POST-TRANSLATIONAL PROCESSING OF THE LOW DENSITY LIPOPROTEIN RECEPTOR

by

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Abbreviations

apo..... apolipoprotein
ARF ..... ADP-ribosylation factor
BFA..... brefeldin A
BiP ..... immunoglobulin heavy chain binding protein.
BSA ..... bovine serum albumin
bp..... base pairs
CHO..... Chinese hamster ovary
DMEM..... Dulbecco's modified Eagle's minimum essential medium
DTT..... dithiothreitol
ECL..... enhanced chemiluminescence
EGF ..... epidermal growth factor
Endo H..... Endoglycosidase H (Endo-ß-N-acetylglucosaminidase H)
ER..... endoplasmic reticulum
FCS ..... foetal calf serum
FH..... familial hypercholesterolaemia
HDL ..... high density lipoprotein
HMGCoA ..... 3-hydroxy-3-methylglutaryl coenzyme A
HSPG ..... heparan- and dermatan-sulfate proteoglycans
IDL..... intermediate density lipoprotein
LDL..... low density lipoprotein
LPDS ..... lipoprotein-deficient serum
MHC ..... major histocompatibility complex
NEM..... N-ethyl maleimide
O-Glycanase..... Endo-α-N-acetyl-galactosaminidase
PBS ..... phosphate-buffered saline
PMSF..... phenylmethylsulfonyl fluoride
SREBP..... sterol regulatory element binding protein
TGN..... trans-Golgi apparatus

Summary

The low density lipoprotein (LDL) receptor is a transmembrane glycoprotein that mediates the uptake of plasma LDL and thereby provides cholesterol to cells. During its synthesis in the endoplasmic reticulum, the LDL receptor folds and forms disulfide bonds in multiple cysteine-rich repeats. N- and O-linked oligosaccharide chains are added in the endoplasmic reticulum and processed during passage through the Golgi apparatus, en route to the cell surface. The aim of this thesis was to study the influence of post-translational events on the synthesis of the LDL receptor. Experiments addressed: 1) the necessity of the compartmental organisation of the secretory pathway for the glycosylation of the LDL receptor; 2) the requirements for the formation of disulfide bonds; 3) the role for the chaperone, calnexin, in the folding of the LDL receptor; and 4) the manner in which folding was disrupted by mutations. Experiments were performed in cultured cells that were incubated with [^{35}S]methionine. Biosynthetically-labelled LDL receptor was immunoprecipitated and was analysed by SDS polyacrylamide gel electrophoresis.

Treatment with brefeldin A fused the compartments necessary for glycosylation, the endoplasmic reticulum and the Golgi apparatus, and trapped the LDL receptor in the secretory pathway. The glycosylation of the LDL receptor was retarded and a series of partially-processed glycosylation intermediates were synthesised. Processing continued over 4 hours, but fewer than normal O-linked oligosaccharide chains were added to the LDL receptor. The normal secretory pathway was restored when brefeldin A was removed. The abnormal LDL receptors were transported efficiently to the cell surface, but their glycosylation remained irreversibly altered. These findings demonstrate that normal glycosylation requires the secretory pathway to be organised into separate membrane compartments.

The disulfide bonds of the LDL receptor were assessed with a conformation-specific antibody and by electrophoresis under non-reduced conditions. In intact cells, the disulfide bonds of the mature, endocytic form of the LDL receptor were resistant to dithiothreitol-induced reduction, except in the presence of denaturants, as were the solubilised forms of the LDL receptor. In contrast, while in the endoplasmic reticulum,

the disulfide bonds of the LDL receptor precursor were reduced by dithiothreitol, indicating that they were accessible and were prevented from being buried in the folded protein core, despite the precursor having a compact, disulfide-bonded structure that resisted reduction in vitro. When dithiothreitol was removed from intact cells, disulfide bonds reformed rapidly in the endoplasmic reticulum and the refolded receptors were transported to the cell surface. A number of novel findings emerged from these studies. Disulfide bonds were essential for the folding of the LDL receptor. Disulfide bonds did not have to form co-translationally - normal folding occurred when they formed post-translationally. Folding required metabolic energy which implicated a requirement for associated chaperone protein(s). Ca^{2+} was required for LDL receptor folding in a manner that was different to its role in ligand binding to the mature LDL receptor. The glycosylation of the LDL receptor did not influence the formation of disulfide bonds. Similarly, reduction of the disulfide bonds of the LDL receptor did not irreversibly alter its glycosylation. LDL receptor transport from the endoplasmic reticulum was impaired when ATP or Ca^{2+} was depleted, when disulfide bonds were reduced and when cysteine residues were mutated in the 5th binding repeat of the ligand binding domain. All of these changes were shown to affect the folding of the LDL receptor. These findings clearly show the inter-relationship between LDL receptor folding and transport. A missense mutation causing the conservative substitution of an aspartate residue by a glutamate residue (FH Afrikaner-1, Asp206Glu) was shown to impair the disulfide bonding of the mutant LDL receptor in the endoplasmic reticulum. The disulfide bonds of the LDL receptor were not affected by all missense mutations that are known to retard transport from the endoplasmic reticulum. Similarly, mutations (truncations and missense) in the cytoplasmic tail of the LDL receptor retarded processing, but did not affect the disulfide bond structure of the LDL receptor. The chaperone, calnexin, did not show quantitatively significant association with the LDL receptor. Together with other results, this suggested that calnexin was not critical for the folding of the wild type LDL receptor. Calnexin also was not involved in the retention of reduced or mutant forms of the LDL receptor in the endoplasmic reticulum.

Mutant LDL receptors were constructed to individually substitute (to alanine) each of the six cysteine residues in the 5th binding repeat of the ligand binding domain. Expressed in CHO cells, the disulfide bond structures of the mutant LDL receptors were altered as assessed by electrophoresis under non-reduced conditions, and the rates of

transport from the endoplasmic reticulum were retarded. Differences were observed in the rates of processing and stability of the mutants, which, in all instances, resulted in an extremely low number of surface molecules. These and other results indicate that all 6 cysteine residues are required for the folding of the 5th binding repeat, and are involved in disulfide bonds, though they prevent the 5th repeat from achieving the most compact folded conformation.

Chapter 1

Introduction

Introduction

1.1. Cholesterol and the LDL receptor

Cholesterol is required by all cells as a constituent of membranes, it is also the precursor for the synthesis of bile acids in the liver and the precursor for the synthesis of steroid hormones in the adrenal gland. Cholesterol is transported in plasma lipoproteins in a form esterified to fatty acids, and delivered to cells by the process of receptor-mediated endocytosis. The LDL receptor is a membrane glycoprotein which binds the predominant cholesterol-containing lipoprotein, LDL, and transports it from the plasma into the cell. This cell-surface receptor performs a key role in cholesterol homeostasis and its regulation controls both cellular and plasma cholesterol levels. Mutations that disrupt the LDL receptor gene cause familial hypercholesterolaemia, where the impaired clearance of intermediate density and low density lipoproteins causes the accumulation of cholesterol in the plasma and premature atherosclerosis.

Many features of the cell biology of the LDL receptor have been identified by Brown and Goldstein, in Dallas, who began their studies in 1972 and received the Nobel prize in physiology or medicine in 1985 (Brown and Goldstein, 1986). In their studies using cultured human fibroblasts, they demonstrated that added sterols stimulated cholesterol esterification and suppressed cholesterol synthesis within the cell. The regulatory effects involved a selective, high affinity receptor that was characterised using ^{125}I-labelled lipoproteins: lipoprotein binding at the surface was followed by internalisation and the release of regulatory-cholesterol in the lysosomes. In cells obtained from patients with FH, the regulatory effects were not observed, unless the cholesterol was added in a form which was able to diffuse directly through the cell membrane: the underlying defect in FH was the absence of the surface receptor for LDL.

These elegant studies were extended: the binding activity was used to purify the LDL receptor to homogeneity; antibodies were produced and used to track the movement of this migrant protein through the cell (the LDL receptor is the prototype for the paradigm of receptor-mediated endocytosis); heterogeneous defects causing FH were identified, including defects of binding and internalisation. Cloning of the gene outlined the structure of the LDL receptor and revealed the molecular basis for mutations causing FH. The LDL receptor pathway was confirmed in other cultured cells, in an animal model of FH (the Watenabe heritable hyperlipidaemic rabbit) and in transgenic mice.

Its discovery has advanced the treatment of hypercholesterolaemia, in particular, through the development of the HMGCoA reductase-inhibitor class of drugs, the ability to genetically-council FH patients and through the use of gene-therapy based on the LDL receptor.

1.2. Itinerary of the LDL receptor

The LDL receptor protein is translated by polyribosomes attached to the rough endoplasmic reticulum as a polypeptide of 860 amino acids (Yamamoto et al., 1984). Synthesis is initiated with a 21 amino acid signal sequence which is co-translationally inserted into the ER-lumen and then cleaved from the receptor. In the ER the residual protein of 839 amino acids folds and forms disulfide bonds and is glycosylated with core O-linked sugar chains and the high mannose, N-linked chains characteristic of an ER protein (Cummings et al., 1983). This form of the LDL receptor appears as a protein with an apparent molecular mass of 120 kDa on SDS polyacrylamide gels. The normal LDL receptor is transported from the ER to the cell surface within 15 to 30 minutes (Tolleshaug et al., 1982). En route, the LDL receptor passes through the Golgi apparatus where the N- and the O-linked oligosaccharide chains are processed and are terminated by the addition of galactose and sialic acid residues. These changes modify the apparent molecular mass of the LDL receptor by 40 kDa, from 120 kDa to the 160 kDa of the mature LDL receptor. The transport of the LDL receptor from the ER is prevented by certain mutations which alter the structure of the LDL receptor (Hobbs et al., 1992). This retention is not mediated by specific retention motifs (Pelham, 1991); it probably is due to interaction of the partially-folded, exposed, hydrophobic domains of the LDL receptor with resident chaperone proteins.

The LDL receptors are not distributed evenly on the cell surface. They are inserted as a dispersed population (Sanan et al., 1989) and migrate laterally to be concentrated within clathrin-coated pits: in fibroblasts, about 70% of surface LDL receptors are clustered in clathrin-coated pits, through which efficient receptor-mediated endocytosis occurs (Anderson et al., 1982). In the hepatocytes of transgenic mice, the LDL receptors are confined to the basolateral (sinusoidal) surface of the polarised cell membrane, and are not detected on the apical (cannalicular) surface (Yokode et al., 1992; Pathak et al., 1990). Signals in the cytoplasmic tail of the LDL receptors cause this distribution by targeting transport from the trans-Golgi apparatus to the basolateral

membrane (Matter et al., 1992; Matter et al., 1993; Matter et al., 1994). LDL receptors re-enter the cell when the coated pits invaginate to form coated vesicles with the extracellular domain of the LDL receptor oriented within their interior. Endocytosis occurs constitutively and does not require ligand to be bound to trigger lateral migration to coated pits or internalisation. The LDL receptor and the ligand part company in the acidic endosome (Brown et al., 1983): ligand is delivered through late endosomes to lysosomes, where the apoprotein is degraded and the cholesterol ester is hydrolysed to release free cholesterol for use within the cell; the LDL receptor is concentrated within membrane-rich, tubular elements and recycles to the cell surface for further rounds of endocytosis. In fibroblasts, the half-time for a round of endocytosis is about 12 minutes (Brown et al., 1983), which implies that each receptor performs about 60 rounds, given a half-life of about 12 hours (Casciola et al., 1988). At steady-state, about 25 - 50% of the population of LDL receptors are within the endosomal pathway and the remainder are on the surface (Hare, 1990). It is presumed that all receptors are active and recycling; there is no evidence for an inactive or a sequestered population of LDL receptors, as has been described for the asialoglycoprotein receptor (Weigel and Oka, 1993). In polarised cells, the itinerary of LDL receptors is more complex than in fibroblasts (Li et al., 1991; Matter et al., 1993). LDL receptors require sorting in endosomes, either to recycle back to the surface from where they were internalised, or to cross the cell in a transcytosis pathway. The path followed depends on the surface from which the LDL receptor entered the cell: from basolateral endosomes LDL receptors recycle to the basolateral surface, while delivering ligand to the lysosomes; by contrast, apically-derived LDL receptors are directed to transcytose ligand to the basolateral surface. Thus, from either source of endosomes, the LDL receptors are sorted to the basolateral surface, though via different pathways. Disruption of the targeting signals causes missorting in endosomes, with both the basolateral and the apical LDL receptors being targeted to the apical surface. These basolateral targeting signals are the same as those which direct the sorting of newly-synthesised LDL receptors within the trans-Golgi network, indicating that a common sorting mechanism probably operates at both sites (Matter et al., 1993).

1.3. Structure of the LDL receptor gene and protein

The gene coding for the LDL receptor is located on the distal short arm of chromosome 19 (p13.1-p13.3) (Hobbs et al., 1990). The locus spans 45 kilobases and includes the

known upstream promoter-elements which are found within 200 base pairs 5` of the methionine codon for translation initiation. The gene is well conserved (amphibians to man) (Mehta et al., 1991) indicating that it encodes an ancient protein utilised in diverse animals.

A striking feature is that the division of the gene into 18 exons corresponds to the organisation of the protein into domains and subdomains (Figure 1.1). In many instances, single exons, or groups of exons, encode distinct domains of the protein. These units are found to be repeated within the structure of the LDL receptor, other members of the LDL receptor gene family and in the genes encoding other proteins, which suggests that the exons were duplicated and exchanged (shuffled) through the evolution of these genes (Sudhof et al., 1985).

The LDL receptor is an 839 amino acids protein which contains 2 N-linked and about 18 O-linked oligosaccharides (Cummings et al., 1983). The calculated molecular mass of the glycosylated receptor is about 115 kDa (Tolleshaug et al., 1982). The protein spans the cell membrane once and, at the cell surface, is orientated with its N-terminus being extracellular and its C-terminus in the cytoplasm (Schneider et al., 1983b). In total, 768 amino acids are extracellular, 22 amino acids span the cell membrane and 50 amino acids form the intracellular, cytoplasmic tail (Russell et al., 1984; Yamamoto et al., 1984). Overall the LDL receptor is a modular protein composed of distinct domains (Figure 1.2). The extracellular portion consists of the ligand binding domain, a domain with homology to the epidermal growth factor (EGF) precursor and an O-linked sugar domain (Sudhof et al., 1985). Exon 1 encodes a signal sequence which inserts the nascent polypeptide into the membrane of the ER and thus initiates its journey through the secretory pathway to the cell surface. The signal sequence is cleaved from the LDL receptor in the endoplasmic reticulum. Exons 2-6 encode the ligand binding domain of 292 amino acids which is composed of 7 repeats of a single unit of about 40 amino acids that is homologous to the C9 component of complement. Each repeat contains 6 cysteine residues and has a characteristic central hydrophobic region flanked by hydrophilic ends (Kyte and Doolittle, 1982). The repeats are not perfectly homologous: optimum alignment shows that the spacing of the cysteine residues, and 19 of the 41 amino acids are conserved, including a carboxy-terminal acidic sequence, asp-X-ser-asp-glu, thought to be important for ligand binding (Sudhof et al., 1985). The cysteines are thought to be fully disulfide-bonded within their given repeat and crucial

for maintaining the folded structure of the domain, especially during rounds of endocytosis through the acidic environment of the endosomal pathway. The disulfide bond pattern was identical in 2 peptides, corresponding to the 1st (Daly et al., 1995; Bieri et al., 1995a) or the 2nd (Bieri et al., 1995b) ligand binding repeats, respectively. Within each peptide, cysteine 1 paired with cysteine 3, cysteine 2 paired with cysteine 5, cysteine 4 paired with cysteine 6. Due to the homology between the repeats, is probable that the cysteines in the other binding repeats pair in a similar order.

The domain with homology to the epidermal growth factor (EGF) precursor extends from amino acid 293-692 and is encoded by exons 7-14. The domain contains 3 growth factor repeats (A-C) which are found in 4 copies in the precursor for EGF. The A and B repeats of the LDL receptor are separated from the C repeat by 5 copies of a 40-60 amino acid repeat, each of which contains a conserved YWTD (tyrosine-tryptophan-threonine-aspartate) motif. The EGF precursor homology domain is needed for the binding of LDL on the cell surface, as well as for the dissociation of ligand from the receptor in endosomes.

The O-linked sugar domain is encoded by exon 15. This 57 amino acid domain (amino acids 693 to 750) contains a cluster of 18 serine or threonine residues which are potential sites for the addition of O-linked oligosaccharide chains. The function of this domain has not been clearly defined: FH patients homozygous for a deletion of the entire exon 15 and identified in the Japanese and the Finnish populations, express a mild form of FH (Kajinami et al., 1988; Koivisto et al., 1993). However, deletion of the domain by site-directed mutagenesis did not affect the synthesis, transport, binding, recycling, nor the degradation of the receptor when transfected into hamster fibroblasts (Davis et al., 1986a). It has been speculated that the extensive O-linked glycosylation of the O-linked sugar domain may hold the receptor in an extended conformation, so as to facilitate ligand binding, but this has not been shown directly (Goldstein et al., 1985).

The membrane-spanning domain anchors the receptor in the membrane of the various compartments of the cell. It consists of a hydrophobic, 22 amino acid sequence which is encoded by exon 16 and the 5` part of exon 17. The 50 amino acid cytoplasmic tail of the LDL receptor (amino acids 790-739) is encoded by the 3` end of exon 17 and exon 18. This domain is well conserved between different species and contains signals to direct the receptor to the basolateral domain in the plasma membrane of polarised cells, and a separate signal necessary for the efficient internalisation of clustered LDL receptors through coated pits (Figure 1.3). This domain also is required for the

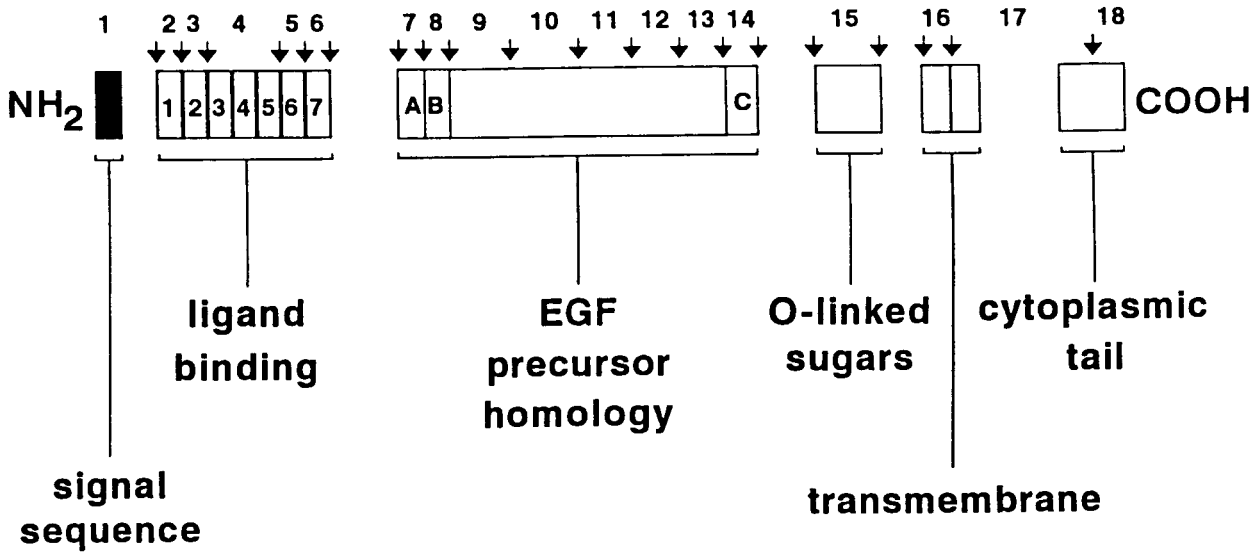


Figure 1.1 Exon organisation and protein domains in the human LDL receptor.

The six domains of the LDL receptor are labelled in the lower portion. The cysteine-rich, 40-amino acid repeats in the binding domain are numbered 1 to 7. The three growth factor repeats in the EGF precursor homology domain are lettered A to C. The positions at which introns interrupt the coding region are indicated by arrows. Exon numbers are shown between the arrows.

(Figure modified from Sudhof et al., 1985).

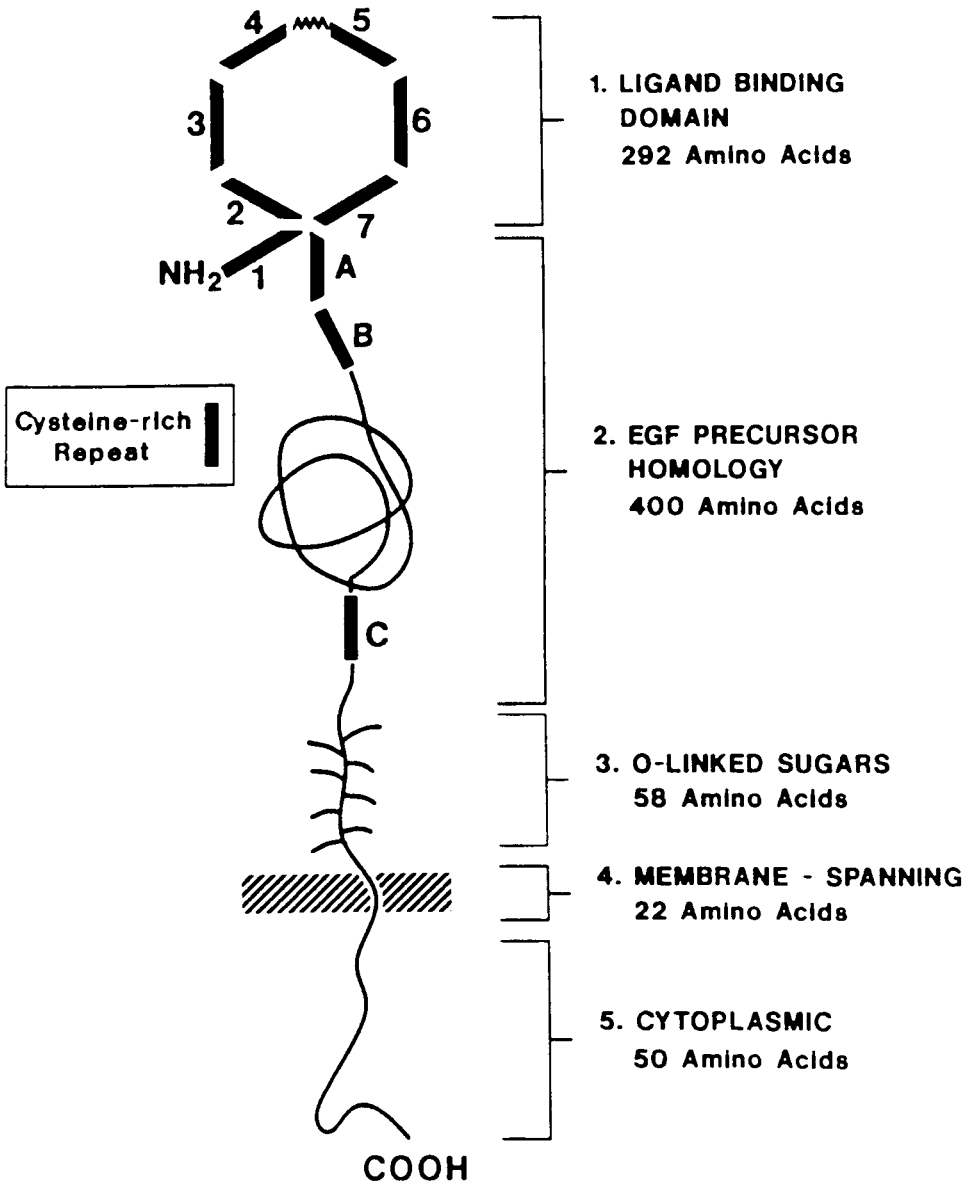


Figure 1.2 Model of the human LDL receptor

The domains of the LDL receptor are indicated. The 40-amino acid ligand binding repeats are numbered 1 to 7, and the growth factor repeats of the EGF precursor homology domain are labelled A to C.

