I-β-VLDL, to FH3b cells were determined. β-VLDL binds to the LDL receptor via apolipoprotein E, rather than apolipoprotein B-100, as for LDL (discussed in section 1.2.1.1), and has been shown to bind to some mutant LDL receptors which are unable to bind LDL (31,89). High-affinity binding values for LDL, IgG-C7 and β-VLDL to FH3b cells were similar or higher than those observed in downregulated normal cells, which presumably do express some residual receptor activity. Thus, the results

in Table 4.1 indicate that FH3a and 3b may express a very low level of surface LDL receptors which is sometimes lower than the limits of detection of the assay.

High-affinity cell-association (bound and intracellular) and metabolism of ^{125}I-LDL and ^{125}I-IgG-C7 at 37°C in FH3b cells (shown in Table 4.2) were below detectable limits, except the value of 10% of normal for degradation of ^{125}I-IgG-C7. The high value (<14%) for limits of detection of cell-associated ^{125}I-IgG-C7 was due to considerable variation in the non-specific background. The apparently significant level of ^{125}I-IgG-C7 degradation may reflect a sensitive measurement of a low level of receptor activity, since degraded products accumulate linearly over the 4-hour incubation period. Since no detectable ^{125}I-LDL degradation was observed for FH3b, the results may indicate the presence of abnormal receptor protein. However, no indication for disproportionately higher IgG-C7 versus LDL binding at 4°C was observed.

Brown and Goldstein originally defined "receptor-negative" cells as those which bound and metabolized less than 2% of normal levels of ^{125}I-LDL, as this represented the detection limits of the assay (74). However, Funahashi et al. (128) have recently shown that cells which synthesized no immunoprecipitable LDL receptors bound 1% to 9% of normal ^{125}I-LDL levels, and up to 4% of normal ^{125}I-IgG-C7 levels. They propose that these percentages represent background binding and that patients whose fibroblasts bound less than 10% of normal levels be considered "receptor-negative". This higher minimum detection limit may reflect greater non-specific background variability, but this aspect was not addressed.

By the criteria of Funahashi et al. (128), the LDL receptor status in FH3a and 3b can be designated "receptor-negative", although, by the original criteria of Brown and Goldstein (74), a low but significant level of surface receptor

Table 4.1 Surface binding of ^{125}I-labelled-LDL and ^{125}I-labelled IgG-C7 (a) (and ^{125}I-labelled β-VLDL (b)) to fibroblasts from normal subjects and FH3b (overleaf).

After 48 hours in lipoprotein-deficient medium to induce maximal LDL receptor expression (or medium containing 2 μg/ml 25-hydroxycholesterol to downregulate receptor expression), fibroblasts from normal subjects and FH3b received 1.5 ml of ice-cold medium containing 7 μg/ml (14 nM) ^{125}I-LDL or 1 μg/ml (6.7 nM) ^{125}I-IgG-C7. In the experiment shown in (b), some cells received medium containing 1 μg/ml ^{125}I-β-VLDL. After 2 hours at 4°C, the amount of ^{125}I-LDL or ^{125}I-IgG-C7 (or ^{125}I-β-VLDL) bound to the cells was determined. The data are means of duplicate or triplicate (indicated in parentheses) incubations and represent high-affinity binding, calculated by subtracting the non-specific values of ^{125}I-ligand or -antibody binding in the presence of excess unlabelled LDL (300 μg/ml, 600 nM), IgG-C7 (50 μg/ml, 330 nM) or β-VLDL (90 μg/ml) from total binding. The difference between the individual non-specific background values (in the presence of excess unlabelled ligand or antibody) determined the limits of detection for each experiment. Specific binding values for FH3b cells were considered significant only if the total binding levels from which they were calculated exceeded non-specific binding by a greater percentage (of normal specific binding levels) than the difference between the individual non-specific values. For example, in experiment 2, the difference between ^{125}I-IgG-C7 binding values in the presence of unlabelled IgG-C7 was 4% (non-specific = 10-14% of total binding to normal cells). Therefore, specific binding in FH3b cells of <5% of normal (i.e. total binding of <15-19% of normal specific binding level) was not considered significant. (Previous results for binding levels in FH3a cells are included in (a) for comparison).

(a)

Expt.	^{125}I-LDL bound (ng/ml)			^{125}I-IgG-C7 bound (ng/mg)		
	Normal	FH3a/b	% Normal	Normal	FH3a/b	% Normal
FH3b 1	377(3)	n.d.(3)	<2	85(3)	3	4
2	189(3), 486(3) 338	27(3)	8	29(3), 81(3) 55	1.6(3)	<5
3	182	n.d.	<2	53	n.d.	<1
FH3a Ref. 103 *	103	4.5	4	28	0.2	<1
	187	n.d.	<3	39	4.4	11

*van der Westhuyzen, D.R. and Coetzee, G.A. (unpublished results)

n.d. = not detectable.

(b)

Cells	^{125}I-LDL bound (ng/mg)	% Normal	^{125}I-IgG-C7 bound (ng/mg)	% Normal	^{125}I-β-VLDL bound (ng/mg)	% Normal
Normal (upregulated)	155	100	39	100	28	100
Normal (downregulated)	14	9	1	3	0.8	3
FH3b	14	9	2	5	1.5	5

Table 4.2 Binding and metabolism of ^{125}I-LDL and ^{125}I-IgG-C7 at 37°C by normal and FH3b fibroblasts (overleaf).

LDL receptor activity in fibroblasts was either upregulated for 48 hours in lipoprotein-deficient medium (5 mg/ml LPDS) or downregulated in medium containing 1 µg/ml 25-hydroxy-cholesterol plus 12 µg/ml cholesterol. The cells were then incubated for 4 hours at 37°C with either 12 nM (6 µg/ml) ^{125}I-LDL (256 cpm/ng) or 2.4 nM (3.6 µg/ml) ^{125}I-IgG-C7 (1925 cpm/ng). High-affinity binding and metabolism of ^{125}I-LDL were calculated by subtraction of non-specific values determined either in the presence of excess unlabelled LDL (600 nM, 300 µg/ml) or in down-regulated cells. In the case of ^{125}I-IgG-C7, high-affinity values were calculated by subtraction of the non-regulatable binding and metabolism in down-regulated cells. Non-specific values for ^{125}I-LDL represented less than 10% of the total, and for ^{125}I-IgG-C7 the non-specific component was less than 20% of the total. Each value is the average of duplicate determinations from a single experiment and did not differ by more than 5% from the mean values. The limits of detectable receptor activity were determined as described in the legend to Table 4.1. (n.d. = not detectable).

Cell type	Ligand/ Antibody	High-affinity value	Cell-associated (ng/mg)	%	Degraded (ng/mg)	%
Normal	^{125}I-LDL	Specific	1074	100	1623	100
		Regulatable	1105	100	1684	100
	^{125}I-IgG-C7	Regulatable	296	100	213	100
FH3b	^{125}I-LDL	Specific	18	<3	13	<3
		Regulatable	7	<3	20	<3
	^{125}I-IgG-C7	Regulatable	n.d.	<14	21	10

activity may be present in these cells. The ability of LDL to stimulate the incorporation of ^{14}C-oleate into cholesteryl oleate, as an index of cellular acyl-CoA cholesterol acyltransferase (ACAT) induction is believed to be a sensitive assay for distinguishing between receptor-negative and receptor-defective cells (103). In previous studies (103) FH3a and 3b cells showed no detectable LDL-induced ACAT activity, thus supporting their classification as receptor-negative. Western blotting of solubilized extracts from FH3b cells (see section 5.3.6) showed that the level of total cellular receptors was undetectable, excluding the possibility of a large proportion of intracellular receptors as the explanation for negligible surface expression.

Thus FH3a and 3b cells express virtually no LDL receptors, as measured either by LDL- or IgG-C7-binding activity, despite the fact that IgG-C7 was able to immunoprecipitate newly-synthesized receptors from these cells. The apparent lack of receptor expression could be due to severely decreased synthesis of receptor precursors, lack of processing to mature receptors, or abnormally high rates of degradation of precursors and/or mature receptors. The respective contributions of receptor synthesis, processing and degradation rates to this lack of expression were therefore determined.

4.2.2 LDL receptor synthesis

To assess how synthetic rates might influence the steady-state LDL receptor levels in FH3a and 3b, initial rates of [^{35}S]-methionine incorporation into immunoprecipitable LDL receptors were measured, relative to synthesis of total trichloroacetic acid-precipitable protein and transferrin receptor synthesis, and compared to the rates in normal cells. The results of 2 such determinations are summarized in Table 4.3. Cells from FH3a and 3b showed reduced, but significant rates of LDL receptor synthesis relative to normal cells (40-60% of normal) when expressed either in

Table 4.3. LDL receptor synthesis rates in normal, FH3a and FH3b fibroblasts (overleaf).

Fibroblasts from normal subjects and from FH3a and FH3b were incubated in lipoprotein-deficient medium for the indicated time to induce LDL receptor synthesis. The cells were pre-incubated in methionine-free medium for 30 minutes, and then pulse-labelled with [^{35}S]-methionine (64 μCi/ml (Expt. 1) or 30 μCi/ml (Expt. 2)) for 30 minutes and 60 minutes (Expt. 1), or 30 minutes and 45 minutes (Expt. 2). At each time point, duplicate dishes of cells were solubilized and an aliquot (10 μl) was taken from each cell lysate for determination of ^{35}S-incorporation into total trichloroacetic acid-precipitable protein. LDL receptors and transferrin receptors were co-immunoprecipitated from the lysates and subjected to SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography. The initial rates of ^{35}S-incorporation into LDL receptor bands (120 kDa + 160 kDa) and transferrin receptor bands were quantified by densitometric scanning. Rates of incorporation into total protein were determined by scintillation counting of trichloroacetic acid-precipitable material. Initial synthesis rates were calculated by linear regression through zero. The LDL receptor synthesis rates (in arbitrary integration units (discussed in section 2.2.7.5, p. 64) per hour) are expressed relative to rates of transferrin receptor synthesis (arbitrary integration units/hour) and total protein synthesis (dpm [^{35}S]-methionine incorporated x 10^{-5}/hour).

Expt.	Upregulation time (h)	Cell type	LDL receptor synthesis/ Transferrin receptor synthesis		% Normal	LDL receptor synthesis/ Total protein synthesis		% Normal
1	30	Normal#1	2.00	1.83 ± 0.17	100 ± 9%	3.09	4.34 ± 1.54	100 ± 35%
		Normal#2	1.67			3.88		
		Normal#3	1.83			6.06		
		FH3a	0.68		37%	2.70		62%
		FH3b	1.03		56%	2.26		52%
2	16	Normal	1.82		100%	3.71		100%
		FH3b	0.89		49%	1.88		51%

terms of total protein synthesis or the synthesis of transferrin receptors. These results confirmed that the "receptor-negative" phenotype in FH3a and 3b was not the result of severely decreased synthesis of receptors.

4.2.3 Biosynthesis and processing of LDL receptors

Post-translational processing of newly-synthesized receptors was studied by [^{35}S]-methionine pulse-chase experiments. Fibroblasts from a normal subject, FH3a and FH3b were induced for LDL receptor synthesis by incubation for 24 hours in lipoprotein-deficient medium, and then pulse-labelled for 1 hour with [^{35}S]-methionine, followed by various chase periods. At the end of the pulse-labelling, approximately equal amounts of ^{35}S-label were present in the precursor (120 kDa) and mature (160 kDa) LDL receptors in normal cells (Figure 4.1(a)). During the chase, the normal precursor was rapidly and quantitatively converted ($t_{½}$ approximately 15 minutes) to the mature form by oligosaccharide processing reactions (discussed in section 1.3).

The precursor receptor in FH3a (not shown) and 3b (see figure 3.1F) had a normal apparent molecular weight (120 kDa), indicating that the mutation caused no large deletion or insertion in the protein. However, the conversion of precursor into mature 160 kDa receptor, which accompanies transport to the Golgi complex and maturation of core sugars, was slower than in normal cells, with a half-time for conversion of about 1.5 hours (Figures 4.1(b) and (c)). Further evidence for slow processing was the fact that only the precursor form of the receptor in FH3a and 3b was labelled after the pulse period, whereas 50% of the ^{35}S-labelled precursor had already been converted to mature receptor in normal cells. Although post-translational processing in cells from FH3a and 3b was slower than normal, significant processing took place nevertheless, and conversion to the mature form was essentially complete within approximately 3 hours.

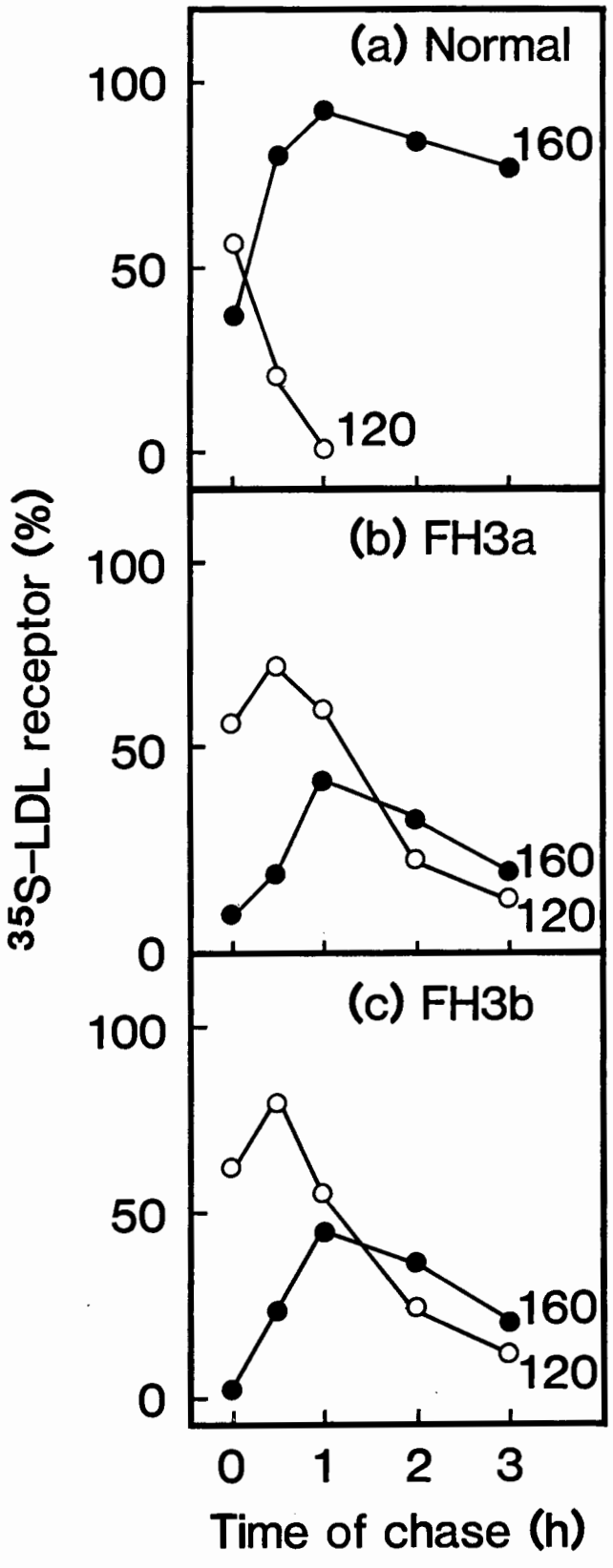

Figure 4.1 (legend overleaf)

Figure 4.1 Processing of LDL receptors in normal, FH3a and FH3b fibroblasts

Fibroblasts from a normal subject (a), FH3a (b) and 3b(c) were induced for LDL receptor synthesis by incubation for 24 hours in lipoprotein-deficient medium. The cells were pre-incubated for 30 minutes in methionine-free EMEM/LPDS, and then pulse-labelled with [^{35}S]-methionine (50 μCi/ml) for 1 hour, at 37°C. The medium was then changed to DMEM/LPDS containing 200 μM unlabelled methionine and the fibroblasts were incubated at 37°C for various chase times. The cells were then washed and solubilized and ^{35}S-labelled LDL receptors were immunoprecipitated. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (5-20% acrylamide gradient) and fluorography (Normal - overnight, FH3a and 3b - 2 day exposure, -70°C). The ^{35}S-radio-activity in the 120kDa precursor (O) and 160 kDa mature (●) receptor bands in the fluorograms was quantitated by densitometric scanning and expressed as a percentage of the maximum ^{35}S-incorporation into total LDL receptors. "100%" incorporation into receptors in FH3a and 3b represented 60% of the maximum incorporation into normal receptors. The data points are averages of duplicates (See Figure 3.1 for fluorograms of processing in normal cells (A) and FH3b (F)).

The most striking feature of the processing in the kindred 3 mutants, apart from the retarded conversion to the mature receptor, was the rapid loss of ^{35}S-label from the receptor bands. In normal cells only a 20% loss of the total incorporated [^{35}S]-methionine (present in the precursor and mature bands) was observed during the 3 hour time-course. However, cells from FH3a and 3b showed disappearance of ^{35}S-labelled receptors with $t_{\frac{1}{2}}$ values of less than 2 hours, suggesting accelerated receptor degradation at some stage. The possibility that this rapid loss represented mature receptor degradation was therefore investigated.

4.2.4 Half-life of mature LDL receptors in FH3a and 3b

The degradation rate of a protein is an important factor in determining its steady-state level in the cell. In the case of certain proteins, such as HMG-CoA reductase (72), alterations in the degradation rate have a regulatory function. However, Casciola *et al*. (73) have shown that regulation of LDL receptor activity in normal fibroblasts is solely the result of changes in the rate of receptor synthesis. The half-life of the LDL receptor in these cells was shown, by [^{35}S]-methionine pulse-chase measurements, to be 11.7 ± 2.2 hours, regardless of the level of LDL receptor up-regulation or the presence of ligand (73). The same procedures were used to compare the half-life of the mature LDL receptor in normal, FH3a and FH3b cells.

A typical example of a determination of the half-life of the LDL receptor in normal cells by IgG-C7 immunoprecipitation of ^{35}S-labelled receptors is shown in Figure 4.2. The half-life of another membrane receptor, the transferrin receptor, was determined in the same experiment by a second immunoprecipitation from the same cell lysate with an anti-transferrin receptor antibody. Data from 4 separate experiments yielded a half-life for the normal mature LDL receptor of 12.6 ± 1.3 hours, which agrees well with the

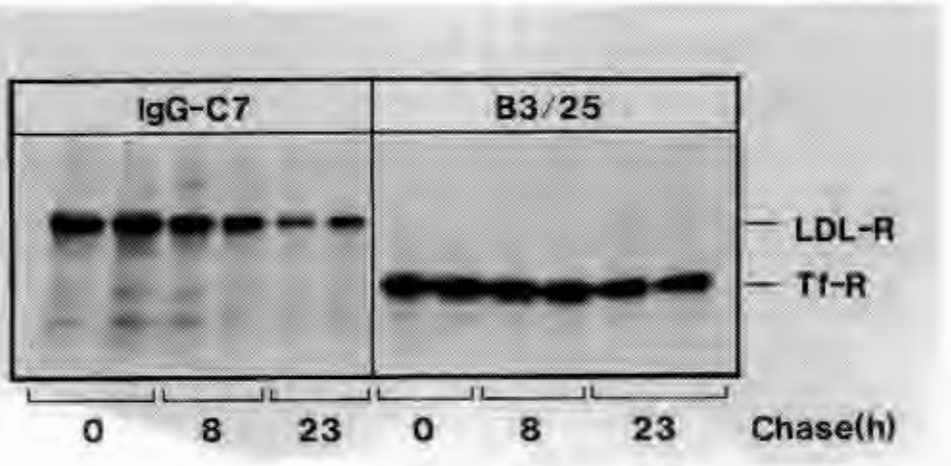

Figure 4.2 Degradation of ^{35}S-labelled LDL- and transferrin receptors in normal fibroblasts

Human skin fibroblasts from a normal subject were incubated for 24 hours in lipoprotein-deficient medium and then pulse-labelled for 9 hours at 37°C with [^{35}S]-methionine (30 μCi/ml). The medium was then changed to DMEM/LPDS containing 200 μM unlabelled methionine and the cells were incubated at 37°C for various chase times ("zero" = 1 hour chase). The cells were then washed and solubilized and LDL receptors (LDL-R), and then transferrin receptors (Tf-R), were isolated by sequential immunoprecipitations, with IgG-C7 followed by anti-transferrin receptor B3/25, from the cell lysates. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (5-20% polyacrylamide gradient) and fluorography. Non-linear regression analysis of the data (duplicates), obtained from densitometric scanning of the fluorographic bands, yielded $t_{½}$ values (mean ± S.E.M.) of 16.6 ± 2.9 hours for the LDL receptor and 55.9 ± 11.9 hours for the transferrin receptor.

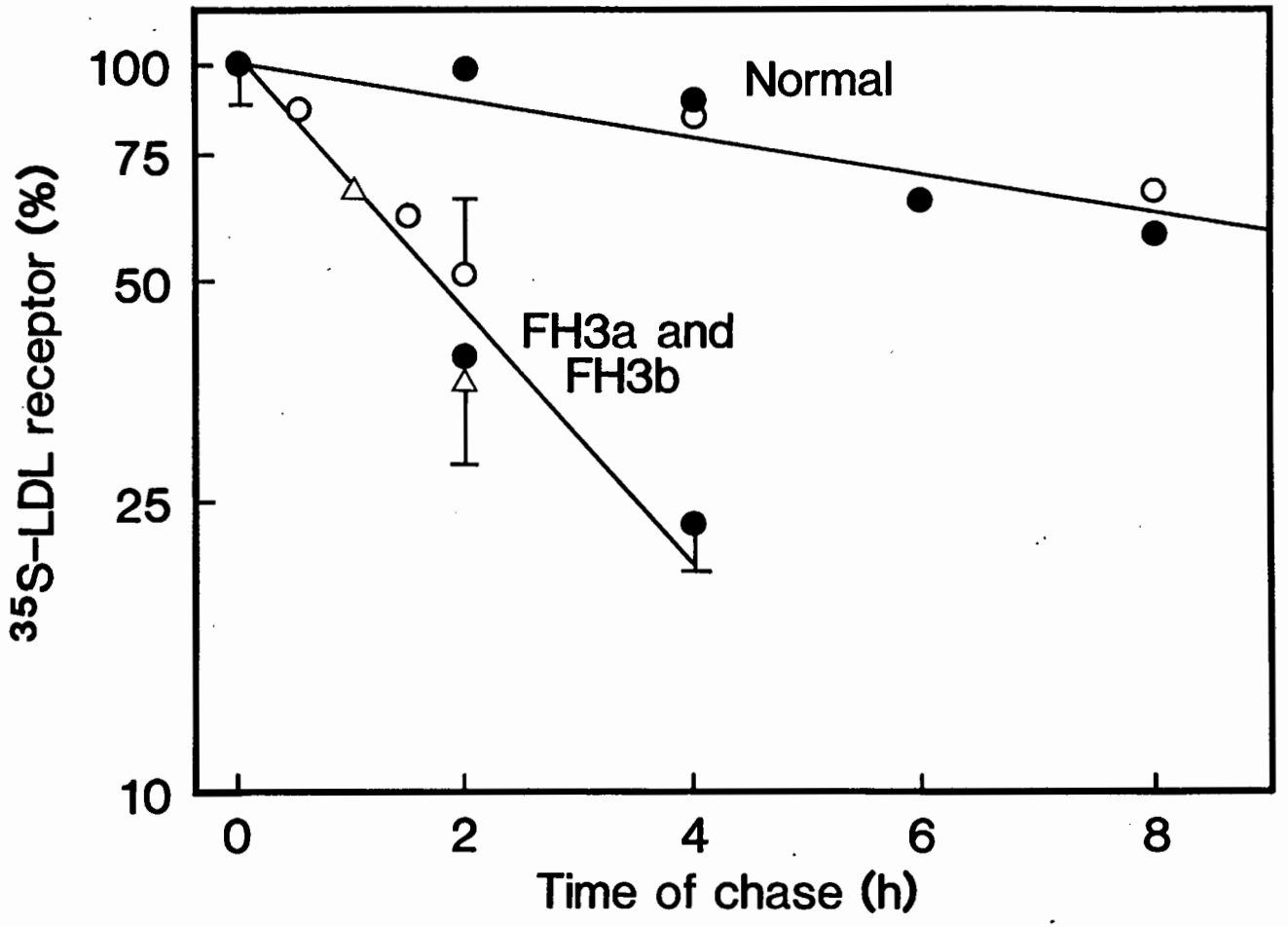

Figure 4.3 (legend overleaf)

Figure 4.3 Degradation of ^{35}S-labelled mature LDL receptors in normal, FH3a and FH3b fibroblasts

Cells from a normal subject, FH3a and FH3b were induced for LDL receptor synthesis by incubation for 16 hours in lipoprotein-deficient medium. The cells were pre-incubated for 30 minutes in methionine-free EMEM/LPDS, after which they were pulse-labelled with [^{35}S]-methionine (40-80 μCi/ml) for 2 hours, at 37°C. The medium was then changed to DMEM/LPDS, containing 200 μM unlabelled methionine, and the fibroblasts were incubated at 37°C until virtually none of the ^{35}S-label remained in the precursor (120 kDa) band. This was assumed to be the "zero" chase time-point (Normal, 1 hour ; FH3a and 3b, 3 hours). After subsequent chase periods, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography were performed. The ^{35}S-label in fluorographic receptor bands was quantified by densitometric scanning. The data points are averages of duplicates and the bars represent variations greater than 10% from the mean values. The "100%" value at zero time represents the mean ± standard deviation of 4 values for normal, 4 for FH3a and 2 for FH3b cells. The data from 3 separate experiments (○, normal and FH3a; ● normal and FH3b; △, FH3a only) yielded $t_{½}$ values (means ± S.E.M.) for LDL receptor degradation of 12.0 ± 1.8 hours for normal cells, and 1.7 ± 0.2 hours for the combined data from FH3a and FH3b.

values obtained by Casciola et al. (73) and Knight et al (129). The $t_{½}$ of the transferrin receptor (from quantitation of results in Figure 4.2) was 55.9 ± 11.9 hours, thus illustrating the unique turnover rates of two different membrane proteins.

Because of the retarded processing to the mature form of the receptor in FH3a and 3b, an initial chase period of at least 3 hours, following pulse-labelling, was necessary to ensure that virtually none of the ^{35}S-label remained in the precursor (120 kDa) band. The half-life of the mature receptor could then be determined from the rate of disappearance of ^{35}S-label from the mature (160 kDa) receptor band. As shown in Figure 4.3, the half life of the mature LDL receptors was markedly shorter than in normal cells. The receptor in FH3a and 3b was degraded with a half-life of approximately 1.7 hours, compared to a $t_{½}$ of 12 hours for the receptor in normal cells analyzed at the same time. This extremely rapid degradation can to a large extent account for the very low surface receptor activity observed in FH3a and 3b (section 4.2.1) and represents a mutant phenotype not previously described in the classification of naturally-occurring LDL receptor mutations. Thus, the mutations in FH3a and 3b (and 2 other mutations discussed later in section 4.3) represent a new class of LDL receptor defects.

A possible alternative mechanism for the rapid loss of mature receptors from FH3a and 3b cells was secretion of receptors from the cells, as seen in the class 4 "variants" (35,36). Therefore, after ^{35}S-labelling of receptors, immunoprecipitations with monoclonal (IgG-C7) and polyclonal (489-1) antibodies were carried out on the medium from FH3b cells, to detect possible secreted receptors (see section 2.2.7.4).