(Figure modified from Esser et al., 1986).

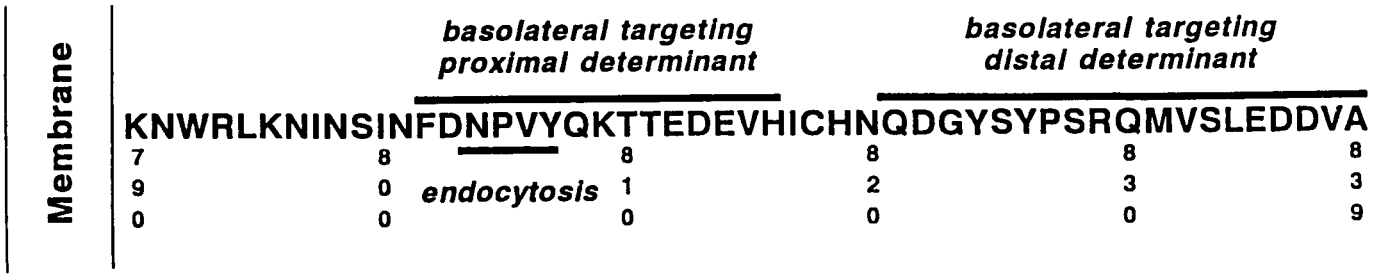


Figure 1.3 Localisation of the signals for internalisation and targeting in the cytoplasmic tail of the LDL receptor.

The sequence of the LDL receptor cytoplasmic tail is given in single letter code. The position of the signal for internalisation through clathrin-coated pits is indicated, as are the positions for the proximal and distal signals for basolateral targeting in polarised cells.

self-association of receptors into multimeric structures (van Driel et al., 1987a). The carboxy-terminus of the cytoplasmic tail is conserved almost perfectly between species and encodes a polarised targeting signal (Matter et al., 1992; matter et al., 1993).

1.4. Regulation of LDL receptor expression

The LDL receptor promoter consists of three imperfect repeats and two TATA boxes. Repeats 1 and 3 are binding sites for the transcription factor, Sp1, which constitutively promotes transcription (Dawson et al., 1988; Sudhof et al., 1987a; Sudhof et al., 1987b). The activity of the LDL receptor promoter is regulated by oxysterols derived from the intracellular pool of unesterified cholesterol: low intracellular sterols stimulate maximal positive transcriptional activity, but its positive effect is lost when intracellular sterols accumulate. These effects are mediated through repeat 2, which is a conditional-positive, sterol regulatory element (Dawson et al., 1988; Briggs et al., 1993). Two transcription factors which bind repeat 2, sterol regulatory element binding protein-1 (SREBP-1) and SREBP-2, have been isolated (Briggs et al., 1993; Hua et al., 1993; Wang et al., 1993; Yokoyama et al., 1993) and the mechanism of their regulation by sterols has been determined (Wang et al., 1994). The SREBPs are transmembrane proteins which are attached to the ER membrane. When sterols are depleted from cells, a protease cleaves the amino-terminal fragment of each SREBP and releases an active leucine-zipper type transcription factor which translocates into the nucleus and stimulates transcription by binding to the sterol regulatory element. SREBPs also bind to the sterol regulatory element of 3-hydroxy-3-methylglutaryl coenzyme A and stimulate its transcription: the combined effect is to increase the intracellular pool of cholesterol which prevents the proteolytic release of the SREBPs from the ER This feedback mechanism serves to limit the accumulation of cholesterol within cells. Recently the model of transcription stimulation has been refined to include an interaction between the SREBPs at repeat 2 and the binding of Sp1 to repeat 3: SREBPs are weak stimulators of transcription; their binding increases Sp1 binding to repeat 3, and together they activate expression from the LDL receptor promoter (Sanchez et al., 1995; Yieh et al., 1995).

1.4.1 Post-transcriptional control of LDL receptor activity

The action of many hormones regulate the level of LDL receptor activity expressed in different cells (Soutar and Knight, 1990). In general, these effects have been characterised poorly in comparison to the sterol effects described above. These hormones act through transcription mechanisms: the LDL receptor activity being regulated directly through its mRNA levels. Interestingly, insulin, human chorionic gonadotropin and calmodulin antagonists are able to exert stimulatory effects which override the effects of sterols (either suppression by sterols or apparent full stimulation by sterols). Their mechanisms of action are not known. Convincing evidence for the post-translational control of LDL receptor activity has not been apparent (Brown and Goldstein, 1986) until recently. This was demonstrated by the sterol-mediated down-regulation of a retroviral vector-encoded transcript of the normal LDL receptor, in receptor-deficient cells (Sharkey et al., 1990). This vector contained a strong, constitutive promoter which was unresponsive to sterols and did not alter the mRNA levels, indicating that the control was at the level of translation or protein stability.

1.5. LDL receptor mutations

The Dallas collection of fibroblast cultures from FH patients probably is the largest collection of receptor mutants expressed in animal cell (Hobbs et al., 1992; Hobbs et al., 1990). This database has enabled the systematic dissection of structure-function relationships of the LDL receptor. More than 150 mutations have been identified in the LDL receptor genes of hypercholesterolaemic patients. Together with artificial mutant-constructs, these alleles have provided a wealth of information on the function of membrane proteins. The mutations are classified based on their protein phenotype. Class 1 mutants do not have detectable receptor protein. They are caused by mutations affecting the promoter and by mutations which produce unstable mRNA molecules: many are missense mutations which produce mRNA molecules of the correct size; the instability is due to translation stopping prematurely. This class would also include those alleles producing protein-products which have an extremely short half-life possibly due to rapid degradation in the ER. Class 2 mutants are transported slowly from the ER to the cell surface and are detected by retarded conversion from the precursor to the mature form (typically in biosynthetic pulse-chase experiments). This phenotype is probably caused by impaired folding of the LDL receptor and its subsequent association with chaperones, which promote folding partly by preventing

the aggregation of partially-folded proteins. Other defined catalytic activities promote disulfide bond formation and the cis-trans isomerisation of peptidyl-prolyl bonds. Class 2 has been divided into those alleles having an apparent complete block in processing and those where the rate only is retarded. These distinctions are largely facile as other effects, such as the stability of either the precursor or the mature LDL receptor, often are not considered and complicate the interpretation of pulse-chase experiments. Many mutations which are classified in other classes, especially class 3 mutants (binding defective), overlap with class 2. Overall, more than 50% of the mutant alleles belong to his class. An understanding of the folding of the normal LDL receptor and its disruption by class 2 mutations is a major theme of this thesis and is discussed further in Section 1.8.2.

Class 3 mutants have a binding defect and are caused by mutations in the ligand binding domain or the domain with homology to the EGF precursor. Study of mutants of this class have been particularly useful to define the manner in which a receptor single binding domain is able to bind structurally unrelated ligands. The manner in which the LDL receptor binds to its ligands is described below under "ligand binding to the LDL receptor". All mutants described to date have a reduced number of surface binding sites; no mutants with a reduced binding affinity have been described. Class 4 mutants have internalisation defects due to mutations within the cytoplasmic tail of the LDL receptor. These mutants range from point mutations within the internalisation motif, to larger deletions which truncate the tail. They are discussed in detail under "internalisation of the LDL receptor" (Section 1.7). Class 5 mutants are unable to recycle during rounds of endocytosis. These mutants have defects within the domain with homology to the EGF precursor which prevents the acid-induced dissociation from the ligand within the endosomes. These mutants are trapped within the cell and are rapidly degraded. Mutants with defects within the polarised targeting signals of the cytoplasmic tail would also be classified in this class, but have not been detected. They would be unable to recycle back to the correct surface domain of polarised cells. A 6th class has been proposed to group the diverse set of mutants which are unstable and have a rapid rate of degradation (Fourie et al., 1988). The normal route of degradation of the LDL receptor has not been identified. The site and the mechanism of the degradation of normal LDL receptors has not been identified. In fibroblasts, LDL receptors are turned over with a half-life of about 12 hours (Casciola et al., 1988). This rate is not changed by conditions that alter the number of receptors present within the

cell, indicating that the number of receptors is regulated solely by the rate of synthesis, and not through degradation. Lysosomes, responsible for the degradation of the LDL ligand, appear not to be the primary site of receptor turnover, since their activity can be inhibited without affecting the half life of the LDL receptor (Casciola et al., 1989). The presence of ligand, though, has no effect on the stability of normal LDL receptors (Casciola et al., 1988). These results indicate that the receptor and the ligand follow independent routes to their sites of degradation (Casciola et al., 1988). The initial degradation events of the LDL receptor may occur on the cell surface, as mutations which prevent receptor internalisation do not alter their rate of degradation (Casciola et al., 1989). The stability of pre-existing LDL receptors is prolonged when new protein synthesis is inhibited by cycloheximide (Casciola et al., 1988). This suggests that the turnover of the LDL receptor requires a short-lived protein, though its function is not known. In the case of the normal LDL receptor expressed in cells defective in glycosylation-ability, rapid receptor degradation occurs with the release of a large soluble receptor fragment into the medium (Kozarsky et al., 1988). Sometimes, mutant LDL receptors are degraded at an enhanced rate due to their being unable to follow the normal LDL receptor itinerary. However, the features which dictate the sites at which LDL receptors are rendered unstable, are not always clear. Thus certain LDL receptors are retained within the ER, probably due to impaired folding (Hobbs et al., 1990). Some of these mutant LDL receptors are degraded from this site, but others are stable while being retained in the ER. LDL receptors with certain other mutations escape from the ER and reach the cell surface, where they exhibit their unstable phenotype (Fourie et al., 1988). In all these instances, the mechanisms of degradation are not known. LDL receptors with mutations in the EGF precursor homology domain are prevented from recycling, as they are unable to dissociate from ligand (van der Westhuyzen et al., 1991; Davis et al., 1987a). These intracellularly trapped LDL receptors are rendered unstable in the presence of ligand, indicating that it is their inability to dissociate from ligand which targets them to degradation. Normal LDL receptors can also be prevented from dissociating from ligand by incubation with NH_4Cl which prevents the acidification of endosomes. This too enhances the degradation of LDL receptors in a ligand-dependent manner (Grant et al., 1990). NH_4Cl inhibits the function of lysosomes, indicating that they are not involved in this enhanced degradation process.

1.5.1. Mechanism of mutation in the LDL receptor gene

FH is one of the commonest autosomal dominant genetic disorders in humans, affecting about 1 in 500 individuals, though the frequency is much higher in certain populations (see below). The factors that influence the frequency of a genetic change in a population are: i) the rate at which new mutations occur at a particular locus (and the time-span involved); ii) the reproductive advantage (or disadvantage) experienced; iii) the chance events, such as founder effects, that affect sampling.

The molecular mechanisms that underlie the mutagenicity at this locus involve recombination events at Alu repeats and cytosine to thymidine transitions at CpG dinucleotides. Alu repeats are the commonest "middle repetitive" sequences in the human genome: they consist of a 300 base pair sequence which is repeated to about 910 000 copies (Hwu et al., 1986; Hobbs et al., 1990). In the LDL receptor gene, the frequency is about double that of the average for the rest of the genome. Alu repeat sequences are present at 9 of the 10 known endpoints of the 45 large deletions or insertions reported in the LDL receptor gene (Hobbs et al., 1992). 2 mechanisms underlie their effect: first, and commonly, an unequal crossover event may occur during homologous recombination at meiosis, between daughter chromatids containing Alu repeats oriented in the same direction; and second, the formation of a stem-loop structure within the same chromosomal strand may cause a mutagenic event between Alu repeats oriented in opposite directions (Lehrman et al., 1985a and 1987a). Cytosine to thymidine transitions occur at CpG dinucleotides: deamination of cytosine forms uracil, which is corrected back to cytosine by uracil glycosidase; deamination of methyl-cytosine forms thymidine which is maintained by replication (Cooper and Youssoufian, 1988). These changes are implicated in causing 16 % of the missense mutations in the LDL receptor locus, and include the recurrence of the same mutation on a separate haplotype, indicating a separate mutagenic event (Hobbs et al., 1992). In FH, there is no recognised survival advantage associated with the heterozygous state, which also does not overtly compromise gene transmission, as the disease usually manifests after the reproductive ages. Disease alleles are excluded from the population by homozygous patients who commonly die prior to attaining a reproductive age. In the Afrikaner, French Canadian, Finnish, Ashkenazi Jews, and Christian Lebanese populations, the frequency of FH is amplified by founder effects. These arose when certain mutant FH alleles were over-represented in the initial pool of settlers, compared to their frequency in the general population (Hobbs et al., 1992). This higher allele frequency was maintained as these populations grew in relative

isolation, and furthermore were subjected to the random genetic drift characteristic of small populations. Characteristically, the FH individuals in a founder population express the same mutation (or few mutations), unlike the general population where many different mutations cause FH. This is unlike cystic fibrosis, where a single mutation causes about 70% of cases (Lemna et al., 1990). Such a pattern could arisen either by an ancient mutation having occurred in the context of a very low mutation rate, or by recurrent mutations occurring at a particular site ('hot spot'). Other more complex models are also possible, such as those which include a survival advantage for patients with this particular mutation.

Finally, the relative ease of diagnosis of FH has facilitated the identification of patients and thus the detection of mutations. The LDL receptor gene and the apoB gene are the two candidate genes recognised as having the potential to cause a monogenic pattern of hypercholesterolaemia.

Krieger and colleagues have developed an artificial system to select for cells with defects in the LDL receptor pathway (Krieger, 1986). After exposing cell to various mutagens, toxins were delivered, within LDL, to kill cells with intact LDL receptors and to select for cells with defective LDL receptor activity. In this manner, several different classes of mutant cells were identified to affect the LDL receptor, itself, and other protein involved in protein secretion (Krieger et al., 1981; Kingsley and Krieger, 1984). The ldlA class (defective LDL receptor gene) have been used to determine the effects of cloned LDL receptor genes (also used in this thesis) (Sege et al., 1984). The ldlC and the ldlF class involve defective peripheral Golgi coat proteins necessary for protein secretion (Guo et al., 1994; Podos et al., 1994). The ldlD class have defects in UDP-galactose and UDP-N-acetylgalactosamine 4-epimerase activity (Kingsley et al., 1986a), causing reversible defects in N- and O-linked glycosylation (see Section 1.8.1,"glycosylation of the LDL receptor")(Kozarsky et al., 1988). LDL receptor glycosylation also is affected in the ldlB class, though through undefined mechanisms. Recently, several more classes have been identified to affect LDL receptor stability and intracellular membrane transport (Hobbie et al., 1994).

1.6. Binding to the LDL receptor

The binding of lipoproteins to cells may be initiated by an interaction with heparan- and dermatan-sulfate proteoglycans (HSPG), prior to the involvement of a specific (high

affinity) surface receptor, such as the LDL receptor (Saxena et al., 1993). The HSPGs might act as an abundant source of low affinity sites to initially concentrate ligand at the cell surface and thus facilitate binding to the LDL receptor. Although the binding of lipoproteins directly to HSPGs may be poor, two factors increase this binding by up to 80 fold (Mulder et al., 1993). First, lipoprotein lipase (LPL) acts as an intermediary in associating lipoproteins (LDL, Lp(a) and VLDL) with HSPGs (Mulder et al., 1993; Williams et al., 1992; Rumsey et al., 1992; Saxena et al., 1993). Second, apoE-enriched chylomicron remnants (and VLDL) associate with HSPGs without the involvement of LPL (Ji et al., 1993). However, the physiological relevance of these findings is unclear as the level of LPL used to demonstrate enhanced binding of lipoproteins to HSPGs is 2 orders of magnitude higher than the concentration of LPL in the circulation (Goldberg et al., 1986; Mulder et al., 1993; Williams et al., 1992). The LPL concentration, though, may be far greater in confined compartments such as the site of lipoprotein-clearance in the liver (the space of Disse) (Williams et al., 1992). In such a localised environment, the interaction of lipoproteins with HSPGs would be influenced not only by the availability of LPL, but also by the availability of apoE, which inhibits lipoprotein binding to LPL (Saxena et al., 1993). The role of the LDL receptor in mediating the endocytosis of HSPG-associated lipoproteins, is controversial (HSPGs themselves do not mediate endocytosis). The internalisation of lipoprotein was not affected by the down-regulation of LDL receptor activity (with sterols) (Williams et al., 1992), nor was it reduced in the fibroblasts from FH patients (lacking functional LDL receptors) (Rumsey et al., 1992). However, another study indicated a role for the LDL receptor when FH fibroblasts exhibited less than 10% of the HSPG-associated LDL uptake of normal fibroblasts, and the internalisation activity was suppressed in parallel to the down regulation of LDL receptor activity in normal fibroblasts (Mulder et al., 1993).

The LDL receptor binds lipoproteins containing apolipoprotein B-100 (apoB) or apolipoprotein E (apoE) with high affinity (Goldstein and Brown, 1977), including LDL, VLDL, IDL, chylomicron remnants and the lipoproteins derived from cholesterol-fed animals, ßVLDL (rabbits)and HDLc (dogs). Binding occurs by ionic interactions between basic residues on the apolipoproteins and acidic residues of the 7 repeats in the ligand binding domain of the LDL receptor, and is Ca^{2+}-dependent (Goldstein and Brown, 1977). Mutagenic studies have revealed the manner in which a receptor utilises multiple repeats, within a single binding domain, to recognise more than one

ligand: different combinations of the repeats are required to bind the different ligands (Esser et al., 1988; Russell et al., 1989). In these studies where the binding repeats were individually deleted, deletion of repeat 5 reduced apoE binding by 60%, whereas any other repeat could be removed without affecting binding. The simplest interpretation of these findings is that apoE only binds to repeat 5, though other more complex models are possible. Repeat 5 has the unique presence of 4 acidic amino acid compared to a triplet cluster of acidic amino acids found in the other repeats (serine-aspartate-glutamate-glutamate vs. serine-aspartate-glutamate) which are important for ligand binding (Goldstein et al., 1985). Interestingly, the binding of apoE-containing ligands occurs with a 10-fold higher affinity than apoB-containing ligands. This is thought, in part, to be due to the many apoE molecules of a single lipoprotein particle being able to interact simultaneously, with several LDL receptors gathered together in an oligomeric partnership, with each receptor providing a single apoE binding site (the stoichiometry of apoB to LDL receptor binding is 1:1). The binding of apoB (LDL) tolerated only the deletion of repeat 1, indicating that repeats 2-7 were all required for apoB binding. In addition to the ligand binding domain, repeat A of the EGF precursor homology domain also is required for the binding of apoB (Esser et al., 1988); its deletion reduced LDL binding by 75%. However, repeat A is not required for the binding of LDL to receptors which are immobilised on nitrocellulose (ligand blot), and thus appears to perform a permissive role in LDL binding at the cell surface. The receptor binding domain of apoB is composed of 2 clusters of basic amino acids together with a flanking region of about 800 amino acids (Milne et al., 1989): one of the clusters is homologous to the receptor binding domain of apoE. Due to the large size of apoB (4536 amino acids) and its binding domain, apoB is probably relatively rigid on the surface of the lipoprotein particle which might explain its poor tolerance to mutations in the binding repeats of the LDL receptor. By contrast apoE is a far smaller protein (299 amino acids) (Lalazar et al., 1988) which possibly is more mobile on the surface of the lipoprotein particle, and thus more able to adapt to distortions introduced into the LDL receptor (with the exception of repeat 5) by mutations (induced fit model).

1.7. Internalisation and recycling of the LDL receptor

The first signal directing the internalisation of a surface protein, was identified in the LDL receptor (Davis et al., 1986b; Lehrman et al., 1985b): it was localised to the first 22 amino acid (amino acids 790-812) of the 50 amino acid tail (Figure 1.3) and defined to

include amino acids 804-807 (the motif NPXY, where X could be any amino acid) (Davis et al., 1987b; Chen et al., 1990). This sequence is conserved in the LDL receptor tail across 6 species and is found in the cytoplasmic tail of several other proteins which are known to be internalised via coated pits, though it is not the only amino acid motif able to direct internalisation through coated pits. These internalisation signals are all assumed to interact with the same cytoplasmic components, and are assumed to adopt a similar conformation (Collawn et al., 1990): structural studies on a nonapeptide containing the NPVY LDL receptor signal show that it forms a reverse ß turn (Bansal and Gierasch, 1991). Peptides derived from LDL receptors that are defective in internalisation do not form reverse turns. The aromatic amino acid (tyrosine 807) is crucial to the structural integrity of the turn, there is a lax requirement for the amino acid at position 806, in agreement with the earlier mutagenic studies (Davis et al., 1987b; Chen et al., 1990), and the region on the COOH-terminal side of the signal does not affect the structure of the turn (Bansal and Gierasch, 1991). The importance of the turn conformation was emphasised by the correlation of the efficiency of internalisation with the propensity of the nonapeptide to assume a reverse turn; this also has been determined for other internalisation signals without sequence similarity to the signal of the LDL receptor (lysosomal acid phosphatase) (Eberle et al., 1991). Thus the internalisation signal is expressed in the structural context of a tight reverse turn conformation, which is presented to a putative cytoplasmic acceptor. The adaptor protein complex (adaptors) that are localised to the plasma membrane (classified as hydroxyapatite type II) recognise the internalisation signal (Pearse and Robinson, 1990). Adaptors bind to the cytoplasmic tail of different receptors including the LDL receptor and are also required for receptor inclusion into coated pits (Pearse, 1988; Smythe et al., 1992). These complexes (molecular mass of 250-300kDa) are composed of two adaptin subunits (α,ß adaptins) of 100kDa each, together with one 50kDa protein and another 20kDa protein. The interaction between the adaptor and the LDL receptor tail can be prevented by competition by other receptors which are internalised via coated pits, indicating the common role of a single protein complex in recognising diverse internalisation signals. Importantly a receptor which is not internalised via coated pits, the haemaglutinin receptor, does not compete with the LDL receptor for the binding of adaptors (Pearse, 1988; Lazarovits and Roth, 1988). The actual binding site for adaptors on the LDL receptor has not been studied; on other receptors, adaptors bind to the aromatic internalisation signal (Pearse and Robinson,

1990). Adaptors also bind clathrin and promote the assembly of clathrin triskelions. Thus they have a central role in receptor mediated endocytosis, both by localising specific receptors within coated pits and by promoting the formation of the clathrin coat.