After FH3b cells were pulse-labelled overnight in the presence of 5μM unlabelled methionine, immunoprecipitation

with IgG-C7 (and anti-transferrin receptor antibody) from the cells showed a very small amount of ^{35}S-labelled LDL receptor, particularly compared to the amount of ^{35}S-labelled transferrin receptor in the same cells (Figure 4.4(a)). However, only non-specific bands were present in the immunoprecipitate from the medium, suggesting that no significant secretion of LDL receptors had occurred from FH3b cells.

When FH3b cells were labelled for 2 hours with [^{35}S]-methionine, and chased in the presence of 200 μM unlabelled methionine for 4 hours (Figure 4.4(b)), the amount of ^{35}S-labelled LDL receptor immunoprecipitable from the cells, particularly by the polyclonal antibody, 489-1 (Figure 4.4(b)(ii)) was virtually undetectable. As found after labelling overnight, only non-specific bands were present in immunoprecipitates from the medium. Since the half-life of the immunoprecipitable mutant receptors in these cells is 1.7 hours (discussed above), after 4 hours of chase, only approximately 20% of the original ^{35}S-labelled receptors would be expected to remain. Since no ^{35}S-labelled receptors were immunoprecipitated from the medium with either IgG-C7 or polyclonal antibody, it could be concluded that the receptor loss (80%) was due to degradation rather than secretion. Similar results (not shown) were obtained for IgG-C7 immunoprecipitation from the medium of FH3a cells. Since the polyclonal antibody also immunoprecipitated virtually no ^{35}S-labelled mature receptors from FH3b cells after the 4 hour chase period, the rapid loss of labelled receptors observed using monoclonal IgG-C7 appeared to be due to degradation, and not due to loss of the antigenic site for IgG-C7 through receptor denaturation. The ^{35}S-labelled bands between the 120 kDa and 160 kDa positions, immunoprecipitated by IgG-C7 and polyclonal antibody 489-1 from FH3b cells (Figure 4.4(b)(i) and (ii)), may represent degradation fragments of the mature receptor and are discussed later in section 4.2.7.

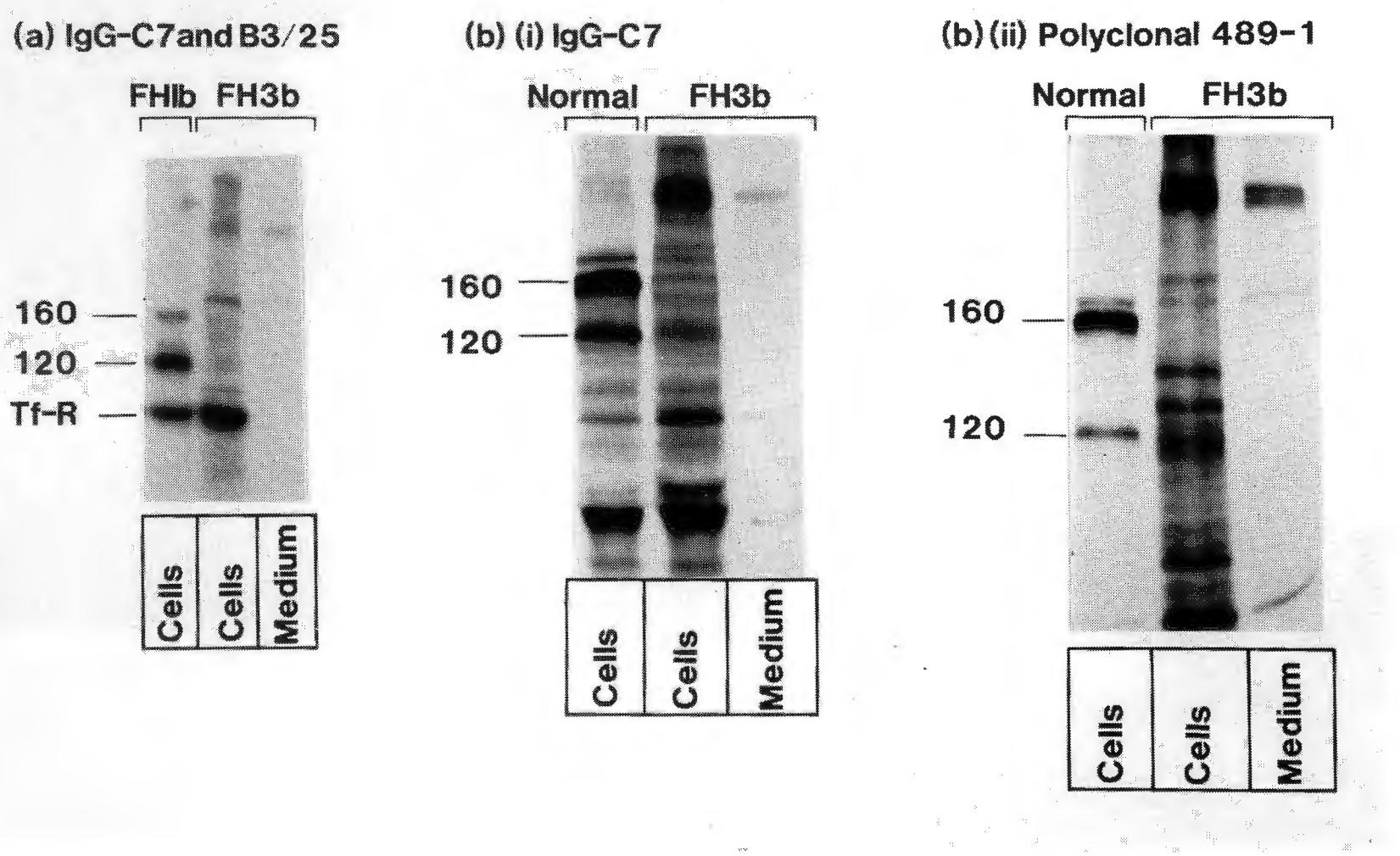

Figure 4.4 (legend overleaf)

Figure 4.4 Immunoprecipitation from medium of FH3b cells

Fibroblasts from FH3b were induced for LDL receptor synthesis by incubation in lipoprotein-deficient medium for 24 hours.

(a) Cells were pulse-labelled with [^{35}S]-methionine (50 µCi/ml) in EMEM containing 5 µM unlabelled methionine for 18 hours at 37°C. Immunoprecipitation with IgG-C7 was then performed on half the total pulse medium and on the total cell lysate.

(b) The fibroblasts were pulse-labelled in methionine-free medium with [^{35}S]-methionine (80 µCi/ml) for 2 hours at 37°C, and then chased in medium containing 200 µM unlabelled methionine for 4 hours. Immunoprecipitation was performed on the total chase medium or on the cell lysate, using IgG-C7 (i) or polyclonal 489-1 (ii).

All immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis ((a) 5-20%. (b) (i) 5-12%. (ii) 7% acrylamide) and fluorography.As references for the positions of the 120 kDa and 160 kDa bands, LDL receptors from FH1b (2 h pulse, 1 h chase) (a) or normal cells (2 h pulse) (b) were immunoprecipitated and electrophoresed with the immunoprecipitates from FH3b. The bands just above the 160 kDa band, and at ±200 kDa (possibly myosin) and ±40 kDa (possibly actin) represent non-specific contaminants.

4.2.5 Biosynthesis, processing and degradation of LDL receptors in the heterozygous subject, 3c

Since FH3a and 3b have been shown by haplotype analysis to be homozygous for a single type of mutant allele, represented by haplotype 15, each of their heterozygous parents must therefore carry one copy of this mutant allele, together with a normal allele. To examine the phenotypic expression of the mutant allele, in the presence of a normal allele, the synthesis, processing and degradation of ^{35}S-labelled LDL receptors were studied in the heterozygous mother of FH3a and 3b, subject 3c (103).

Previous studies of LDL receptor activity in 3c (103) showed approximately 50% of the normal levels of ^{125}I-LDL binding and metabolism, intermediate between that obtained for normal cells, and for FH3a and 3b. Consistent with these findings, the phenotypic expression of cellular LDL receptors in 3c showed synthesis, processing and degradation rates intermediate between those observed in normal cells and FH3b (Figure 4.5). After a 2 hour pulse, the distribution of ^{35}S-label between mature and precursor bands in 3c resembled that in the normal cell more closely than that in FH3b, possibly as a result of the reduced translation rate of the mutant allele (section 4.2.2). After 1 hour of chase, the conversion to the mature receptor was essentially complete in normal cells, whereas ^{35}S-labelled precursor persisted in 3c, presumably representing the slowly-processed product of the mutant allele as observed in FH3b cells. After a 4-hour chase period, the product of the mutant allele in FH3b had virtually disappeared, while ^{35}S-labelled mature receptor was still observed in 3c, at a level of approximately half that produced by two normal alleles in the normal control. These results are consistent with the interpretation that, in the heterozygous cells, the product of the normal allele is synthesized, processed and degraded independently at

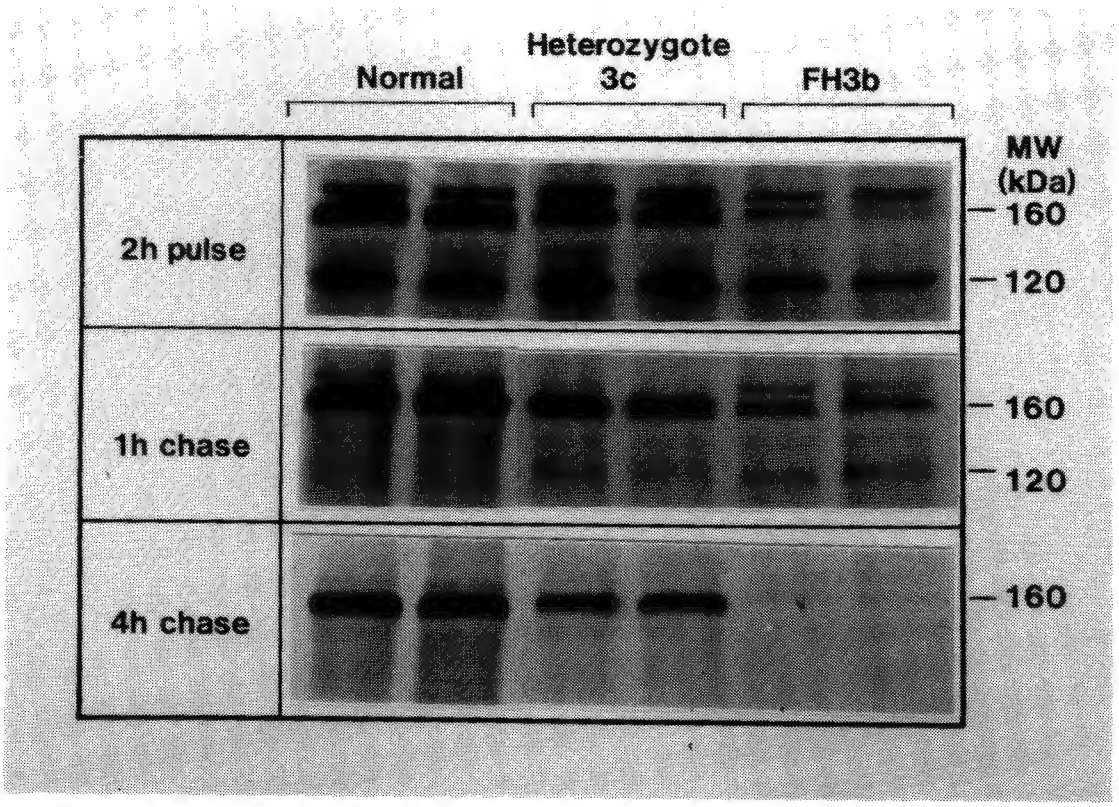

Figure 4.5 Biosynthesis, processing and degradation of ^{35}S-labelled LDL receptors in cells from a normal subject, FH heterozygote 3c, and FH homozygote, 3b

Fibroblasts from a normal subject, FH heterozygote 3c, and FH homozygote 3b were incubated in lipoprotein-deficient medium for 24 hours, pre-incubated for 30 minutes in methionine-free EMEM and pulse-labelled with [^{35}S]-methionine (80 μCi/ml) for 2 hours, at 37°C. Cells were harvested after pulse-labelling, and after 1 hour and 4 hour chase periods in DMEM/LPDS containing 200 μM unlabelled methionine. ^{35}S-labelled LDL receptors were immuno-precipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography. The band above the 160 kDa band is a non-specific contaminant.

normal rates, despite the presence of the abnormal receptor produced by the mutant allele, and vice versa. This strongly suggests that the slow processing and rapid degradation of the receptor observed in FH3a and 3b are due to a defect in the receptor per se, and not in another protein(s) responsible for its intracellular transport and/or metabolism.

4.2.6 Sensitivity of mature LDL receptors to Pronase and neuraminidase.

Although rapid degradation of mature LDL receptors in FH3a and 3b was shown by [^{35}S]-methionine pulse-chase measurements, this method gives no indication of the site of degradation, and it was not known whether the mature receptors were degraded before or after reaching the cell surface. Pronase is known to degrade surface LDL receptors (19), whereas neuraminidase causes a small decrease in apparent molecular weight of the receptors on SDS polyacrylamide gel electrophoresis through removal of sialic acid residues (33). Pronase and neuraminidase sensitivity were therefore used to determine whether mature receptors in FH3b were degraded intracellularly before reaching the plasma membrane, or were transported to the surface and degraded thereafter. In normal cells, the mature receptor band showed a slight but significant decrease in molecular weight after neuraminidase treatment and complete susceptibility to Pronase digestion (Figure 4.6). The same result was observed for the mature receptor band in FH3b cells, and thus all the mature FH3b receptor observed in the control incubations without neuraminidase (lanes 6 and 7) or Pronase (lanes 12 and 13) must have reached the cell surface. The precursor receptor in FH3b would not be expected to be susceptible to neuraminidase treatment as it presumably does not contain sialic acid residues. The lack of susceptibility of the precursor to Pronase digestion confirms that it is intracellular, and that no contaminating Pronase activity was present in the cell lysate.

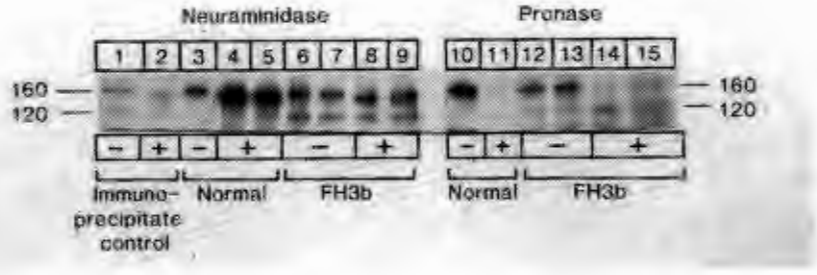

Figure 4.6 Sensitivity of LDL receptors in normal and FH3b cells to treatment with neuraminidase and Pronase

Lanes 1 and 2: upregulated normal fibroblasts were pulse-labelled with [^{35}S]-methionine (80 μCi/ml) for 30 minutes, followed by a 30 minute chase period. ^{35}S-labelled LDL receptors were then immunoprecipitated, and the immuno-precipitates were incubated overnight at 4°C in the absence or presence of neuraminidase (0.8 units/ml) (44). Lanes 3 to 15: upregulated cells from a normal subject or FH3b were pulse-labelled with [^{35}S]-methionine (80 μCi/ml) for 60 minutes, at 37°C, followed by a chase for 60 minutes in medium containing 200 μM unlabelled methionine. The chase medium was removed and the cells were washed and incubated at 37°C in the absence or presence of neuraminidase (Calbiochem-Behring, 0.05 units/ml) for 30 minutes (33) or Pronase (20 μg/ml) for 20 minutes (19). The cells were then washed and solubilized, after which ^{35}S-labelled LDL receptors were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (10-20% acrylamide) and fluorography.

The sensitivity of the mature receptors in FH3b to neuraminidase and Pronase treatment of intact cells shows that ^{35}S-labelled mature receptors in these cells all apparently reach the surface. Thus, the short $t_{½}$ observed for these receptors (section 4.2.4) is the result of degradation of mature receptors after they have reached the cell-surface. The results also show that there is some, albeit very low, expression of LDL receptors on the surface of these cells, which may account for periodic detection of significant LDL- and IgG-C7-binding activities in these cells (section 4.2.1). Although all the ^{35}S-labelled mature receptors in FH3b appear to reach the cell-surface before being rapidly degraded, the results do not exclude the possibility of another population of mature receptors, degraded intracellularly so rapidly that they are not detected.

4.2.7 Effect of lysosomal inhibitors on rapid degradation of LDL receptors in FH3b

The degradation of membrane proteins, and more specifically cell-surface receptors, is not well understood. Protein degradation can be broadly classified into lysosomal and non-lysosomal processes. Receptors which do not recycle in the presence of ligand are thought to be degraded in lysosomes along with their ligands (130). However, the role of the lysosome in the breakdown of recycling receptors, such as the LDL receptor, remains uncertain. Degradation in the lysosome is catalyzed by a number of thiol proteases (cathepsins B, H and L) and a carboxyl protease, cathepsin D (131). Lysosomal involvement in degradative processes has traditionally been demonstrated by the prevention of proteolysis through inhibition of the lysosomal enzymes, either through the use of relatively specific inhibitors such as leupeptin for cathepsin B, or pepstatin for cathepsin D (132), or by non-specific inhibition using lysosomotropic agents such as ammonium chloride and chloroquine. The latter compounds are weak

bases which accumulate in the lysosomes once they become protonated, and thereby increase intralysosomal pH, preventing the action of the acidic proteases (133). In addition to disrupting lysosomal pH, chloroquine specifically inhibits cathepsin B activity (132). Although lysosomal inhibitors, such as ammonium chloride, chloroquine and leupeptin, do not inhibit normal LDL receptor turnover (Casciola, L.A.F.. et al., manuscript in preparation), the possibility that the abnormally rapid receptor degradation in FH3a and 3b occurred in lysosomes was investigated by examining the effects of various lysosomal inhibitors.

The effects of ammonium chloride (10 mM), leupeptin (150 μg/ml) and chloroquine (50 μM) on total receptor degradation, and more specifically, mature 160 kDa receptor degradation, in FH3b cells were investigated. After pulse-labelling for 2 hours with [^{35}S]-methionine, the cells were chased for 1 hour, at which stage approximately 50% conversion of precursor to mature receptor had occurred (Figure 4.7, "zero" chase). The chase medium was then changed to one containing either ammonium chloride, leupeptin, chloroquine or combinations of these agents, at the concentrations given above, and the chase incubation continued for 2 hours, after which LDL receptors were immunoprecipitated. The results in Figure 4.7 suggest that the presence of ammonium chloride in the chase medium markedly inhibited the rapid loss of ^{35}S-label, from both precursor (120 kDa) and mature (160 kDa) receptor bands, observed in the absence of additions. The combination of ammonium chloride, leupeptin and chloroquine had an even more dramatic inhibitory effect on receptor degradation.

The decreased loss of ^{35}S-label in the precursor band in the presence of the lysosomal inhibitors may suggest that the decrease in intensity of the 120 kDa band in the control, attributed to processing to the mature form, is also partially due to degradation of precursor receptors. Alternatively, ammonium chloride and/or chloroquine may

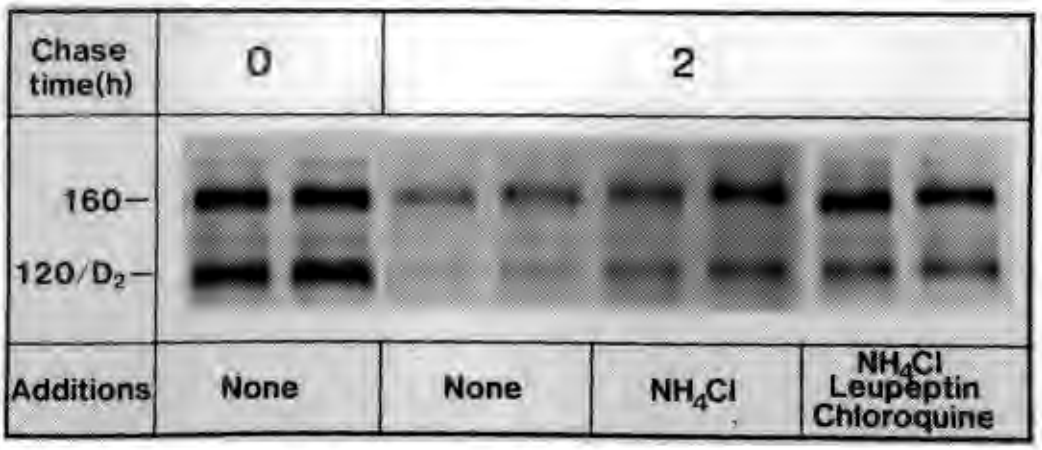

Figure 4.7 Effect of ammonium chloride, plus or minus leupeptin and chloroquine, on total LDL receptor degradation in FH3b cells

Fibroblasts from FH3b were incubated for 18 hours in lipoprotein-deficient medium to induce LDL receptor synthesis. The cells were pre-incubated for 30 minutes at 37°C in methionine-free EMEM and then pulse-labelled for 2 hours with [^{35}S]-methionine (112 µCi/ml). After an initial chase incubation for 1 hour in DMEM/LPDS (containing 200 µM unlabelled methionine), the medium was changed to DMEM/LPDS supplemented with no additions, ammonium chloride (10 mM), or ammonium chloride plus leupeptin (150 µg/ml) plus chloroquine (50 µM) for a further 2 hour chase period. The cells were then solubilized and ^{35}S-labelled LDL receptors immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide gradient) and fluorography.

retard processing. However, this possibility seems unlikely since an inhibition of processing would cause a concomitant decrease in ^{35}S-label transferred to the mature form of the receptor and this is not observed. The apparently higher level of ^{35}S-label relative to the control precursor band may represent an increased level of a putative degradation fragment observed at approximately the same molecular weight in a number of experiments (see Figure 4.8 and discussion later). Accumulation of such a degradation intermediate would imply that ammonium chloride inhibits a degradation step subsequent to the formation of this fragment. Ammonium chloride, alone or in combination with leupeptin and chloroquine, apparently showed a dramatic inhibitory effect on loss of ^{35}S-labelled mature receptor in FH3b cells. However, this could possibly have been an effect secondary to prevention of precursor degradation, thus making more precursor available for conversion to mature receptor. The results of another experiment to test the effects of the three agents, alone or in different combinations, on LDL receptor degradation in FH3b cells are shown in Table 4.4. Chloroquine and leupeptin alone each had similar effects to ammonium chloride on the levels of precursor and mature receptors. Combinations of these agents, particularly ammonium chloride plus chloroquine or leupeptin, were apparently more effective. The results suggest that at least some of the steps in the degradation of the precursors and/or mature receptors and/or degradation intermediates may take place in lysosomes (or other acidified vesicles, for example, endosomes) and may involve cathepsin B activity.

To examine more specifically the effects of these agents on degradation of the mature receptor, the initial chase period in the absence of agents was extended to 3 hours, to allow more complete conversion of precursor to mature receptors (see section 4.2.3). The chase medium was then changed to one containing no additions, ammonium chloride, leupeptin, or a combination of these two agents for further 2-hour and 4-hour chase periods. The results are shown in Figure 4.8.

Table 4.4 Effect of ammonium chloride, chloroquine and leupeptin, alone or in combination, on total LDL receptor degradation in FH3b cells

FH3b cells were incubated for 18 hours in lipoprotein-deficient medium to induce LDL receptor synthesis. The cells were then pulse-labelled with [^{35}S[-methionine (70 µCi/ml) for 2 hours and chased for one hour in the absence of added agents (= "zero" chase). After this initial chase period, the medium was changed to one supplemented with no additions, ammonium chloride (10 mM), chloroquine (50 µM), leupeptin (150 µg/ml), or combinations of these agents for a further 2-hour chase period. The cells were then solubilized and ^{35}S-labelled LDL receptors immuno-precipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography. The amount of ^{35}S-label in the precursor (120 kDa) and mature (160 kDa) receptor bands was quantified by densitometric scanning and expressed as a percentage of the total ^{35}S-radioactivity after the initial one-hour chase period ("zero"). The values are averages of duplicates.

Chase	Additions	^{35}S-LDL Receptor (%)		
		120 kDa	160 kDa	Total
"Zero"	-	53	47	100
2 h	-	14	30	44
	NH_4Cl	29	41	70
	Chloroquine	26	48	74
	Leupeptin	24	49	73
	NH_4Cl + chloroquine	33	68	101
	NH_4Cl + leupeptin	27	64	91
	Chloroquine + leupeptin	29	43	72
	NH_4Cl, chloroquine, leupeptin	24	54	78

In the absence of agents, a marked loss of ^{35}S-label from the mature receptor band, i.e. degradation, took place during the first 2 hours, despite the fact that some processing from the precursor still occurred. In addition, the appearance of possible degradation fragments, at apparent molecular weights slightly (± 20 kDa) lower than the mature receptor (D1) and slightly (± 10 kDa) greater than the precursor receptor (D2) was observed. By 4 hours of chase, the mature receptor band had essentially disappeared, and an increase in the intensity of the putative degradation bands, particularly D1 was observed. Both D1 and D2 were observed in at least 3 other experiments (see, for example, Figure 4.6(b) (i) and (ii)). The bands did not appear to be non-specific contaminants, since they were not present at early chase times, but only appeared later when significant receptor degradation had taken place, thus supporting the possibility that they might be degradation fragments. Although similar bands to D1 and D2 were sometimes observed in immunoprecipitates from normal cells, they were always present in very low amounts relative to the amount of intact 160 kDa receptor, whereas in FH3a and 3b they were often present in amounts equal to or greater than the mature receptor band (see section 4.3 for further discussion).

Rapid receptor degradation was observed even in the presence of the lysosomal inhibitors. However, ammonium chloride, particularly in the presence of leupeptin, appeared to inhibit loss of ^{35}S-label from the 160 kDa band, suggesting inhibition of receptor degradation. Densitometric scanning of the fluorograms of the 4 hour chase point showed clearly discernible 160 kDa peaks for incubations with ammonium chloride in the absence (B) and presence (D) of leupeptin, whereas essentially no peaks above background were observed for incubations in the absence of agents (A) or in the presence of leupeptin alone (C). The apparent decrease in background of the fluorogram in the region below the 160 kDa peak position is probably due to the presence of residual

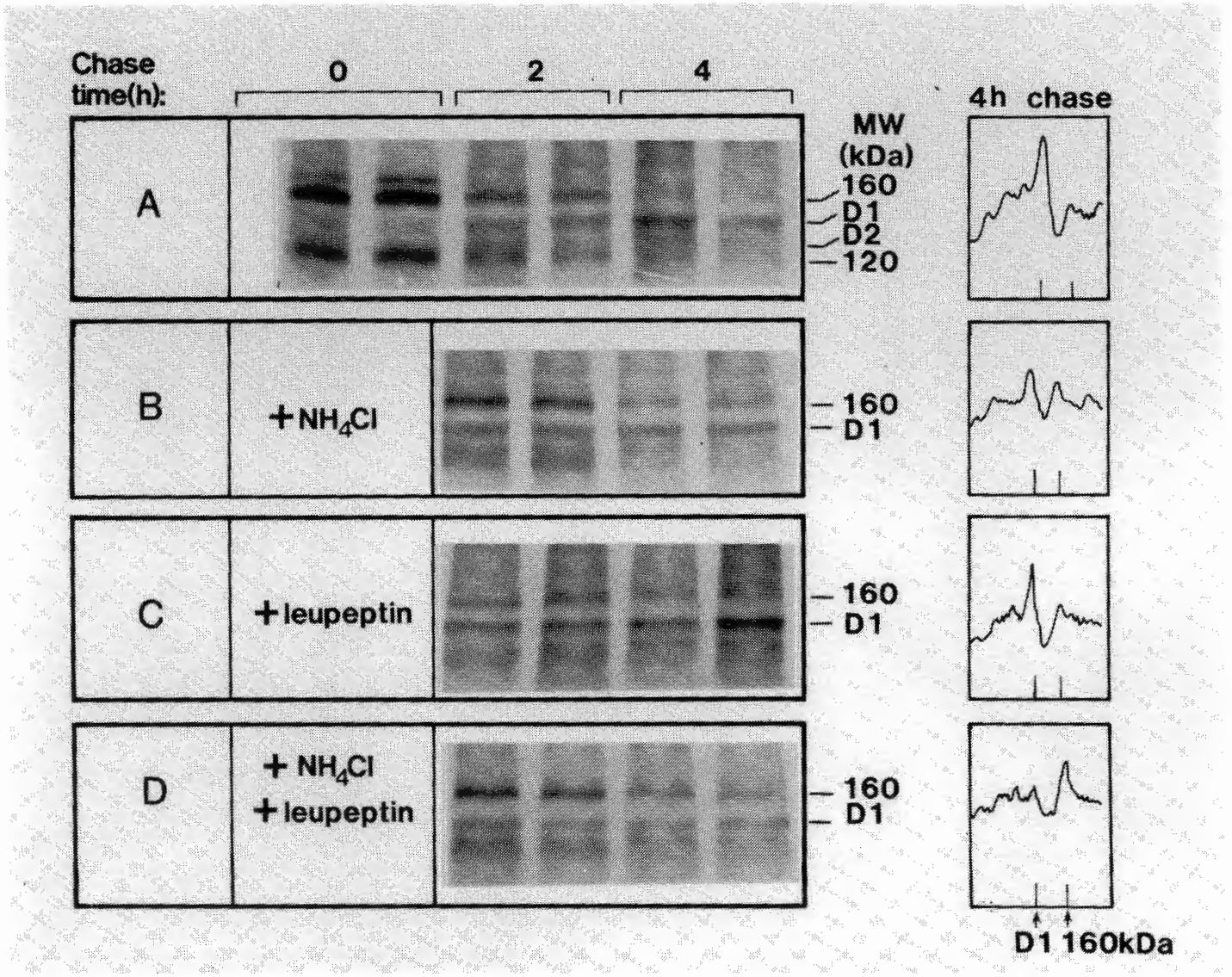

Figure 4.8 (legend overleaf)

Figure 4.8 Effect of ammonium chloride and leupeptin on degradation of the mature LDL receptor in FH3b cells

Fibroblasts from FH3b were induced for LDL receptor synthesis by incubation for 18 hours in lipoprotein-deficient medium. The cells were then pre-incubated for 30 minutes at 37°C in methionine-free EMEM, followed by pulse-labelling with [^{35}S]-methionine (60 μCi/ml) for 2 hours. The medium was then changed to DMEM/LPDS (containing 200 μM unlabelled methionine) for an initial 3 hour chase period at 37°C ("zero" time) and then to DMEM/LPDS supplemented with ammonium chloride (10 mM), leupeptin (150 μg/ml), the combination of these two agents, or no additions, and the chase was continued for a further 2 hours or 4 hours. The cells were lysed and subjected to immunoprecipitation with IgG-C7. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide gradient) and fluorography. Densitometric scans of the fluorographic bands after 4 hours of chase are shown. The band above the 160 kDa band at zero time is a non-specific contaminant.

non-reduced IgG (150 kDa), which is not radioactively labelled. The 160 kDa peaks in (B) and (D) were comparable in size or greater than the degradation fragment (D1) peak, in contrast to the relatively large D1 peaks in (A) and (C), supporting the idea that ammonium chloride, particularly in the presence of leupeptin, inhibited the degradative process giving rise to fragment D1. It was not possible to accurately quantitate the fluorographic bands corresponding to the mature receptor and degradation fragments, as their intensities were below the linear response range of the X-ray film. Although leupeptin and ammonium chloride in combination appeared to have additive inhibitory effects on the degradation, leupeptin alone did not inhibit the rapid loss of ^{35}S-label from the mature receptor. Possibly, leupeptin inhibited a step subsequent to an initial proteolytic cleavage. Similar observations were made by Ascoli (134) for degradation of receptor-bound human choriogonadotropin. It was found that ammonium chloride inhibited the formation of all intracellular degradation products, while leupeptin allowed the formation of the first product to occur, but blocked any further degradation.

The half-life of the LDL receptor in FH3b cells is less than 2 hours in the absence of added inhibitors (section 4.2.4). If the combination of ammonium chloride and leupeptin was able to significantly increase this half-life by inhibition of degradation (without any effect on synthesis rates), an "accumulation" of receptor protein levels, relative to those in untreated FH3b cells, would be expected after incubation with the agents for a period encompassing a number of half-lives. Therefore, the effects of pre-incubation of FH3b cells for 13 hours in the presence of ammonium chloride and leupeptin on steady-state receptor levels, as measured by ^{125}I-LDL and -IgG-C7 binding, were determined. The results (Table 4.5) showed no significant increase in receptor levels, relative to normal cells, after this treatment. This is consistent with the observation that although ammonium chloride and leupeptin showed inhibition of mature

Table 4.5 Effect of pre-incubation with ammonium chloride and leupeptin on LDL receptor activity in FH3b cells (overleaf)

After incubation for 30 hours in lipoprotein-deficient medium, normal and FH3b fibroblasts were incubated at 37°C for 13 hours in the same medium, in the absence or presence of 10 mM ammonium chloride and 150 µg/ml leupeptin. LDL receptor activity was then determined by ^{125}I-LDL (629 cpm/ng, 10 µg/ml) binding and uptake during 30 minutes at 37°C, or by the amount of ^{125}I-IgG-C7 (1272 cpm/ng, 1 µg/ml) bound during 2 hours at 4°C. Cells were then washed as described in section 2.2.5 and dissolved in 1 M NaOH for determination of the ^{125}I-radioactivity associated with the cells. The data are averages of duplicate determinations and represent high-affinity values, calculated by subtracting non-specific values obtained in the presence of excess unlabelled LDL (350 µg/ml) or IgG-C7 (50 µg/ml).

LDL receptor assay	NH_4Cl + leupeptin	Normal (ng/mg)	FH3b (ng/mg)	% Normal
^{125}I-LDL, 30 minutes at 37°C	-	669	18	3%
	+	522	19	4%
^{125}I-IgG-C7, 2 hours at 4°C	-	63	5	8%
	+	45	5	11%

receptor degradation, turnover of receptors in FH3b cells was considerably more rapid than in normal cells, even in the presence of these agents (Figure 4.8).

Although the results are still somewhat preliminary and quantitatively variable between experiments, ammonium chloride (and other lysosomal inhibitors) consistently showed inhibition of the rapid receptor degradation in the kindred 3 mutant cells, in the experiments described above and others not shown. Thus, although the results are not yet conclusive, a strong indication for the involvement of proteolysis in lysosomes or other acidified vesicles (such as endosomes) in the abnormal receptor degradation, was obtained. Some of the variability in the experimental results, and the inability to totally inhibit the rapid degradation of the mutant receptors, may be related to the problem that lysosomotropic agents and proteolytic inhibitors (even in combination) incompletely inhibit lysosomal function (135) and therefore may complicate interpretation. In addition, since the inhibitors take some time to accumulate substantially in the cells, their use in experiments on short half-life proteins, such as the rapidly-degraded mutant LDL receptors, may be unsatisfactory (136). An alternative approach for future studies on the site and mechanism of receptor degradation in FH3a and 3b cells may be the use of temperatures below 20°C to inhibit lysosomal proteolysis, presumably by prevention of endosome-lysosome fusion and thus of delivery of material to the lysosome for degradation (137,138). Since all processes will be slowed considerably at this temperature, this manipulation may allow a clearer dissection of the cellular events involved in the very rapid degradation (at 37°C) of the mutant receptors.

4.3 Discussion

LDL receptors in FH3a and 3b were shown to be synthesized at reduced but significant rates, and then processed more slowly than normal to mature forms which undergo rapid degradation after reaching the cell surface. Although the post-translational processing of these mutant receptors is slower than normal, their half-time for processing of 1.5 hours is much faster than typical Class 2 "variants", where conversion to the mature receptor is retarded approximately ten-fold (19,77).

The most striking feature in the phenotype of FH3a and 3b is the rapid degradation of the mature LDL receptor (described in ref. 139). This type of defect was not previously described in the phenotypic classification of LDL receptor mutations, which included defects in synthesis, processing, ligand binding, internalisation or receptor secretion (section 1.5). Thus, at the time when their mutant receptor phenotype was first reported (102), FH3a and 3b were the first examples of a new class of naturally-occurring LDL receptor mutations. Recently, however, two other mutations giving rise to rapidly-degraded mature receptors have been characterized. One of these has been described by Funahashi *et al.* (128) in three siblings from a Japanese kindred. Cells from these homozygous FH subjects produce precursor and mature LDL receptors which have apparent molecular weights about 5 kDa less than normal. The precursors are converted at a normal rate to mature forms, which are rapidly degraded with a half-life of 0.5 to 1 hour. A similar phenotype is produced by the mutation in South African FH subjects, RI and TD (section 3.2.3), which results in removal of the first two cysteine-rich repeats (A and B) from the EGF precursor homologous domain of the LDL receptor. The smaller precursor (105 kDa) is processed normally to a mature receptor (145 kDa) that is degraded with a half-life of 2 hours (van der Westhuyzen *et al.*, manuscript in preparation).

Although the phenotypes of the two mutations discussed above are similar to that of FH3a and 3b, they have other distinguishing features. Both the Japanese mutants and RI and TD express measurable, immunologically-detectable, levels of surface receptors (Table 3.1, ref. 128), whereas FH3a and 3b are essentially receptor-negative (section 4.2.1). In addition, the receptors synthesized in FH3a and 3b cells have normal apparent molecular weights and are slowly processed, whereas the Japanese subjects, and RI and TD, all produce abnormally small receptors which are processed at a normal rate. Presumably the Japanese, RI and TD mutations do not disrupt protein folding, and thus transport and processing, because they involve deletions of entire receptor units, such as cysteine-rich repeats, whereas FH3a and 3b may have small deletions or substitutions within such repeats, causing abnormal folding and retarded processing (discussed in section 3.3).

Accelerated turnover of mature LDL receptors has also been observed in site-directed mutations where the whole EGF precursor-like domain, or only cysteine-rich repeats A and B (as in RI and TD), have been deleted (31). In transfected Chinese hamster ovary cells, these mutant receptors are rapidly degraded in the presence of the ligand, β-VLDL, but not in its absence, presumably because the receptor is unable to undergo the acid-dependent conformational changes required to allow dissociation of the ligand in the endosome. The mutation in FH3a and 3b leads to rapid receptor degradation, even in the absence of ligand. There is no evidence for ligand-induced acceleration of receptor turnover in these cells since (i) pulse-chase studies show no dramatic increase in receptor degradation in the presence of LDL (not shown), and (ii) no significant difference is detected between receptor activity measured at 37°C after 30-minute or 4-hour incubations with LDL (compare results in Tables 4.2 and 4.5). It is of interest to note that, although the mutations in RI and TD are very similar (if not

identical) to the constructed mutation lacking cysteine-rich repeats A and B, the mature receptors in RI and TD cells turn over rapidly even in the absence of ligand, as observed for FH3a and 3b. However, this rapid degradation is further accelerated in the presence of ligand (van der Westhuyzen et al., manuscript in preparation).

In their studies of experimentally-induced mutations in the LDL receptor in Chinese hamster ovary cells (section 1.5.2), Kozarsky et al. (66) showed that O-linked sugar-deficient receptors reached the cell-surface but were abnormally unstable due to proteolytic cleavage and release into the medium of most of the N-terminal extracellular domain. The proteolytic site was postulated to be within or near the domain usually containing clustered O-linked oligosaccharides, which presumably protect the cleavage site in the normally-glycosylated receptor. The unstable receptors in FH3a and 3b cells appear to undergo retarded but normal glycosylation, since the expected 120 kDa to 160 kDa apparent size increase, associated with maturation of oligosaccharide residues, is observed (section 4.2.3) and the mature surface receptors contain terminal sialic acid residues (section 4.2.6). Thus, a glycosylation-related defect in the LDL receptor does not appear to be responsible for the rapid degradation of mature receptors in FH3a and 3b.

However, the appearance of putative proteolytic receptor fragments D1 and D2, at apparent molecular weights slightly (± 20 kDa) lower than the mature receptor and slightly (±10 kDa) greater than the precursor receptor, respectively, was observed in immunoprecipitates from FH3a and 3b cells. Lehrman et al. (37) and Kozarsky et al. (93) have identified degradation products of the normal LDL receptor with apparent molecular weights of 125 kDa in fibroblasts and 118 kDa in Chinese hamster ovary cells, respectively. These fragments were detected by antibodies to the C-terminal region of the receptor, and were not recognized by IgG-C7

(37) or another antibody directed against the N-terminal domain of the receptor (93), thus suggesting that these degradation products had been formed by proteolytic removal of an N-terminal fragment. Casciola et al. (73) identified a putative high molecular weight (120 kDa) degradation intermediate using a polyclonal antibody to the LDL receptor, although this fragment was not detected by IgG-C7. Since the degradation products observed in FH3a and 3b were detected by IgG-C7, they cannot correspond to the above receptor fragments. However, Lehrman et al. (36) identified a fragment, slightly larger than the precursor receptor, that was specifically immunoprecipitated by IgG-C7, from fibroblasts of a normal subject, homozygote FH274, and his heterozygous parents. The band obtained in FH3a and 3b approximately 10 kDa above the precursor position (D2) may represent a similar fragment. A band at this molecular weight was also observed periodically in normal cells in these studies.

The only precedents for N-terminal, IgG-C7-recognisable, fragments of slightly lower molecular weight than the mature form of the receptor, are those which are secreted from cells into the medium, as found by Kozarsky and co-workers for O-linked sugar-deficient receptors (66) and Lehrman et al. (36) for the secreted form of the truncated receptor in FH274. It seems unlikely that the fragments in FH3a and 3b which migrate at approximately 20 kDa smaller than the mature receptor (D1) have lost a C-terminal fragment, as they would then be expected to lack the membrane-spanning region and be secreted as for the abnormal receptors described above (36,66). One possible explanation is that proteolysis of the mutant receptors may cleave the receptor molecules at a site in the N-terminal region, but that inter-repeat disulphide bonding, possibly abnormal, maintains the receptor "intact", including the first cysteine-rich repeat at the N-terminus, containing the epitope for IgG-C7 (29). After immunoprecipitation, the disulphide bonds of the receptor are reduced prior to

electrophoresis, and, at this stage, the N-terminal fragment could be lost.

Alternatively, fragment D1 may represent receptors which have lost a C-terminal fragment (including the membrane-spanning region) by proteolysis in intracellular vesicles, such as endosomes, which have been shown to contain certain proteolytic enzymes (140,141). If such a proteolytic step affected all endocytosed mutant receptors, thus converting a large percentage of the receptor population to the proteolyzed form trapped in intracellular vesicles, and if this form were then degraded rather than "recycled" and secreted, this could explain why the N-terminal fragment could be detected in cell lysates and was not found as a secreted form of the receptor.

The site(s) and mechanisms(s) for the rapid receptor degradation in FH3a and 3b, like that of the normal LDL receptor, remain obscure. Some lines of evidence suggest that the LDL receptor in normal cells is degraded by a non-lysosomal mechanism (Casciola, L.A.F.. et al., manuscript in preparation) which may involve a short-lived mediator protein (73) since inhibition of protein synthesis by cycloheximide dramatically inhibits receptor degradation. Lysosomotropic agents, such as ammonium chloride, do not inhibit normal LDL receptor turnover. In the presence of ligand (LDL), these agents actually lead to a marked acceleration of degradation, presumably because of receptor trapping by inhibition of acid-dependent ligand dissociation (see section 2.2.9), similar to that observed when the EGF precursor-like region of the receptor is deleted (31). In contrast, lysosomal inhibitors which raise intravesicular pH, cause partial inhibition of the rapid degradation in FH3a and 3b (section 4.2.8). This suggests that proteolysis in the lysosome or in the acidic endosome (140,141) may play a role in turnover of the mutant receptors.

It is not known whether degradation of the mutant receptors in FH3a and 3b is mediated by a short-lived mediator protein, as for the normal LDL receptor (73). Since the receptors in FH3a and 3b are themselves short-lived, inhibition of synthesis of another rapidly turned-over protein may not have a dramatic effect on their short half-life. No evidence for any effect of cycloheximide on receptor degradation in FH3a was obtained in a preliminary experiment (not shown).

Since the site(s) of rapid degradation and the molecular defect in the mutant FH3a and 3b receptors have not yet been elucidated, the reason for the short half-life of the receptor protein is open to speculation. The mutation may create a new site for protease activity or may lead to an abnormal protein conformation that renders the protein more susceptible to proteolysis. The retarded post-translational transport and processing of the mutant receptors suggest that they are abnormally folded (78-80). Recycling of LDL receptors may require a relatively stable structure to allow repeated passage through the acidic endosome without irreversible denaturation of receptors taking place (3). The mutant receptors in FH3a and 3b may have a mutation which renders their structure unstable to continual recycling and results in rapid degradation (139).

Although relatively little is known about the turnover of membrane receptors, it is believed that receptors which do not recycle in the presence of ligand, such as those for EGF and insulin, and the macrophage Fc receptor, are degraded in the lysosome along with ligand (130). These receptors therefore have half-lives of approximately 1 to 2 hours, similar to those of endocytosed ligands. Receptors which recycle on the other hand, have relatively long half-lives, between 10 and 80 hours (130). It is therefore interesting to note that the $t_{\frac{1}{2}}$ of less than 2 hours for mature LDL receptors in FH3a and 3b would be typical for a receptor which did not recycle, in contrast to the half-life of 12

hours for the normal, recycling LDL receptor (73,129). Similarly, typical "non-recycling" $t_{½}$ values have been observed for (i) a site-directed mutant LDL receptor (lacking the EGF precursor-like region) which is unable to undergo acid-dependent dissociation of bound ligand, and is therefore rapidly degraded (31) and thus does not recycle (ii) mutants RI and TD, which lack part of the EGF-precursor-like region (discussed earlier), and (iii) the normal LDL receptor in the presence of LDL and ammonium chloride, which is degraded with a $t_{½}$ of about 2 hours, presumably because of intracellular receptor trapping by inhibition of acid-dependent ligand dissociation (section 2.2.9). The appearance in FH3a and 3b cells of an N-terminal, IgG-C7 recognisable fragment (D1) of slightly lower molecular weight than the mature LDL receptor may represent a prelysosomal, proteolytic product which would not be recycled but, having lost its membrane-spanning region, be segregated from recycling components, as for dissociated ligand, and possibly delivered eventually to the lysosome for degradation. This possibility is purely speculative, but should be considered in future work. Elucidation of the mechanism for the rapid degradation of the LDL receptor in FH3a and 3b cells will provide valuable information on how a small mutation converts a recycling receptor, with a relatively long half-life, to one which is rapidly degraded, with a much shorter half-life, and thus possibly does not recycle.

CHAPTER 5

PHENOTYPIC CHARACTERIZATION OF THE MUTATION PRODUCING "HIGH-DEFECTIVE" LDL RECEPTOR LEVELS IN AFRIKANER FH KINDRED 1

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5.1 Introduction

Analysis of LDL receptor activity in cells from 23 FH homozygotes from 17 Afrikaner kindreds indicated the predominance of a "receptor-defective" type of mutation (98,103). The receptor-defective subjects could be sub-divided into two groups: "low" receptor-defective (1% to 10% of normal receptor activity) and "high" receptor-defective (11% to 25% of normal). FH siblings 1a and 1b exhibited the highest LDL receptor activity (25% normal) in the latter category and clinical symptoms typical of the homozygous state of FH i.e. plasma cholesterol concentrations greater than 14.3 mmol/l and the presence of xanthomata in their first decade of life (103). No signs of coronary heart disease were shown by FH1a or 1b at ages of 14 and 11 years, respectively.

Haplotype analysis on the basis of 10 polymorphic restriction sites (section 3.2.1) showed that FH1a and 1b were true genetic homozygotes for a specific mutant allele represented by haplotype 3. This haplotype accounted for approximately 65% of the mutant alleles analyzed from Afrikaner FH subjects (Leitersdorf E, Hobbs H H, et al., manuscript in preparation) thus representing a prevalent, possibly founder, mutation in this population. Since FH1a and 1b expressed relatively high levels of the mutant receptor encoded by the allele associated with haplotype 3, the phenotypic expression of this mutation could be studied in these subjects, without interference from another normal or mutant allele.

Preliminary phenotypic studies on several homozygous FH subjects (including FH1a) suggested that haplotype 3 was associated with severely impaired post-translational receptor transport and processing (Class 2 mutation) and defective LDL binding (Table 3.1, section 3.2.1). "Class 2", or transport-deficient LDL receptor mutants such as these are potentially of great cell biological interest. Many of these mutations involve subtle changes to the

receptor protein, such as small deletions or substitutions, preserving recognition by antibodies, and, to some extent, ligands, but disrupting transport from the endoplasmic reticulum to the Golgi apparatus, a complex and poorly-understood process. In order to gain information on the characteristics of, and mechanisms responsible for, the delayed transport and reduced receptor level and function, the phenotypic expression of the mutation represented by haplotype 3 was studied in fibroblasts from the genetically-homozygous siblings FH1a and 1b. ^{125}I-LDL and ^{125}I-IgG-C7 binding and metabolism were used to characterize surface receptor activity in terms of affinity and maximum levels, and how these levels changed during up- and down-regulation. LDL receptor synthesis, post-translational processing and degradation rates were determined using [^{35}S]-methionine pulse-chase studies and glycosylation of the precursor and mature receptors was characterized using glycosidic enzymes and glycosylation inhibitors.

5.2 Results

5.2.1 LDL receptor activity

Previous assessments of LDL receptor levels in homozygous siblings FH1a and 1b were based on ^{125}I-LDL binding and metabolism at 37°C at non-saturating LDL concentrations (10 µg/ml) (103). The possibility thus existed that the apparently reduced levels (relative to normal) of LDL receptor activity in these subjects could represent somewhat higher levels of binding-deficient receptors. This was suggested by the higher proportion of antibody (IgG-C7) versus ligand (LDL) binding at 4°C observed for FH1a (103). Therefore, LDL receptor activity in fibroblasts from FH1a and 1b was characterized on the basis of affinity and maximum binding levels for ^{125}I-LDL and ^{125}I-IgG-C7 at 4°C, and in terms of the biological activity of these receptors at 37°C in binding, and subsequent internalisation and degradation, of ligand and antibody.

5.2.1.1 Surface binding activity of ^{125}I-labelled LDL and ^{125}I-labelled IgG-C7 at 4°C

Surface LDL receptors in normal, FH1a and FH1b cells were characterized by ^{125}I-LDL and ^{125}I-IgG-C7 binding at 4°C. The results of a typical experiment are shown in Figure 5.1. ^{125}I-LDL binding to cells from FH1a showed a binding affinity (mean ± S.D., n=3) for LDL (K_d = 2.2 ± 0.9 µg/ml) which was similar to that of normal controls (K_d = 1.5 ± 0.8 µg/ml), whereas the maximum number of receptors in up-regulated cells, as assessed by high-affinity LDL binding, was approximately 20% of normal (Figure 5.1(a) and Table 5.1), in agreement with previous findings (103).

Surface LDL receptors were also assessed on the basis of high-affinity binding of the monoclonal antibody, IgG-C7. This antibody recognizes an epitope distinct from the LDL binding site, in the first cysteine-rich repeat of the N-terminal binding domain (29). High-affinity binding of ^{125}I-IgG-C7 to FH1a cells showed a significantly greater number of receptors (approximately 80% of normal) than found for ^{125}I-LDL binding, with an affinity for IgG-C7 similar to normal (Figure 5.1(b)). As shown in Table 5.1, in 6 separate experiments, the ^{125}I-labelled IgG-C7 binding to FH1a cells (54 ± 14% [mean ± S.D.] of receptor-normal controls) was 3.2 ± 0.4 times higher than the ^{125}I-labelled LDL binding (17 ± 3% of normal). Similarly, in two separate experiments, FH1b yielded values for IgG-C7 binding of 3.2-fold and 3.3-fold higher than LDL binding, respectively.

Total binding was always corrected for non-specific, low-affinity values obtained in the presence of excess unlabelled ligand or antibody, or calculated by non-linear regression of concentration curves. Non-specific values were similar for normal and FH1a or FH1b cells and represented 10 ± 4% of total ^{125}I-LDL binding, and 13 ± 4% of total ^{125}I-IgG-C7 binding, in normal cells in 10 different determinations. Specificity of the high-affinity component was indicated by the fact that down-regulation of

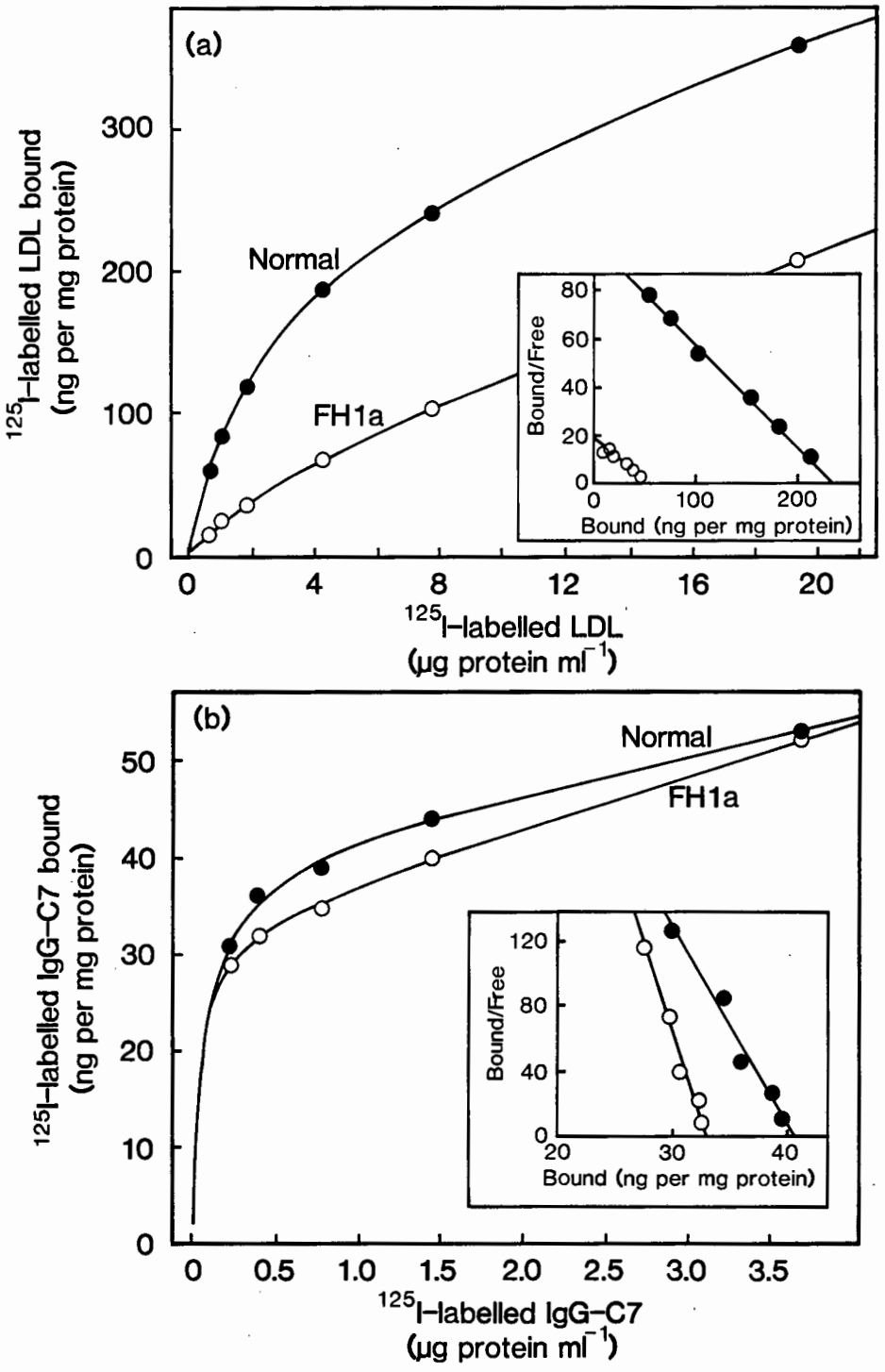

Figure 5.1 (legend overleaf)

Figure 5.1 Surface binding at 4°C of ^{125}I-labelled LDL and ^{125}I-labelled IgG-C7 to normal and FH1a fibroblasts.