1.8. Post-translational processing of the LDL receptor

During their transit through the ER and the Golgi apparatus, secretory proteins are covalently modified in many ways: disulfide bonds form, sugar chains are added and processed, some proteins are proteolytically cleaved and so on. For the LDL receptor, the most prominent changes are its glycosylation and the formation of disulfide bonds. These and other less well characterised modifications, including sulfation, phosphorylation, oligomerisation and proteolytic cleavage are considered in this section.

1.8.1. Glycosylation

As has been described under "Biosynthesis of the LDL receptor", the protein molecule is modified by the addition of N- and O-linked oligosaccharide chains after its insertion into the ER. In general, the multistep assembly of N-linked chains has been well characterised, though for the LDL receptor, the number of N-linked chains has not been determined. Cummings has estimated that 1 or 2 biantennary, N-linked chains are added in fibroblasts, though this could vary in different cell-lines as there are 5 potential sites with the glycosylation motif, asparagine—X—serine/threonine (where X could be substituted by any amino acid) (Cummings et al., 1983). Three of the potential sites are in the binding domain and two are in the EGF precursor homology domain: it has been suggested that the compact folding of the binding domain may prevent its glycosylation (Goldstein et al., 1985). N-linked chains are known to be assembled while attached to the membrane lipid, dolichol, and then transferred to the receptor molecule (Kornfeld and Kornfeld, 1985). Initially, the chain is assembled on the cytoplasmic face of the ER and then flipped into the ER-lumen. The chain is assembled by the sequential addition of 14 hexose monosaccharides derived from nucleotide-and dolichol-P sugars, until it has the structure shown in Figure 1.4. As the LDL receptor is translated and translocated through into the ER, the 14-hexose chain is transferred to the asparagine of the glycosylation-motif. In total, 9 of the hexose sugars are removed by glycosidases: in the ER, glucose residues are removed (glucosidase I and II) to form the high mannose chain typical of an ER protein, while mannose

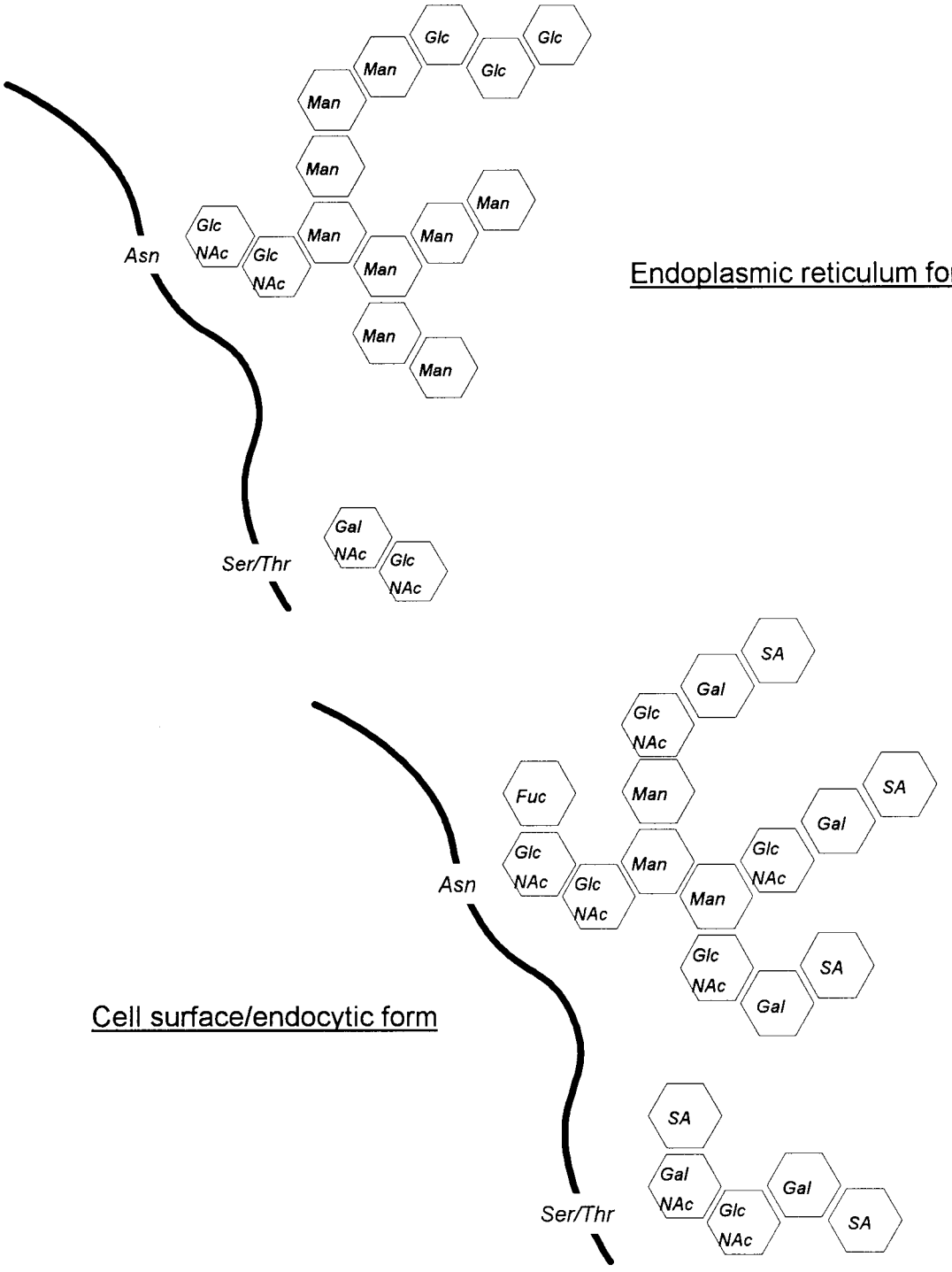


Figure 1.4 Typical structures of the oligosaccharide chains of the LDL receptor.

The structures of the oligosaccharide chains of the endoplasmic reticulum and the cell surface/endocytic forms of the LDL receptor are shown. The N-linked chains are attached to asparagine (Asn) residues, and the O-linked chain are attached either to serine (Ser) or threonine (Thr) residues.

Abbreviations used: GlcNAc, N-acetyl-glucosamine; Man, mannose; Glc, glucose; GalNAc, N-acetyl-galactosamine; Fuc, fucose; Gal, galactose; SA, sialic acid.

residues are removed in the cis (mannosidase I) and medial Golgi (mannosidase II). The chain structure is completed by the addition of N-acetyl-glucosamine and fucose in the medial Golgi, and galactose and sialic acid in the trans Golgi. The incorporation of ^{35}S-sulfate into the LDL receptor was blocked by treatment with tunicamycin, indicating that the N-linked chains are sufated (Cummings et al., 1983). O-linked chains are attached to the LDL receptor mainly at the 18 serine and threonine residues clustered in the O-linked sugar domain which lies adjacent to the cell membrane. Up to one quarter of the O-linked chains are dispersed in other regions of the extracytoplasmic domain of the LDL receptor (Davis et al., 1986a). The structure of O-linked chains have not been determined precisely. There is the possibility of considerable heterogeneity: N-acetylgalactosamine is added to the serine/threonine residue in the ER to initiate the chain, followed by the addition of N-acetylglucosamine to some of the chains (Figure 1.4)(Cummings et al., 1983). In the Golgi apparatus, galactose and sialic acid residues complete the O-linked structures, forming tri- and tetrasaccharide chains (Pathak et al., 1988). Sialic acids may be added at 2 sites on each chain: to the galactose or to the N-acetylgalactosamine. The heterogeneity in composition of the O-linked chains also is not limited to variation at different sites on the same LDL receptor molecule, but probably also varies between different LDL receptors. A prominent feature of the glycosylation, is that it dramatically alters the apparent molecular weight of the LDL receptor when electrophoresed on SDS polyacrylamide gels (Cummings et al., 1983; Davis et al., 1986a). The precursor has the electrophoretic mobility of a protein with a molecular weight of 120 000, while it has a calculated molecular weight of 93 102 for the protein component, and about 4 000 for the carbohydrate component. The processing in the Golgi apparatus contributes the net addition of sugars with an estimated molecular weight of 8 000-16 000 (depending on assumptions about the number of N- and O-linked chains), giving the mature LDL receptor an estimated molecular weight of about 115 000 (Goldstein et al., 1985), while its electrophoretic mobility indicates a molecular weight of 160 000. The mobility shift is largely due to extension of the O-linked chains: its magnitude is not altered by tunicamycin which reduces the apparent molecular weight of both the precursor and the mature LDL receptor by about 8 000. The effects of the O-linked chains was shown in a mutant strain of CHO cells, deficient in the ability to add galactose residues, which minimised the molecular weight change (Cummings et al., 1983). These chains lacked both galactose and terminal sialic acid residues. The removal of sialic acids (using

neuraminidase) reduced the apparent molecular weight of the mature LDL receptor by only 10 000, indicating that the galactose residues were the prime determinant of the molecular weight change, despite the presence of only a single galactose residue per chain (compared to 2 sialic acid residues). Deletion of the O-linked sugar glycosylation domain also minimised the molecular weight change, localising the main cause of the apparent molecular weight change to the addition of galactose residues to the clustered O-linked chains (Davis et al., 1986a). The shift in the electrophoretic mobility of the LDL receptor facilitates the identification, in biosynthetic studies, of retarded processing from the precursor to the mature forms.

1.8.1.1 Functional effects of LDL receptor glycosylation

The role of glycosylation in LDL receptor function is unclear, despite several studies that have determined the behaviour of LDL receptors with altered sugars. Treatment of the LDL receptor with sialidase, which removes sialic acid residues from both N- and O-linked chains, has no effect on ligand binding (Schneider et al., 1982). A role for the N-linked chains is indicated by the reduced binding affinity of LDL receptors synthesised in the presence of tunicamycin (without N-linked chains), and the 40% decrease in LDL binding (without a change in LDL receptor number) caused by impaired processing in the presence of castanospermine (Edwards et al., 1989; Filipovic, 1989). However, substantial LDL receptor activity is expressed in CHO cells with 17 different types of defect predominantly affecting N-linked glycosylation (Kingsley et al., 1986b). Similarly, the role of the O-linked chains are not clear. The LDL receptor is unstable and cleaved in the region of the O-linked sugar domain in CHO cells with a reversible defect in O-linked glycosylation which prevents the initiation of the O-linked chains (Kozarsky et al., 1988). Deletion of the O-linked sugar domain (site of attachment of the clustered chains) does not alter the behaviour of the LDL receptor in fibroblasts (Davis et al., 1986a), though it does cause a mild form of familial hypercholesterolaemia through a subtle disturbance of receptor function (Koivisto et al., 1993). The dispersed O-linked sugar chains (not in the clustered O-linked domain) are involved in ligand binding; their absence in a monensin-resistant cell line reduces the binding affinity for LDL and its internalisation rate (Kuwano et al., 1991; Seguchi et al., 1991; Shite et al., 1988).

1.8.2. LDL receptor folding and retention in the ER

Disulfide bonds cross-link the folded structure of the cysteine-rich domains (binding domain and the EGF precursor-homology domain) of the LDL receptor. 63 cysteines are present in the LDL receptor: all but 3 are found in the cysteine-rich repeats of the extracellular domain and are thought to be involved in disulfide bonding (Figure 1.5). One is present in the EGF domain between repeats B and C, one is present in the membrane spanning domain and another is in the cytoplasmic tail: these are not involved in disulfide bonds (Lehrman et al., 1987b). The extensive disulfide bonding is confirmed by the inability to alkylate the LDL receptor without first reducing the bonds (Lehrman et al., 1987b; Daniel et al., 1983). Overall, 30 disulfide bonds form in the ER during the folding of each LDL receptor molecule; folding probably occurs co-translationally with bonding starting as the nascent chain is translocated into the ER. Bonds form between cysteines probably situated within the same repeat (see section 1.6, "Binding to the LDL receptor"). The modular character of the folding, and the function, of each binding repeat is emphasised by the finding that point mutations within different repeats cause the same disruption to binding as deletion of the corresponding entire repeat (Esser et al., 1988; Russell et al., 1989). The effects of mutations extend only within their own repeat and do not disrupt the function of neighbouring repeats. The effects of all of the numerous mutations characterised within the ligand binding domain, all conform to these general principles for the role of the different repeats in the binding of ligand (Hobbs et al., 1992). Folding is probably aided by chaperone proteins that prevent the aggregation of partially-folded intermediates and catalyse the formation of the many disulfide bonds (protein disulfide isomerase) (Gething and Sambrook, 1992). Of particular interest is the manner in which putative, gate-keeper proteins (possibly the same chaperones) might prevent the passage of mutant LDL receptors from the ER, probably by recognising a small region of misfolding in the LDL receptor. The retarded processing of mutant alleles was described in the initial report of LDL receptor processing (Tolleshaug et al., 1982): overall, 54% of the mutant alleles in the Dallas collection are classified as class 2 (Hobbs et al., 1992). Of these, two thirds affect the binding domain, and one third affect the EGF precursor homology domain. In general, the deletion of domains or subdomains do not affect processing; rather it is the small deletions and missense mutations which have the greatest effects on the structure of the binding domain. Small deletions affect the spacing of the cysteine residues which is conserved in the

repeats and is apparently crucial for proper folding. Missense mutations cause the substitution of amino acids: these changes are dependent on the structure in the local environment of the mutation - certain sites are exquisitely sensitive (space-restricted regions) and tolerate substitutions poorly; sometimes, these include even substitutions which maintain the character (charge, hydrophobicity) of the substituted amino acid, such as the FH-Afrikaner-1 mutation where aspartate is substituted by glutamate (Fourie et al., 1988; Leitersdorf et al., 1989). Frequently, changes in the space-restricted regions involve the substitution of glycine residues by residues with longer side-chains which are not able to be packed in the confined interior of the cross-linked repeats or in tight turns (Esser and Russell, 1988). These changes also may impair the disulfide bonding of cysteine residues.

The role of cysteine residues in slowed processing was addressed using the Lebanese allele, where a missense mutation terminates translation at the third cysteine of repeat C in the EGF domain (Lehrman et al., 1987b). The pattern of disulfide bond pairing in this repeat has been identified in prothrombin and EGF (Esser and Russell, 1988): cysteines 1 and 3, 2 and 4, 5 and 6 pair, suggesting that in the truncated mutant, cysteines 1 and 2 probably are not involved in intramolecular bonds, but may bond to other proteins and prevent transport from the ER. Substitution of these two cysteines (by alanine) does not improve the slow processing of the Lebanese mutant in monkey kidney (COS) cells (Esser and Russell, 1988) indicating that retention does not necessarily involve inappropriate disulfide bonds cross-linking of the LDL receptor with other proteins or with other LDL receptor molecules (Tolleshaug et al., 1983; Yamamoto et al., 1986; Lehrman et al., 1987b). Clearly these findings do not imply that disulfide bonds are not required for successful folding and processing - they are; these results only indicate that free cysteine residues are not essential for retention in the ER. Possibly not all of the cysteines are required for successful folding: in a monkey kidney (COS) cell system, substitution of cysteines 6 and 18 (by alanine) in the first binding repeat does not retard processing, though this system is not optimal for the study of processing as the normal LDL receptor takes up to 4.5 hours to be converted to the mature form (Esser et al., 1988).

Chaperone proteins retain malfolded proteins, in the ER, by associating with exposed hydrophobic regions (Gething and Sambrook, 1992). The specific chaperones important for LDL receptor folding and retention have not been identified. A protein

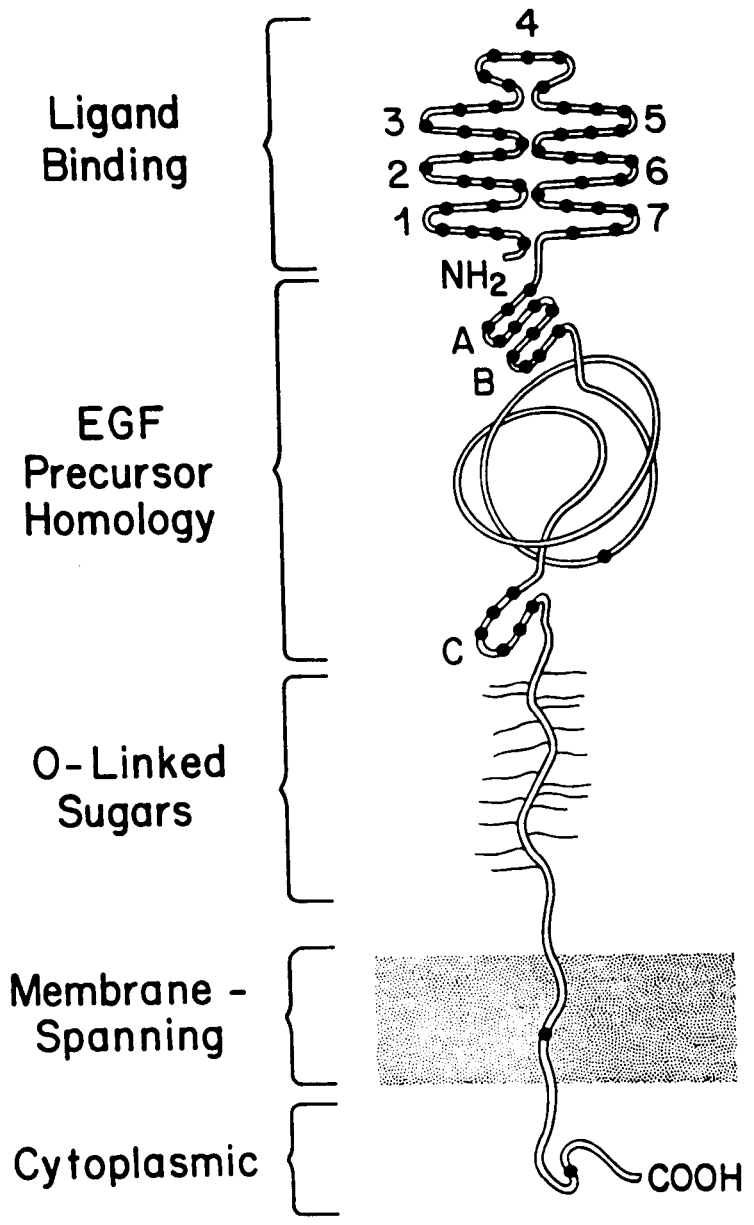


Figure 1.5 Positions of the cysteine residues in the LDL receptor.

Each black dot indicates the position of a cysteine residue in the LDL receptor. The 40-amino acid ligand binding repeats are numbered 1 to 7, and the growth factor repeats of the EGF precursor homology domain are labelled A to C.

(Figure modified from Lehrman et al., 1987).

with the molecular mass of BiP (a major chaperone for secreted proteins) was co-immunoprecipitated with LDL receptors containing defects in their binding domain (Esser and Russell, 1988), but was not characterised further. By immunohistochemistry, retained LDL receptors have been localised to the rough ER and irregular extensions of the rough ER, (Pathak et al., 1988): sugar analysis indicates that they have not passed through the Golgi apparatus, though the retention mechanism may involve recycling through a compartment intermediate between the ER and the Golgi apparatus (Pelham, 1991).

The fate of the retained proteins vary: as has been described under 'mutations in the LDL receptor ' certain proteins escape the retention and appear at the cell surface, while others do not seem to exit the ER; some of the retained proteins are remarkably stable, though many are degraded at an enhanced rate. Degradation systems operate in the ER (Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991), but the site and mechanisms of LDL receptor degradation have not been determined. The inter-relationship between retention and degradation is crucial to the retention mechanism preventing the expression of malfolded proteins at the cell surface. For retention to be effective, the number of LDL receptors arriving at the cell surface should be reduced. However, without degradation of the precursor LDL receptor, this is not the case. In the absence of degradation, retention causes the pool of LDL receptor molecules in the ER to increase until the absolute rate of transport is restored. Thus, certain transport-impaired mutants (FH-Afrikaner-1) are converted quantitatively (without degradation) from the precursor to the mature form - the retarded transport does not reduce the number of receptor molecules on the cell surface (Fourie et al., 1988). It has been proposed that one role of the quality control mechanism is to limit the induction of an immune response directed against the abnormal proteins (Gething et al., 1986). This function requires that the malfolded proteins do not reach the cell surface - they are retained and degraded.

1.8.2.1. Transport-defective mutations in human disease

The processes involved in LDL receptor transport are of general relevance in cell biology and medicine. Several different genetic diseases have been identified to involve mutations which disrupt the transit of specific membrane or secreted proteins through the secretory pathway; these proteins include receptors (both transport-mediators and signalling), ion channels, adhesion molecules, and secreted enzymes

and structural components (Table 1.1) (Hobbs et al., 1992; Hobbs et al., 1990). It is anticipated that many more members of this group remain to be identified and that the group could include the mutant-forms of most surface molecules.

Table 1.1 Human diseases causes by defects in the transport of membrane and secreted proteins.

Membrane protein	Disease
LDL receptor	FH
cystic fibrosis transmembrane conductance regulator	cystic fibrosis
rhodopsin	retinitis pigmentosa
insulin receptor	insulin-resistant diabetes
sucrase-isomaltase	sucrase-isomaltase deficiency
common ß-subunit of LFA-1, Mac-1 and p150,95	leukocyte adhesion deficiency
Secreted protein	**Disease**
α1-antitrypsin	emphysema
pro-α1-collagen and pro-α2-collagen	osteogenesis imperfecta

1.8.3. Oligomerisation

About 25% of LDL receptors at the cell surface are self-associated into dimers and trimers (van Driel et al., 1987a) which are able to bind ligand and recycle through the endocytic pathway (Grant et al., 1990). These oligomers may have a higher avidity for ligands containing apoE (see Section 1.6, "binding to the LDL receptor"), but, in general, the role of oligomers in LDL receptor function has been poorly characterised and the site of assembly has not been determined. Classically, the oligomeric assembly of proteins occurs during folding and is required for transport from the ER, though, for the LDL receptor, it is possible that it could occur at the cell surface. The oligomeric structure of the LDL receptor involves non-covalent interactions (not

disulfide bonds) and depends on amino acids 812 to 839 of the cytoplasmic tail (van Driel et al., 1987a).

1.8.4. Phosphorylation

The LDL receptor is phosphorylated at a serine residue in the cytoplasmic tail (amino acid 833) (Kishimoto et al., 1987a). Although a candidate kinase was identified, its physiological location and its function were not determined (Kishimoto et al., 1987b). A similar phosphorylation event regulates the distribution of proteins in polarised cells (Casanova et al., 1990), but has no discernible effect on the targeting of the LDL receptor (Yokode et al., 1992). A phosphorylation-dephosphorylation cycle commonly regulates the activity of proteins involved in cell signalling; there is no evidence for a similar modulation of LDL receptor activity (Lin et al., 1986).

1.8.5. Proteolytic cleavage

Apart from cleavage of the signal sequence, the LDL receptor does not normally undergo proteolytic processing during its biosynthesis. The cleavage of the LDL receptor is induced at a site in the O-linked sugar domain, by preventing the normal O-linked glycosylation of the LDL receptor (Kozarsky et al., 1988), or by treatment with interferon A, which releases a soluble product consisting of most of the extracellular domain of the LDL receptor and mediates part of interferon A's antiviral activity (Fischer et al., 1994; Fischer et al., 1993). In both these cases, the cellular site of release has not been determined.

1.8.6. Hydroxylation

The LDL receptor undergoes a low degree of ß-hydroxylation at 2 sites in the EGF precursor-homology domain (aspartic acid-310 in repeat A and asparagine-349 in repeat B) which contain a motif found in other hydroxylated proteins (Stenflo et al., 1988). Substitution of these residues by alanine suggest that the aspartic acid 310 may be hydroxylated and influence LDL binding, while asparagine 349 is dispensable (Esser et al., 1988). Hydroxylated residues are involved in calcium binding in other proteins which may account for the effect on LDL binding (Schneider, 1989). A hydroxylase (not identified) may maintain and regulate the low degree of hydroxylation of the LDL receptor.

1.9. Research objectives

The broad aim of this thesis was to study the influence of post-translational alterations on the synthesis of the LDL receptor. The revelation of the LDL receptor pathway and the recognition of numerous mutations that disturb its function, together have offered a means to probe the folding of the LDL receptor. Interest was focused on the glycosylation and the folding of the LDL receptor, its transport from the ER and the manner in which these processes are disrupted by mutations.

Experiments were performed to determine:

1. The influence of the disruption of the compartmental organisation of the secretory pathway, with brefeldin A, on the glycosylation of the LDL receptor.
2. The requirements for the folding of the LDL receptor in the ER.
3. The effect of mutation of cysteine residues on the folding of the 5th repeat of the binding domain of the LDL receptor.
4. The role of the chaperone, calnexin, in the synthesis of the LDL receptor.

Chapter 2

Experimental procedures

2.1. Materials

The anti-LDL receptor monoclonal antibody, IgG-C7, was prepared from hybridoma cells obtained from ATCC (CRL/691) and iodinated using Iodogen (Pierce Chemical Co.) according to the method of Beiseigel et al. (Beisiegel et al., 1981). The anti-LDL receptor monoclonal antibody, IgG-HL1, was a gift from Drs Brown and Goldstein (Dallas, Texas). The polyclonal antibodies (rabbit serum) directed at either of 2 epitopes in the cytoplasmic tail of calnexin (amino acids 487-505 or amino acids 555-573) were a gift from Dr JJM Bergeron (Montreal, Canada). The polyclonal antibodies (rabbit serum) directed against α1-antitrypsin and transferrin were purchased from Dako. Goat anti-mouse IgG was purchased from Cappel Laboratories (Malvern, PA, USA). Ham's F-12, Dulbecco's modified Eagle's minimum essential medium (DMEM) and foetal calf serum (FCS) were purchased from Highveld Biologicals (Kelvin, South Africa). LDL and lipoprotein-deficient serum (LPDS) were prepared from human plasma (Goldstein et al., 1983), and ßVLDL was prepared from the plasma of cholesterol-fed rabbits (Kovanen et al., 1981). These lipoproteins were iodinated by the iodine-monochloride method (Goldstein et al., 1983). Ca^{2+}-free DMEM was purchased from Gibco (Life Technologies). Methionine-free and cysteine-free medium and TRAN[^{35}S]methionine were obtained from ICN (Irvine, CA). Na[^{125}I] and the enhanced chemiluminescence immunoblotting kit were obtained from Amersham (Buckinghamshire, England). M13BM21, DTT, Endo-α-N-acetyl-galactosaminidase (O-Glycanase, a trademark of Genzyme Corporation, Cambridge, MA, USA) (from Diplococcus pneumoniae), endoglycosidase H, as well as pronase (from Streptomyces griseus) was obtained from Boehringer Mannheim. Geneticin, N-ethyl maleimide (NEM), 2-deoxy-D-glucose, neuraminidase (from Clostridium perfringens), protein A-sepharose and brefeldin A were obtained from Sigma. Brefeldin A was stored at -20°C as a 10 mg/ml stock solution in methanol. Ca^{2+} ionophore, A23187, was purchased from Calbiochem.

2.1. Methods

2.2.1. Cell culture

Human skin fibroblasts were seeded (day 0) at 20000 cells per 35mm dish and incubated at 37°C in 2 ml of full medium (DMEM + 10% foetal calf serum + penicillin (60µg/ml) + streptomycin (100 µg/ml)). The medium was changed on day 2 and day 4. On day 6 the cells were washed once with Dulbecco's phosphate buffered saline and

then incubated for 24 hours in medium (DMEM + penicillin + streptomycin) containing 5% LPDS, in order to up-regulate LDL receptor activity. Experiments were performed on day 7.

The Chinese hamster ovary cell (CHO) line, TR-715, was a gift from Brown and Goldstein (Dallas, Texas). It is a stable line of ldlA7 cells (a mutant cell line lacking endogenous, functional LDL receptors) ((Kingsley and Krieger, 1984), which was transfected with a plasmid (pLDLR2) encoding the gene for the human LDL receptor under the control of a constitutive SV40 promoter (Davis et al., 1986a). Similar ldlA7 CHO cell lines transfected with the FH Afrikaner-1 (Leitersdorf et al., 1989), FH Afrikaner-2 (Leitersdorf et al., 1989) and the FH Afrikaner-3 mutant LDL receptors (Graadt van Roggen et al., 1995) were constructed in the Van der Westhuyzen laboratory (Cape Town). LdlA7 CHO cells expressing mutant LDL receptors with cytoplasmic tail mutations (Stop792, Stop812 and Phe807Ala) were constructed by Drs Graadt van Roggen and Davies, also in the Van der Westhuyzen laboratory. Cells were seeded (day 0) at 60 000 cells per 35mm dish (for pulse-chase experiments) or at 160 000 cells per 60mm dish (for binding experiments) in full medium (Ham's F-12 + 5% FCS + penicillin (60µg/ml) + streptomycin (100µg/ml)). The medium was changed on day 2 and experiments usually were performed on day 3.

HepG2 cells were seeded (day 0) in 35mm dishes and incubated at 37°C in 2 ml of full medium (DMEM + 10% foetal calf serum). The medium was changed every second day and experiments were performed when cells were arranged in sinusoids which were not yet coalesced. The cells were washed once with Dulbecco's phosphate buffered saline and then incubated for 12 hours in DMEM containing 5% LPDS in order to up-regulate LDL receptor activity.

2.2.2. Pulse-chase experiments

The cell layers were washed once with phosphate-buffered saline and then incubated in methionine- and cysteine-free medium for 30 minutes, prior to being pulsed with 50-100µCi/ml TRAN[^{35}S]methionine for the indicated time periods. For the chase-incubation, the cell layers were washed once with chase medium (DMEM containing 5% LPDS and supplemented with 100µM cycloheximide to ensure an abrupt termination of protein translation), and then incubated at 37°C for the indicated times in

fresh chase medium. DTT (5mM), BFA (0.05µg/ml or 5µg/ml), A23187 (5µM), EDTA (10mM) or 2-deoxy-D-glucose (20mM) and sodium azide (10mM) were included either in the pulse- and/or chase-incubations, as indicated in the figure legends. To enable oxidative conditions to be re-established after DTT treatment, cells were washed twice with chase medium and then incubated for the indicated times in fresh chase medium.

2.2.3. Immunoprecipitation of TRAN[^{35}S]methionine-labelled LDL receptors

After the pulse or chase period, dishes were cooled to 4°C by washing twice with buffer A (150mM NaCL, 2mM $CaCl_2$, 10mM Hepes), and then lysed in buffer B (10mM Hepes, 200mM NaCl, 2mM $CaCl_2$, 2.5mM MgCl, 1mM PMSF, 0.1mM leupeptin, 1% Triton X-100). 20mM NEM was included in both these solutions to alkylate reduced cysteine residues in order to prevent disulfide bond shuffling. All subsequent steps were carried out at 4°C. LDL receptor was immunoprecipitated from the post-nuclear supernatants (12 000g for 10 minutes) using a preformed immune complex as described by Tolleshaug et al. (Tolleshaug et al., 1982), or precipitated using protein A-sepharose. After incubating the post-nuclear supernatant with 6µg of IgG-C7 or IgG-HL1 for 1 hour, 15µl of protein A-sepharose was added for 1 hour. Immunoprecipitates were washed three times with 10mM Tris pH 8.0: wash 1 contained 0.5% Chaps; wash 2 contained 0.2% Nonidet P40 and 500mM NaCl; wash 3 contained 0.2% Nonidet P40 and 0.1% SDS. Detergents were removed in a single wash with buffer A. Immunoprecipitates were then solubilised in gel-loading buffer (10% glycerol, 2.4% SDS, 75mM Tris, 100mM DTT and 5% 2-mercaptoethanol), boiled for 3 minutes and then separated on 7% SDS polyacrylamide gel electrophoresis with a 5% stack (Laemmli, 1970). For electrophoresis under non-reduced conditions, DTT and 2-mercaptoethanol were omitted from the gel-loading buffer. After fixing (10% methanol and 10% acetic acid), the gels were enhanced using sodium salicylate ((Chamberlain, 1979), dried and subjected to fluorography at -70°C using Kodak BioMax MR-1 or XAR-5 film. Typical exposure times ranged from 16-48 hours.

2.2.4. Analysis of LDL receptor glycosylation

2.2.4.1. Neuraminidase and O-Glycanase treatment of the LDL receptor

For treatment with neuraminidase, TRAN[^{35}S]labelled, immunoprecipitated LDL receptor was resuspended in 50µl of a buffer containing 20mM sodium citrate, 20mM Tris-maleate (pH 6.0) and incubated for 18 hours at 20°C, in the absence or presence

of neuraminidase (50milliunits) (Davis et al., 1986a). Certain neuraminidase-treated samples were then treated with 4milliunits of O-Glycanase for 24 hours at 20°C. After neuraminidase or O-Glycanase treatment, 50µl of buffer containing 20% glycerol, 125mM Tris-HCl (pH 6.8), 4.6% SDS and 0.2M dithiothreitol was added to each sample, followed by analysis by SDS polyacrylamide gel electrophoresis, as described above.

2.2.4.2. Endoglycosidase H treatment of the LDL receptor

Immunoprecipitated [^{35}S]methionine-labelled LDL receptor was resuspended in 50µl of a buffer containing 30mM sodium citrate(pH 5.5), 0.75mM SDS, 1mM phenylmethylsulfonylfluoride and 1mM DTT, and incubated for 18 hours at 20°C, in the absence or presence of Endoglycosidase H (10milliunits) (Lehrman et al., 1987b). After the incubation, 50µl of buffer containing 20% glycerol, 125mM Tris-HCl (pH 6.8), 4.6% SDS and 0.2M dithiothreitol was added to each sample, followed by analysis by SDS polyacrylamide gel electrophoresis, as described above.

2.2.5. Pronase treatment

Pronase treatment of intact cells was performed as described previously (Tolleshaug et al., 1983). Cells were incubated at 37°C in the presence of pronase (10µg/ml) for 20 minutes. Cells were washed, the LDL receptors were solubilised and analysed as described above.

2.2.6. Binding of ^{125}I-labelled lipoproteins or ^{125}I-labelled IgG-C7 at 4°C

Semi-confluent 60mm dishes of Chinese hamster ovary cells were incubated in Ham's F-12/LPDS for 1 hour at 37°C to clear lipoproteins from LDL receptors at the cell surface. DTT (5mM or 50mM) was added for 5 minutes at 37°C and the dishes were cooled to 4°C by washing twice with phosphate-buffered saline containing 50mM NEM in order to quench the DTT and alkylate reduced cysteine residues. All subsequent steps were performed at 4°C. Surface binding assays with ^{125}I-ligands (Fourie et al., 1992) or ^{125}I-IgG-C7 antibody (Tolleshaug et al., 1982) were performed for 2 hours at the concentrations indicated in Table 1. Incubations were done in DMEM/LPDS buffered at pH 7.4 with 20mM Hepes. After the incubation the cells were washed 4 times with phosphate-buffered saline containing 0.2% bovine serum albumin, followed by 3 washes with phosphate-buffered saline. Total surface-bound radioactivity was

measured after the cells were dissolved in 1N NaOH. Specific values were determined by subtracting from the total activity the non-specific value obtained in the presence of an excess of unlabeled LDL (200µg/ml), or IgG-C7 (50µg/ml) or ßVLDL (100µg/ml).

2.2.7 Lipoprotein and immunoblotting of LDL receptors

Semi-confluent fibroblasts or CHO cells were cooled to 4°C by washing (50mM Hepes and 100mM NaCl) and then scraped from 10cm dishes in buffer A (50mM Hepes, 100mM NaCl, 0.5mM leupeptin, 10mM NEM, 1mM PMSF). Cells were collected by centrifugation (2min at 10 000g) and lysed over 20 min in 100µl buffer A containing 1% Triton X-100. The post-nuclear supernatant (10min at 10 000g) was adjusted to 1% SDS and 10% glycerol, and subject to SDS polyacrylamide gel electrophoresis (5% stack and 7% separating gel) without boiling or the addition of reducing agents. Electrophoresed proteins were transferred onto a nitrocellulose membrane in a sandwich transfer apparatus submerged in 20mM Tris, 150mM glycine and 20% methanol (v/v). Transfer was performed overnight at 100mA at 4°C. Immobilised protein was detected either with enhanced chemiluminescence using horseradish peroxidase-labelled antibodies (Amersham) or ^{125}I-labelled ßVLDL or ^{125}I-labelled antibodies.

2.2.7.1. Detection using ^{125}I-labelled antibodies

The membrane was blocked by incubation in buffer B (50mM Tris, 80mM NaCl, 2mM $CaCl_2$ and 0.05% Tween 20) containing 3% BSA for 1 hour at 37°C. After incubation with primary antibody, 5µg/ml IgG-C7 for the LDL receptor, or a 1:2000 dilution of polyclonal rabbit serum against calnexin, in buffer B containing 3% BSA for 1 hour at 20°C, the membrane was washed 3 times (15min each) with buffer B. The membrane was incubated in buffer B containing 3% BSA and $3x10^6$cpm/ml ^{125}I-labelled goat anti-mouse antibody (LDL receptor) or $3x10^6$cpm/ml ^{125}I-labelled goat anti-rabbit antibody (calnexin) for 1 hour, and then washed 3 times (15min each) with buffer B. The dried membrane was exposed to Kodak Biomax film at -70°C and the position of the LDL receptor or calnexin was confirmed in relation to molecular weight markers.

2.2.7.2. Detection of the LDL receptor using ^{125}I-labelled ßVLDL

The membrane was blocked in buffer B (50mM Tris, 80mM NaCl, 2mM $CaCl_2$ and 0.05% Tween 20) containing 3% BSA and 3% dried milk powder for 1 hour at 37°C.

The membrane was incubated in buffer B (with 3% BSA and 3% dried milk powder) containing 5µg/ml ^{125}I-ßVLDL for 1 hour, and then washed 4 times (1 rapid wash, 2 washes of 15min and a final rapid wash) with buffer B containing 0.3% BSA and 0.3% dried milk powder. The dried membrane was exposed to Kodak Biomax film at -70°C and the position of the LDL receptor was confirmed in relation to molecular weight markers.

2.2.7.3. Detection of the LDL receptor using enhanced chemiluminescence

The membrane (pure nitrocellulose, Amersham) was blocked in buffer C (20mM Tris, pH7.6, 137mM NaCl, 2mM $CaCl_2$ and 0.05% Tween 20) containing 5% dried milk powder for 1 hour at 20°C. The membrane was washed 5 times with buffer C containing 0.3% dried milk powder and then incubated with primary antibody (2µg/ml) for 1 hour at 20°C. The membrane was washed as before, and incubated with horseradish peroxidase-labelled sheep anti-mouse antibody at a 1:1000 dilution for 1 hour at 20°C. The membrane was washed as above, dried and then the horseradish peroxidase substrate was added as described in the protocol accompanying the enhanced chemiluminescence (ECL) immunoblotting kit. Light emission was detected with Kodak Biomax film after typical exposes ranging from 5 seconds to 15 minutes.

2.2.8. Immunoprecipitation of TRAN[^{35}S]methionine-labelled calnexin

This procedure was performed as described by Bergeron (Ou et al., 1993). After the pulse or chase period, dishes were cooled to 4°C by washing twice with buffer A (150mM NaCL, 2mM $CaCl_2$, 10mM Hepes pH7.4), and then lysed in buffer B (50mM Hepes pH 7.5, 200mM NaCl, 2mM $CaCl_2$, 2.5mM MgCl, 1mM PMSF, 5µg/ml leupeptin, 5µg/ml aprotinin and 2% cholate). 20mM NEM was included in both these solutions to alkylate reduced cysteine residues in order to prevent inappropriate disulfide bond linkage between calnexin and the LDL receptor. Lysates were centrifuged (12 000g for 10 minutes) and the supernatants were retained. For immunoprecipitation under non-denaturing conditions the supernatants were diluted by the addition of an equal volume of buffer B (without cholate) and were mixed (rotary shaker) with 3µl of anti-calnexin polyclonal antibody for 2 hours at 4°C. 20µl protein A-sepharose beads were added and mixed for 1 hour at 4°C. Protein A-sepharose beads were collected by centrifugation (12 000g for 30 seconds) and washed 4 times with 800µl of buffer B containing 0.5% cholate. For immunoprecipitation under denaturing conditions, lysates

were adjusted to 1% SDS and heated at 90°C for 3 minutes. They were diluted by the addition of 20-volumes of buffer B containing 1% Triton X-100 and immunoprecipitated with calnexin as described above. Sequential immunoprecipitations were initially done under non-denaturing conditions as described above. 100µl of buffer B with 1% SDS (without cholate) was added to the protein A-sepharose beads after the 4th wash, and heated at 90°C for 3 minutes. Following centrifugation (12 000g for 30 seconds) the supernatant was diluted by the addition of 2ml of buffer B (without cholate) containing 1% Triton X-100. The second immunoprecipitation was performed using specific antibodies to proteins possibly associated to calnexin. The immunoprecipitated proteins were analysed by electrophoresis and fluorography.

2.2.9. Immunoblotting of calnexin

CHO and HepG2 cells were collected, electrophoresed and transferred to a nitrocellulose membrane as described in section 2.2.7. The membrane was washed with buffer A (10mM Tris pH 8.5, 300mM NaCl and 0.05% Tween-20) for 30 minutes at 20°C and then blocked in buffer A with 5% skim milk for 1 hour at 37°C. The membrane was incubated with the primary antibody (1:1500 dilution) in buffer A with 5% skim milk for 2 hours at 20°C. The membrane was washed 3 times (each for 10 minutes) in buffer A with 0.3% skim milk. ^{125}I-labelled goat anti-rabbit IgG ($1.5x10^6$cpm/ml) was incubated with the membrane in buffer A with 5% skim milk for 1 hour at 20°C. 3 washes, each of 30 minutes were performed with buffer A containing 0.3% skim milk. The dried membrane was exposed to Kodak Biomax film at -70°C.

2.2.10. Construction of LDL receptors with cysteine residues substituted in the 5th binding repeat

Standard molecular biology techniques were used to prepare and manipulate DNA for cloning (Ausubel et al., 1994). The mutagenesis reactions were performed in the bacteriophage, M13BM21, and required subcloning of cDNA fragments from the plasmid, pLDLR2, the vector containing the entire cDNA of the human LDL receptor. 2 overlapping DNA fragments were used to construct the 6 mutants, each corresponding to the substitution of a single cysteine residue of the 5th binding repeat by alanine. The mutant LDL receptors were designated mutant 1-6, dependent on the cysteine residue mutated: mutant 1, Cys176Ala; mutant 2, Cys183Ala; mutant 3, Cys188Ala; mutant 4, Cys195Ala; mutant 5, Cys201Ala; mutant 6, Cys210Ala. The substitution of

cysteine residues in mutants 1 and 2 were performed on a HincII-EcoRI fragment (483bp) that was released from pLDLR2 and cloned directly into the polylinker of M13BM21. The substitutions of the cysteine residues in the other 4 mutants were performed on a TaqI-EcoRI fragment (119bp) that was initially released as a 758bp XbaI-EcoRI fragment from pLDLR2, and then cleaved with TaqI. The residual 639bp XbaI-TaqI fragment was preserved for later re-assembly of pLDLR2 (see below). The restriction enzymes, AccI and EcoRI, were used to generate the cloning site for the TaqI-EcoRI fragment in M13BM21; TaqI could not be used as it has multiple cleavage sites in M13BM21. Oligonucleotide-directed mutagenesis was performed without phenotypic selection according to the method of Kunkel (Kunkel et al., 1987). A single strand bacteriophage template containing uracil was prepared from the culture supernatant of the CJ236 strain of *Esherichia coli.* Unlike the wild type bacteria (strain JM109) used in the transformation reactions, these bacteria tolerate the presence of uracil in DNA due to the absence of the enzymes uracil N-glycosylase and dUTPase (*E. coli dut⁻ ung⁻*). An oligonucleotide 26 nucleotides in length was used to mutate 2 nucleotides of the codon for cysteine to code for alanine. The entire cloned fragment was sequenced (dideoxy method) to confirm that mutagenesis was successful and that no other mutations were introduced. In order to reassemble the cDNA for mutants 1 and 2, the mutated HincII-EcoRI fragment (483bp) was released from M13BM21 and cloned directly into pLDLR2. For the other 4 mutants, the mutated TaqI-EcoRI fragment (119bp) was introduced into pLDLR2 in a single-step ligation reaction together with the corresponding 639bp XbaI-TaqI fragment (see above) in order to restore the 758bp XbaI-EcoRI fragment. For all 6 mutant constructs, the presence of the mutation in pLDLR2 was confirmed by subsequent subcloning of the 758bp XbaI-EcoRI fragments into M13BM21 and resequencing. Concatamers were excluded by the demonstration that each of the enzymes used for the cloning steps successfully linearised pLDLR2 without the release of DNA fragments.