Cells from a normal subject (●) and FH1a (○) were analyzed for cell-surface binding at 4°C of ^{125}I-labelled LDL (a) or ^{125}I-labelled IgG-C7 (b) as described in section 2.2.5. Each value represents the average of duplicate incubations from a typical experiment. The K_d values for ^{125}I-labelled LDL in normal and FH1a cells were 2.4 and 3.0 μg of LDL protein/ml respectively. Maximum high-affinity binding of ^{125}I-labelled LDL in normal and FH1a cells was 237 and 54 (23% of normal) ng/mg of cell protein respectively. Maximum high-affinity binding of ^{125}I-labelled IgG-C7 was 41 and 33 (80% of normal) ng/mg of cell protein, respectively.

Table 5.1 LDL receptor activity in FH1a cells as a percentage of normal at 4°C and 37°C, assessed using ^{125}I-LDL and ^{125}I-IgG-C7 (Overleaf)

After incubation for 48 hours (or 24 hours, experiment 5) in lipoprotein-deficient medium, each cell monolayer received 2 ml (37°C) or 1.5 ml (4°C) of medium containing ^{125}I-LDL (7 μg/ml at 4°C, 10 μg/ml at 37°C) or ^{125}I-IgG-C7 (1 μg/ml at 4°C, 5 μg/ml at 37°C). After 2 hours at 4°C or 4 hours at 37°C the amount of ^{125}I-LDL and ^{125}I-IgG-C7 bound (4°C) or cell-associated and degraded (37°C) were determined. High-affinity values were determined by subtraction from total values of the values obtained in the presence of excess unlabelled LDL or IgG-C7, or, for IgG-C7 at 37°C, by subtracting the values obtained for sterol-treated cells (down-regulated by incubation with 1 μg/ml 25-hydroxycholesterol and 12 μg/ml cholesterol for 48 hours) or for receptor-negative cells (FH3b, experiment 9). In experiment 10, 80 μg/ml ^{125}I-LDL and 30 μg/ml ^{125}I-IgG-C7 were used for the assays at 37°C. ^{*125}I-β-VLDL was also used to assess LDL receptor activity in experiments 5 (4°C) and 9 (37°C). The value for suramin-releasable ^{125}I-β-VLDL binding at 4°C in experiment 5 was 34% of normal, and the values for cell-associated and degraded ^{125}I-β-VLDL at 37°C in experiment 9 were 54% and 31% of normal, respectively.

Expt.	Bound (4°C) (% normal)		Cell-associated (37°C)(% normal)		Degradation (37°C) (% normal)	
	^{125}I-LDL	^{125}I-IgG-C7	^{125}I-LDL	^{125}I-IgG-C7	^{125}I-LDL	^{125}I-IgG-C7
1	15	44				
2	22	82				
3	19	47.5				
4	15	51				
*5	13	44				
6	17	56	20	37	19	35
7			19	32	22	32
8			22	46	28	38
*9			32	49	23	30
Mean ±S.D.	17±3	54±14	23±6	41±8	23±4	34±4
10	17	44	20	41	29	45

the LDL receptor by sterols (18) decreased total binding of ^{125}I-LDL or ^{125}I-IgG-C7 by an amount equal to the high-affinity binding, i.e. down to 10% and 13% of total binding, respectively (data not shown).

In all experiments with cells from FH1a or 1b, the maximum high-affinity binding of IgG-C7 was several-fold higher than that of LDL. The results suggest the presence in FH1a and 1b of one receptor population, comprising about 20% of the normal number of fully active receptors possessing normal high affinity for LDL, and a second, consisting of inactive receptors which fail to bind LDL, but which can nevertheless be recognized immunologically by IgG-C7. This would explain the disproportionately high IgG-C7 versus LDL binding observed previously for FH1a and other homozygous FH subjects believed to carry the same haplotype (see Table 3.1).

Further evidence supporting the existence of an abnormal population of receptors in FH1a cells was obtained from the binding, at 4°C, of an additional ligand, ^{125}I-labelled β-VLDL. β-VLDL, which binds to the receptor via apoprotein E, has been shown to have less stringent requirements of receptor structure for binding (28,31). As described in the legend to Table 5.1 (Exp. 5), FH1a cells bound a higher proportion of ^{125}I-β-VLDL (similar to ^{125}I-IgG-C7 binding) than ^{125}I-labelled LDL at 4°C, suggesting that the abnormal receptors were able to recognize β-VLDL.

Since FH1a and 1b are homozygous for a single type of mutant allele, represented by haplotype 3, the results suggest that the same receptor protein is present in two forms, an active species, which binds LDL, and an inactive form, recognized by IgG-C7 (and β-VLDL). This differs from the idea, originally proposed by Goldstein, Brown *et al.* (3), that in class 2 variants, the slowly-processed receptors which eventually reach the surface constitute a homogeneous receptor population which binds LDL "inefficiently". FH1a

and 1b appear to express a functionally heterogeneous surface receptor population, consisting of the two forms described above.

5.2.1.2 Binding and metabolism of ^{125}I-labelled LDL and ^{125}I-labelled IgG-C7 at 37°C

When LDL receptor activity in FH1a was assessed on the basis of ^{125}I-LDL binding and metabolism at 37°C, approximately 20% of normal activity was observed (Table 5.1) with a normal affinity for LDL (see Figure 5.2,A and B). Mean apparent K_d values (± S.D.) from 3 experiments in normal cells were 6.9 ± 0.8 µg/ml for cell-associated (bound plus intracellular) ^{125}I-LDL, and 5.0 ± 1.7 µg/ml for degraded ^{125}I-LDL. FH1a cells yielded apparent K_d values of 7.7 ± 1.0 µg/ml and 9.0 ± 1.8 µg/ml for cell-associated and degraded ^{125}I-LDL, respectively. Similar results were obtained for FH1b (not shown).

FH1a cells bound and metabolized 30 to 40% of the normal level of ^{125}I-IgG-C7 at 37°C (Table 5.1) with an affinity similar to normal (see legend to Figure 5.2). The amount of ^{125}I-IgG-C7 cell-associated (bound and intracellular) by FH1a cells was 1.8 ± 0.2 (mean ± S.D.) times the amount of cell-associated ^{125}I-LDL (relative to normal), while the ratio of degraded ^{125}I-IgG-C7 to ^{125}I-LDL was 1.5 ± 0.2, compared to the ratio for 4°C binding of 3.2 ± 0.4. Thus, the same level of active LDL binding receptors as at 4°C was observed, but the apparent activity of the IgG-C7-recognizing receptors was diminished relative to that measured at 4°C. This was well-demonstrated in experiment 6 (Table 5.1) where assays at 4°C and at 37°C were performed in the same experiment.

One possible explanation for the apparently lower IgG-C7 binding and metabolism at 37°C versus the binding activity at 4°C in experiments 1 to 9 is that, if FH1a receptors have a lower affinity for IgG-C7 than normal at 37°C, estimation of the receptor level versus normal at a non-saturating

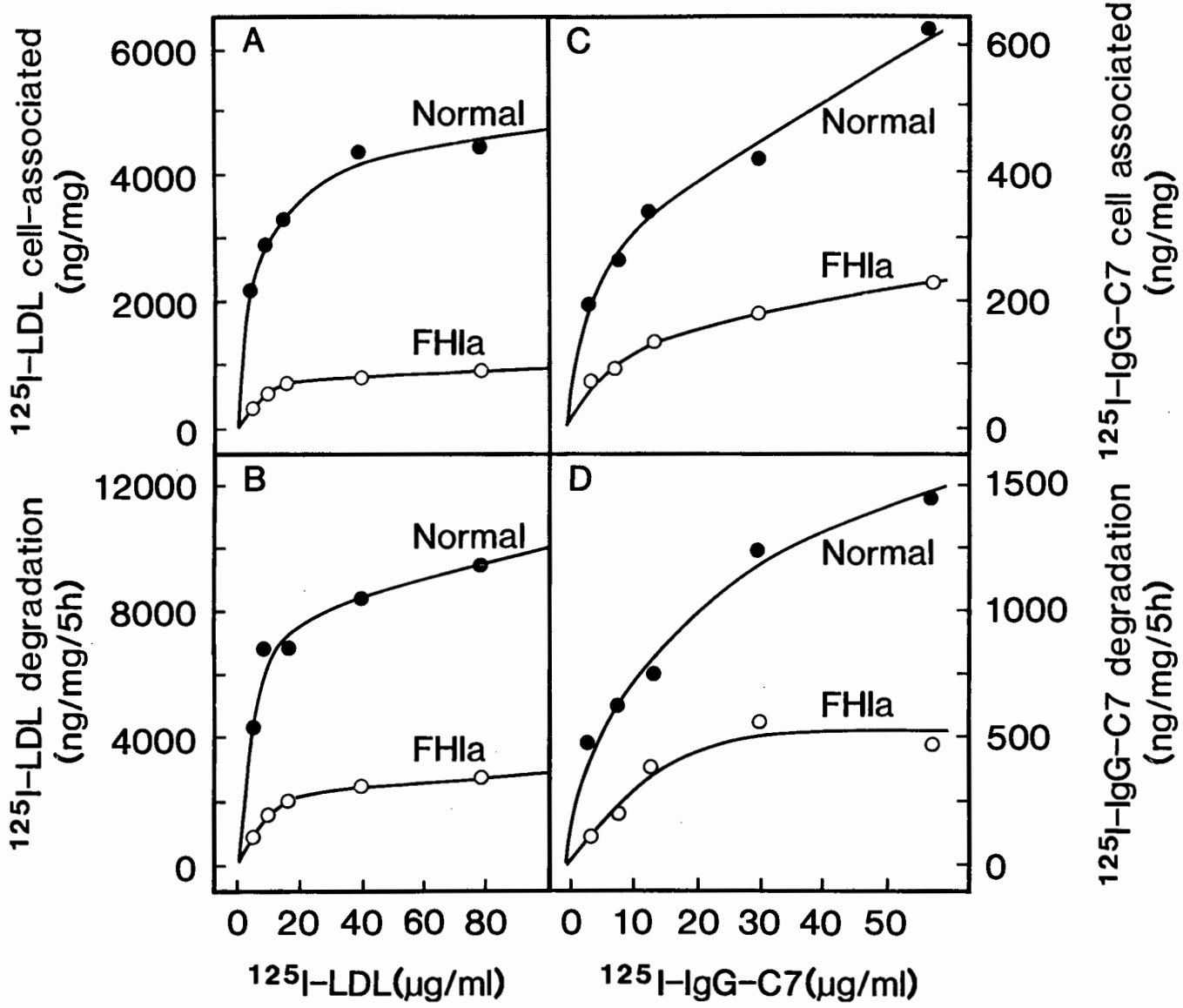

Figure 5.2 (legend overleaf)

Figure 5.2 Cell-associated and degraded ^{125}I-LDL and ^{125}I-IgG-C7 at 37°C as a function of concentration in normal and FH1a fibroblasts.

After incubation for 48 hours in lipoprotein-deficient medium, each cell monolayer received 2 ml of medium containing the indicated concentrations of ^{125}I-LDL (385 cpm/ng) [A and B] or ^{125}I-IgG-C7 (854 cpm/ng) [C and D]. After incubation for 5 hours at 37°C, the amounts of cell-associated (surface-bound plus intracellular) [A and C] and degraded [B and D] ^{125}I-LDL and ^{125}I-IgG-C7 were determined. The data are averages of duplicate determinations from a typical experiment and represent high-affinity values calculated by subtracting non-specific values, determined from cells which had been down-regulated by incubation for 48 hours in the presence of 1 µg/ml 25-hydroxycholesterol and 12 µg/ml cholesterol, from total values. The K_d values (calculated by non-linear regression) for cell-associated and degraded ^{125}I-LDL were 6.2 and 5.4 µg/ml, respectively, for normal cells, and 8.4 and 8.9 µg/ml, respectively, for FH1a cells. The K_d values for cell-associated ^{125}I-IgG-C7 were 14.4 and 14.3 for normal and FH1a fibroblasts, respectively, and were 15.6 and 12.5 for ^{125}I-IgG-C7 degradation in normal and FH1a cells, respectively. The maximum levels of cell-associated and degraded ^{125}I-LDL for FH1a cells were 21% and 31% of normal values, respectively, while those for cell-associated and degraded ^{125}I-IgG-C7 were 37% and 35% of normal, respectively.

concentration (5 μg/ml) could be falsely low. However, LDL receptors in FH1a cells showed a similar IgG-C7 affinity to normal at 37°C (Figure 5.2, C and D), and when saturating concentrations of ^{125}I-LDL and ^{125}I-IgG-C7 were used at 37°C (Experiment 10, Table 5.1), the amount of ^{125}I-IgG-C7 cell-associated in FH1a cells (as a percentage of normal) was 2.1 times the amount of cell-associated ^{125}I-LDL, while the ratio of degraded ^{125}I-IgG-C7 to ^{125}I-LDL was 1.6. (In the same experiment, the ratio of ^{125}I-IgG-C7 to ^{125}I-LDL binding at 4°C was 2.6). The ratios at 37°C agree well with those obtained at non-saturating IgG-C7 concentrations in experiments 6 to 9.

Since the amount of ligand or antibody that is cell-associated (bound and intracellular) or degraded at 37°C depends both on the number of receptors and their functional ability to internalise and recycle, the difference in the results at 4°C and 37°C suggests that, although the abnormal receptors bound IgG-C7 (but not LDL), their subsequent internalisation and, as a result, degradation of IgG-C7 was inefficient. The LDL internalisation index (intracellular+degraded / bound[heparin-releasable]) for the LDL binding receptor population in FH1a was previously shown to be normal (103). A similar internalisation index for IgG-C7 (i.e. for normal plus abnormal FH1a receptors) cannot be determined, as there is no satisfactory method for distinguishing surface-bound from intracellular IgG-C7.

The abnormal receptor population in FH1a cells was also able to bind and metabolize ^{125}I-labelled β-VLDL at 37°C, as shown by the higher level of cell-associated and degraded ^{125}I-β-VLDL (similar to ^{125}I-IgG-C7) relative to that for ^{125}I-LDL (see legend to Table 5.1, experiment 9).

5.2.2 LDL receptor synthesis rates

Studies of LDL receptor activity in FH1a and 1b (section 5.2.1) showed that although functional LDL receptor levels were 20% of normal values, the actual amount of

Table 5.2 LDL receptor synthesis rates in normal, FH1a and FH1b fibroblasts

[^{35}S]-methionine incorporation rates into immunoprecipitable LDL receptors and transferrin receptors and into total trichloroacetic acid-precipitable protein were determined in experiments 1 and 2 as described in the legend to Table 4.3. See Figure 5.13A for results of another experiment comparing synthesis rates in normal and FH1a cells.

Expt.	Upregulation time (h)	Cell type	LDL receptor synthesis/ Transferrin receptor synthesis		% Normal	LDL receptor synthesis/ Total protein synthesis		% Normal
1	30	Normal#1	2.00			3.09		
		Normal#2	1.67		100	3.88		100
				1.83±	±9%		4.34±	±35%
				0.17			1.54	
		Normal#3	1.83			6.06		
		FH1a	1.48		81%	3.40		78%
2	16	Normal	1.82		100%	3.71		100%
		FH1b	1.43		79%	2.07		56%

immunologically-detectable receptor protein in these cells was approximately 50% of normal (see Table 5.1). The contribution of LDL receptor synthesis rates to this somewhat lower receptor level was assessed by measuring initial rates of [^{35}S]-methionine incorporation into immunoprecipitable LDL receptors (120 kDa precursor + 160 kDa mature), relative to synthesis of total trichloroacetic acid-precipitable protein and immunoprecipitable transferrin receptor synthesis. The results in Table 5.2 show that LDL receptor synthesis rates in FH1a or 1b were always within 10% of the normal range (shown in experiment 1). Furthermore, LDL receptor synthesis rates were similar to normal at all levels of upregulation (see Figure 5.13A). Thus, the synthesis rates in FH1a and 1b cells were not sufficiently reduced relative to normal rates to account for the 50% lower surface LDL receptor levels.

5.2.3 Biosynthesis and processing of LDL receptors

The LDL receptor is synthesized in the endoplasmic reticulum as a precursor, with an apparent molecular weight of 120 kDa, that is transported to the Golgi apparatus, where carbohydrate processing reactions convert the receptor to the mature form, with an apparent molecular weight of 160 kDa (section 1.3). In normal cells, this conversion takes place with a half-time of approximately 15 minutes (see Figure 3.1A). However, preliminary biosynthetic studies on FH1a cells showed a marked delay ($t_{½}$ greater than 5 hours) in processing, and hence in transport to the Golgi, of the LDL receptor precursor (Figure 3.1C and Table 3.1).

Post-translational processing of newly-synthesized receptors in normal, FH1a and FH1b cells was compared. Fibroblasts were induced for LDL receptor synthesis by incubation for 18 hours in lipoprotein-deficient medium, and then pulse-labelled for 2 hours with [^{35}S]-methionine, followed by chase periods of one to nine hours. ^{35}S-labelled LDL receptors were then immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and

fluorography. The results obtained by densitometric scanning of the fluorograms are shown in Figure 5.3. In normal cells, complete conversion of the precursor (120 kDa) to mature (160 kDa) LDL receptors had taken place by the end of the one-hour chase period (Figure 5.3(a)). However, this maturation process, indicating rate of transport to the Golgi complex, was severely retarded in FH1a and 1b (Figures 5.3(b) and (c)). The half-time for processing in these cells was approximately 5 hours, and complete conversion to the mature forms required longer than 9 hours. FH1a and 1b therefore have LDL receptor mutations typical of Class 2 variants, i.e. ten-fold slower processing than normal (19,77).

Pronase digestion of intact FH1a cells was carried out after a 4-hour chase period (approximately equal amounts of ^{35}S-label in precursor and mature bands) to differentiate surface, Pronase-sensitive, LDL receptors from intracellular Pronase-resistant receptors. As was shown in Figure 4.6, p.127, for normal and FH3b cells, all the ^{35}S-labelled mature receptor in FH1a cells was susceptible to Pronase digestion and thus on the cell surface, whereas the long-lived precursor was Pronase-resistant and therefore remained intracellular (results not shown). This result suggests that although conversion to the mature form, and thus transport of receptors from the endoplasmic reticulum to the Golgi complex, was severely retarded, once the maturation was complete, subsequent transport to the cell-surface was rapid, since no intracellular (i.e. pronase-resistant) pool of mature receptors was detected.

The initial amounts of ^{35}S-label in the total LDL receptors after one hour of chase was the same in normal, FH1a and FH1b cells in the experiment shown in Figure 5.3, consistent with the essentially normal synthesis rates observed in these mutants (section 5.2.2). However, after 9 hours of chase, the total receptors in FH1a and 1b contained only approximately half as much ^{35}S-label as the normal

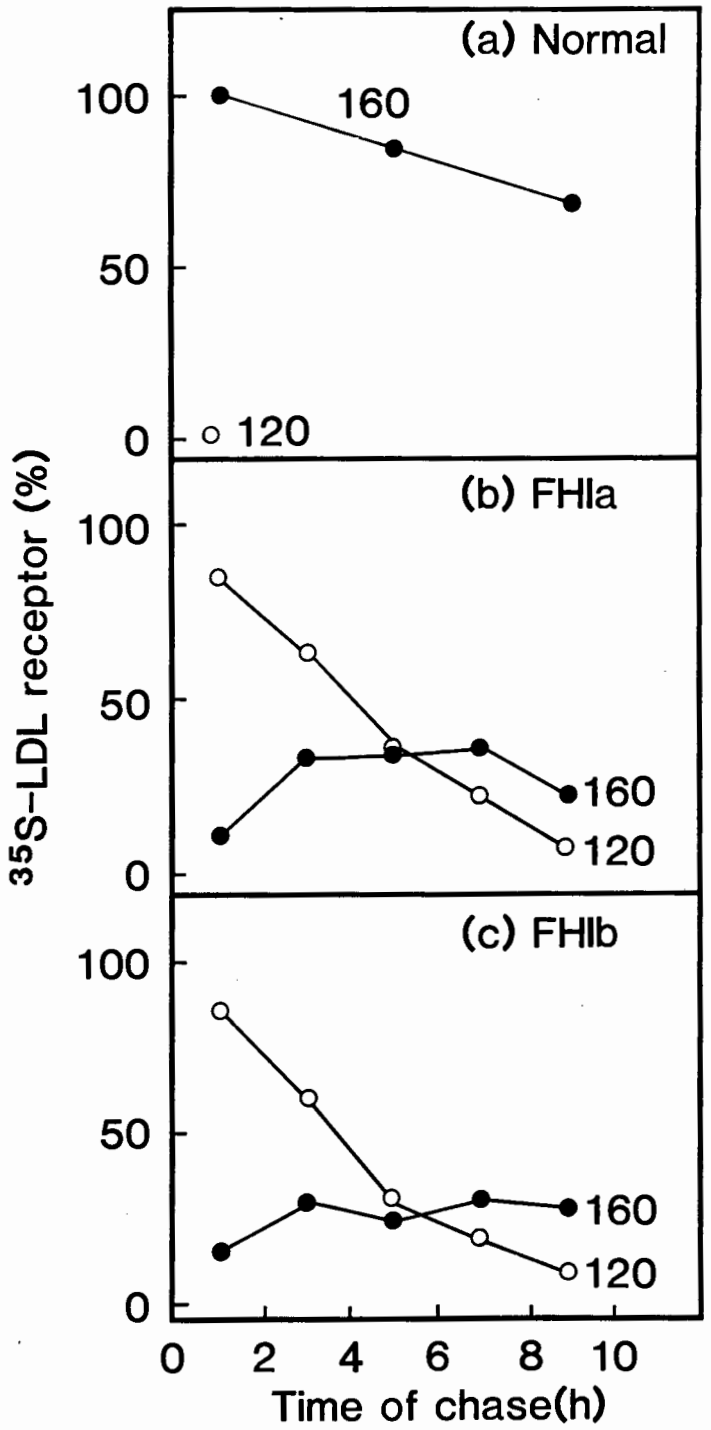

Figure 5.3 (legend overleaf)

Figure 5.3 Processing of LDL receptors in fibroblasts from a normal subject (a), and from FH1a (b) and FH1b (c).

Upregulated fibroblasts from a normal subject (a) and from FH1a (b) and 1b (c) were pre-incubated for 30 minutes at 37°C in methionine-free EMEM/LPDS and then pulse-labelled with [^{35}S]-methionine (30-40 μCi/ml) for 2 hours at 37°C. The medium was then changed to DMEM/LPDS containing 200 μM unlabelled methionine and the fibroblasts were incubated at 37°C for various chase times. The cells were then washed and solubilized, and ^{35}S-labelled receptors immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography. The ^{35}S-radioactivity in the 120 kDa precursor (O) and 160 kDa mature (●) fluorographic bands was quantified by densitometric scanning and is expressed as a percentage of the maximum ^{35}S-incorporation into total LDL receptors. The data points are averages of duplicates. In normal cells, complete conversion of precursor to mature receptors had occurred by the first chase point (1 h). (See Figure 3.1B for fluorogram of processing in FH1a, and Figures 3.1A (fluorogram) and 4.1 (a) (quantitation) for processing in normal cells).

receptors, suggesting abnormal receptor degradation. This is discussed later in the light of the measured half-life of the mature receptors in FH1a and 1b (section 5.2.4.1).

The electrophoretic analysis of post-translational processing of precursor receptors shown in Figure 5.3 (see also fluorogram of FH1a, Fig. 3.1C) was performed in 5-20% acrylamide gradients. To achieve better separation in the region of migration of the LDL receptor, 5-12% gradients and 7% acrylamide gels were used. Although 7% acrylamide achieved better separation, 5-12% gradients yielded sharper bands.

Analysis of post-translational processing of LDL receptors on 7% acrylamide gels (Figure 5.4A) showed that after a 30 minute pulse-labelling period for FH1a cells and a one-hour pulse for normal cells, the apparent molecular weights of the normal and mutant precursors were essentially identical. The normal cells also showed labelled mature receptor (160 kDa) due to rapid processing, and both cell types exhibited a non-specific contaminant band above 160 kDa found frequently in immunoprecipitations in this laboratory and others (36,93). Closer examination revealed that in FH1a cells, the slowly-processed precursor receptor showed a progressive increase in apparent molecular weight with time of chase, reaching a maximum apparent increase of 3 to 4 kDa after 6 hours of chase, at which stage approximately 80% conversion to the mature form had taken place. The precursor band also appeared to become more diffuse, and, by implication, more heterogeneous, with time.