2.2.11. Stable transfection of CHO cells

The LDL receptor-deficient CHO line, ldlA7, was transfected with the mutant forms of the plasmid, pLDLR2 using the calcium phosphate precipitation method (Davis et al., 1986a). The plasmid, pSV3-Neo, which contains the neomycin-resistance gene was co-transfected (1:10) with the mutants forms of pLDLR2, and the antibiotic, geneticin (G418), was used to select for transfected cells. Geneticin was included in the medium

(700µg/ml) for approximately 3 weeks, during which time clones of surviving cells were isolated with cloning rings and expanded into cell lines. Subsequently, cells were further cloned by limiting dilution and LDL receptor expression was confirmed by immunoprecipitation. The established CHO cell lines expressing the mutant forms of the LDL receptor were maintained in cell culture in the same manner as TR-715, the cell line expressing the wild type LDL receptor, as described above. All experiments were repeated in mutant cell lines obtained from at least 2 independent transfection reactions.

2.2.12. Scanning and printing of fluorograms and autoradiograms

After fluorography or autoradiography using either Kodak XAR-5 or BioMax MR-1 film, the fluorograms and autoradiograms were scanned with a hand-held, 256 grey scale scanner (Logitech ScanMan 256) at a resolution of 100 dots per inch. The scanned image was edited using FotoTouch (Logitech) software: undesired regions were cropped from the scanned image and in certain instances, the order of the lanes was rearranged. The brightness, contrast and tonal curve (linearity of response) of the scanned image were not altered. The image was exported in tagged image file format to a word processor (Microsoft Word, version 6) and labelled. The final image was printed on a laser printer (Hewlett Packard, LaserJet 4P) at a resolution of 600 dots per inch.

Chapter 3

The effects of brefeldin A on the post-translational processing of the LDL receptor.

3.1. Introduction

The movement of proteins from one membrane compartment to another in the secretory pathway is mediated by the budding (formation) and fusion of transport vesicles. These processes are regulated by proteins which coat the cytoplasmic face of the membranes: budding involves the assembly of the protein coat from cytoplasmic components, and fusion requires the coat to be detached from the membranes of the transport vesicles (3155, 3019). Assembly of the coat is initiated when the small guanine nucleotide-binding protein, ADP-ribosylation factor (ARF), is triggered to bind the membrane by binding GTP in exchange for GDP (Donaldson et al., 1992; Palmer et al., 1993). The GDP-form is unable to bind the membrane: indeed, the cycle is further regulated as GTP-hydrolysis triggers uncoating (Tanigawa et al., 1993). The mechanism of regulation of the coat was identified through the effects of brefeldin A, an isoprenoid antibiotic derived from fungi, which prevents nucleotide exchange by ARF and thus prevents the attachment of the coat proteins onto the membrane (3046, 3011). In the presence of brefeldin A, vesicles do not form; instead, tubules form and extend from the Golgi, along microtubules, to fuse with the ER and redistributes the contents of the Golgi into the ER (Klausner et al., 1992; Lippincott-Schwartz et al., 1990; Lippincott-Schwartz et al., 1989). In addition, protein transport from this hybrid compartment is prevented.

The effects of brefeldin A are not limited to the secretory pathway. The clathrin coat of vesicles involved in endocytosis and in transport from the trans-Golgi network (TGN), are also displaced from the membranes by brefeldin A; an elaborate tubular network forms and fuses the early endosomes with the TGN (Lippincott-Schwartz et al., 1991; Hunziker et al., 1992). Brefeldin A-treatment thus results in the formation of 2 separate hybrid organelles, the ER/Golgi and the TGN/endosomal pathway.

Protein glycosylation in the secretory pathway is catalysed by multiple enzymes in the various compartments of the endoplasmic reticulum and the Golgi apparatus(Kornfeld and Kornfeld, 1985; Dunphy and Rothman, 1985). The step-wise processing of sugar chains also has been implicated in the quality control mechanism that retains partially-folded proteins within the ER (Hammond et al., 1994): for example, proteins associate with calnexin (an ER chaperone) depending on the extent of processing of their N-linked chains, while the processing enzyme, UDP-glucose:glycoprotein glucosyltransferase, is specific for denatured or unfolded substrates (Sousa et al.,

1992). Thus, folding, glycosylation and retention/transport are inter-related. While ordered glycosylation generally has been thought to be dependent on the vectorial movement of proteins through the compartments of the pathway, the role of compartmentalisation in these events has not been defined clearly (Mellman and Simons, 1992). In fact, recent evidence suggests that due to vesicular traffic and tubular connections, the compartments of the Golgi may not be as functionally, nor structurally distinct as previously thought (Mellman and Simons, 1992; Orci et al., 1991). The finding that brefeldin A causes the appearance of active, Golgi-derived glycosylation enzymes in the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Ulmer and Palade, 1989), provides a tool to question the role of compartments in glycosylation, and the influence of glycosylation on the folding and transport of the LDL receptor.

The influence brefeldin A on the processing of the LDL receptor is described in this section.

3.2. Results

3.2.1. LDL receptor glycosylation in the presence of brefeldin A

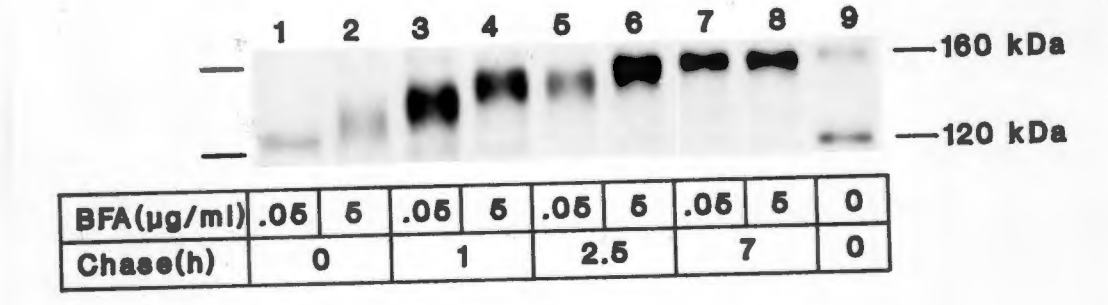
After the addition of [^{35}S]methionine, biosynthetically-labelled LDL receptors are processed through glycosylation, from an ER precursor form (apparent molecular mass of 120 kDa) to a cell-surface, mature form (apparent molecular mass 160 kDa) (Tolleshaug et al., 1983; Beisiegel et al., 1981) (Figure 3.1, lane 9). The absence of processing-intermediates between the precursor and the mature forms of the LDL receptor is in line with rapid transport to the cell surface, once the LDL receptors had exited from the ER. The absence of these intermediates also indicated that the Golgi apparatus contained a small pool of LDL receptors.

The effect of brefeldin A on the processing of LDL receptors was determined (Figure 3.1). Cells were treated with Brefeldin A (0.05µg/ml or 5µg/ml) during the labelling period, and processing was monitored for 7 hours in the continued presence of brefeldin A. Under these circumstances, neither the 120 kDa precursor, nor the 160 kDa mature receptor were detected (Figure 3.1, lanes 1 and 2). Instead, a range of heterogeneous intermediates were observed (lanes 1-6), which were reproducibly distinct, at the earliest (lanes 1 and 2) or latest time-points (lanes 7 and 8), from the normal precursor and mature bands, respectively (lane 9). The observation of the range of processing-intermediates indicated a general slowing of all stages of

processing in the fused ER/Golgi compartment. The effects of the different doses of brefeldin A was noted: at each time of chase (less than 7 hours), processing at the high brefeldin A dose (5µg/ml)(lanes 2, 4 and 6) was more extensive than at the lower one (0.05µg/ml)(lanes 1, 3 and 5). At the lower dose, LDL receptors were apparently processed through the same intermediates as at the higher dose, but at a reduced rate. This somewhat surprising result suggests that the rate of brefeldin A-induced redistribution of Golgi enzymes to the ER was dose-dependent in the concentration range tested, while the block in secretion from the ER was not. However, we were not able to find a dose of brefeldin A which dissociated these separate pathways - no doses was identified which gave an unmodified precursor form alone, which would have indicated a block to transport without redistribution of the Golgi enzymes into the ER.

The nature of the sugars added to the LDL receptor in the presence of brefeldin A were assessed using glycosidase enzymes. Much of the difference in apparent molecular mass between the precursor and mature forms of the LDL receptor is accounted for by the addition (in the trans-Golgi) of galactose and sialic acid sugars to the O-linked chains (Cummings et al., 1983). As expected, the precursor was not affected by neuraminidase-treatment which removed sialic acids from the mature LDL receptor and altered its molecular mass (Figure 3.2, lanes 1 and 2). By contrast, all the intermediates synthesised in the presence of brefeldin A were sialylated, as assessed by neuraminidase, even at the earliest time-points after the addition of brefeldin A. (lanes 3-8). Neuraminidase-treatment also indicated that LDL receptors were sialylated heterogeneously shortly after the addition of brefeldin A (lanes 3 to 6) - the heterogeneity in gel-mobility was largely abolished when the sialic acids were removed.

During the chase in brefeldin A, sugars other than sialic acids were added to the LDL receptor and progressively retarded the gel-mobility of the desialylated forms at the different chase times (lanes 4, 6 and 8). Much of this chain extension probably is due to the addition of galactose residues to the O-linked chains, because the overall contribution of the N-linked chains to the mobility-shifts were small (assessed by treatment with tunicamycin) and similar in the presence or absence of brefeldin A (Figure 3.3). The N-linked chains were processed in the presence of brefeldin A, and changed from sensitivity to resistance to endoglycosidase H within a chase period of 1



BFA(µg/ml)	.05	5	.05	5	.05	5	.05	5	0
Chase(h)	0		1		2.5		7		0

Figure 3.1 The effect of brefeldin A on the post-translational processing of the LDL receptor.

Fibroblasts were labelled with [^{35}S]methionine for 45 minutes, and chased in the presence of unlabelled methionine for the indicated times. Brefeldin A (0,05 µg/ml or 5 µg/ml) was present at the indicated concentrations during both the pulse and chase periods. LDL receptors were immunoprecipitated using a preformed immune-complex with IgG-C7, followed by analysis by 7% SDS polyacrylamide gel electrophoresis and fluorography. The positions of the 120 kDa precursor and the 160 kDa mature forms of the LDL receptor are shown.

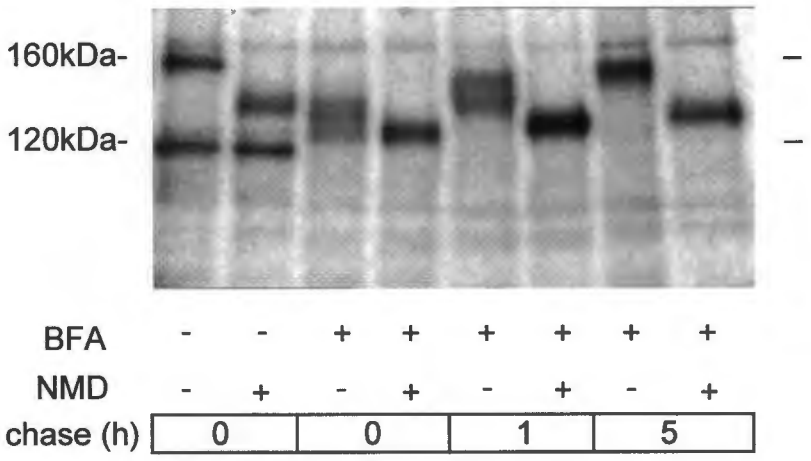


Figure 3.2 The effect of neuraminidase on LDL receptors synthesised and processed in the presence of brefeldin A.

Fibroblasts were labelled with [^{35}S]methionine for 45 minutes and chased, where indicated, in the presence of unlabelled methionine. Brefeldin A (5µg/ml) was present, where indicated, during the pulse and chase periods. LDL receptors were immunoprecipitated with IgG-C7, treated with or without neuraminidase (NMD) for 18 hours at room temperature, followed by analysis by 7% SDS polyacrylamide gel electrophoresis and fluorography. The positions of the 120 kDa precursor and the 160 kDa mature forms of the LDL receptor are shown.

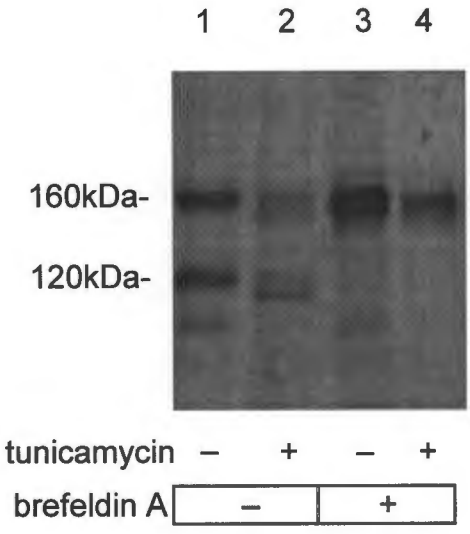


Figure 3.3 Effects of tunicamycin on the LDL receptor synthesised in the presence of brefeldin A.

Fibroblasts were preincubated in the presence of 5µg/ml tunicamycin (lanes 2 and 4) for 3 hours prior to being labelled with [^{35}S]methionine for 45 minutes and chased for 5 hours (lanes 3 and 4). Tunicamycin (5µg/ml) and brefeldin A (5µg/ml) were present in the pulse and chase medium, as indicated. LDL receptors were immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis and fluorography.

hour (results not shown). The maximal extent of processing in the presence of brefeldin A was observed after a chase of about 4 hours; this form of the LDL receptor, designated the pseudomature form, was slightly, but reproducibly smaller than the mature form of 160 kDa, and did not attain that expected molecular mass even after 9 hours within the ER-Golgi compartment (see Figure 3.1, compare lanes 8 and 9, or Figure 3.4, lanes 1 and 2). This interesting finding suggested that the oligosaccharide chains formed in the presence of brefeldin A were either shorter than normal or fewer in number. An altered protein conformation (in an SDS micelle) was considered less likely to be the cause of the altered gel-mobility, since the LDL receptor was boiled and reduced prior to electrophoresis. Sialic acids also did not seem to be involved as the cause of the altered mobility, as the desialylated form of the brefeldin A-treated LDL receptors (Figure 3.4, lane 4) were smaller than the desialylated, normal LDL receptors (lane 3). N-linked glycosylation also did not seem to be involved, because, as described above, the effect of tunicamycin was similar in the presence or absence of brefeldin A. O-linked glycosylation was assessed further with O-Glycanase: once the sialic acids have been removed with neuraminidase, O-Glycanase is known to cleave sugars with the disaccharide structure (Umemoto et al., 1977):

serine/threonine—N-acetyl-galactosamine—galactose

O-Glycanase treatment caused substantial band-shifts to the desialylated LDL receptors and abolished the difference between the normal and the brefeldin A-treated LDL receptors (Figure 3.4, lanes 3-6). The most likely explanation was that fewer O-linked carbohydrate chains were added to the LDL receptors synthesised in the presence of brefeldin A. This finding confirmed that a glycosylation defect, and not a proteolytic or other post-translational processing event, accounted for their enhanced mobility. In summary, the effect of brefeldin A was to retard the overall rate of glycosylation of the LDL receptor which made apparent the processing intermediates. Sialic acid residues were added to the O-linked chains of the LDL receptor shortly after its synthesis and may have contributed to the addition of fewer than normal O-linked chains. The N-linked glycosylation was relatively unaffected.

3.2.2. Reversibility of the effects of brefeldin A

The effects of brefeldin A on the morphology of the cell are reversible. Therefore, the processing of the LDL receptor was assessed after cells were labelled in the presence of brefeldin A, washed and then chased for a 4 hour period without brefeldin A (Figure

3.5). During this period in the absence of brefeldin A, the LDL receptor failed to attain the apparent molecular mass of the normal mature 160 kDa form (lane 2), and did not process beyond the pseudomature form seen in the continued presence of brefeldin A (lane 3). The same result was obtained when the chase period in the absence of brefeldin A was extended to 12 hours (data not shown). In these experiments, the washout of brefeldin A was complete, since the synthesis, as well as the glycosylation and the transport of newly-synthesised LDL receptors, resumed within 30 minutes after brefeldin A removal (data not shown). The lack of reversibility of the glycosylation defect of the LDL receptor in the presence of brefeldin A was unexpected: previous studies have shown that brefeldin A does not redistribute the TGN to the ER and thus causes incomplete chain processing (Chege and Pfeffer, 1990) with the absence of N-linked sialylation (Sampath et al., 1992; Spiro et al., 1991; Chege and Pfeffer, 1990; Shite et al., 1990; Doms et al., 1989). In these instances, processing was completed when brefeldin A was removed (Sampath et al., 1992; Ulmer and Palade, 1989; Spiro et al., 1991) and transport through the TGN was restored. By contrast, for the LDL receptor, the glycosylation in the presence of brefeldin A was not simply interrupted, rather, together with the O-Glycanase result, it was demonstrated that the O-linked glycosylation was irreversibly altered.

3.2.3. The effect of brefeldin A on the function of the LDL receptor

The nature of the glycosylation defect was not assessed more definitively. The following functional properties of the brefeldin A-treated LDL receptors were investigated: 1) LDL receptor transport to the cell surface; 2) LDL receptor stability and 3) ligand binding by the LDL receptor.

3.2.3.1. LDL receptor transport to the cell surface

After the LDL receptors were synthesised in the presence of brefeldin A, brefeldin A was removed and the LDL receptors' ability to be transported to the cell surface was assessed. The assay used was the ability of pronase (added to the medium) to cleave the molecules on the cell surface. The rationale was that in the continued presence of brefeldin A, protein transport through the secretory pathway would be blocked, and the LDL receptors would be resistant to pronase. Once brefeldin A was removed, transport through the secretory pathway would be restored and pronase would cleave the LDL receptors when they appeared at the cell surface. A limitation of this assay was that

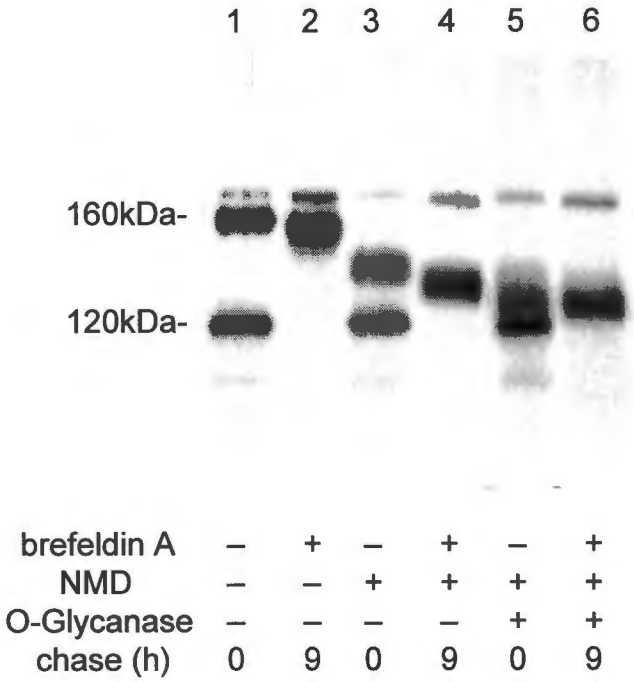


Figure 3.4 The effect of O-glycanase on LDL receptors synthesised and processed in the presence of brefeldin A.

Fibroblasts were labelled with [^{35}S]methionine for 45 minutes and chased (lanes 2, 4 and 6) in the presence of unlabelled methionine. Brefeldin A (5µg/ml) was present during the pulse and chase periods in lanes 2, 4 and 6. LDL receptors were immunoprecipitated with IgG-C7, treated with neuraminidase (NMD) for 18 hours at 20°C (lanes 3-6), followed by treatment with O-Glycanase (lanes 5 and 6) for 24 hours at 20°C. Samples were then analysed by 7% SDS polyacrylamide gel electrophoresis and fluorography. The positions of the 120 kDa precursor and the 160 kDa mature forms of the LDL receptor are shown. The 120 kDa precursor, detected in the absence of brefeldin A, facilitates a comparison of band positions between different lanes.

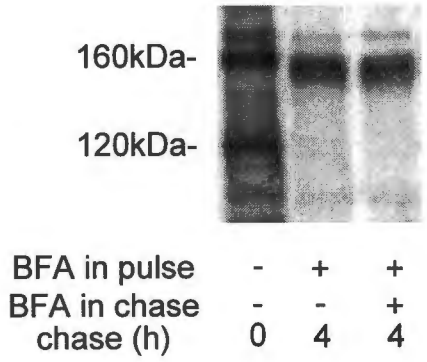


Figure 3.5 The effect of removal of brefeldin A on the post-translational processing of LDL receptors.

Fibroblasts were labelled with [^{35}S]methionine for 45 minutes and chased in the presence of unlabeled methionine for 60 minutes (labelled as pulse). Brefeldin A (0,05µg/ml) was present where indicated. The cells were then washed four times and chased for a further 4 hours (lanes 2 and 3) in the continued presence (lane 3) or absence of brefeldin A (lane 2). LDL receptors were immunoprecipitated with IgG-C7 and analysed by 7% SDS polyacrylamide gel electrophoresis and fluorography. The positions of the 120 kDa precursor and the 160 kDa mature forms of the LDL receptor are shown.

pronase would enter the cell by fluid phase endocytosis as the treatment was performed over 20 minutes at 37°C. In intact cells, the LDL receptors synthesised in the continued presence of brefeldin A were unaffected by pronase (Figure 3.6, lanes 1-4), indicating that they were located intracellularly. As expected, the endocytosed pronase was unable to access the ER-Golgi (Lippincott-Schwartz et al., 1991; Hunziker et al., 1992). In contrast, the LDL receptors became progressively more sensitive to pronase during the chase following removal of brefeldin A (Figure 3.6, lanes 3-10.). Within one hour of the removal of brefeldin A, more than half of the labelled receptors were accessible to pronase which indicated their transport to the cell surface or into the endocytic pathway (Figure 3.6, lanes 5 and 6); by 2 hours, virtually all the labelled LDL receptors were degraded. Thus, removal of brefeldin A did affect the transport of LDL receptors and enabled them to reach the cell surface (Figure 3.6), though without correcting their aberrant glycosylation (Figure 3.5).

3.2.3.2 LDL receptor stability

Abnormal protein structure is often associated with enhanced protein breakdown [3414]. In the case of secretory proteins such as LDL receptors, certain mutant proteins are retained and degraded in the ER, while others reach the cell surface but are unstable (Hobbs et al., 1992; Hobbs et al., 1990) . LDL receptors lacking O-linked sugars were previously shown to be rapidly degraded on the cell surface (Davis et al., 1986a). As shown in Figure 3.5, the abnormally glycosylated LDL receptors synthesised in the presence of brefeldin A, appeared to be stable in the brefeldin A-fused ER/Golgi compartment and were efficiently transported to the cell surface following brefeldin A removal. Thus, no obvious instability was apparent, though these results should be interpreted with caution as it is difficult to know what the "normal" rate of turnover would be in the ER: the apparent turnover of the precursor is due to processing to the mature form of the LDL receptor which seems to be quantitative (without degradation). It also probably is not appropriate to compare the stability of the LDL receptor in the ER/Golgi (in the presence of brefeldin A) to that of the mature, endocytic LDL receptor (in the absence of brefeldin A) as they are in different locations within the cell. A better assessment of the protein stability of the brefeldin A-altered LDL receptors was made once brefeldin A was removed and transport to the cell surface was restored. During the early phase (less than 2 hours) after removal of brefeldin A, the brefeldin A-altered LDL receptors were not unstable while en route to

the cell surface (Figure 3.7A). Similarly, once on the cell surface (Figure 3.7B), the brefeldin A-altered LDL receptors were not more unstable than the receptors which had not been treated with BFA. Thus, the subtle structural changes in the brefeldin A-affected LDL receptors did not appear to cause the protein to be unstable.

3.2.3.3. Ligand binding by the LDL receptor

In order to determine the ligand binding ability of the LDL receptor synthesised in the presence of brefeldin A, it was necessary to exclude the binding activity of the pre-existing, normal LDL receptor population. When cells were pre-incubated for 12 hours in the presence of brefeldin A, LDL receptor synthesis was severely impaired (results not shown). Thus it was not possible to use a long pre-incubation to replace the total LDL receptor pool with brefeldin A-modified LDL receptors, given that normal LDL receptors are removed with a half-time of about 12 hours (Casciola et al., 1988). Similarly, the alternative strategy of first removing the pre-existing LDL receptors population with a protease, prior to re-populating the cell with brefeldin A-modified LDL receptors, was doomed by the limited period during which cells tolerated brefeldin A-treatment.

Ligand binding to LDL receptors immobilised on nitrocellulose membranes (ligand blot) also was assessed (Figure 3.8). After a 12 hour incubation in the presence of brefeldin A, pronase was added which could not access the intracellular, brefeldin A-modified LDL receptors, while the pre-existing, normal LDL receptors were cleaved at the cell surface (and in endosomes). The use of pronase was similar to the use described above to assay for LDL receptor transport to the cell surface (section 3.2.3.1.) After electrophoresis and protein transfer to nitrocellulose membranes, IgG-C7 and ßVLDL was used to detect the LDL receptor. As expected, the pronase treatment abolished the signal corresponding to the normal, mature LDL receptor at the cell surface/endocytic pathway (compare lanes 1 and 2; or lanes 5 and 6). Unexpectedly, after pronase treatment, ßVLDL did detect a heterogeneous population of LDL receptors (lane 6) with a mobility faster than the mature LDL receptor (lane 5). These possibly represented partially-degraded LDL receptor, though they were not detected by IgG-C7 (lane 2), (blot was exposed longer -not shown). This difference in detection by ßVLDL and IgG-C7 possibly was explained by pronase having cleaved the IgG-C7 epitope (in the 1st ligand binding repeat), leaving the remaining part of the LDL

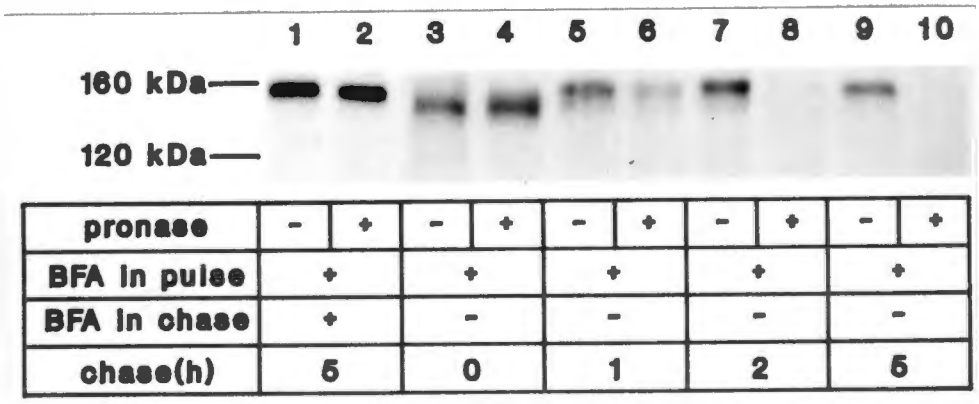


Figure 3.6 The effect of brefeldin A and its removal on the transport of the LDL receptor to the cell surface.

Fibroblasts were labelled with [^{35}S]methionine for 45 minutes and chased in the presence of unlabelled methionine for 60 minutes (labelled as pulse), in the presence of brefeldin A (0,05 µg/ml). The cells were then washed four times and chased, for the indicated time periods, in the continued presence or absence of brefeldin A. Cells were then incubated at 37°C for 20 minutes in the presence or absence of pronase. LDL receptors were immunoprecipitated with IgG-C7 and analysed by 7% SDS polyacrylamide gel electrophoresis and fluorography. The positions of the 120 kDa precursor and the 160 kDa mature forms of the LDL receptor are shown.

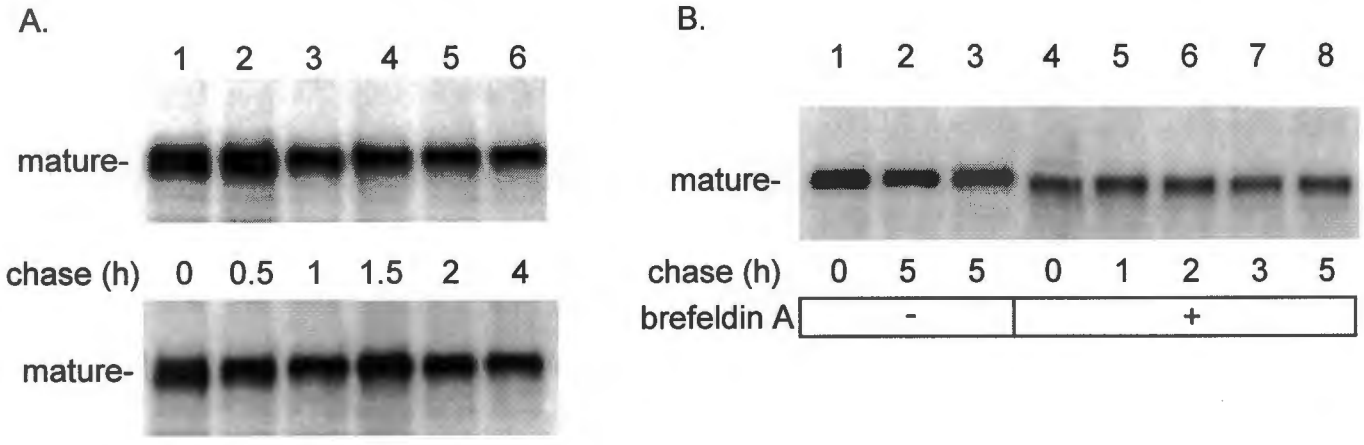


Figure 3.7 Stability of the LDL receptor after removal of brefeldin A.

Panel A: Fibroblasts were labelled with [^{35}S]methionine for 1 hour and chased for 45 minutes in the presence of 0.05µg/ml brefeldin A. Cells were washed 4 times and chased for the indicated times in medium without brefeldin A. LDL receptors were immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis and fluorography. The results of duplicate dishes from a single experiment are shown.

Panel B: Fibroblasts were labelled with [^{35}S]methionine for 1 hour and chased for 45 minutes in the presence (lanes 4-8) or absence (lanes 1-3) of 0.05µg/ml brefeldin A. Cells were washed 4 times and chased for a further 2 hours without brefeldin A to enable the labelled LDL receptors to reach the cell surface and equilibrate in the recycling endocytic pathway. Cells were then chased for the indicated times in medium without brefeldin A. LDL receptors were immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis and fluorography.

The results presented are representative of 2 independent experiments.

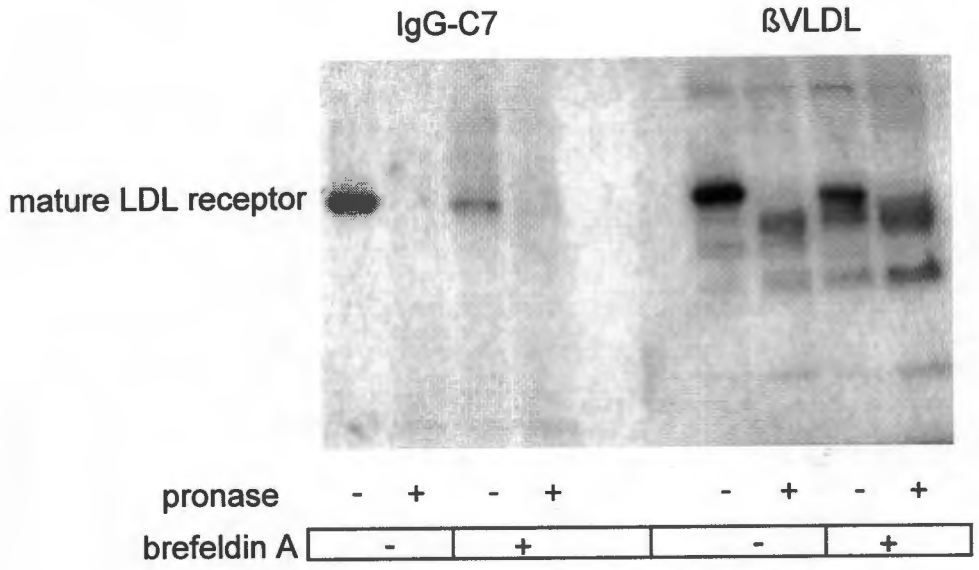


Figure 3.8 IgG-C7 and ßVLDL blotting of brefeldin A-treated LDL receptors immobilised on nitrocellulose membranes

Fibroblasts were preincubated with brefeldin A (5µg/ml) for 12 hours and then treated with pronase at 37°C for 20 minutes, as described in section 2.2.5. Cells were lysed, proteins were subject to SDS polyacrylamide gel electrophoresis under non-reduced conditions and transferred to nitrocellulose membranes, as described in section 2.2.7. The LDL receptor was detected with either IgG-C7 (5µg/ml) and ^{125}I-labelled goat anti-mouse ($3x10^6$cpm/ml) (lanes 1-4) or ^{125}I-labelled ßVLDL (5µg/ml) (lanes 5-8) (described in sections 2.2.7.1 and 2.2.7.2). The specific activity of the ^{125}I-labelled ßVLDL was $3.7x10^5$cpm/µg).

receptor able to bind ßVLDL.

Disappointingly, after brefeldin A preincubation, pronase removed the total signal corresponding to the mature LDL receptor (detected by IgG-C7) (lanes 3 and 4). The expected intracellular pool of LDL receptors that were synthesised in the presence of brefeldin A were not detected after pronase treatment. This could be accounted for by impaired LDL receptor synthesis during the preincubation with brefeldin A (see above). This, together with the pronase-induced heterogeneous LDL receptor forms (lanes 6 and 8) prevented an interpretation of the ßVLDL blot of the brefeldin A-treated LDL receptor.

An indirect assay to determine LDL receptor binding activity took advantage of the observation that NH_4Cl prevents the recycling of LDL receptors in a ligand-dependent manner (Grant et al., 1990). In the endosome, the acid-induced dissociation of the LDL receptor from ligand is neutralised by NH_4Cl, which traps the LDL receptor within the cell and causes its rapid degradation. Those LDL receptors which were internalised while unoccupied by ligand, were not trapped and recycled normally: trapping (and degradation) was ligand-dependent (Figure 3.9, lanes 1 and 2). The ability of NH_4Cl and ligand to trap and degrade LDL receptors that were synthesised in the presence or the absence of brefeldin A, was determined (Figure 3.9). The hypothesis being that brefeldin A-treated LDL receptors would have an enhanced rate of degradation only if able to bind ligand. The results indicated that as for the normal LDL receptors (lanes 2-4), ligand did enhance the rate of degradation of brefeldin A-treated LDL receptors in the presence of NH_4Cl, suggesting that they were able to bind ligand (lanes 6-8). However, a major weakness in this experiment was the realisation that ligand bound to a normal LDL receptor possibly could trap other LDL receptors of an oligomeric partnership, which could include brefeldin A-treated LDL receptors which were unoccupied by ligand. Thus trapping (and degradation) of brefeldin A-treated LDL receptors might not necessarily indicate their ability to bind ligand. This limitation assumes the formation of mixed oligomers composed of normal and brefeldin A-modified LDL receptors, which has not been verified. Such mixed oligomers would have to have been assembled at the cell surface or within the endocytic pathway, since the monomers would have been synthesised (in the ER) at different times. It is not known whether the members of an oligomeric group are able to be shuffled at the cell surface or within the endocytic pathway.

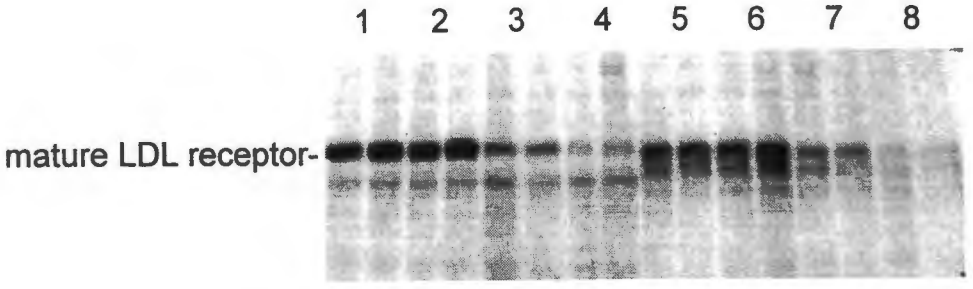
As a result of these difficulties in assessing the functional consequence(s) to the LDL receptor of the glycosylation defects induced by brefeldin A-treatment, more extensive sugar analysis was not performed to precisely characterise the altered sugar chain structure.

The partially processed sugar chains generated in the presence of brefeldin A were used in section 4.2.8 to assess the influence of glycosylation on the folding and the disulphide bond formation of the LDL receptor.

3.3. Discussion

There are several potential causes of the altered glycosylation of LDL receptors in the presence of brefeldin A. First, the TGN does not redistribute to the ER/Golgi compartment in the presence of brefeldin A, and therefore not all glycosyltransferases would be present in the fused ER/Golgi compartment (Lippincott-Schwartz et al., 1989; Chege and Pfeffer, 1990; Lippincott-Schwartz et al., 1991; Wood et al., 1991; Bosshart et al., 1991). Brefeldin A has been shown to give rise to incomplete N-linked oligosaccharide chains in other proteins, an effect that is reported to be reversible (Sampath et al., 1992). In the case of LDL receptors, abnormal processing involves O-linked carbohydrates. In contrast to N-linked glycosylation, all steps of normal, O-linked processing are thought to be possible in the fused ER/Golgi compartment found in the presence of brefeldin A (Chege and Pfeffer, 1990; Shite et al., 1990; Cummings et al., 1983; Lippincott-Schwartz et al., 1990). Indeed, the presence of N-acetylgalactosamine, galactose and sialic acid residues in the O-linked sugars of the LDL receptor indicated that the relevant glycosyltransferases were present in the fused ER/Golgi. Despite this, the O-linked glycosylation of the LDL receptor is here shown to be abnormal under such conditions.

A second general cause for abnormal glycosylation might be that altered conditions within the fused ER/Golgi compartment may affect enzyme specificities or substrate availability in such a way as to change the structure or numbers of oligosaccharide chains. Competition between processing enzymes normally found in separate compartments might also be a factor. Altered conditions such as pH, ionic strength and possibly geometric differences might influence enzyme specificities (Griffiths et al., 1984; Zizi et al., 1991). The same conditions might affect the folding and conformation of LDL receptor processing-intermediates. Another feature, the prolonged residence



NH_4Cl	-	+	+	+	-	+	+	+
LDL (µg/ml)	0	0	5	40	0	0	5	40
brefeldin A	-				+			

Figure 3.9 Incubation of the brefeldin A-treated LDL receptor with LDL and NH_4Cl

Fibroblasts were labelled with [^{35}S]methionine for 90 minutes in the presence (lanes 5-8) or absence of 5µg/ml brefeldin A (lanes 1-4). Cells were washed 4 times and chased for 3 hours to enable the labelled LDL receptors to reach the cell surface and equilibrate in the recycling endocytic pathway. Cells were incubated at 37°C for a further 4 hours in the presence of 5µg/ml (lanes 3 and 7) or 40 µg/ml LDL (lanes 4 and 8) and 10mM NH_4Cl (lanes 2-4 and 6-8). LDL receptors were immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis and fluorography.

time of LDL receptors within the fused ER/Golgi compartment might also influence their folding and, in turn, their glycosylation. In this regard, ribophorins, which normally do not contain O-linked chains, are O-glycosylated in the presence of brefeldin A (Ivessa et al., 1992). Interestingly, the ribophorins are transiently accessible to the glycosyltransferases in the presence of brefeldin A, possibly as a consequence of a conformational change during their maturation. In the presence of brefeldin A, even the earliest LDL receptor intermediates were found to be sialylated, and such early sialylation may have affected subsequent folding and processing. One hypothesis is that in the presence of brefeldin A, premature sialylation prevents (by conformational hindrance) certain serine or threonine residues of the LDL receptor from serving as substrates for N-acetylgalactosamine-transferase. This could result in the addition of fewer O-linked oligosaccharide chains to the LDL receptor than in the absence of brefeldin A.

In summary, fusion of the ER and Golgi compartments, by brefeldin A, caused the slowed and the abnormal glycosylation of the LDL receptor. These results indicate that one of the most compelling structural features of the secretory pathway - the presence of discrete compartments - may indeed have a functional role.

Chapter 4

Requirements for the folding of the LDL receptor

4.1. Introduction

In the cell, secretory and membrane proteins fold and form disulfide bonds in the endoplasmic reticulum (ER). In this compartment many factors have the potential to influence the folding process. Topologically, the ER is equivalent to the extracellular space with which it shares millimolar levels of Ca^{2+} compared to the micromolar levels found in the cytosol. The redox enzymes and the oxidising milieu of the ER promote the formation of disulfide bonds (Hwang et al., 1992). Post-translational modifications such as glycosylation are initiated in the ER and influence the folding of certain proteins (Helenius, 1994). Proteins may begin to fold as they enter the ER and before translation completes the elongation of the nascent chain (Chen et al., 1995). Chaperone proteins are present at high concentrations and facilitate productive folding reactions while preventing the transport of partially folded and malfolded proteins from the ER (Gething and Sambrook, 1992). The regulated transport ensures that migrant proteins are retained in this specialised folding environment until their folding is complete.

In the present chapter, requirements for the folding of the wild type LDL receptor are examined. The folding of the LDL receptor has been reviewed in the introductory chapter (section 1.8.2). The LDL receptor contains 63 cysteine residues and forms 30 disulfide bonds during its folding in the ER. Disulfide bonds cross-link the 7 cysteine-rich repeats of the ligand binding domain and the 3 cysteine-rich repeats in the EGF precursor homology domain. The reducing agent, DTT, has been used to study protein folding in living cells (Alberini et al., 1990; Braakman et al., 1992a). DTT-treatment reduces the disulfide bonds of newly synthesised proteins without disrupting other critical actions of the secretory pathway (Lodish and Kong, 1993; Tatu et al., 1993; Opstelten et al., 1993; Jamsa et al., 1994; Losch and Koch-Brandt, 1995). After DTT-treatment, proteins reform disulfide bonds as oxidising conditions are re-established in the ER.

The ability of DTT to reduce the disulfide bonds of the precursor and the mature forms of the LDL receptor were compared both in intact cells and in vitro. This assay (sensitivity to reduction by DTT) was used to assess the folding of the precursor form of the LDL receptor. The relationship between the formation of disulfide bonds and LDL receptor translocation into the ER was determined. Ca^{2+} and ATP were depleted in the

ER of intact cells, and their effects on the structure of the LDL receptor were determined. The effect of LDL receptor glycosylation of the formation of disulfide bonds was determined. The relationship between the folding of the LDL receptor and its transport from the ER was assessed.

4.2. Results

4.2.1. LDL receptor precursor was sensitive to DTT in the ER

The disulfide bonds of the human LDL receptor were studied in Chinese hamster ovary cells. Cells were biosynthetically-labelled with [^{35}S]methionine, LDL receptors were isolated by immunoprecipitation and then analysed by electrophoresis under reduced and non-reduced conditions (Figure 4.1). The difference in the electrophoretic mobilities of the LDL receptor under reducing or non-reducing conditions demonstrates, as previously shown (Tolleshaug et al., 1982; Beisiegel et al., 1982; Schneider et al., 1982; Daniel et al., 1983; Yamamoto et al., 1984), that both the precursor and mature forms are extensively disulfide-bonded and that this bonding contributes to their folding into a compact structure which migrated further into the gel (Figure 4.1, compare lanes 1 and 4). The earliest precursor form that was identified (5 to 15 minutes after the addition of [^{35}S]methionine) had formed extensive disulfide bonds (results not shown). DTT treatment of cells (5mM for 5 minutes) caused extensive reduction of disulfide bonds of the precursor LDL receptor as witnessed by the mobility shifts. The precursor from DTT-treated cells exhibited a mobility on non-reducing gels (lane 3, arrow) that was similar to the fully-reduced precursor (lane 1). The precursor from treated cells had a slightly altered mobility on reducing gels (Figure 4.1, lanes 1 and 2; Figure 4.2, lanes 1 and 3). This altered mobility (detected under reducing electrophoretic conditions) was apparent after EndoH-treatment, indicating that N-linked sugar chains were not involved (Figure 4.2, lanes 3 and 4). This altered mobility also was induced by DTT treatment at 4°C and was not altered by the inclusion of 4M urea in the gel application buffer (results not shown). When DTT was removed, and the cells were incubated in the presence of fresh medium for 10 minutes, the DTT-treated LDL receptor regained the wild type LDL receptor mobility (see below, section 4.2.5). Together, these results indicate an additional DTT-induced covalent alteration (apart from disulfide bond reduction) of the precursor, as yet not identified.