A similar result was obtained for the experiment shown in Figure 5.4B (5-12% acrylamide). Comparison of the normal precursor band after a one-hour pulse-labelling period, with that in FH1a cells after a 2-hour pulse, showed heterogeneity in the mutant precursor band, suggesting a number of species with molecular weights ranging upwards from 120 kDa. When the 2-hour pulse was followed by a 4-hour chase period,

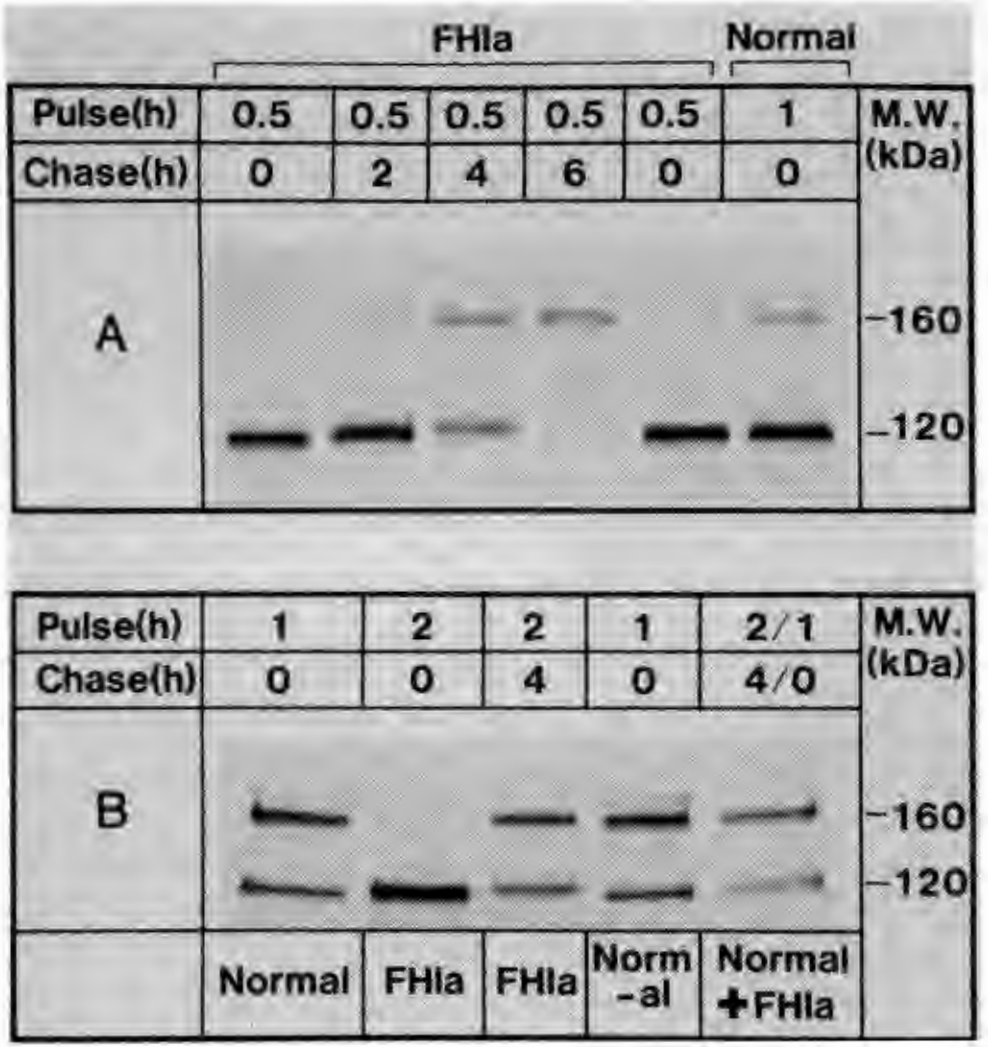

Figure 5.4 (legend overleaf)

Figure 5.4 Post-translational processing of LDL receptor precursors to mature forms in FH1a cells.

After 16 hours incubation in lipoprotein-deficient medium, fibroblasts from a normal subject and from FH1a were pre-incubated for 30 minutes in methionine-free EMEM/LPDS and then:

(A) pulse-labelled at 37°C for 30 minutes (FH1a) or 1 hour (normal) with [^{35}S]-methionine (60 μCi/ml). For FH1a cells, the medium was then changed to DMEM/LPDS (containing 200 μM unlabelled methionine) and the cells were incubated at 37°C for various chase times. The cells were then washed and solubilized. ^{35}S-labelled LDL receptors were immuno-precipitated and subjected to SDS-polyacrylamide gel electrophoresis (7% acrylamide) and fluorography.

(B) pulse-labelled at 37°C with [^{35}S]-methionine (30 μCi/ml) for 1 hour (normal) or 2 hours (FH1a) or 2 hours, followed by a 4-hour chase period (FH1a). ^{35}S-labelled receptors were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide) and fluorography. In lane 5, a combination of immunoprecipitates from normal cells pulse-labelled for 1 hour, and FH1a cells, pulsed for 2 hours and chased for 4 hours, was run in the same lane to illustrate the difference in the size of the respective precursor forms.

a sharper precursor band was obtained, corresponding to the highest apparent molecular weight of the range, thus illustrating again the time-dependent increase in apparent molecular weight. The difference in apparent molecular weight between the precursor in normal cells and the slowly-processed FH1a precursor receptor was evident from the combination of immunoprecipitates from normal and FH1a cells, giving rise to a doublet precursor band (Figure 5.4B, lane 5).

In the experiments shown in Figure 5.4, it was not possible to clearly distinguish whether or not the small increase in apparent precursor molecular weight gave rise to a concomitant increase in the molecular weight of the mature receptor, since the resolving power of the gel at higher molecular weights was not as good as in the region of the precursor.

The normal increase in apparent molecular weight of the LDL receptor from 120 kDa to 160 kDa is due to the addition of carbohydrate residues to the receptor, and occurs largely because of conformational changes induced by the glycosylation (see section 1.3). Thus, the small increase in apparent molecular weight observed for the mutant precursor in FH1a may be due to abnormal glycosylation and/or conformational changes in the receptor.

5.2.3.1 N-linked glycosylation of the LDL receptor

The LDL receptor contains 5 potential sites for asparagine- or N-linked glycosylation, of which only 2 sites (in the EGF-precursor-homologous region - see Figure 1.1) are glycosylated in the normal receptor (3). Since the other 3 sites are in the highly-repeated binding domain, where other transport-deficient receptors have been shown to have mutations (10,23,87), the possibility existed that abnormal conformation or unfolding of this region in the slowly-processed precursor in FH1a could lead to abnormal glycosylation of these sites, giving rise to the observed

increase in molecular weight. Alternatively, N-linked oligosaccharides with different, larger structures could be attached to the normal glycosylation sites. The role of N-linked glycosylation in the increase in precursor apparent molecular weight in FH1a cells was therefore investigated by (i) removal of "high mannose" N-linked oligosaccharides from the precursor by Endoglycosidase H (section 2.2.12.4) and (ii) inhibition of N-linked glycosylation by tunicamycin (section 2.2.12.3). Endoglycosidase H treatment of immunoprecipitated LDL receptors from normal and FH1a cells (shown in Figure 5.5) caused a decrease of approximately 4 kDa in apparent molecular weights of the normal and slightly larger mutant precursor receptors, by removal of high mannose N-linked oligosaccharides. However, no discernible effect on the difference in apparent molecular weight between normal and FH1a precursors was observed, since the Endoglycosidase H-treated mutant precursor still appeared to be about 3 kDa larger than the normal precursor which had been similarly treated. Thus, removal of N-linked oligosaccharides did not appear to abolish the increased apparent molecular weight of the FH1a precursor.

Figure 5.6 shows the effects of inhibition of N-linked glycosylation by pre-incubation with tunicamycin, on LDL receptors in normal and FH1a cells. During a 2-hour pulse in the absence of tunicamycin, the normal cells synthesized both precursor (120 kDa) and mature (160 kDa) receptors. As observed before (Figure 5.4B), after 2 hours of ^{35}S-labelling, a diffuse precursor band was produced in FH1a cells, apparently containing both newly-synthesized receptors of normal molecular weight, and slowly-processed precursors of slightly larger apparent molecular weight. After a 4-hour chase period, approximately 50% conversion to mature receptors had occurred, and only the slowly-processed precursors of slightly increased molecular weight remained in the precursor band. In the presence of tunicamycin, normal and FH1a cells both synthesized precursor receptors which were approximately 7 kDa smaller than normal.

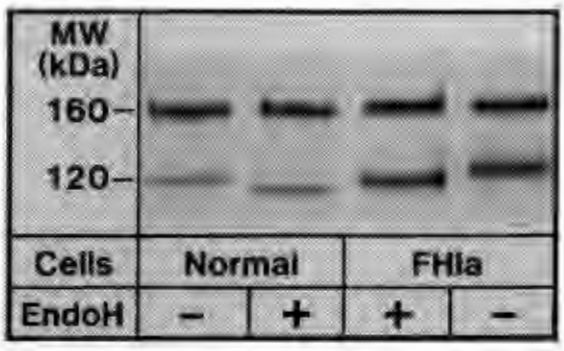

Figure 5.5 Effect of Endoglycosidase H treatment on ^{35}S-labelled LDL receptor precursors in normal and FH1a cells.

After 16 hours incubation in lipoprotein-deficient medium, fibroblasts from a normal subject and from FH1a were pre-incubated for 30 minutes in methionine-free medium, and then pulse-labelled at 37°C with ^{35}S-methionine (60 µCi/ml) for 1 hour (normal) or 2 hours followed by a chase for 4 hours in medium containing 200 µM unlabelled methionine (FH1a). ^{35}S-labelled LDL receptors were immunoprecipitated from solubilized cells, treated in the absence or presence of Endoglycosidase H as described in section 2.2.12.4, and then subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide gradient) and fluorography. Molecular weights of receptor bands were determined relative to molecular weight standards. The results are from one of two identical experiments which yielded similar results.

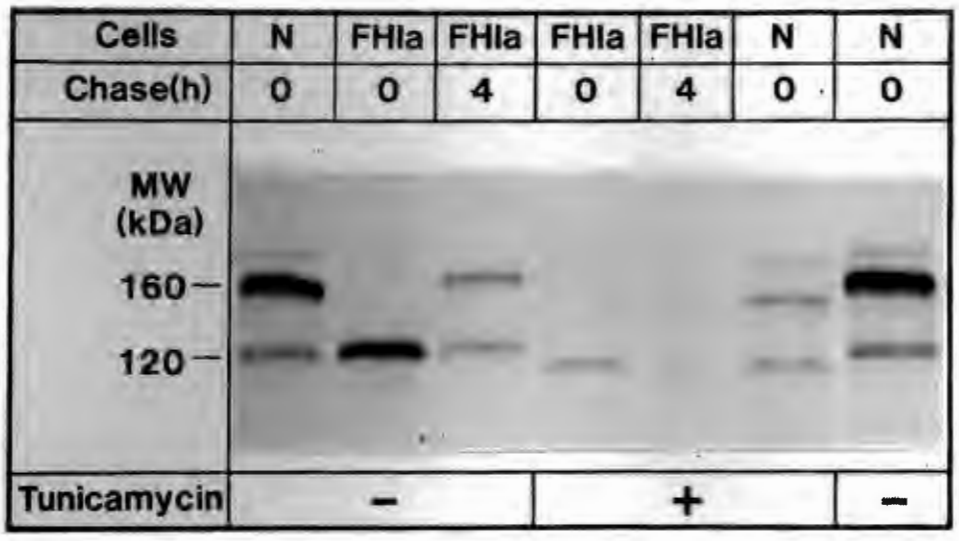

Figure 5.6 Effect of tunicamycin on the post-translational processing of the LDL receptor in FH1a and normal fibroblasts.

After incubation for 20 hours in lipoprotein-deficient medium, cells from a normal subject (N) and from FH1a were pre-incubated in the absence or presence of 10 μg/ml tunicamycin for 3 hours at 37°C. The cells were then pre-incubated for 30 minutes in methionine-free EMEM/LPDS, and pulse-labelled with [^{35}S]-methionine for 2 hours at 37°C, in the absence (40 μCi/ml) or presence (60 μCi/ml) of 10 μg/ml tunicamycin. After the pulse, some FH1a cells were incubated for a chase period of 4 hours in medium containing 200 μM unlabelled methionine, in the absence or presence of tunicamycin. The cells were solubilized and LDL receptors were immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide gradient) and fluorography. Molecular weights of LDL receptor bands were determined relative to molecular weight standards. The band above the 160 kDa band is a non-specific contaminant.

Although the precursor band after the pulse-labelling in FH1a appeared to be less diffuse than in the absence of tunicamycin, an increase in molecular weight (± 4 kDa) still occurred during the chase period (see faint band in lane 5). Processing appeared to be inhibited since there was clearly less than 50% conversion to the mature form in FH1a cells.

The N-linked sugar-deficient mature receptors in both cell types were 13 to 16 kDa smaller than normal. Since inhibition of N-linked glycosylation decreased the apparent molecular weight of the mature receptor more significantly than that of the precursor, it would appear that maturation of N-linked sugars contributes to the anomalously low mobility of the mature receptor on SDS-polyacrylamide gels, possibly as a result of terminal sialylation of N-linked oligosaccharides (see Figure 1.3). Kozarsky et al. (93) found that tunicamycin treatment of human fibroblasts decreased the apparent molecular weight of the precursor by approximately 8 kDa and that of the mature receptor by 10 kDa. Similar results were obtained by Cummings et al. (32) for fibroblasts and A431 cells, and by Davis et al. (33) for transfected Chinese hamster ovary cells.

Thus, removal of N-linked oligosaccharides from the precursor receptor in FH1a cells, or prevention of N-linked glycosylation of the receptor did not abolish the increase in apparent molecular weight of the slowly-processed precursor. The results suggest that the observed increase was not due to abnormal N-linked glycosylation. Therefore, the role of O-linked glycosylation was investigated.

5.2.3.2 O-linked glycosylation of the LDL receptor

In other transport-deficient mutant LDL receptors, the stalled precursor has been shown to contain N-linked sugars, which are added co-translationally, in addition to core O-linked sugars, which are added post-translationally, either in the endoplasmic reticulum (ER) or in a transitional zone between the ER and Golgi apparatus (63,64). Since these

are the sites where the blockage of transport of the mutant receptors takes place (64), abnormal post-translational O-linked glycosylation is more likely than abnormal N-linked glycosylation.

As shown in Figure 5.7 (last 2 lanes) when normal and FH1a cells were pulsed for 30 minutes with [^{35}S]-methionine, the newly-synthesized precursors had the same apparent molecular weight. However, comparison of a one-hour pulse in normal cells with a 2-hour pulse, followed a 4-hour chase period, in FH1a cells showed an increase in the apparent molecular weight of the slowly-processed precursor. No obvious increase in molecular weight of the mature FH1a receptor could be detected, but as previously discussed, this may have been due to the lower resolving power of the polyacrylamide gel in that particular range of molecular weights. Removal of sialic acid residues by neuraminidase treatment decreased the apparent molecular weight of the mature LDL receptors in both normal and FH1a fibroblasts by approximately 24 kDa (Figure 5.7), without affecting the precursor receptors which do not contain sialic acid. This is similar to the results of Cummings *et al*. (32), who showed decreases of 20 kDa in normal human fibroblasts and 25 kDa in A431 cells, while Kozarsky *et al*. (93) showed a decrease of greater than 17 kDa in fibroblasts.

Subsequent treatment of the desialylated, immuno-precipitated receptors with O-glycanase, which liberates the disaccharide Gal(β1$\rightarrow$3) GalNAc from O-linked oligo-saccharides, decreased the apparent molecular weight of the normal receptor by 8 kDa and that of the FH1a receptor by 14 kDa, in the experiment shown in Figure 5.7. However, in a subsequent experiment, O-glycanase decreased the molecular weights of both the normal and FH1a receptors by approximately 11 kDa. Thus, it was not clear whether or not O-glycanase caused a greater decrease in the molecular weight of the mutant receptor, which would have implied abnormal O-linked glycosylation. However, whereas the

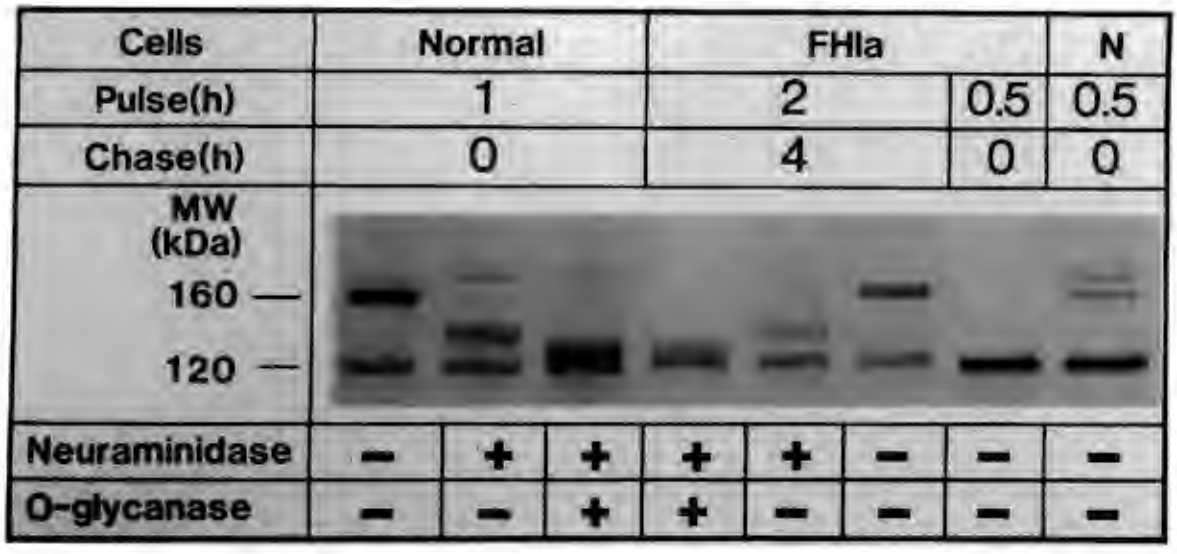

Figure 5.7 Effect of neuraminidase and O-glycanase treatment on ^{35}S-labelled LDL receptors in normal (N) and FH1a fibroblasts.

After incubation for 16 hours in lipoprotein-deficient medium, cells from a normal subject and FH1a were pre-incubated at 37°C for 30 minutes in methionine-free EMEM/LPDS, followed by pulse-labelling with [^{35}S]-methionine (30 μCi/ml) for 1 hour (normal) or 2 hours followed by a chase for 4 hours in medium containing 200 μM unlabelled methionine (FH1a). Normal and FH1a cells were also pulse-labelled for 30 minutes at 110 μCi/ml. ^{35}S-labelled LDL receptors were immunoprecipitated from solubilized cells and then treated in the absence or presence of neuraminidase, or neuraminidase followed by O-glycanase, as described in section 2.2.12.2. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis (5-12% acrylamide gradient) and fluorography. Molecular weights of receptor bands were determined relative to molecular weight standards. The band above the 160 kDa band is a non-specific contaminant.

precursors from normal and FH1a cells showed a clear difference in molecular weight, their corresponding O-linked sugar-deficient mature receptors migrated, in the same region of the gel as the precursors, with apparently identical molecular weights. This suggests that if the fully-glycosylated normal and mutant mature receptors had different molecular weights (which could not be distinguished because of insufficient resolution), then removal of O-linked oligosaccharides from the mature receptors had abolished their size difference. Unfortunately, a specific inhibitor of O-linked glycosylation (such as tunicamycin for N-linked glycosylation) or an enzyme for removal of core O-linked sugars from the precursor is not available to directly test the role of O-linked glycosylation in the increase in apparent molecular weight of the FH1a precursor.

It was interesting to note that O-glycanase treatment of the desialylated receptors in both cell types apparently did not remove all the O-linked oligosaccharides, since the receptors still migrated with slightly lower mobility than the precursors. If all the O-linked sugars were removed, the receptor should contain only two desialylated N-linked sugars, and should thus migrate at an apparent molecular weight of approximately 100 kDa, as found by Davis et al. (33) for the human LDL receptor transfected into receptor-deficient Chinese hamster ovary cells, or 110 kDa, as found by Yoshimura et al. (155) for the endogenous Chinese hamster ovary cell receptor. Cummings et al. (32) found that the O-linked sugars on the LDL receptor from fibroblasts, but not A431 cells, contained an additional monosaccharide postulated to be N-acetylglucosamine. Since O-glycanase is unable to remove the disaccharide of N-acetylgalactosamine and galactose if an N-acetylglucosamine substituent is present, this could explain why removal of the O-linked sugars from the fibroblast receptor was incomplete. However, the results suggest that the O-linked oligosaccharides are heterogeneous, since, as shown in

Figure 4.7, some removal occurred and thus some of the disaccharides must have been free of substituents.

The effects of N- and O-linked glycosylation of the LDL receptor on LDL- and IgG-C7 binding activity in normal and FH1a cells, are discussed in section 5.2.8.

5.2.4 Degradation rate of mature LDL receptors

Although the degradation rate of certain proteins has a regulatory function in determining the steady-state level in the cell, regulation of LDL receptor expression in normal fibroblasts is solely the result of changes in the rate of receptor synthesis (73). The steady-state level of surface LDL receptors in FH1a and 1b cells, determined by ^{125}I-IgG-C7 binding (section 5.2.1) was approximately 50% of normal, although the receptor synthesis rates in these cells were not significantly reduced (section 5.2.2). In post-translational processing studies (section 5.2.3), a more rapid loss than normal of ^{35}S-label from LDL receptors in FH1a cells was observed, suggesting abnormal receptor degradation, albeit far less dramatic than in the case of the receptor-negative mutants, FH3a and 3b (sections 4.2.3 and 4.2.4). To further investigate the possibility of abnormal receptor degradation rates in FH1a and 1b, the half-life of the mature LDL receptor was determined by [^{35}S]-methionine pulse-chase experiments, and from down-regulation kinetics of surface receptor activity measured by ^{125}I-LDL and ^{125}I-IgG-C7 binding.

5.2.4.1 Half-life of ^{35}S-labelled mature receptors

After induction of LDL receptor synthesis by incubation for 24 hours in lipoprotein-deficient medium, fibroblasts from a normal subject, FH1a, and FH1b were pulse-labelled with [^{35}S]-methionine and then chased until virtually none of the ^{35}S-label remained in the slowly-processed mutant precursors. This was assumed to be the "zero" chase time-point, and the rate of disappearance of ^{35}S-label from the mature receptor band thereafter was taken to be a measure of

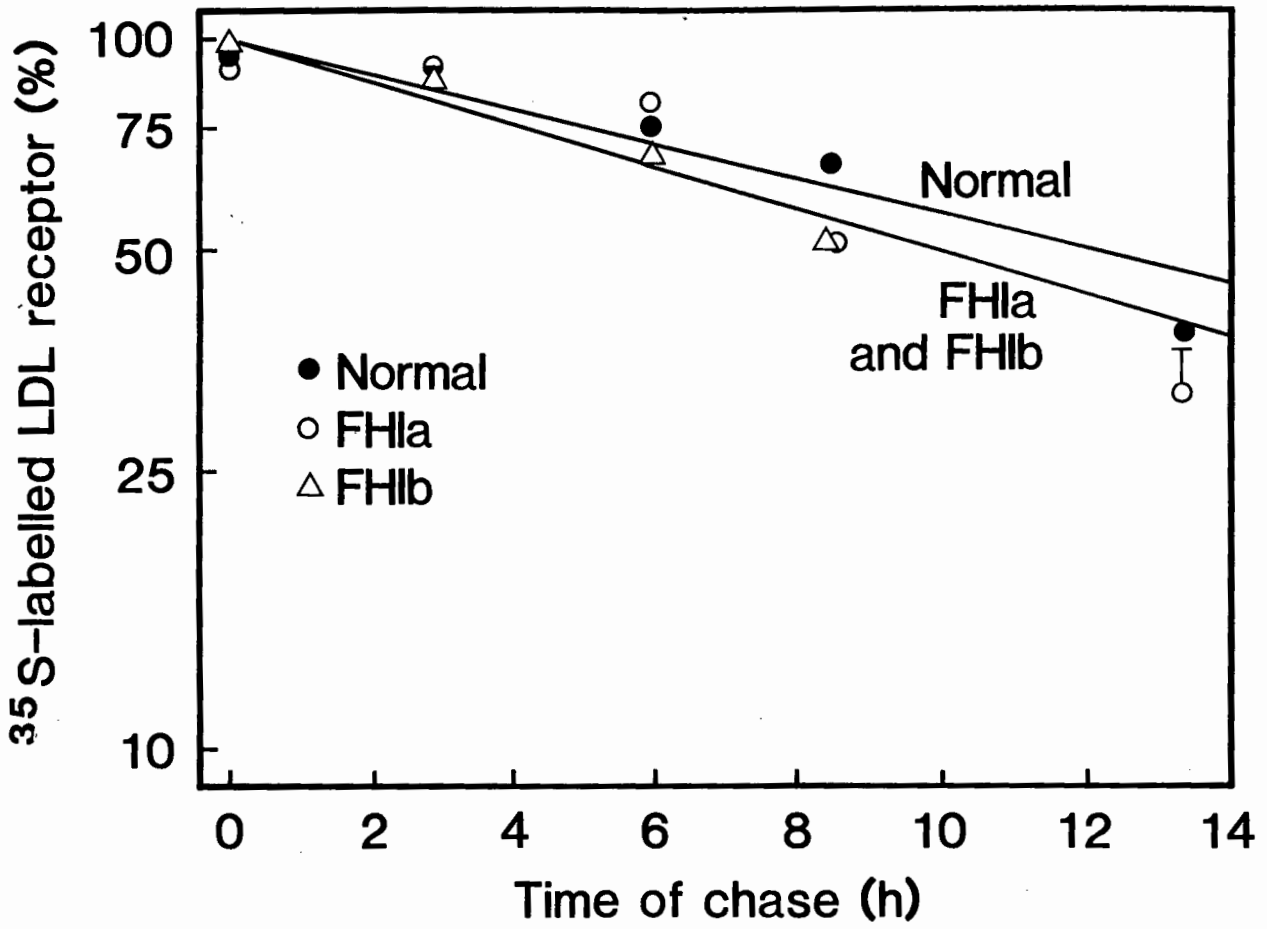

Figure 5.8 (legend overleaf)

Figure 5.8 Degradation of ^{35}S-labelled mature receptors in cells from a normal subject, FH1a and FH1b.

Fibroblasts from a normal subject, FH1a, and FH1b were incubated in lipoprotein-deficient medium for 24 hours to induce LDL receptor synthesis. The cells were pre-incubated for 30 minutes at 37°C in methionine-free EMEM, and then pulse-labelled with [^{35}S]- methionine (20 μCi/ml) for 4 hours. The medium was then changed to DMEM/LPDS (containing 200 μM methionine) and the fibroblasts were incubated at 37°C for 11.5 hours to ensure that virtually none of the ^{35}S-label remained in the precursor band. This was assumed to be the "zero" chase time point. After subsequent chase periods, immunoprecipitation, SDS-polyacrylamide gel electrophoresis (5-20% acrylamide) and fluorography of ^{35}S-labelled receptors were performed. The ^{35}S-label in fluorographic receptor bands was quantified by densitometric scanning. The data points are averages of duplicates from a single experiment and did not differ by more than 10% from the means, except where indicated by the error bar. The $t_{½}$ values (means ± S.E.M.) for LDL receptor degradation (determined by non-linear regression) were: normal 12.7 ± 1.6 hours, FH1a 10.3 ± 2.0 hours, FH1b 10.3 ± 1.1 hours.

its rate of degradation. Non-linear regression (section 2.2.8) of the data (Figure 5.8) from densitometric scanning of the fluorograms yielded $t_{½}$ values (mean ± S.E.M.) of 12.7 ± 1.6 hours for the normal receptor, and 10.3 ± 2.0 hours and 10.3 ± 1.1 hours, for FH1a and 1b, respectively. (These values were confirmed by down-regulation kinetics reported in section 5.2.4.2). Thus, the mature LDL receptors in FH1a and 1b, including both the LDL-binding population and the abnormal population which recognized only IgG-C7 (discussed in section 5.2.1) were turned over with apparently normal half-lives. To confirm this result, we attempted to measure the degradation rates of the LDL-binding and IgG-C7-binding populations separately, as discussed below (section 5.2.4.2).

The fact that the ^{35}S-labelled mature receptors in FH1a and 1b were apparently degraded at normal rates, suggested that the greater than normal loss of ^{35}S-label from total receptors (120 kDa + 160 kDa) observed during post-translational processing (Figures 5.3B and C) could be due to precursor degradation, as observed for other transport-deficient mutant receptors (23,82,88). A computer simulation of the time-course of processing in FH1a, assuming a half-life of 10.3 hours for the mature receptor, showed that the disappearance of ^{35}S-labelled precursors was more rapid than could be accounted for by processing to mature forms (Figure 5.9). The best correlation (solid line) with the experimental data points (from Fig. 5.3(b)) was generated by assuming that processing of precursor to mature receptors took place with a half-time of 5.1 hours, while precursor degradation occurred with a $t_{½}$ of 7.6 hours. The latter value is approximately 60% of the $t_{½}$ value for the normal LDL receptor, and the mature (160 kDa) receptor population in FH1a cells appeared to reach about 60% of the level that it should have, if no precursor degradation had occurred (dotted line). Thus, turnover of precursor receptors during slow processing may account for the lower than normal steady-state level of surface receptors in FH1a (54 ± 14% of normal) despite normal rates

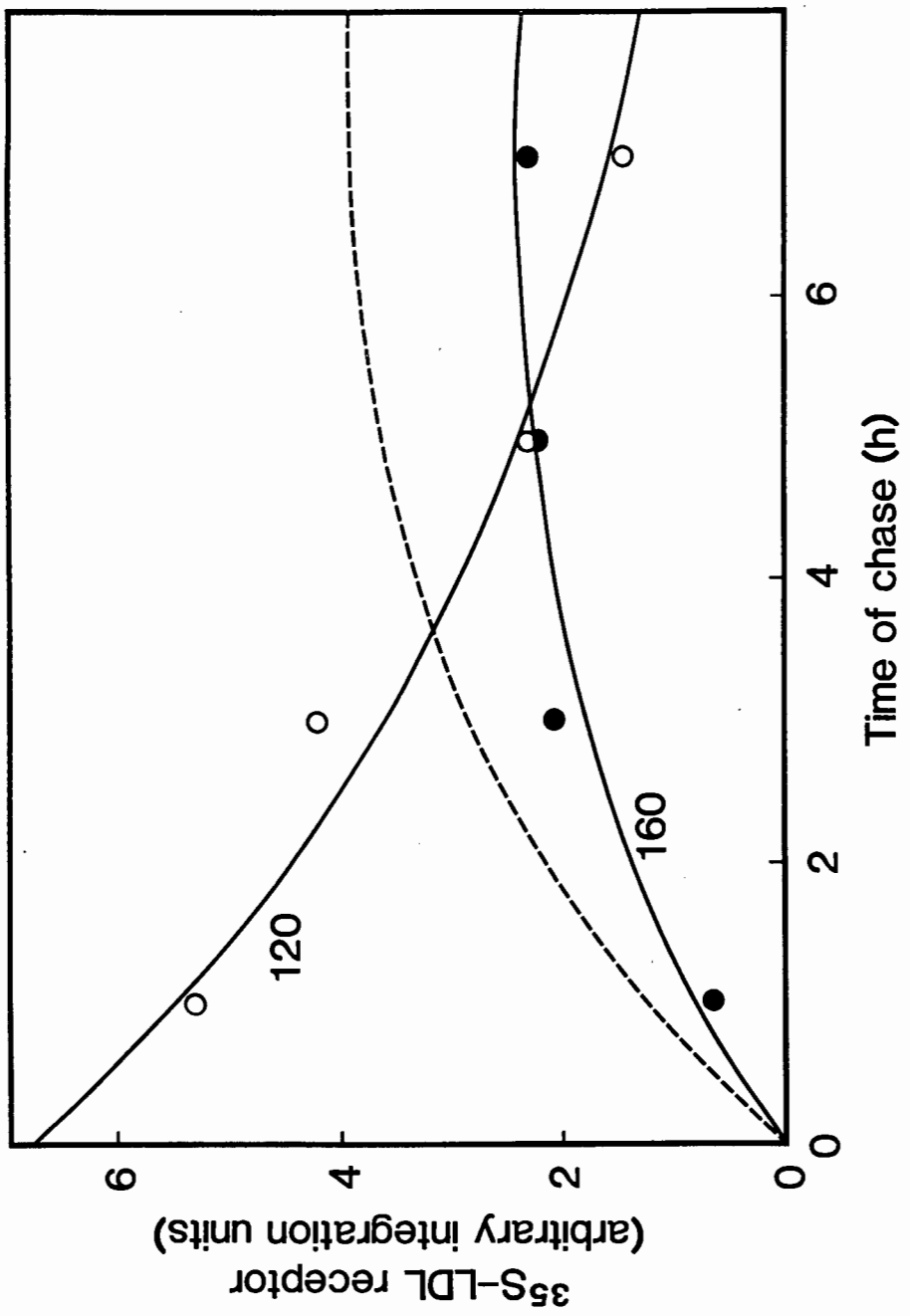

Figure 5.9 (legend overleaf)

Figure 5.9 Computer simulation of LDL receptor processing in FH1a cells.

The data points from Figure 5.3(B) (mature 160 kDa (O) and precursor 120 kDa (O) LDL receptors) were used for a computer simulation of LDL receptor processing. Assuming a $t_{½}$ of 10.3 hours for the degradation of the mature (160 kDa) receptor (from results in Figure 5.8), the amounts of mature receptor that would be generated if the precursor were quantitatively processed to the mature form (dotted line), or if processing and degradation of the precursor took place (solid line), were determined by fitting a single ($t_{½(processing)}$ = 3.3 h) or a double exponential decay curve ($t_{½(processing)}$ = 5.1h, $t_{½(degradation)}$ = 7.6 h), respectively, to the precursor data points (O).

of receptor synthesis (section 5.2.2) and mature receptor degradation.

5.2.4.2 Degradation rates of the LDL-binding and IgG-C7-binding receptor populations in FH1a.

LDL receptor activity in normal and FH1a fibroblasts was maximally upregulated by incubation for 48 hours in lipoprotein-deficient medium. The cells were then incubated in the presence of 40 μg/ml LDL to suppress receptor synthesis, and the decline in surface binding activity for ^{125}I-labelled-LDL and -IgG-C7, in the absence of synthesis, was then determined as a measure of degradation rates of the "normal" and "abnormal" receptor populations, respectively. As shown in Figure 5.10, the downregulation kinetics of the ^{125}I-LDL and ^{125}I-IgG-C7 binding activities, in both normal and FH1a cells, were virtually identical. Surface binding activity for both LDL and IgG-C7 decreased with a half-life of approximately 11 hours, which was consistent with the results from [^{35}S]-methionine pulse-chase experiments (section 5.2.4.1). At all stages of downregulation, FH1a cells bound 3 times more ^{125}I-IgG-C7 (60-70% normal) than ^{125}I-LDL (20-25% normal). [See Table 5.3].

Table 5.3 ^{125}I-LDL and ^{125}I-IgG-C7 binding at 4°C to fibroblasts from a normal subject and FH1a during downregulation shown in Fig. 5.10

Down-regulation (h)	Normal		FH1a			
	^{125}I-LDL Binding (ng/mg)	^{125}I-IgG-C7 Binding (ng/mg)	^{125}I-LDL Binding (ng/mg)	% of Normal	^{125}I-IgG-C7 Binding (ng/mg)	% of Normal
0	215 (100%)	35 (100%)	40 ("100%")	19	25 ("100%")	62
8.7	131 (61%)	23 (66%)	22 ("56%")	17	15 ("65%")	65
20.7	38 (18%)	7 (20%)	10 ("20%")	26	5 ("23%")	71

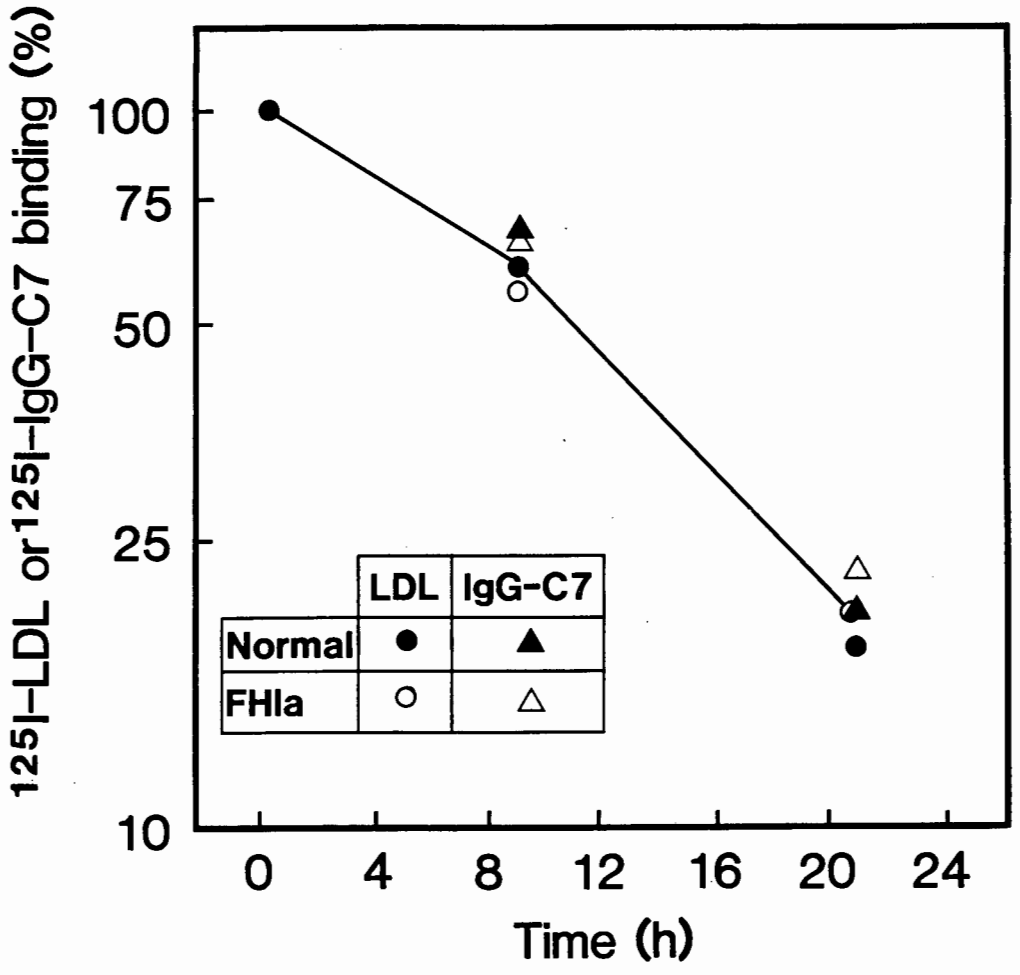

Figure 5.10 Downregulation kinetics of IgG-C7 and LDL-binding populations in normal and FH1a fibroblasts.

After incubation for 48 hours in lipoprotein-deficient medium, each monolayer received 2 ml of medium containing 40 µg/ml LDL. At zero time and after 8.7 hours and 20.7 hours incubation at 37°C, the cells were analyzed at 4°C for cell-surface binding of ^{125}I-LDL (407 cpm/ng, 5.5 µg/ml) and ^{125}I-IgG-C7 (2812 cpm/ng, 0.8 µg/ml). The data shown from a single experiment represent high-affinity values calculated by subtracting non-specific values (means of duplicates), obtained in the presence of excess unlabelled LDL (300 µg/ml) or IgG-C7 (50 µg/ml), from total values (means of triplicates), and are expressed as percentages of the values at zero time. The line joins the mean percentage values at each time point.

Thus, despite their functional differences, the LDL- and IgG-C7-binding receptor populations were degraded at the same rate, similar to the rate for normal LDL receptors. In addition, the results of the downregulation showed that, at a receptor level where ^{125}I-LDL binding in normal cells was 20% of the usual upregulated level (as in FH1a), ^{125}I-IgG-C7 binding to the same cells was also down to 20% of the normal level. Thus the higher ^{125}I-IgG-C7 binding relative to ^{125}I-LDL binding in FH1a was not an artifact of low receptor levels.

Whereas the same kinetics for LDL receptor downregulation were observed in normal and FH1a cells in the presence of 40 µg/ml LDL, at a level of 10 µg/ml LDL, FH1a cells still exhibited 60% of the upregulated receptor activity even after 20 hours of incubation, while normal cells showed a similar time-course of downregulation to that observed for 40 µg/ml LDL (results not shown). LDL receptor activity is suppressed in response to cellular cholesterol levels (6-9). Goldstein et al. (142) have shown that in cells from an FH heterozygote, a 2.5-fold higher LDL concentration than for normal cells was required for 50% suppression of the LDL receptor level within three days. This was presumably because of the diminished rate of delivery of LDL-cholesterol to the cells via the lower receptor number (approximately 50% of normal) in the FH heterozygote cells. Consistent with these results, FH1a cells required a 4-fold higher concentration of LDL (40 µg/ml) to achieve the same level of suppression within 20 hours as normal cells at 10 µg/ ml LDL, because they express only approximately 25% of the normal level of LDL-binding receptors.

5.2.5 Phenotypic expression of LDL receptors in cells from FH heterozygous subject, 1d.

On the basis of ^{125}I-LDL binding and metabolism at 37°C, cells from heterozygous FH subject, 1d (the father of FH1a

and 1b) were previously shown to express 60 to 80% of normal LDL receptor activities (103), consistent with expression of one normal allele, and one mutant allele giving rise to a "high-defective" receptor level. Biosynthetic studies, using [^{35}S]-methionine labelling and immunoprecipitation of receptors, showed that subject 1d expressed a LDL receptor phenotype intermediate between normal and FH1a cells (Figure 5.11). After a 2-hour pulse, approximately 90% of the ^{35}S-label was in the mature receptor band in normal cells, and after a 1-hour chase period, no label remained in the precursor form. In contrast, in FH1a cells, the vast majority of the label remained in the precursor receptor band, even after the 1 hour chase period, only reaching an equal distribution between precursor and mature receptors after 4 hours of chase. In cells from subject 1d, 70% of the ^{35}S-label was in the precursor after pulse-labelling, but within one hour of chase, the mature receptor band contained 65% of the label, presumably mainly due to rapid processing of the normal gene product. At the end of the 4-hour chase period, the proportion of ^{35}S-label in the mature receptors had increased to 85%, consistent with additional slow processing of the abnormal receptors. The results suggest that the receptor proteins, produced by the normal and mutant alleles in the heterozygous cells, are synthesized and processed independently, so that the phenotypic expression of the normal allele is unaffected by the abnormal allele in the same cell, and vice versa.

5.2.6 Immunoblotting of solubilized LDL receptors from normal, FH1b and FH3b cells.

The WHHL rabbit produces transport-deficient LDL receptor precursors which undergo essentially no processing to mature receptors (23). Ligand-blotting of solubilized LDL receptors from these cells visualized a significant cellular precursor pool and no detectable mature receptor pool, in contrast to normal cells where at least 90% of the cellular

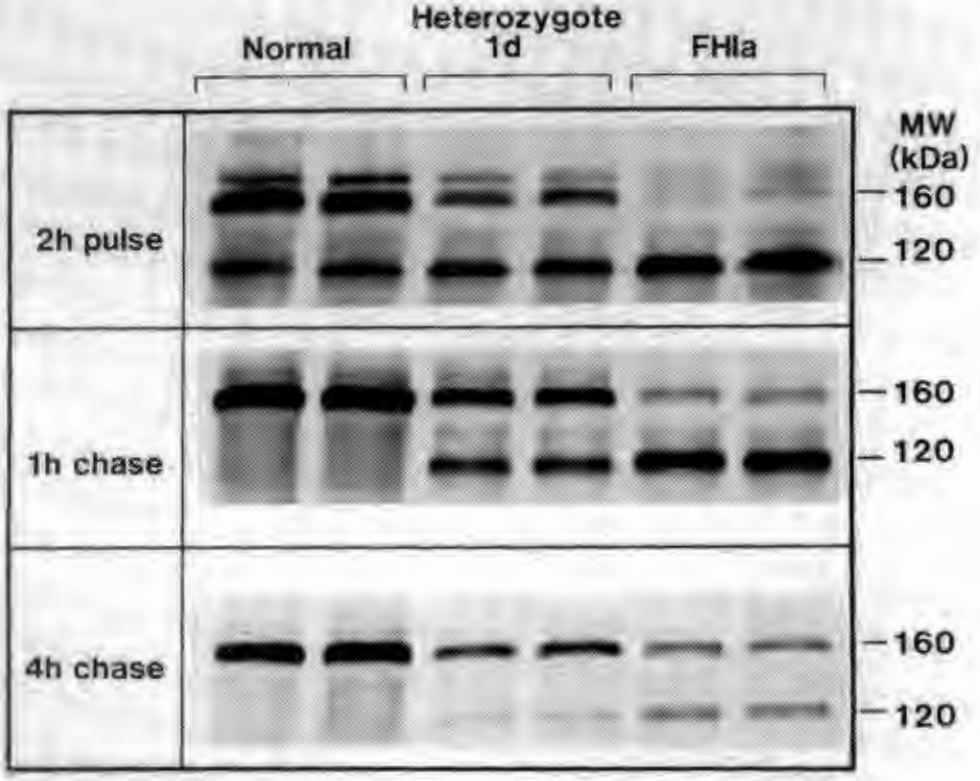

Figure 5.11 Biosynthesis and processing of ^{35}S-labelled LDL receptors in cells from a normal subject, FH heterozygote, 1d and FH homozygote, 1a.

Fibroblasts from a normal subject, FH heterozygote, 1d and FH homozygote, 1a, were incubated in lipoprotein-deficient medium for 24 hours, pre-incubated for 30 minutes in methionine-free EMEM, and pulse-labelled with [^{35}S]-methionine (80 μCi/ml) for 2 hours, at 37°C. Cells were harvested after pulse-labelling, or after 1-hour and 4-hour chase periods in DMEM/LPDS containing 200 μM unlabelled methionine. ^{35}S-labelled LDL receptors were immuno-precipitated and subjected to SDS-polyacrylamide gel electro-phoresis (5-12% acrylamide gradient) and fluorography. The band above the 160 kDa band is a non-specific contaminant.

receptors were visualized in the mature form (23). Since FH1a cells exhibited retarded precursor processing and approximately 50% of normal mature surface receptor levels, the possibility existed of a precursor pool, comparable in size to the mature receptor pool, in these cells. The precursor receptors in FH1a fibroblasts were known to bind IgG-C7 (from immunoprecipitation studies) and immunoblotting with IgG-C7, followed by detection with ^{125}I-labelled goat-anti-mouse IgG, was therefore carried out on normal, FH1a and FH3b (receptor-negative) cells, to determine whether or not FH1a cells had a large, intracellular LDL receptor precursor pool.

Immunoblotting of receptors from the three cell types, in which LDL receptor activity had either been upregulated or downregulated for 48 hours, was performed as described in section 2.2.13. In normal cells, a distinct band, at a molecular weight of 130 kDa, was immunologically detected in upregulated, but not downregulated cells (Figure 5.12, lanes 1 and 2). This corresponds to the molecular weight of the LDL receptor under non-reducing conditions (161). In contrast, in FH3b cells, no receptor band could be detected and no difference between up- and down-regulated cells could be seen (lanes 5 and 6), consistent with the "receptor-negative" phenotype of these cells (section 4.2.1). An intermediate amount of 130 kDa receptor was observed in FH1a cells, in addition to a faint band at approximately 92 kDa (lane 3), which was not present in down-regulated cells (lane 4), and presumably represented a small, intracellular precursor pool, not detected in normal cells. The fact that very low levels of precursor receptor, relative to the amount of mature receptor, were visualized by immunoblotting in FH1a cells, lends further support to the idea of degradation of the transport-deficient precursors retained in the endoplasmic reticulum in these cells (section 5.2.4.1).

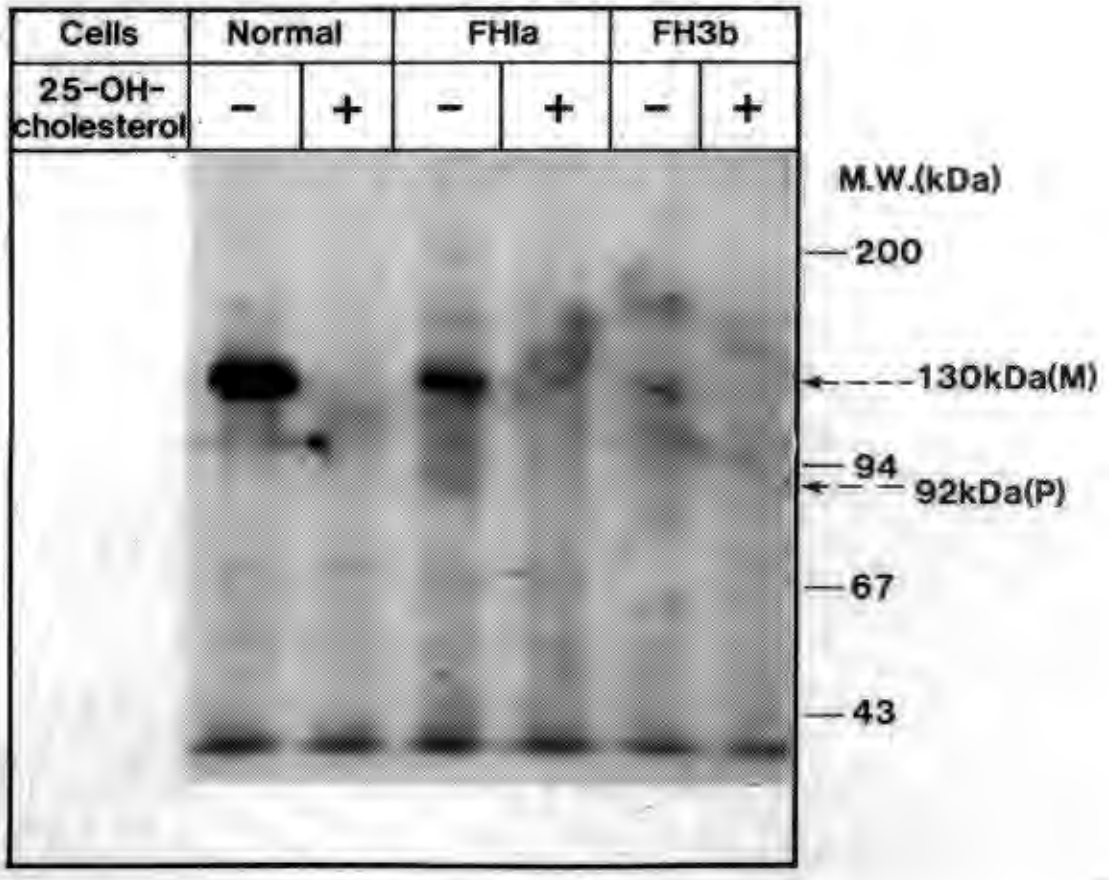

Figure 5.12 Immunoblotting of solubilized LDL receptors from normal, FH1a and FH3b fibroblasts

After incubation in lipoprotein-deficient medium for 48 hours in the absence (upregulation) or presence (downregulation) of 2 µg/ml 25-hydroxycholesterol, the monolayers were washed, scraped from the dishes, collected by centrifugation, solubilized, and subjected to SDS-polyacrylamide gel electrophoresis in a 7% acrylamide gel under non-reducing conditions. Proteins and molecular weight standards were transferred electrophoretically to a Hybond-C nitrocellulose membrane at 100 mA for 16 h at 4°C. The membrane was then incubated, as described in section 2.2.13, with IgG-C7 and ^{125}I-labelled goat-anti-mouse IgG, washed, dried, and subjected to autoradiography for 3 days at -70°C.

If the blotting technique is performed quantitatively, particularly in terms of the amount of solubilized cell protein electrophoresed, it may be possible, by using both immunoblotting with IgG-C7, and ligand blotting with LDL, to comparatively demonstrate the small "normal", LDL-binding, and much larger "abnormal" IgG-C7 binding, receptor pools in FH1a (and 1b) cells.

5.2.7 Appearance at the cell-surface of newly-synthesized LDL- and IgG-C7-binding receptor populations in FH1a cells

It has been shown (section 5.2.4.2) that the LDL- and IgG-C7-binding receptor populations in FH1a cells were degraded at the same rates. Although initial receptor synthesis rates in FH1a and 1b cells were shown to be normal (section 5.2.2), no information was available on the formation of the functionally distinct forms which originate from the same newly-synthesized protein. To address this aspect, normal and FH1a cells, which were growing in medium containing 10% foetal calf serum, were incubated for 72 hours in lipoprotein-deficient medium to upregulate LDL receptor activity. During upregulation, initial LDL receptor synthesis rates and ^{125}I-LDL and -IgG-C7 binding were measured to monitor induction of receptor synthesis and the appearance of newly-synthesized receptors at the cell surface. Upregulation of synthesis rates (Figure 5.13A) and receptor levels (Figure 5.13B) in FH1a cells followed a similar time-course to that in normal cells, reaching maximum levels after 48 hours in lipoprotein-deficient medium. Throughout the incubation, FH1a cells exhibited approximately 3-fold higher IgG-C7- than LDL-binding activity, suggesting that the abnormal form of the mutant receptors predominated even in newly-synthesized surface receptors.

The results of the computer simulation in Figure 5.9 (p.180) suggested degradation of slowly-processed precursor receptors with a half-life of approximately 60% of the

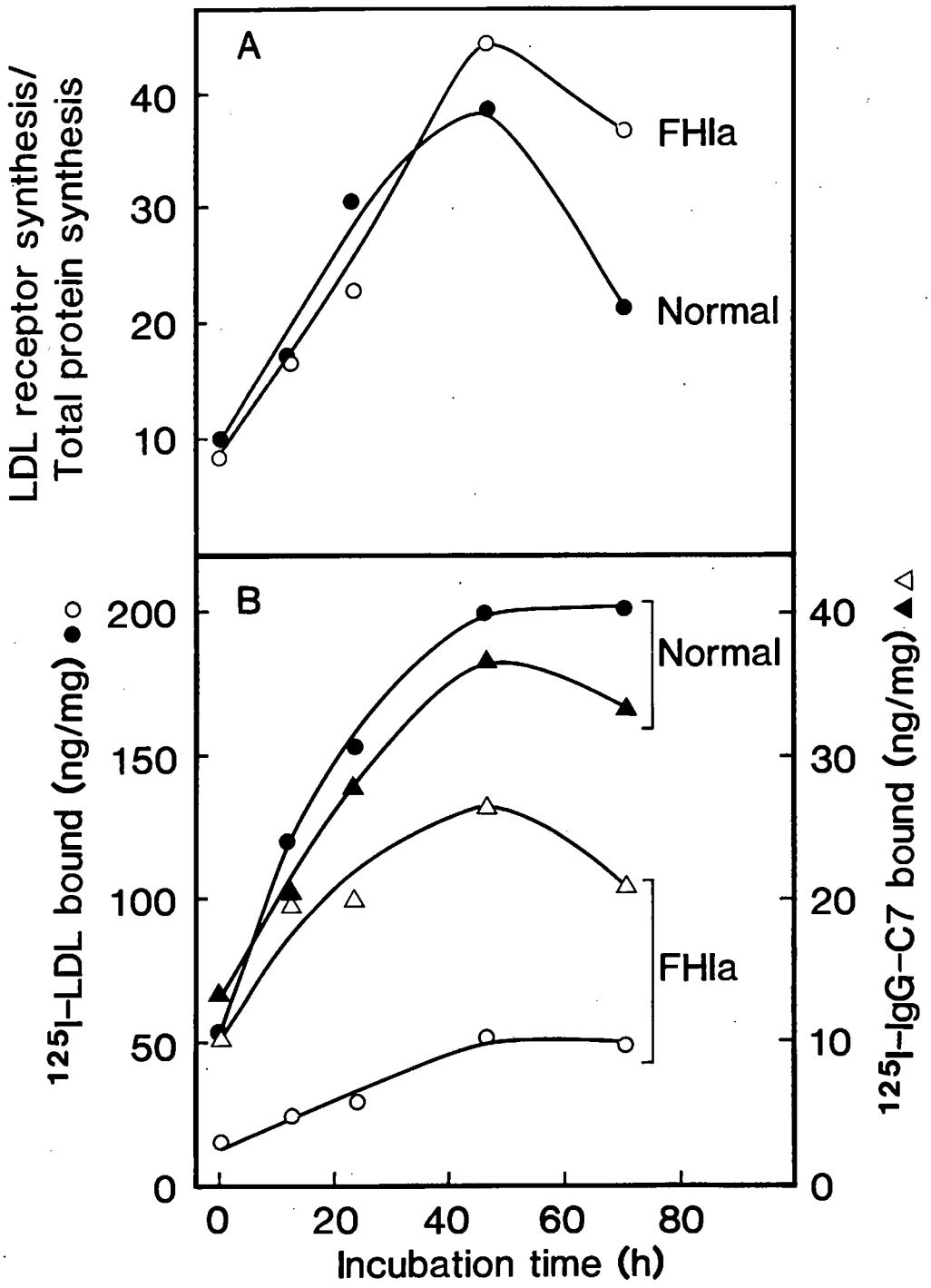

Figure 5.13 (legend overleaf)

Figure 5.13 LDL receptor synthesis rates (A) and surface expression (B) in normal and FH1a cells during a 72-hour period of incubation in lipoprotein-deficient medium.