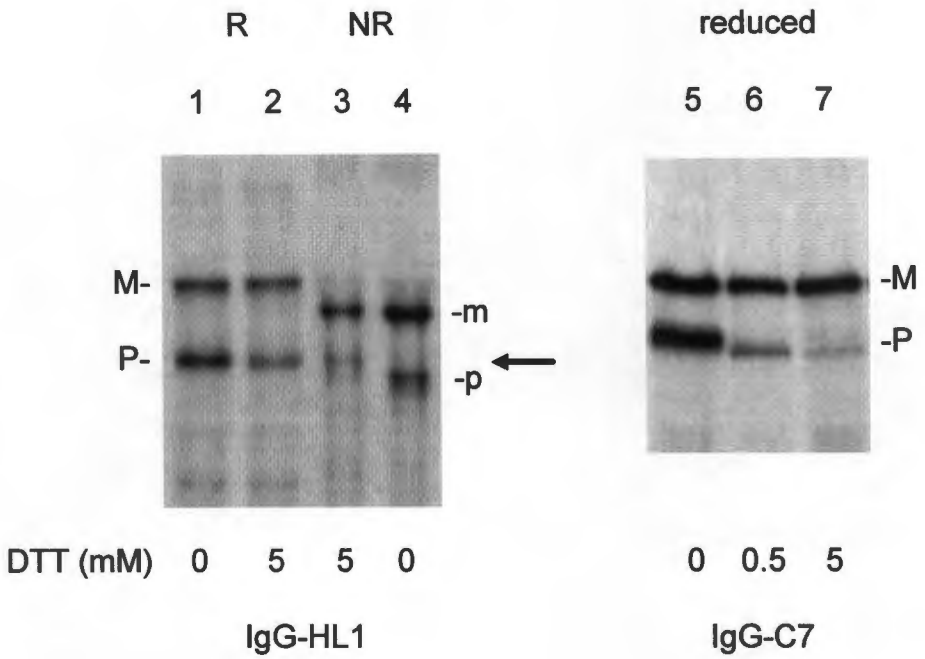


Figure 4.1 Reduction of the disulfide bonds of the LDL receptor in intact cells and in vitro.

Dishes were pulsed for 1 hour with 50μCi/ml Tran[^{35}S]methionine and then were treated with the indicated concentration of DTT for 5 minutes. LDL receptor was immunoprecipitated with IgG-HL1 (lanes 1-4) or IgG-C7 (lanes 5-7) and was analysed by electrophoresis under reduced (R) (lanes 1, 2 and 5-7) or non-reduced conditions (NR) (lanes 3 and 4). The positions of the precursor (p, P) and the mature (m, M) LDL receptor are indicated under non-reduced (p, m) and reduced (P, M) conditions. The arrow identifies the precursor LDL receptor which was reduced in intact cells (lane 3).

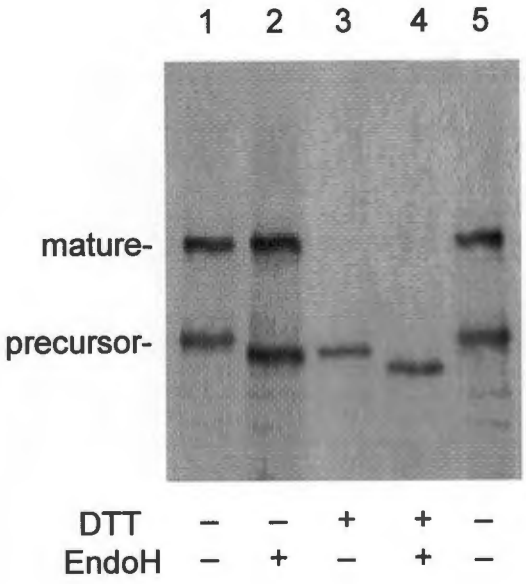


Figure 4.2 Effects of Endoglycosidase H and DTT on the LDL receptor.

CHO cells were pulsed for 45 minutes with 50μCi/ml [^{35}S]methionine. 0.5mM DTT was included in the pulse medium of dishes 1 and 2. The LDL receptor was immunoprecipitated with IgG-C7 and treated in the presence (lanes 2 and 4) or absence (lanes 1, 3 and 5) of 10 milliunits Endoglycosidase H (Endo H) for 18 hours. The LDL receptor then was analysed by SDS polyacrylamide gel electrophoresis and fluorography. The positions of the mature and the precursor forms of the LDL receptor are indicated.

The unfolding of the precursor LDL receptor was further monitored using the conformation-specific antibody, IgG-C7, which binds the first repeat of the ligand binding domain of the LDL receptor only if properly folded. DTT (added to cells) reduced the precursor and caused the loss of its immuno-reactivity. Therefore there was a marked decrease in precursor signal detected in the gel (Figure 4.1, lanes 5-7). Unexpectedly, DTT treatment of cells did not result in the complete reduction of the mature LDL receptor, assessed either by electrophoretic mobility (Figure 4.1, lane 3) or immuno-reactivity (Figure 4.1, lanes 6 and 7). This indicated that the mature receptor was more resistant to unfolding caused by disulfide bond reduction, than the precursor form.

To determine whether DTT had any detectable structural effect on the mature LDL receptor in intact cells, the ability of the LDL receptor to bind antibody and lipoprotein ligands was measured. After treatment with DTT (5 minutes at 37°C), cells were cooled to 4°C, washed with alkylating agent and then incubated for 2 hours in the presence of ^{125}I-labelled ligands. (Table 4.1). At a saturating ligand concentration, the binding of the antibody, IgG-C7, to DTT-treated cells was between 40% (at 50mM DTT) and 60% (at 5mM DTT) of control cells. The binding of lipoproteins to DTT-treated cells varied in three experiments, but was always more severely impaired than the binding of IgG-C7. At the higher concentrations of DTT (50mM), LDL binding was reduced to about 20% or less compared with normal cells, while the effect of 5mM DTT was less marked. The binding of ßVLDL was reduced to an intermediate degree compared to that of IgG-C7 or LDL (experiment 2). These results indicate that some, but not all of the disulfide bonds of the mature LDL receptor, were reduced by DTT. Different combinations of the repeats are required to bind the different ligands ((Russell et al., 1989). Thus sensitive disulfide bonds were detected in repeat 1 (IgG-C7 binding), and in repeat 5 (ßVLDL binding), and among repeats 2 to 7 (LDL binding). In all of the experiments, except for the use of LDL in experiment 1, a saturating concentration of ligand was used to determine the effects of DTT on LDL receptor binding. It was not possible from this data to determine an effect on binding affinity, as binding studies at a range of ligand concentrations (binding curves) were not performed. The loss of IgG-C7 immunoreactivity detected by the binding experiment (Table 4.1) was not matched by a similar loss of mature LDL receptor in the immunoprecipitation experiments (Figure 4.1). The cause of this difference in sensitivity between the 2 assays is not known.

Table 4.1: Binding of ^{125}I-labelled IgG-C7, ^{125}I-labelled LDL and ^{125}I-labelled ßVLDL to DTT-treated cells at 4°C.

Experiment 1

DTT	IgG-C7	LDL
0 mM	76ng/mg	242ng/mg
5 mM	63%	41%

Experiment 2

DTT	IgG-C7	LDL	ßVLDL
0mM	61ng/mg	310ng/mg	380ng/mg
5mM	59%	23%	45%
50 mM	41%	5%	21%

Experiment 3

DTT	IgG-C7	LDL
0mM	50ng/mg	853ng/mg
5mM	66%	63%
50mM	50%	22%

Binding studies were performed as described in Methods. 1.25µg/ml ^{125}I-labelled LDL was used in experiment 1, and 10µg/ml ^{125}I-labelled LDL was used in experiments 2 and 3. 1µg/ml ^{125}I-labelled IgG-C7 was used in each experiment, and 5µg/ml ^{125}I-labelled ßVLDL was used in experiment 2. Binding values obtained after DTT-treatment are expressed as a percentage of the value obtained (ng/mg cell protein) when DTT was not added. Each value is the average of duplicate dishes.

4.2.2. Reduction of solubilised LDL receptor by DTT

The DTT sensitivity of the solubilised LDL receptor was tested. Immunopurified LDL receptor was boiled in SDS polyacrylamide gel electrophoresis sample buffer, in the presence of increasing doses of DTT and then electrophoresed on a non-reducing gel (Figure 4.3). Addition of 0.5mM DTT increased the mobilities of both the precursor and the mature receptor compared with the mobilities of the non-reduced forms, suggesting the formation of more compact structures. Higher doses of DTT progressively retarded the mobility of both the precursor and mature LDL receptor, indicative of an increasing degree of reduction. Importantly, the effect of increasing doses of DTT appeared to be identical for the precursor and the mature receptor forms. The resistance of the mature receptor to reduction as observed in intact cells was therefore abolished under these in vitro conditions.

4.2.3. LDL receptor must be denatured to be reduced in vitro

The effect of denaturants (boiling and the detergent, SDS) on the DTT-induced reduction of the LDL receptor was assessed (Figure 4.4) DTT was added to immunopurified LDL receptor for 5 minutes at 37°C, in the presence of either Triton X-100 or SDS. Samples were alkylated with N-ethyl maleimide (NEM), in order to quench the DTT and to prevent shuffling of disulfide bonds. Samples were then boiled, and electrophoresed without the addition of further reducing agents. The non-reduced, folded LDL receptor is seen in lanes 1 and 6 and the fully-reduced LDL receptor is seen in lane 4. Compared to the non-reduced forms, the mobility of the precursor and the mature LDL receptor were slightly slower in the DTT-treated samples, alone (lane 5) or together with either 1% Triton X-100 (lane 3) or 1% SDS (lane 2). Receptors were fully-reduced only when the alkylating agent was not added, causing active DTT to be present when the sample was boiled (lane 4). Under these conditions, there was no difference between the sensitivity to DTT of the precursor and the mature LDL receptor. These results show that it was necessary to denature (boil) the LDL receptor to allow complete reduction by DTT. The sensitivity of the precursor LDL receptor, in the ER of living cells, was dependent on it being maintained in a partially folded or denatured state. Its sensitivity was dependent on its localisation within the ER; in contrast, fully-folded mature receptors in the endocytic pathway, were relatively resistant to unfolding caused by addition of a reducing agent.

4.2.4. LDL receptor folding and processing in the presence of DTT

When cells are labelled in the presence of DTT, protein synthesis continues and the secretory pathway remains functional (Braakman et al., 1992b; Tatu et al., 1993; Opstelten et al., 1993; Jamsa et al., 1994; Losch and Koch-Brandt, 1995). However, in the ER, the folding of certain proteins is prevented since they are unable to form disulfide bonds. The effect of DTT on LDL receptor folding and transport was assessed in Figure 4.5. In the presence of DTT, LDL receptor precursor was synthesised; at doses greater than 0.15mM, LDL receptor transport out of the ER was impaired and less mature form was detected (compare lanes 2 and 3). Increasing doses of DTT had further effects: 0.5mM DTT (lane 4) caused the covalent change described in section 4.2.1; 1.5mM DTT destroyed the IgG-C7 epitope (lane 5). Thus LDL receptor transport from the ER was retarded at a lower dose of DTT (0.15mM DTT) than the unfolding of the IgG-C7 epitope (1.5mM DTT). The mobility of the LDL receptor precursor was altered on non-reduced gels at 0.5mM DTT (not shown), which correlated with the complete block to processing from the ER (lane 4). Similar results were obtained in a repeat experiment (and see Figure 4.1). These experiments show that the formation of disulfide bonds was required for the folding of the LDL receptor and its transport out of the ER.

4.2.5. Post-translational folding of the LDL receptor.

Next, DTT was removed, and the post-translational folding and disulfide bond formation of the unfolded LDL receptor was assessed in living cells. Cells were labelled in the presence of DTT and then chased in fresh medium without DTT, for varying times, to enable oxidative conditions to be re-established in the ER (Figure 4.6). Cycloheximide was included during the chase period to prevent protein translation from continuing after the pulse period. In this manner, the folding of the LDL receptor molecules labelled during the pulse-period were monitored during the chase period. After 1 minute of chase, a disulfide-bonded, folded form of the precursor LDL receptor was detected (Figure 4.6B, lane 4). After longer chase times (30 minutes) a mature form of the receptor was observed (lanes 7-9). Thus, the LDL receptor formed disulfide bonds post-translationally with sufficient fidelity to escape the quality control mechanism that normally retains malfolded proteins in the ER. A mutant LDL receptor (FH Afrikaner-1) that is known to be slowly-processed under normal conditions (Fourie et al., 1988) was retained when DTT was reversed, confirming that the normal quality-control mechanism was functional in the ER (see chapter 6). During the chase period, there was an

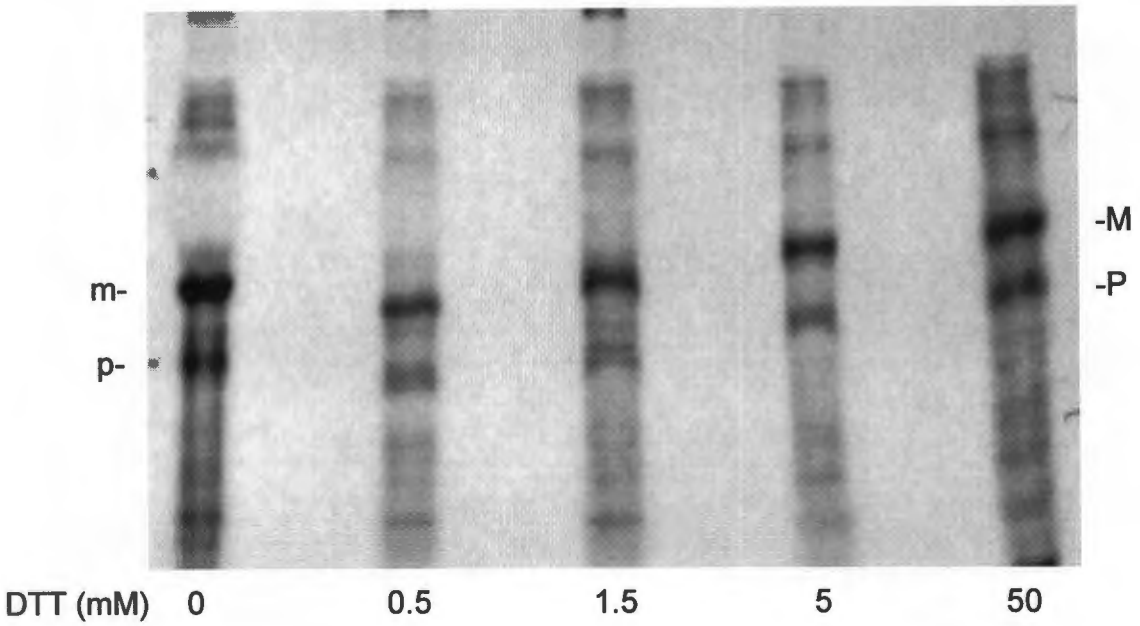


Figure 4.3 Reduction of solubilised LDL receptor.

Dishes were pulsed for 1 hour with 50µCi/ml Tran[^{35}S]methionine and LDL receptor was immunoprecipitated with IgG-C7. Immunoprecipitates were boiled for 3 minutes in SDS-containing gel loading buffer containing DTT at the concentrations indicated, and then analysed by electrophoresis. The positions of the non-reduced forms of the precursor (p) and the mature (m) LDL receptor are indicated. In each lane, the "upper" band corresponds to the mature LDL receptor and the "lower" band corresponds to the precursor LDL receptor.

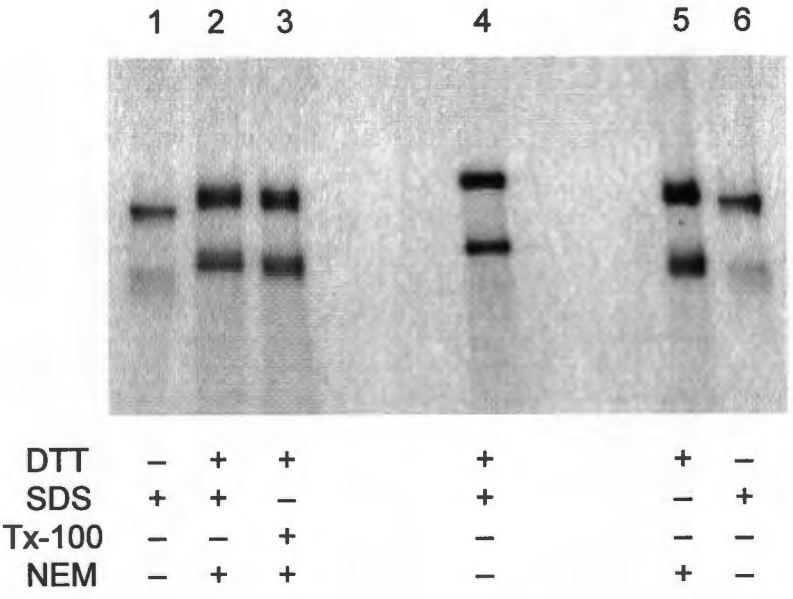


Figure 4.4 Effect of denaturation on the reduction of solubilised LDL receptor

Dishes were pulsed for 1 hour with 50μCi/ml Tran[^{35}S]methionine and LDL receptor was immunoprecipitated with IgG-C7. Immunoprecipitates were treated with 5mM DTT (lanes 2-5) for 5 minutes at 37°C in the presence of 1% SDS (lanes 1, 2, 4 and 6) or 1% Triton X-100 (lane 3). NEM (20mM) was added (lanes 2, 3 and 5) and immunoprecipitates were boiled for 3 minutes in gel loading buffer without the addition of further reducing agents. They were then analysed by electrophoresis. In each lane, the "upper" band corresponds to the mature LDL receptor and the "lower" band corresponds to the precursor LDL receptor.

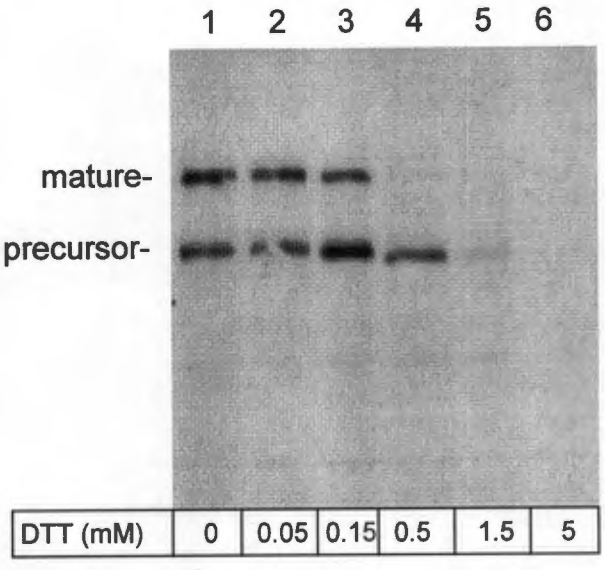


Figure 4.5 LDL receptor synthesis in the presence of DTT.

CHO cells were pulsed for 45 minutes with 50µCi/ml Tran[^{35}S]methionine in the presence of the indicated concentrations of DTT. The LDL receptor was immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis under reducing conditions. Immunoprecipitated protein was detected by fluorography. The positions of the mature and the precursor forms of the LDL receptor are indicated.

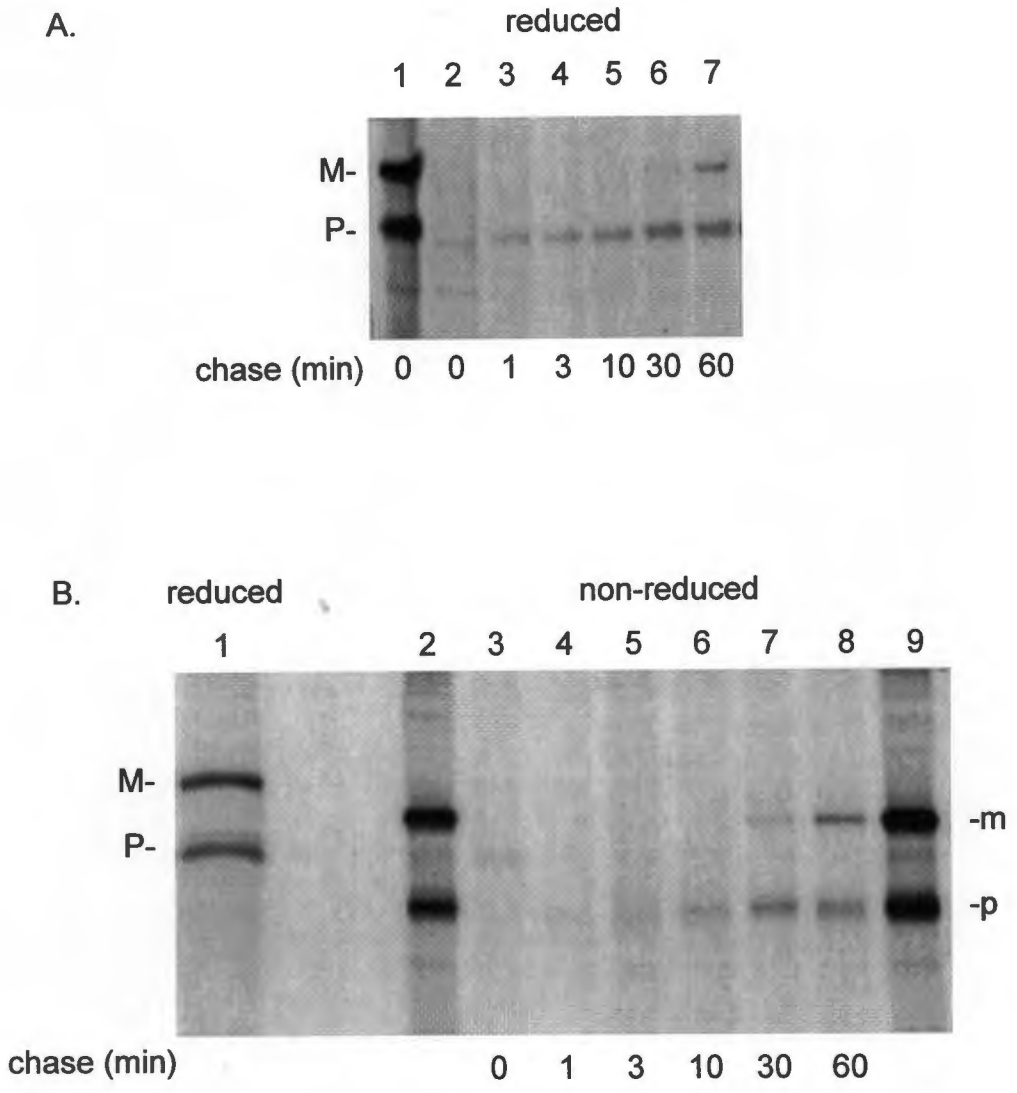


Figure 4.6 Post-translational folding of the LDL receptor.