Fibroblasts from a normal subject and FH1a were seeded (Day 0) at $1x10^5$ cells per 60 mm Petri dish in medium containing 10% foetal calf serum. Medium was changed on the 3rd day and on day 4 was replaced with lipoprotein-deficient medium (DMEM supplemented with LPDS at 2.5 mg protein/ml). Medium was changed every 24 hours thereafter. LDL receptor synthesis rates (A) (arbitrary integration units per mg cell protein) were determined (in duplicate) from the amount of [^{35}S]-methionine incorporated into IgG-C7-immunoprecipitable LDL receptors during a 30 minute pulse-labelling period, and are expressed relative to [^{35}S]-methionine incorporation into total trichloroacetic acid-precipitable protein (dpm ^{35}S-label incorporated x 10^{-5}) during the same incubation. LDL receptor activity was assayed by measuring ^{125}I-LDL (260 cpm/ng) and ^{125}I-IgG-C7 (460 cpm/ng) binding levels (B) after 2 hours at 4°C. The data are averages of duplicates from a single experiment, and did not differ by more than 12% from the means, and represent high-affinity values calculated by subtraction of non-specific binding obtained in the presence of excess unlabelled LDL (300 µg/ml) or IgG-C7 (50 µg/ml). Non-specific binding was approximately 10% of the total binding in normal cells after 47 hours in lipoprotein-deficient medium. High-affinity binding values (ng/mg cell protein) were normalized to the rate of protein synthesis at each time point, taking the rate for normal cells at 47 hours to be unity, in order to express binding in terms of viable cell protein rather than total protein content. Although a decrease in protein synthesis was observed after changing from full medium to lipoprotein-deficient medium, protein synthesis per mg protein between 12 and 72 hours incubation in the latter medium did not vary by more than 10% in normal cells, or by more than 22% in FH1a cells.

normal receptor half-life. Consistent with these results, surface receptor levels in FH1a cells at maximum upregulation after 48 hours, as assessed by ^{125}I-IgG-C7 binding, were only 70% of normal (Figure 5.13B), although initial receptor synthesis rates in FH1a fibroblasts were slightly higher than normal (Figure 5.13A).

5.2.8 Effect of pre-incubation of FH1a cells with ammonium chloride and LDL on the LDL- and IgG-C7-binding receptor populations

As discussed in section 2.2.9, normal LDL receptors are trapped intracellularly in the presence of ammonium chloride and LDL, and rapidly degraded. Since this process is LDL-dependent, it is believed that disruption of endosomal pH by ammonium chloride inhibits the acid-dependent dissociation of bound LDL, and therefore receptor-LDL complexes are trapped and degraded. Because the trapping and degradation appear to depend on LDL binding, it was of interest to determine the effects of ammonium chloride plus LDL on the LDL-binding receptors, and the abnormal receptors that do not recognize LDL, in FH1a cells.

5.2.8.1 Effect of ammonium chloride and LDL on surface receptor activity in FH1a cells

Upregulated fibroblasts from a normal subject and FH1a were pre-incubated for 30 minutes at 37°C with 40 µg/ml LDL, followed by a one-hour incubation in the presence of both 40 µg/ml LDL and 10 mM ammonium chloride. After releasing bound unlabelled LDL with heparin, surface receptor activity was determined from ^{125}I-LDL or -IgG-C7 binding at 4°C. As shown in Table 5.4, ^{125}I-LDL-binding in normal and FH1a cells decreased to approximately 30% of the control value after incubation for 1 hour with ammonium chloride (10 mM) and LDL (40 µg/ml) presumably because of intracellular trapping of undissociated receptor-ligand complexes. However, although ^{125}I-IgG-C7 binding decreased by the same amount in normal cells, 60% of the original IgG-C7 binding receptors in FH1a were detectable at the surface, implying

Table 5.4 Effect of ammonium chloride and LDL on ^{125}I-IgG-C7 and ^{125}I-LDL surface binding in normal and FH1a fibroblasts

After incubation for 48 hours in lipoprotein-deficient medium, fibroblast monolayers from a normal subject and FH1a were either incubated for 30 minutes at 37°C in the presence of 40 µg/ml LDL, or for 30 minutes with 40 µg/ml LDL followed by one hour at 37°C in the presence of 40 µg/ml LDL plus 10 mM ammonium chloride. After releasing bound unlabelled LDL for 40 minutes at 4°C with 0.3% heparin in PBS, the cells were analyzed at 4°C for cell-surface binding of ^{125}I-LDL (406 cpm/ng, 6 µg/ml) and ^{125}I-IgG-C7 (420 cpm/ng, 0.9 µg/ml). The data shown represent high-affinity values calculated by subtraction of non-specific values obtained in the presence of excess unlabelled LDL (300 µg/ml) or IgG-C7 (50 µg/ml) from total values. Values are means of duplicates from a single experiment. In 3 separate experiments ^{125}I-IgG-C7 binding was reduced after 1 hour with ammonium chloride and LDL to 32±4% (mean ± S.D.) in normal cells and to 61±4% in FH1a cells.

Cells	^{125}I-LDL binding (ng/mg)		^{125}I-IgG-C7 binding (ng/mg)	
	30 min + LDL	1 h + NH_4Cl + LDL	30 min + LDL	1 h + NH_4Cl + LDL
Normal	202	74 (37%)	71	24 (34%)
FH1a	50 (25% normal)	15 (30%) (20% normal)	31.5 (44% normal)	18.5 (59%) (77% normal)

that the majority of these receptors did not bind LDL, and were therefore not trapped in the presence of ammonium chloride. In 3 separate experiments, ^{125}I-IgG-C7 binding activity in FH1a cells showed a significantly smaller decrease than in normal cells, after incubation with ammonium chloride and LDL (see legend to Table 5.4). These results therefore supported the postulate of populations of both normal and abnormal receptors in FH1a (and 1b) cells, previously proposed on the basis of ^{125}I-LDL and ^{125}I-IgG-C7 binding curves (section 5.2.1). In addition, the results suggested that the LDL-binding population of receptors in FH1a could function independently of the abnormal population.

5.2.8.2 Effect of ammonium chloride and LDL on ^{35}S-labelled, IgG-C7 immunoprecipitable receptors in FH1a cells

After incubation in lipoprotein-deficient medium to induce LDL receptor synthesis, fibroblasts from a normal subject and FH1a were pulse-labelled with [^{35}S]-methionine, and then chased until virtually none of the ^{35}S-label remained in the slowly-processed mutant precursors. This was assumed to be the "zero" chase-point. The cells were then pre-incubated with 10 mM ammonium chloride in the absence or presence of 40 µg/ml LDL for 3 hours at 37°C (Figure 5.14A). In a second experiment (Figure 5.14B), cells were pre-incubated for 3 hours in the presence of either no additions, ammonium chloride, LDL, or ammonium chloride plus LDL, and, in addition, for 6 hours in the presence of LDL and ammonium chloride.

As shown in Figure 5.14A, incubation of normal cells with ammonium chloride and LDL caused a dramatic decrease in the amount of ^{35}S-labelled mature LDL receptors which could be immunoprecipitated, whereas the presence of ammonium chloride alone had no effect. As discussed previously (section 2.2.9), this was interpreted to indicate that receptors bound to LDL were trapped intracellularly in the

presence of ammonium chloride, and therefore rapidly degraded. However, in FH1a cells, only a small decrease in the level of IgG-C7 immunoprecipitable mature receptors in the presence of ammonium chloride and LDL was observed, once again consistent with the idea of a large proportion of receptors that do not bind LDL, and are therefore not trapped and degraded intracellularly in the presence of ammonium chloride. The fact that a small decrease in the amount of ^{35}S-labelled receptors was observed (relative to incubation with ammonium chloride alone) confirms the existence of a small sub-population of receptors which bind and internalize LDL normally, and are therefore trapped and degraded, as for normal receptors.

In Figure 5.14B, quantitation of the results from a similar experiment are shown. Whereas ammonium chloride or LDL alone had no significant effect on ^{35}S-labelled LDL receptor levels in normal or FH1a cells, the combination of these two agents caused a dramatic decrease after 3 hours in normal ^{35}S-labelled receptors, which decreased even further after 6 hours of incubation. However, even prolonged incubation (up to 6 hours) in the presence of LDL and ammonium chloride did not cause any dramatic decrease in ^{35}S-labelled immunoprecipitable receptors from FH1a cells. The amount of ^{35}S-label in the receptor band in FH1a cells at "zero" time (i.e. "100%") was equal to 51% of that in the normal receptor band. After 6 hours in the presence of ammonium chloride and LDL, the ^{35}S-label in the FH1a receptor band was 2-fold higher than that in the normal receptor band, and represented approximately 60% of the original amount at "zero" time. Since the $t_{½}$ for degradation of normal receptors trapped in the presence of ammonium chloride and LDL is approximately 2 hours, this result suggests that a very small proportion of the FH1a receptors was able to bind LDL, and subsequently to be trapped and degraded.

The selective trapping and degradation of only the LDL-binding receptor population in FH1a cells, demonstrated by

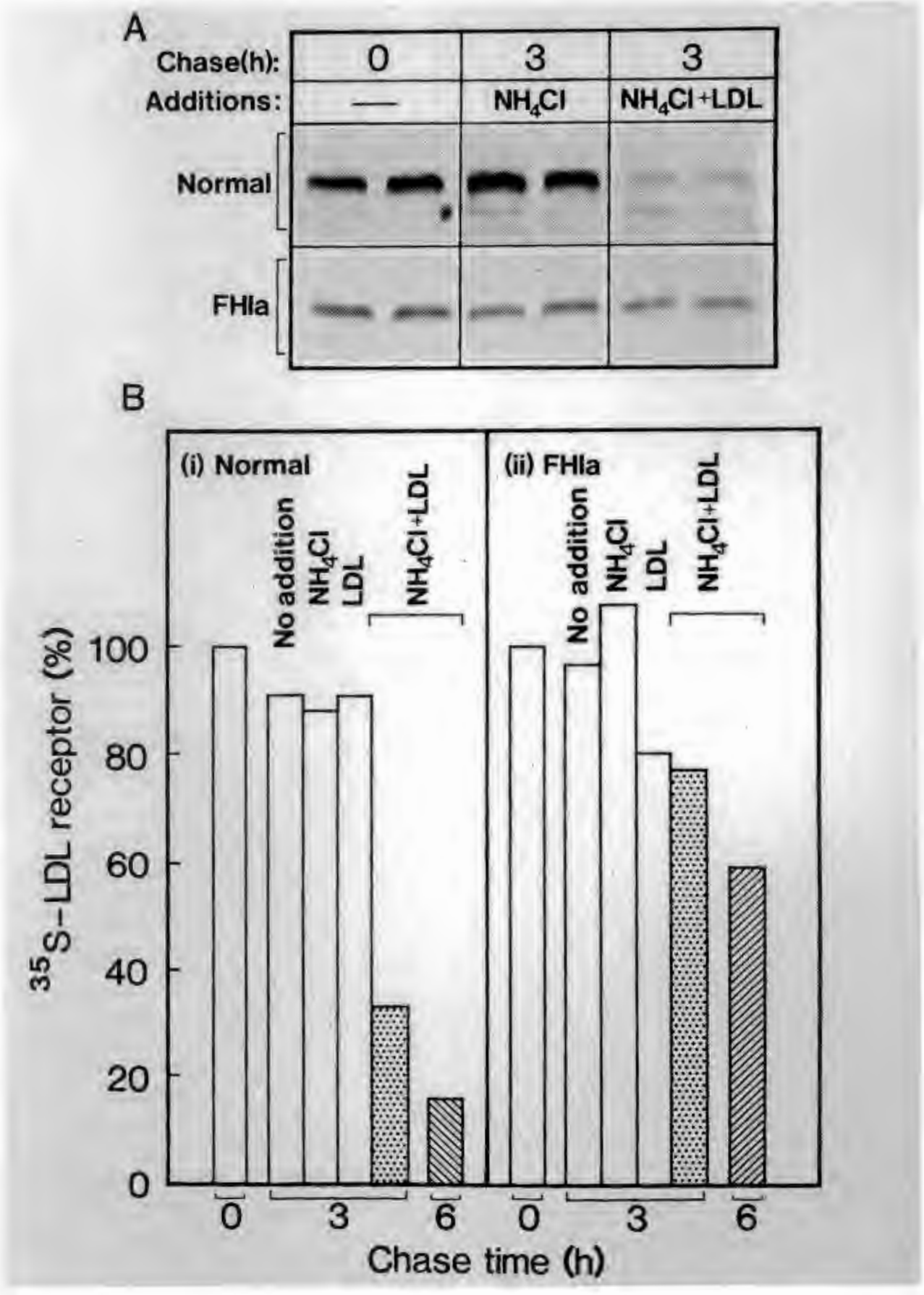

Figure 5.14 (legend overleaf)

Figure 5.14 Effect of ammonium chloride and LDL on [^{35}S]-labelled IgG-C7-immunoprecipitable LDL receptors in normal and FH1a fibroblasts.

A. After 24 hours incubation in lipoprotein-deficient medium, cells from a normal subject and FH1a were pulse-labelled for 2.5 hours at 37°C with [^{35}S]-methionine (30 μCi/ml) in methionine-free EMEM, followed by a 13-hour chase period in medium containing 200 μM unlabelled methionine, to ensure that all the labelled receptors in FH1a had been processed to the mature form. The medium was then changed to one containing 10 mM ammonium chloride plus or minus 40 μg/ml LDL and the cells were incubated for a further 3 hours at 37°C. The cells were then washed, solubilized and LDL-receptors were immunoprecipitated, and subjected to SDS-polyacrylamide gel electrophoresis (7% acrylamide).

B. After upregulation in lipoprotein-deficient medium for 30 hours, normal and FH1a cells were pulse-labelled for 3 hours at 37°C with [^{35}S]-methionine (50 μCi/ml) in methionine-free medium, followed by a 13.5 hour chase period. Duplicate dishes from each cell type were harvested and taken to represent the "zero" chase time point. The medium was then changed to one containing no additions, 10 mM NH_4Cl, 40 μg/ml LDL, or both 10 mM NH_4Cl and 40 μg/ml LDL, and chased for a further 3 hours at 37°C. One set of dishes was chased for 6 hours in the presence of NH_4Cl and LDL. At the indicated time-points, cells were harvested, solubilized and ^{35}S-labelled LDL receptors immunoprecipitated and subjected to SDS-PAGE (5-12% acrylamide) and fluorography. The amount of ^{35}S-labelled LDL receptor in each sample was quantified by densitometric scanning and expressed as a percentage of the amount at "zero" time. The results are averages of duplicates except those for normal cells at "zero" time, and for FH1a cells after 3 hours in the presence of NH_4Cl, which are results of single determinations.

the effects described above of ammonium chloride and LDL on ^{35}S-labelled immunoprecipitable receptors, thus provided more definitive proof for the existence of 2 surface receptor populations, originally distinguished on the basis of ligand and antibody binding (139).

In the pulse-chase experiments described above, long initial chase incubations (> 10 hours) were employed to ensure that all the ^{35}S-label was chased from the slowly-processed precursor into the mature receptor, before the effects of ammonium chloride plus LDL were examined. The effect of these agents on newly-synthesized surface receptors was investigated by chasing in the presence of ammonium chloride and LDL for short periods (3 hours) after pulse-labelling. No dramatic selective loss of mature receptors was observed (results not shown), indicating that the "earliest" detectable, newly-synthesized and processed mature receptors, which reached the cell-surface in FH1a cells, were predominantly in the abnormal form which does not bind LDL, consistent with the results obtained for newly-synthesized receptors during up-regulation (section 5.2.7).

5.2.9 Effect of glycosylation on the LDL- and IgG-C7 binding populations in FH1a

During the retarded post-translational processing of the LDL receptor in FH1a cells, an apparent increase in the molecular weight of the transport-deficient precursor receptor was observed (section 5.2.3), possibly as a result of abnormal glycosylation. To establish whether or not abnormal glycosylation played a role in the formation of the normal and abnormal receptor populations in FH1a, the effects of N-linked and O-linked glycosylation on ^{125}I-LDL and ^{125}I-IgG-C7 binding activity in normal and FH1a cells were determined.

5.2.9.1 N-linked glycosylation

The effect of inhibition of N-linked glycosylation by tunicamycin on the LDL- and IgG-C7 binding activity in

normal and FH1a cells was determined after pre-incubation in the absence or presence of 1 μg/ml tunicamycin in lipoprotein-deficient medium for a 24-hour upregulation period. The results in Table 5.5 show that tunicamycin treatment decreased LDL-binding activity in normal and FH1a cells to approximately 40% of control values, and IgG-C7 binding activity to 60% of control. The decrease in antibody binding may reflect a decrease in the amount of receptor protein, because of inhibition of glycoprotein synthesis by tunicamycin (67). The greater relative decrease in ligand binding may indicate that N-linked sugar-deficient receptors had a lower affinity for LDL, so that the concentration of ^{125}I-LDL used in the assay was no longer saturating, and thus an apparent decrease relative to maximum normal binding levels was observed. Alternatively, if the decreased level reflected LDL binding with normal affinity, the results would imply the existence of abnormal receptors which did not bind LDL, but were recognized by the antibody. Since Chatterjee et al. (67) have shown that receptors synthesized in the presence of tunicamycin had a normal affinity for LDL, the latter explanation seems more likely, suggesting that inhibition of N-linked glycosylation created a population of abnormal receptors, unable to bind LDL, in normal cells. Tunicamycin treatment did not change the ratio of the abnormal to "normal" LDL receptors in FH1a, suggesting that abnormal N-linked glycosylation was not responsible for creating the two populations observed under normal (fully-glycosylated) conditions. Similarly, it was shown that N-linked oligosaccharides did not play a role in the apparent increase in molecular weight of transport-deficient precursor receptors in FH1a (section 5.2.3.1).

5.2.9.2 Sialic acid and O-linked glycosylation

Since there is no specific inhibitor available to prevent the addition of O-linked oligosaccharides, the approach used to examine the role of O-linked glycosylation in the formation of the heterogeneous receptor population in FH1a cells was to remove O-linked oligosaccharides from

Table 5.5 Effect of tunicamycin treatment on ^{125}I-LDL and ^{125}I-IgG-C7 binding at 4°C to normal and FH1a cells

On day 4 of cell growth, the medium was changed and the cells were incubated for 24 hours at 37°C in the absence or presence of 1 µg/ml tunicamycin . The cells were then chilled to 4°C and ^{125}I-LDL (402 cpm/ng, 5.5 µg/ml) and ^{125}I-IgG-C7 (809 cpm/ng, 0.8 µg/ml) binding levels were determined. The data are means of duplicates from a single experiment and represent high affinity values, calculated by subtracting the values from dishes that had 1 µg/ml 25-hydroxycholesterol and 12 µg/ml cholesterol present in the medium during the 24 hour incubation, from total binding values. The non-specific values for normal cells in the absence of tunicamycin represented 11% of total LDL binding and 24% of total IgG-C7 binding.

Cell type	^{125}I-LDL binding (ng/mg protein)			^{125}I-IgG-C7 binding (ng/mg protein)		
	-Tunicamycin	+Tunicamycin	%	-Tunicamycin	+Tunicamycin	%
Normal	341	126	37	34	21	62
FH1a	46 (13 % normal)	19 (15% normal)	41	15 (44% normal)	9 (43% normal)	60

"normally"-glycosylated receptors at the cell surface using the enzyme, O-glycanase, after neuraminidase treatment to first remove terminal sialic acid residues which interfere with the action of O-glycanase. Neuraminidase treatment alone, or followed by O-glycanase treatment, decreased LDL-binding and IgG-C7 binding by different relative amounts (29-45% and 4-18%, respectively), but to the same extent in normal and FH1a cells (Table 5.6). Thus, the ratio of abnormal to LDL-binding receptors (relative to normal cells) was not changed in FH1a cells. However, neuraminidase and O-glycanase treatment of immunoprecipitated, ^{35}S-labelled LDL receptors (section 5.2.3.2) suggested possible involvement of abnormal O-linked glycosylation in the increased molecular weight of the slowly-processed receptor precursor. The results of the binding assays cannot exclude a role for abnormal O-linked glycosylation in the creation of the 2 receptor populations in FH1a cells for 2 reasons: (i) To allow restoration of fibroblast morphology after incubation in the acidic medium required for neuraminidase and O-glycanase activity, measurement of LDL- and IgG-C7 binding activity were performed after a one-hour recovery period during which newly-synthesized, fully-glycosylated receptors could have masked effects of the deglycosylation, and (ii) studies on ^{35}S-labelled receptors (section 5.2.3.2) showed that, possibly due to heterogeneity of substituents, incomplete removal of O-linked oligosaccharides by O-glycanase occurred. In addition, as for tunicamycin treatment, neuraminidase treatment alone, or in combination with O-glycanase, caused a significant decrease in LDL-binding activity in normal and FH1a cells, but virtually no difference in IgG-C7 binding levels. This suggests that (as discussed above for N-linked glycosylation) unless a difference in receptor affinity for LDL is created, defective O-glycosylation (or defective sialylation of both O- and N-linked oligosaccharides) can result in populations of normal and abnormal receptors, as observed in FH1a cells.

Table 5.6 Effect of neuraminidase and O-glycanase treatment on ^{125}I-LDL and ^{125}I-IgG-C7 binding at 4°C to cells from a normal subject and FH1a.

After incubation for 48 hours in lipoprotein-deficient medium, cells from a normal subject and FH1a were washed once with PBS and then incubated for 40 minutes at 37°C in the presence of (i) no additions, (ii) 0.1 units of neuraminidase (Sigma), (iii) 0.1 units of neuraminidase for 20 minutes, followed by the addition of 2.5 milliunits of O-glycanase for the last 20 minutes. The medium was then removed and the cells were incubated in DMEM/LPDS for 1 hour at 37°C. The cells were then chilled to 4°C and ^{125}I-LDL (406 cpm/ng, 6 μg/ml) and ^{125}I-IgG-C7 (420 cpm/ng, 0.9 μg/ml) binding were determined. The data are means of duplicates from a single experiment and represent high affinity values calculated by subtracting values obtained in the presence of excess unlabelled LDL (300 μg/ml) or IgG-C7 (50 μg/ml) from total binding values. The non-specific values for normal cells represented 12% of total LDL binding and 7% of total IgG-C7 binding. (Abbreviations: N'ase = neuraminidase; O-G'ase = O-glycanase)

	Normal		FH1a			
Preincubation	^{125}I-LDL bound (ng/mg)	^{125}I-IgG-C7 bound (ng/mg)	^{125}I-LDL bound (ng/mg)	% Normal	^{125}I-IgG-C7 bound (ng/mg)	% Normal
Control	190 (100%)	42 (100%)	55 (100%)	29	22 (100%)	52
+N'ase	105 (55%)	39 (94%)	33 (60%)	31	18 (82%)	46
+N'ase +O-G'ase	135 (71%)	41 (96%)	34 (62%)	25	20 (91%)	49

5.3 Discussion

5.3.1 Summary/Conclusions

The mutant LDL receptor allele present in the homozygous form in FH1a and 1b, produces a gene product that is evidently slowly processed to one of two mature forms, which constitute a functionally heterogeneous surface receptor population. On the basis of ligand and antibody binding at 4°C, one population, comprising 20% of the normal number of receptors, which bound LDL with normal affinity, was distinguished from a second population (40% of the normal receptor number) which recognized only IgG-C7, but not LDL. Since these two populations are products of a single mutant allele, conformational differences may account for their functional heterogeneity. Abnormal folding or denaturation of receptor precursors may also be responsible for the delay in transport and processing. More definitive proof for two populations of surface receptors was provided by selective trapping and degradation of the LDL-binding population in the presence of ammonium chloride and LDL. Studies of receptor activity at 37°C suggested that the receptor population which recognizes only IgG-C7 does not efficiently internalize and degrade surface-bound IgG-C7. This functionally abnormal form of the receptor predominates even in newly-synthesized receptors, suggesting that an early post-translational event involving protein folding and/or glycosylation, leads to receptors with the same primary sequence appearing on the surface in either the "normal", LDL-binding, or abnormal form. Despite their functional and, presumably, conformational differences, both surface forms of the mutant receptor are degraded at equal rates, similar to degradation rates for normal LDL receptors.

5.3.2 Synthesis and degradation of mutant LDL receptors

The regulation of receptor levels in FH1a is apparently normal. Induction of LDL receptor synthesis by incubation in lipoprotein-deficient medium follows a similar time-course in normal and FH1a cells. The kinetics of up-regulation, and down-regulation in the absence of synthesis,

of steady-state receptor levels for both surface populations in FH1a correspond to those in normal cells.

Although the initial rates of receptor synthesis in FH1a, at all stages of up-regulation in lipoprotein-deficient medium, and the rates of degradation of both the LDL-binding and abnormal receptor populations, are normal, the steady-state level of surface receptors in FH1a cells is approximately 55% of normal. The Pronase sensitivity of mature ^{35}S-labelled receptors in intact cells from FH1a implies that there is no unusually large intracellular pool of mature receptors. Although the newly-synthesized precursor receptors in FH1a are slowly processed to mature forms, Western blotting showed no evidence for a large steady-state precursor pool relative to the mature receptor pool. Thus, in contrast to normal cells (73), it appears that the synthesis rate in FH1a is not the only factor determining the cellular LDL receptor content. One possible explanation for the apparent loss of receptors is degradation of precursors retained in the endoplasmic reticulum. Support for this idea is the more rapid disappearance of ^{35}S-labelled precursors, than could be accounted for by processing to mature forms with normal degradation rates. Degradation of transport-deficient precursors has also been proposed for the mutant LDL receptors in the WHHL rabbit (23), Lebanese FH subjects (88) and FH429 (82). In transfected Chinese hamster ovary cells the mutation in FH429 produced a protein that was not transported to the cell-surface, but degraded intracellularly with a half-life of 5 hours, compared to one of 8.5 hours for the normal LDL receptor protein. Lippincott-Schwartz *et al.* (76) have recently shown that newly-synthesized proteins which failed to meet the structural criteria for transport to the Golgi could be rapidly and efficiently degraded by the cell at a non-lysosomal site closely related to the endoplasmic reticulum. A similar mechanism may lead to degradation of precursor receptors trapped in the endoplasmic reticulum in FH1a and other transport deficient mutants.

5.3.3 The role of protein folding in transport from the ER

Rothman (79), Lodish (80) and Gething *et al.* (81) propose that any mutation that affects protein folding will cause retardation in the endoplasmic reticulum, since the criterion for transport to the Golgi complex appears to be a correct, native protein conformation. The most important rate-determining steps in protein folding are disulphide bond formation, catalyzed by protein disulphide isomerase (60,61) and *cis*-*trans* isomerism of prolyl peptide bonds, which can give rise to the co-existence of alternative, slowly interconverting states of the protein (60). The fact that the receptors in FH1a cells are retarded in the endoplasmic reticulum and eventually processed to two different forms of the receptor is thus strong evidence for conformational abnormality of the mutant receptors. The mutations in the WHHL rabbit (23), FH563 (10) and subject TT (87), which produce receptors exhibiting delayed transport to the cell surface, all result from small deletions in the cysteine-rich binding domain, and could thus lead to incorrect disulphide bonding and protein folding. The Lebanese allele produces a transport-deficient truncated protein that terminates in the middle of a cysteine-rich "growth-factor" repeat, leaving unpaired cysteines and the potential for abnormal disulphide bonding and conformation. The mutation in FH429 substitutes a valine for a glycine residue in a cysteine-poor region of the receptor (82). The deletions in the WHHL rabbit, FH563 and TT also all include glycine residues. Since glycine residues are often found in space-restricted regions in proteins, their deletion or substitution by larger amino acids could potentially disrupt protein conformation, and thus impair intracellular receptor transport. Since the mutant receptors in FH1a have a normal apparent molecular weight, and since the same mutation leads to impaired transport and defective LDL binding, the FH1a (and 1b) mutation can be expected to be a small deletion, insertion or substitution in the cysteine-rich binding domain which disrupts the

normal conformation required for transport and ligand recognition.

Kreis and Lodish (143) and Machamer and Rose (144) have demonstrated temperature-dependence of intracellular transport of vesicular stomatitis virus-G protein mutants from the endoplasmic reticulum to the Golgi. At temperatures of 37-39°C, transport was blocked, but lowering the temperature to 30-32°C allowed transport to the Golgi, processing, and transport to the cell-surface, implying a temperature-dependent polypeptide folding step. However, pre-incubation of FH1a cells for 24 hours at 30°C did not significantly change the ratio of abnormal to normal (LDL binding) receptors on the surface compared to that seen at 37°C (results not shown). There do not seem to be gross disulphide-bonding abnormalities in the FH1a receptor since the non-reduced receptor, on Western blots (section 5.2.6) and electrophoresis of immunoprecipitated receptors (not shown), migrates with apparently normal mobility. However, subtle alterations in disulphide bonding cannot be excluded. Attempts by Esser and Russell (82) to directly demonstrate conformational abnormality in the receptor from FH429 by means of altered protease sensitivity (81,145,146) or aggregation state (81,145) were unsuccessful. Thus, the mutations in FH1a and FH429 which lead to impaired transport of receptors must cause minor or highly localized conformational changes. The mechanism by which denatured proteins are retained in the endoplasmic reticulum is unknown, but a family of proteins, including immunoglobulin heavy-chain binding protein (BiP) (81), have recently been shown to interact with conformationally abnormal proteins, possibly facilitating correct folding and/or preventing export of abnormally folded proteins. No evidence was found for BiP association with immunoprecipitated mutant receptors in FH1a. Esser and Russell (82) have detected variable amounts of a protein, migrating with the molecular weight of BiP, in immunoprecipitation experiments with various transport-deficient LDL receptors.

5.3.4 The role of protein glycosylation in receptor function and intracellular transport

Another factor which can affect protein conformation and function is glycosylation. In a number of *in vitro* studies (147-150), it has been shown that denatured or unfolded proteins can be post-translationally glycosylated at sites not normally glycosylated in the native protein. In this regard, it is of interest that the stalled precursor in FH1a showed a progressive, increase in apparent molecular weight, suggesting abnormal glycosylation. Similarly, Kozarsky *et al*. (93) have shown that in the experimentally-induced class 2 mutant, ldlA-7, the slowly-processed precursor is continually converted to slightly larger, novel, unstable intracellular intermediates. They suggest that the apparent increase in molecular weight may be the result of additional but incomplete processing of O-linked oligosaccharides or other post-translational modifications. The accelerated degradation of the slowly-processed receptors ($t_{½}$ 4-6 hours) in ldlA-7 is similar to that observed in FH429 (82) and proposed for FH1a (section 5.2.4.1) and other transport-deficient mutants (23,88). Naim *et al*. (151) showed that a mutation in the sucrase-isomaltase gene in patients with a congenital deficiency in this enzyme, led to the production of a transport-deficient precursor protein approximately 2 kDa larger than normal. The increased molecular weight could be ascribed to excess N-linked carbohydrate since Endoglycosidase H treatment reduced normal and mutant proteins to the same molecular weight. They favour an interpretation of incomplete carbohydrate trimming in the ER, rather than an additional oligosaccharide chain, since the mature form of the protein showed no increase in apparent molecular weight. The increase in molecular weight in FH1a could not be abolished by prevention or removal of N-linked glycosylation of the precursor. Neuraminidase and O-glycanase treatment reduced the mature receptors from FH1a and normal cells to the same apparent molecular weight, suggesting that altered O-linked glycosylation may be

involved, particularly since recent studies by Pathak et al. (64) have shown accumulation of transport-deficient receptors in cellular compartments where O-linked sugar addition is proposed to take place.

Protein glycosylation has been proposed to have various functions including induction and maintenance of correct polypeptide folding, prevention of intracellular aggregation, protection from proteolysis, and signals for intracellular targeting and cellular recognition (152). Inhibition of N-linked glycosylation of various proteins with tunicamycin has suggested that there is no universal role for N-linked oligosaccharides (152). Chatterjee et al. (67) suggest that tunicamycin affects the level of surface expression of LDL receptors, but not their functional capacity. However, treatment of normal fibroblasts with tunicamycin in the present study (section 5.2.9.1) appeared to result in abnormal surface receptors, as judged by the lower proportion of LDL versus antibody binding. Gallagher et al. (153) recently showed by site-directed mutagenesis that abnormal N-linked glycosylation of influenza virus hemagglutinin caused defects in folding, transport and activity of the molecule. From their studies on glycosylation mutants of vesicular stomatitis virus-G protein, Machamer and Rose have concluded that N-linked carbohydrate plays a role in the intracellular transport of this protein by promoting the correct polypeptide folding and protecting molecules from aberrant disulphide bonding (144,154). Thus, the oligosaccharides promote transport of the protein indirectly through facilitating correct conformation rather than acting as direct "signals" for intracellular transport.

Davis et al. (33) have shown that deletion of the clustered O-linked sugar domain of the LDL receptor has no significant effect on intracellular transport, LDL binding and internalisation, recycling or receptor half-life in cultured hamster fibroblasts. However, Kozarsky et al. (66) showed

that experimentally-induced O-linked sugar-deficient receptors were abnormally unstable due to proteolytic cleavage within or near the domain usually containing clustered O-linked oligosaccharides, which presumably normally render the proteolytic site inaccessible. Yoshimura et al. (155) have suggested that the low binding capacity of the smaller LDL receptor in a monensin-resistant mutant of Chinese hamster ovary cells is due to altered O-linked glycosylation, since treatment of normal and mutant receptors with neuraminidase and O-glycanase almost completely eliminated the difference in their molecular weights. They suggest that the mutant receptors lack some O-linked chains because a mutation in the cells prevents the correct folding of the binding domain necessary for both O-linked glycosylation and normal LDL binding affinity.

Thus, if altered O-linked glycosylation of the LDL receptor in FH1a were present, it could have an effect on the receptor conformation, intracellular transport and LDL binding, and could conceivably lead to the observed functional heterogeneity of the surface receptors. However, treatment of surface receptors with neuraminidase and O-glycanase did not significantly alter the ratio of abnormal to normal (LDL-binding) receptors in FH1a. This observation may not exclude the involvement of abnormal O-linked glycosylation since neuraminidase and O-glycanase were unable to completely remove all the O-linked oligosaccharides (shown by treatment of immunoprecipitated ^{35}S-labelled receptors, section 5.2.3.2) and during the one-hour recovery period after the treatment of the cells in acid medium, newly-synthesized, fully-glycosylated receptors could mask the effects of the deglycosylation. In addition, Lane and co-workers have shown that, although the EGF (156,157) and insulin (158) receptors display glycosylation-dependent acquisition of ligand-binding capacity, subsequent removal of the carbohydrate does not affect the functional activity of the receptor. Thus, oligosaccharides may contribute to tertiary structure to

facilitate further modification such as disulphide bonding, which would not be affected by subsequent removal of the sugar residues.

Tunicamycin, neuraminidase and O-glycanase treatment of normal (and FH1a) cells decreased LDL binding relative to IgG-C7 binding, suggesting that altered glycosylation of LDL receptors can lead to functional heterogeneity. An example where oligosaccharides play a role in functional heterogeneity is the β_2-adrenergic receptors of A431 cells (159). These proteins are present in 2 distinct populations: 55-52 kDa molecules containing complex N-linked chains, and exhibiting a high affinity for the agonist, (-)-isoproterenol, and 65-63 kDa low-affinity receptors, with high-mannose oligosaccharides. Both types of receptors are present in preconfluent cells, but only the high-mannose-containing molecules are found in postconfluent cells. The degree of confluence of FH1a cells did not influence the expression of the two surface receptor populations, since similar ratios of abnormal to LDL-binding receptors were obtained over a 4-fold range of protein content (90 to 330 µg per dish).

5.3.5 Model for creation of "normal" and "abnormal" mutant receptors

A putative model for the creation of functionally distinct surface populations of the same mutant receptor in FH1a would be that, due to a mutation in the amino acid sequence, both "normal" and abnormal conformations are formed, presumably in equilibrium with each other (160). Inability to achieve a "normal" conformation would lead to delayed transport from the ER to the Golgi, and thus create the possibility for abnormal or excessive glycosylation, and possibly degradation. Abnormal glycosylation could play a role in shifting the folding equilibrium to an abnormal conformation, which could be rendered irreversible by incorrect disulphide bonding, thus leading to non-functional protein molecules.

CHAPTER 6

CONCLUDING DISCUSSION

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6.1 Introduction

The aim of this study was to characterize fully the phenotypic expression of LDL receptor mutations in South African FH homozygotes, both for medical and genetic interest, and in order to understand cell-biological changes, as a result of these mutations, in the complex itinerary of the LDL receptor.

A number of novel findings have emerged from detailed studies of selected cells, shown to be homozygous for each of 2 mutant LDL receptor alleles prevalent in Afrikaner FH subjects. In cells from FH3a and 3b, the mature mutant LDL receptors are subject to abnormally rapid degradation, representing a mutant receptor phenotype not previously described in the classification of LDL receptor defects. Although the mutation in Kindred 3 was thus the first example of a new class of naturally-occurring LDL receptor mutations, producing rapidly-degraded receptors (102,139), further examples of this class have subsequently been detected in South African subjects, TD and RI (section 3.2.3) and in three Japanese subjects (128).

A novel aspect of the LDL receptor phenotype in Afrikaner Kindred 1, is that cells homozygous for a single mutant allele apparently display a functionally heterogeneous receptor population on their surfaces (139). One-third of the receptors bind LDL with normal affinity, while two-thirds recognize only IgG-C7. This functional heterogeneity may be caused by conformational differences, which could be the cause or the result of the delayed transport and processing of the mutant precursors. The interesting observation was made that the slowly-processed precursor exhibited a time-dependent increase in apparent molecular weight, possibly suggesting the involvement of abnormal glycosylation in the creation of both "normal" and abnormal forms of the same mutant receptor molecule. The possible role of glycosylation in protein function and

intracellular transport has been discussed (section 5.3.4) and is highlighted by recent work of Gallagher et al. (153), who showed that oligosaccharides at abnormal sites in the influenza virus hemagglutinin (HA) could disrupt folding, transport and activity of the molecule, for example, glycosylation in some regions disrupted functional epitopes on the surface of the protein. The authors postulated that certain regions of the HA molecule must remain devoid of carbohydrate because these areas may be recognition sites for cellular proteins involved in initial folding and mobilization of HA into the secretory pathway, such as BiP (81), or for structural reasons, since attachment of oligosaccharides at some positions might interfere with correct folding or oligomerization of the molecule. Similarly, abnormal glycosylation of the LDL receptor in FH1a and 1b cells could have detrimental effects on folding, transport and activity of the protein.

6.2 Cellular "signals" for transport and degradation of proteins

Major questions in cell biology today are (i) why are cellular proteins degraded at very different rates? and (ii) how are secretory and membrane proteins targeted to the cell-surface and why are they transported from the endoplasmic reticulum (ER) to the Golgi apparatus at different rates? Presumably, some form of biological "signal" targets a protein for degradation, or dictates its rate and destination of intracellular transport. The two LDL receptor mutations examined in detail in the present study alter the protein in such a way that it exhibits either an enhanced degradation rate (FH3a and 3b) or severely retarded intracellular transport and processing to heterogeneous surface receptors (FH1a and 1b). These mutant receptors thus have the potential to elucidate some of the answers to the questions asked above.

Biological "signals" within proteins may comprise specific sequences or may consist of "signal patches" (78) of non-

contiguous regions on the surface of the protein, brought together by protein folding. Sequences which function as signals are easily identified because their function is usually preserved when they are transferred to different proteins, but signal patches are more difficult to identify because they cannot easily be transferred between proteins, and any mutation which directly or indirectly affects protein conformation may eliminate signal patch function.

Various sequences which act as signals for degradation have been identified (reviewed in ref. 162). For example, "PEST" regions, rich in proline, glutamic acid, serine and threonine, have been identified in 23 rapidly-degraded proteins (163). All PEST-sequence-containing proteins appear to be regulatory molecules, which may have fast turnover rates in order to rapidly change their concentrations. A 20-amino acid peptide (S-peptide) of RNase-A, containing the sequence Lys-Phe-Glu-Arg-Gln (KFERQ) has been shown to target various proteins for enhanced lysosomal degradation during nutrient deprivation (164,165). Ubiquitin-mediated proteolysis is enhanced by an unblocked amino-terminus and by the presence of oxidized methionine residues (166,167). The "N-end" rule, proposed by Bachmair *et al*. (168), proposes that the specific amino acid at the amino terminus determines which proteins are targeted for degradation. However, this rule may apply mainly to unfolded proteins during translation (162). There is no consistent experimental evidence for conformational properties (*i.e*. signal "patches") which target proteins for degradation, although there are indications that proteins with altered conformation due to mutation are sometimes more rapidly degraded, and individual subunits are degraded faster than oligomeric proteins. The latter process may have the function of destroying excess subunits when unequal amounts of the different component proteins are produced by the cell. For example, five of the chains of the heptameric T-cell receptor are synthesized in vast excess over the others, and are selectively degraded if they are

not assembled into receptor complexes of the correct stoichiometry (76).

Although some sequences (discussed above) appear to target proteins for degradation, these findings apply mostly to cytoplasmic proteins and very little information on conformational signals for degradation is available. The reasons for differences in degradation rates of membrane proteins, including receptors, are essentially unknown, as are the structural "signals" for a receptor either to recycle or to be degraded along with ligand. Elucidation of the structural details of the LDL receptor mutation in subjects FH3a and 3b, which change the receptor from a relatively long-lived, recycling receptor, to one which is rapidly degraded, probably without recycling, should provide valuable information on the "signals" controlling these processes.

The "signals" that specify the destination and rate of transport of each newly-synthesized protein must be found within its sequence or structure. The ER and Golgi apparatus appear to be involved in recognizing these "signals" and distributing proteins to the cell-surface, secretory storage vesicles and lysosomes. For example, the generation in the Golgi apparatus of mannose-6-phosphate residues on newly-synthesized lysosomal enzymes targets them for delivery to the lysosome via binding to mannose-6-phosphate receptors (78). The rate-limiting step in the transport of most proteins to the cell-surface is their exit from the ER (78). At any given time, the ER contains a great variety of newly-synthesized proteins, only some of which are retained in the ER, while the exported proteins have been shown to be transported at very different rates. Therefore, either (i) exported proteins possess positive signals which control their transport rates from the ER or (ii) proteins are transported constitutively with the "bulk flow", unless they possess signals for retention in, or recycling back to, the ER.

The only evidence to support signal-dependent transport from the ER to the Golgi is the observation that proteins are transported at different rates (169,170). Evidence for signal-dependent retention of resident ER proteins has been obtained from the observation that several luminal ER proteins contain a specific C-terminal sequence, Lys-Asp-Glu-Leu (KDEL), which prevents their secretion (171,172). Attachment of this ER retention signal to a lysosomal protein, cathepsin D, causes its accumulation in the ER via sorting from secretory proteins and retrieval from a post-ER compartment (172). The constitutive "bulk flow" rate out of the ER, measured using synthetic glycopeptides, has been shown by Wieland et al. (173) to have a half-time between 5 and 20 minutes. Since no membrane or secretory proteins are transported faster than the glycopeptides, which are too small to contain a transport signal, it appears that differences in protein transport rates reflect differences in retention in the ER. Elucidation of the LDL receptor mutation in the South African Xhosa subject, TT (section 3.2.2 and ref. 87), has provided additional support for signal-dependent retention, as opposed to signal-dependent transport. Removal of 2 amino acids from the first binding repeat of the LDL receptor in TT cells impaired its transport from the ER to the Golgi apparatus. In contrast, deletion of the entire first repeat by site-directed mutagenesis (29) produced a receptor that was transported and processed at a normal rate. Thus, the small deletion in the TT receptor could not have caused loss of a transport signal, since deletion of the whole repeat did not impair transport. The most likely explanation is that the 2-amino acid deletion within the repeat caused a conformational change in the TT receptor, which was recognized as an ER retention signal or signal "patch".

In general, incorrect tertiary or quaternary structure may constitute a retention "signal", since a variety of mutations in different regions of proteins, which presumably

all lead to alterations in conformation or assembly of subunits, lead to retention of the mutant proteins in the ER (143,146,174-177). Although protein glycosylation is often crucial in promoting export of membrane proteins from the ER, it apparently does not act as a direct "signal", but is required indirectly to promote correct folding of the protein chain (144,154,156-158). Recent studies suggest that although they are pre-requisites for transport, normal folding and assembly of subunits alone may not be sufficient, since mutations of VSV-G protein, which had little effect on folding and trimerization, disrupted transport to the cell-surface (174).

The normal LDL receptor appears to traverse the transport pathway at close to the proposed "bulk flow" rate (173), since the half-time for processing to the mature, surface form of the receptor is only approximately 15 to 30 minutes. Thus, it would appear that the mutation in FH1a and 1b, which causes retardation of receptor transport from the ER, may create a conformation-dependent retention "signal" in the mutant protein. Elucidation of this mutation at the molecular level would thus be of great interest, since correlation of protein folding and conformation with transport may elucidate the factors responsible for directing proteins from the ER, and specifically retaining proteins in the ER, respectively. Future work on the mutant LDL receptor proteins in FH1a and 1b should therefore aim to detect conformational abnormality through loss of recognition by conformation-specific antibodies or ligands, abnormal disulphide bond formation, changes in proteolytic susceptibility, or altered physical properties, such as solubility in detergents, sedimentation pattern, gel mobility or ability to be cross-linked (178). However, attempts to demonstrate conformational abnormality in transport-deficient LDL receptors from FH429 (82), were unsuccessful, suggesting that minor, or highly localized, changes in conformation may be responsible for retention in the ER.

Although the major interest to date has been in understanding the "signals" that control exit of proteins from the ER, another important aspect is the identification of the "receptors" that recognize and bind these putative signals, and of the mechanism(s) for retention of proteins in the ER. Differential transport rates from the ER may simply be due to non-specific "adsorption" by electrostatic and/or hydrophobic interactions of exported proteins with immobile permanent ER resident proteins (78). Alternatively, retention of incorrectly-folded proteins in the ER may be due to their tendency to aggregate, as observed for mutant forms of vesicular stomatitis virus (VSV)-G protein, which were not transported from the ER but formed large aggregates (144,174) that were disulphide-bonded in some cases (144). Incorrectly-folded or unassembled protein subunits may be specifically recognized and retained by resident ER proteins. Certain unfolded proteins associate with a 77 kDa protein, first identified as immunoglobulin heavy-chain binding protein (BiP) (81). BiP may play a role in retention of proteins in the ER, but does not appear to be associated with all misfolded proteins. For example, no consistent indication was found for BiP association with immunoprecipitated transport-deficient mutant LDL receptors (82), and Doms found no evidence for BiP in aggregates of a mutant (ts045) VSV-G protein exhibiting temperature-sensitive transport (178). An example of retention in the ER of a normally-folded protein is that of mouse liver β-glucuronidase, which is retained only when it is complexed with a 64 kDa ER protein called egasyn (78,178). Since only 10% of egasyn was found complexed with β-glucuronidase, it is possible that most egasyn may be complexed with other proteins in the ER (78). No evidence was found for association of either BiP or egasyn with the immunoprecipitated mutant LDL receptors from FH1a and 1b cells. The defective transport of these mutant proteins from the ER to the Golgi may thus be caused by some

of the less specific aggregation/adsorption processes described above.

6.3 Self-association of the LDL receptor

An alternative possible mechanism for delayed transport of the mutant receptors in FH1a and 1b is disruption of correct assembly of oligomeric structures, since correct quaternary structure also appears to be a requirement for transport. In their studies on the role of assembly of hemagglutinin (HA) trimers in intracellular transport, Copeland et al. (145), showed that trimerization occurred post-translationally in the ER, followed immediately by transport to the Golgi complex. However, approximately 10% of the newly-synthesized HA molecules were conformationally altered and formed aberrant trimers that were not transported from the ER. In another study, it was shown that HA monomer folding, trimerization and transport occurred sequentially, and that only fully-assembled trimers, and not monomers, were transported to the Golgi complex (179).

It is not known whether the normal LDL receptor is transported as a monomer or multimer. Van Driel et al. (40) have recently demonstrated self-association of the receptor in noncovalent dimers, and higher order structures, mediated by the cytoplasmic domain. Although the cytoplasmic domain is also required for clustering in coated pits, no causal link between the two processes was established. The functional role, if any, of oligomerization is not to promote ligand binding. One possibility is that it promotes transport of the receptor. Schneider et al. (77) have considered this possibility as an explanation for variability of receptor expression in heterozygous FH. If normal transport requires dimer formation, then in heterodimers of mutant and normal proteins, the transport signal, or lack of a retention signal, in the normal protein may promote transport of the

abnormal one. Alternatively, a retention signal in the mutant protein may prevent transport of the heterodimer.

An hypothesis for the expression of "normal" and abnormal receptor proteins at levels of approximately 20% and 60% of normal, respectively, in FH1a and 1b cells can be proposed based on the above possibilities. If, at any given time, approximately 50% of the mutant precursors in the ER achieve a "normal" conformation, and thus lack a retention signal, those proteins with the normal conformation may promote transport of abnormal proteins in heterodimers. This would lead to expression of approximately 75% of the normal number of receptors on the cell-surface (25% as homodimers between 2 "normally"-folded proteins, and 50% due to heterodimers). Presumably dimers or unassembled monomers of proteins with abnormal conformations would be retained in the ER, and possibly degraded (76). If the assumption is made that the homodimers between 2 mutant proteins which have achieved normal conformation are able to bind LDL, whereas the heterodimers recognize only IgG-C7, then the 3 to 1 ratio of abnormal to normal surface receptors found in FH1a and 1b cells could be explained.

No clear indication for an effect of the normal allele in heterozygote, 1d, on processing of the mutant allele was observed (section 5.2.5) but the extent of conversion to the mature receptor during the 4-hour chase period was approximately 10% higher than that expected if processing of the products of the normal and mutant alleles proceeded independently. In addition, ^{125}I-LDL degradation in subject 1d (84% of normal) suggested surface receptor expression somewhat higher than that expected for an FH heterozygote. However, the possibility of a role for self-association of receptors in the phenotypic expression of the mutation in these mutants remains purely speculative. Receptor cross-linking studies (40) in normal and FH1a (and/or 1b) cells may yield more information on this aspect,

although interpretations should be made with caution due to the inherent inefficiency of cross-linking reactions.

6.4 Future prospects for studies of mutant LDL receptors

The discovery of the LDL receptor was made during studies which began from a purely medical point of view, in order to understand the disease, FH (1). However, because of the complex intracellular itinerary of the LDL receptor, the study of normal and mutant forms of the receptor has, in addition, elucidated fundamental processes in cell biology, such as receptor-mediated endocytosis.

In the South African context, the high incidence of FH in the Afrikaner population can now clearly be ascribed to founder gene effects, particularly on the basis of the high incidence of mutations associated with particular haplotypes in many unrelated individuals with FH. Therefore, genetic counselling to heterozygous FH subjects (1 in 100 Afrikaners) is important to prevent the incidence of the more severe homozygous form of the disease. Future work should involve detailed haplotype analysis of all potential FH subjects, in addition to normal subjects to establish the incidence of specific haplotypes in both normal and FH subjects. Identification of a haplotype associated with FH, or of a common mutation detectable by Southern blotting, possibly after amplification of a specific region of the LDL receptor gene by the polymerase chain reaction technique (87), may allow direct diagnostic testing for certain prevalent mutations in the Afrikaner population, as for the French-Canadian (84), Lebanese (88) and Finnish (91,92) mutations. In the same way, since the mutation in the gene from the South African Xhosa FH subject, TT, creates a new PstI restriction site, this can be used to detect the deletion, and will enable determination of the prevalence of this mutation in the Xhosa community (87).

Although therapeutic approaches to homozygous FH to date have been limited to liver transplants and/or plasmapheresis, the observation that the "high defective" level of mutant LDL receptors in homozygous Afrikaner subject, FH1a (and presumably in other subjects carrying the same mutant allele) is able to be "upregulated" and "downregulated" similarly to that in normal cells (discussed in section 5.3.2), suggests that therapy usually employed for heterozygous subjects, (i.e. upregulation of LDL receptor activity from the normal allele by inhibition of HMG-CoA reductase combined with the use of bile-acid binding resins, see section 1.6) may be effective in these homozygous subjects in increasing hepatic receptor expression, and thus contribute to a decreased plasma cholesterol level.

From a cell biological point of view, LDL receptor mutants have the potential to be very informative. Biochemical analysis of mutations in subjects with FH has revealed much about the structure-function relationships in the LDL receptor protein, and elucidated many steps in its complex intracellular pathways. The combination of the study of naturally-occurring mutations at the phenotypic and molecular level, combined with the ability to create site-directed mutations should enable extensive analysis of the functions of the various protein domains of the receptor, and may shed light on the role of glycosylation in these functions. The findings may yield general concepts which can be applied to understanding structural and functional aspects of other membrane proteins. As discussed above, the two Afrikaner mutations described in this thesis can potentially provide valuable insights into the biological recognition "signals" for protein degradation and intracellular transport. Elucidation of the primary, secondary, tertiary, and possibly quaternary, structural changes responsible for the phenotypic expression of these LDL receptor mutations may lead to an understanding of conformational properties constituting either an ER

retention signal, or a degradation signal for membrane proteins, and, in addition, how these "signals" are recognized in the cell.

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