Dishes were pulsed for 1 hour with 100μCi/ml Tran[^{35}S]methionine. DTT (5mM) was included in the pulse-period of dishes 2-7 (panel A) and of dishes 3-8 (panel B). Dishes were washed and then chased for the times indicated in fresh medium containing cycloheximide. Dishes were cooled to 4°C, alkylated and lysed in detergent. LDL receptor was immunoprecipitated with IgG-HL1 and then divided for electrophoresis under reduced (panel A, all lanes and panel B, lane 1) and non-reduced conditions (panel B, lanes 2-9). As described in Figure 5.1, the positions of the precursor (p, P) and the mature (m, M) LDL receptor are indicated under non-reduced (p, m) and reduced (P, M) conditions.

increase in the amount of LDL receptor detected (Figure 4.6A), indicating that some of the labelled LDL receptors were not detected at the beginning of the chase (Figure 4.6A, lane 2). This was due, in part, to the epitope for the antibody, IgG-HL1, being partially sensitive to DTT. Protein aggregates were not detected during the folding process, but possibly did contribute to the loss of signal observed. In this experiment, refolded LDL receptor was poorly recovered when compared with the untreated controls. In experiments where DTT was added after the LDL receptor had been synthesised, instead of during the pulse period, the post-translational folding-efficiency was close to 100% (see Figure 4.9A, lanes 1-3). These results thus demonstrated that the translation or the translocation of the LDL receptor was impeded when DTT was present during LDL receptor synthesis and that the post-translational folding-process was efficient. In summary, these results demonstrated that precursor LDL receptors were able to form disulfide bonds post-translationally, and, therefore, that co-translational folding was not required for normal LDL receptor synthesis.

4.2.6. Folding of the LDL receptor required Ca^{2+}

The ionophore, A23187, was used to deplete the ER of Ca^{2+} to determine whether the LDL receptor required Ca^{2+} to fold and form disulfide bonds. In the presence of A23187, precursor LDL receptor was synthesised with a relatively normal mobility on reducing gels (Figure 4.7A, lanes 1 and 2). However, transport of the precursor from the ER was prevented and mature LDL receptor was not detected during the 45 minute labelling period. A23187 reportedly does not have a general effect on protein transport from the ER; only malfolded proteins are retained (Lodish and Kong, 1990; Lodish et al., 1992). In the absence of Ca^{2+} the LDL receptor did form disulfide bonds as non-reduced electrophoresis indicated that the mobility of the precursor was enhanced when compared with that of the reduced precursor (Figure 4.7A, lane 2 versus lane 5). However, in the presence of A23187 the mobility of the precursor population was heterogeneous and, in general, was faster than the mobility of the normally-folded precursor, indicating that the folding of the precursor was altered (Figure 4.7A, lane 5 versus lanes 3 and 6). It is not known whether this abnormal mobility reflects incomplete disulfide bonding, or abnormal disulfide bond positioning, or both. Supplementing the medium with Ca^{2+} (up to 10mM), either during or after treating the cells with A23187, did not correct folding of the precursor (data not shown). These

results indicate that precursor LDL receptor requires Ca^{2+} for folding and transport from the ER.

Since the mature LDL receptor requires Ca^{2+} to bind lipoprotein ligands (Goldstein and Brown, 1977) and the conformation-specific, monoclonal antibody, IgG-C7 (van Driel et al., 1987b), its disulfide bond structure was examined after depleting Ca^{2+}. Pulse-labelled cells were washed with Ca^{2+}-free medium containing A23187 and EDTA to remove free Ca^{2+}. This did not alter the electrophoretic mobility of the mature LDL receptor either on reduced (Figure 4.7B, lane 2) or non-reduced gels (Figure 4.7A, lane 4). Thus Ca^{2+} was not needed to maintain the disulfide bonds of the mature LDL receptor. In contrast, the conformation of the precursor LDL receptor was altered by depleting Ca^{2+}. On non-reducing gels, the mobility of the precursor was enhanced and heterogeneous (Figure 4.7A, lane 4), and similar to the mobility of the precursor synthesised in the absence of Ca^{2+} (Figure 4.7A, lane 5). An altered conformation was also evident from the inability of the conformation-specific antibody, IgG-C7, to precipitate the precursor from treated cells (Figure 4.7B, lane 3). Thus depletion of cell Ca^{2+} affected the disulfide bonding of pre-existing, folded precursor LDL receptor, but not that of mature LDL receptor.

To test whether the LDL receptor was able to rebind Ca^{2+}, we replaced Ca^{2+} in the cell lysate in order to be certain that the Ca^{2+} gained access to both the precursor and the mature LDL receptor. IgG-C7 was used to detect whether the LDL receptor had rebound Ca^{2+}. In the absence of Ca^{2+}, IgG-C7 was severely impaired in its ability to immunoprecipitate both the precursor and the mature receptor (Figure 4.7B, lane 3), in comparison with IgG-HL1 which was less affected (Figure 4.7B, lane 2). When Ca^{2+} was replaced in the lysate, the IgG-C7 epitope of the mature receptor was restored (Figure 4.7B, compare lanes 3 and 4), while the precursor remained immunologically undetectable. Ca^{2+} depletion had disrupted the structure of the normal precursor such that replacing Ca^{2+} was not sufficient for the antigenic epitope to reform. In contrast, mature LDL receptor was able to bind Ca^{2+} reversibly (Figure 4.7B, lanes 3 and 4), supporting the finding that its disulfide bonds were not disrupted by Ca^{2+}-removal. Thus, Ca^{2+} was required for the formation of the normal disulfide bonds of the precursor LDL receptor and for the maintenance of the precursor's structure. In contrast,

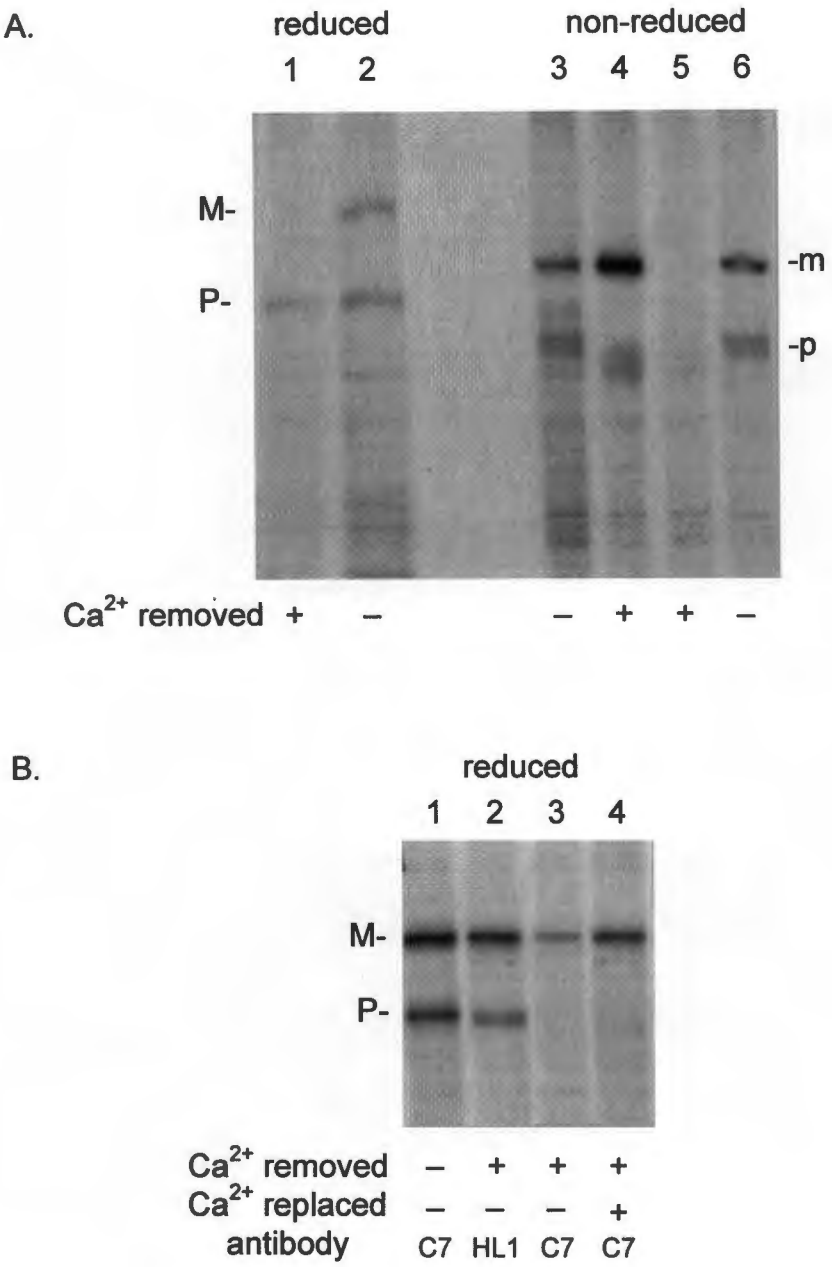


Figure 4.7 Effects of A23187 and Ca^{2+} on the disulfide bonds of the LDL receptor.
Panel A: Dishes were pulsed for 45 minutes with 50μCi/ml TRAN[^{35}S]methionine and the LDL receptor was immunoprecipitated with IgG-HL1. Ca^{2+} was depleted by including A23187 (5μM) during the pulse (lanes 1 and 5) or during a chase (5 minutes) in Ca^{2+}-free medium containing 5mM EDTA and 5μM A23187 (dish 4). Lanes 1, 4 and 5 were lysed in the presence of 150μM EDTA, and lanes 2, 3 and 6 were lysed in the presence of 2mM Ca^{2+}. Immunoprecipitates were analysed by electrophoresis under reduced (lanes 1 and 2) or non-reduced conditions (lanes 3-6).
Panel B: Dishes were pulsed for 45 minutes with 50μCi/ml TRAN[^{35}S]methionine. Ca^{2+} was depleted during a chase (5 minutes) in Ca^{2+}-free medium containing 5mM EDTA and 5μM A23187 (lanes 2-4). Cells were lysed in the presence (lanes 1 and 4) or absence of 2mM Ca^{2+} (lanes 2 and 7). LDL receptor was immunoprecipitated with IgG-C7 (lanes 1, 3 and 4) or IgG-HL1 (lane 2) and analysed by electrophoresis under reduced conditions.

although the mature LDL receptor required Ca^{2+} for ligand binding, it was able to bind Ca^{2+} reversibly without an alteration of its disulfide bonds.

4.2.7. Folding of the LDL receptor requires ATP

The metabolic energy requirement for the formation of disulfide bonds in the precursor LDL receptor was investigated. Metabolic energy is required for protein translation, and thus its role in disulfide bond formation cannot be assessed in normal biosynthetic pulse-labelling experiments. The use of DTT enabled the folding of the LDL receptor to be studied as an isolated, post-translational process, independent of translation or the translocation of the protein into the ER. Newly-synthesised LDL receptor was labelled, treated with DTT to reduce the disulfide bonds and then chased for 20 minutes in medium without DTT either in the presence or absence of ATP (Figure 4.8). ATP was depleted by using glucose-free medium supplemented with 10mM azide and 20mM 2-deoxy-D-glucose (Braakman et al., 1992a). In control cells that were not treated with DTT, about 50% of the pre-existing precursor was converted to mature form during a 20 min chase in the presence of ATP (Figure 4.8A, lanes 1 and 2). In cells that were reduced with DTT (lane 3) and then chased in the absence of ATP, the precursor LDL receptor was almost undetected by IgG-HL1 (lane 4) and IgG-C7 (lane 6). In contrast, significantly more precursor receptor was detected by both antibodies after chasing in the presence of ATP (lanes 5 and 7). Thus, precursor receptor required metabolic energy for refolding after reduction. It was unexpected that after a short chase in the absence of ATP, the precursor was immunoprecipitated poorly by the monoclonal antibody, IgG-HL1, (Figure 4.8A, lane 4), as IgG-HL1 was able to detect the fully-reduced form of the precursor LDL receptor (lane 3). One possibility was that in the absence of ATP, the reduced precursor formed disulfide-bonded aggregates with other proteins which were not detected by the monoclonal antibodies. A prolonged exposure of the non-reduced gel did show that after being reduced (Figure 4.8B, lane 3), the precursor was able to form disulfide bonds in the absence of ATP (Figure 4.8B, lanes 4 and 6). However, these precursors exhibited a heterogeneous and largely abnormal mobility compared to normal precursors (lanes 5 and 7). Together with the poor immuno-reactivity of the ATP-depleted LDL receptor, these results indicated that the correct folding of the LDL receptor was not spontaneous, but required metabolic energy.

4.2.8. Glycosylation does not affect LDL receptor reduction or oxidation

The influence of glycosylation on the folding of the LDL receptor was examined. Brefeldin A, known to fuse the ER with the Golgi apparatus and to prevent protein transport to the cell surface (Klausner et al., 1992), was used to manipulate the glycosylation of the LDL receptor (see Chapter 3). BFA-treatment caused the glycosyltransferases of both the ER and Golgi apparatus to modify the newly-synthesised LDL receptor, resulting in a heterogeneous population of precursors with an electrophoretic-mobility intermediate between the normal precursor and mature forms of the LDL receptor (Figure 4.9A, lane 4 versus lane 1). The processing of the oligosaccharide chains continued during a 4 hour chase-period (Figure 4.9A, lane 8). The DTT-sensitivity of the extensively-glycosylated, intermediate forms of LDL receptor were examined. DTT-treatment prevented IgG-C7 from immunoprecipitating all these intermediate forms of the LDL receptor, including those that were chased for 4 hours in BFA, and that had extensive processing of their oligosaccharide chains (Figure 4.9A, lanes 5 and 9). Thus, the extent of the post-translational modification of the LDL receptor did not influence the sensitivity of the disulfide bonds to DTT. Next, the location of the precursor LDL receptor in the cell was varied with brefeldin A and its sensitivity to DTT was examined. In the presence of brefeldin A, the LDL receptor was trapped in an abnormal, fused ER/Golgi compartment; transport to the cell surface was re-established when cells were incubated in fresh medium without BFA (see section 3.2.3.1.). The DTT-sensitivity of the LDL receptor either in the ER/Golgi or in the cell surface/endocytic pathway was compared. Despite their different locations, these LDL receptors were glycosylated to a similar extent (Figure 4.9A, compare lanes 8 and 12). Importantly, unlike the intracellular form (lanes 8 and 9), the surface/endocytic LDL receptor was resistant to DTT (lanes 12 and 13). Thus, the location of the LDL receptor in the cell determined its sensitivity to DTT, independent of the extent of its glycosylation.

The effect of glycosylation on the formation of disulfide bonds by the LDL receptor was examined. The approach used was to examine the ability of the partially-glycosylated forms of the LDL receptor to refold and reform disulfide bonds after having been reduced with DTT. Brefeldin A was used to manipulate the extent of glycosylation of the LDL receptor. The extensively-glycosylated, intermediate-forms of the LDL receptor, which were produced in the presence of brefeldin A and reduced with DTT

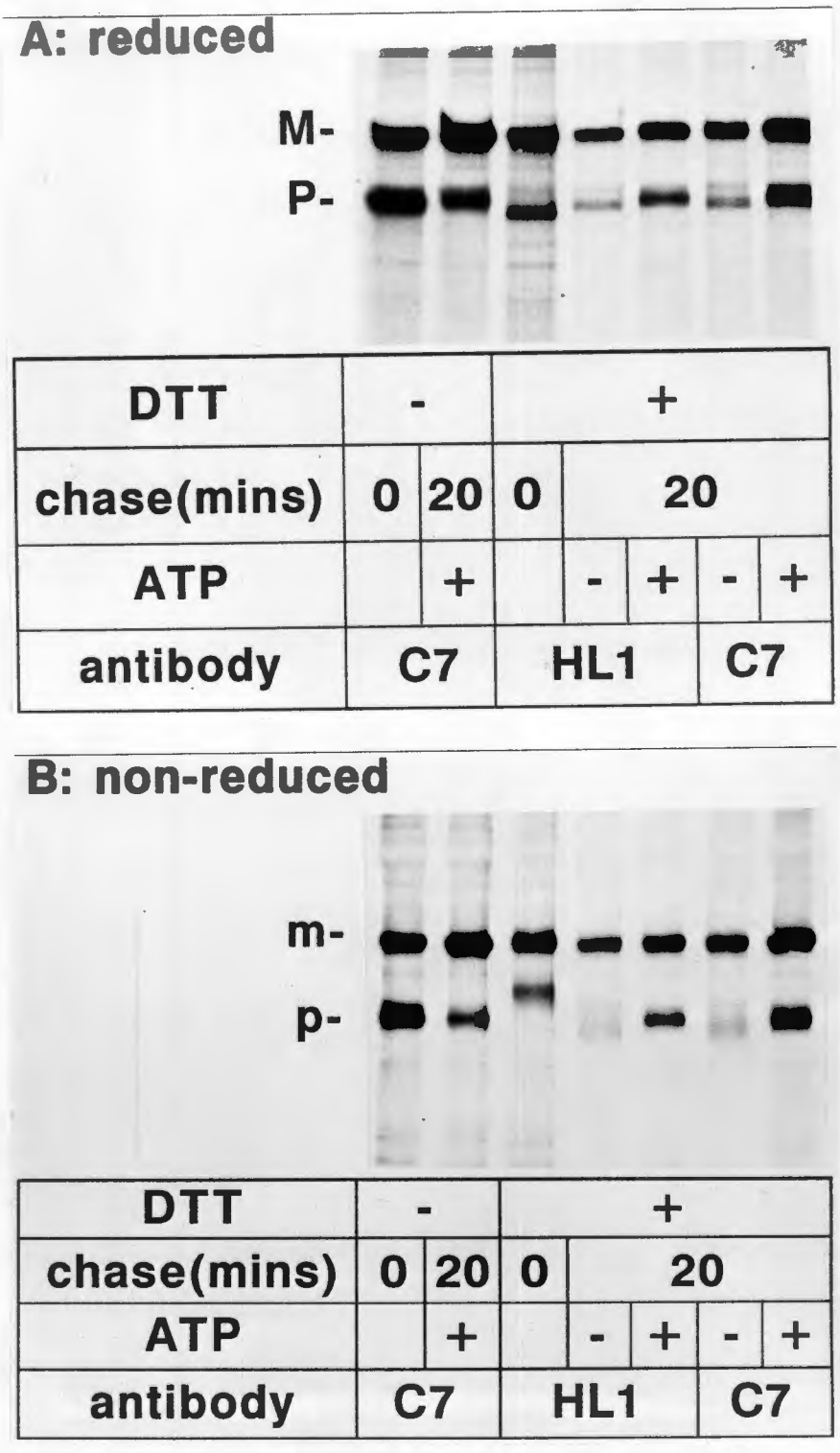
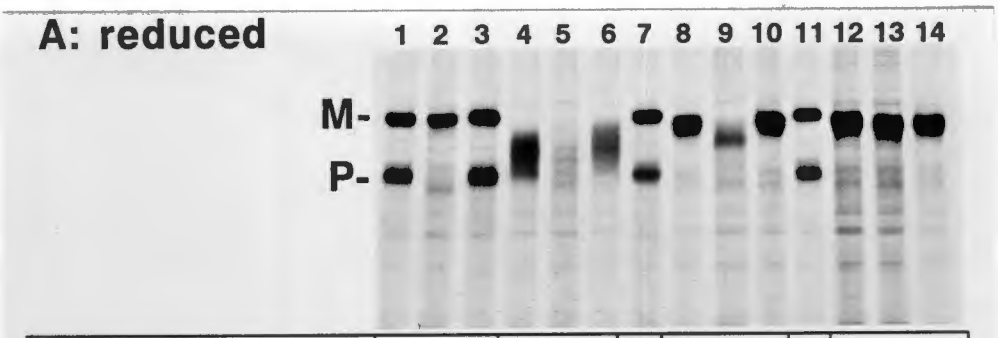
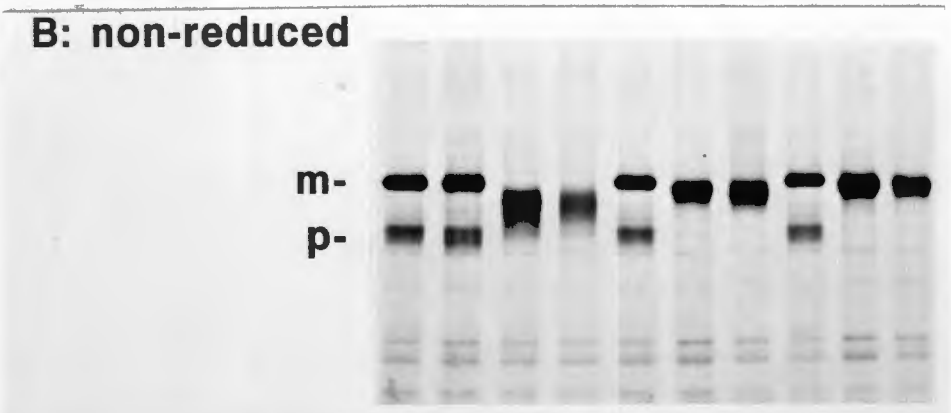


Figure 4.8 Formation of disulfide bonds by the LDL receptor in the absence of ATP.

Dishes were pulsed for 35 minutes with 100µCi/ml Tran[^{35}S]methionine, after which DTT (5mM) was added to dishes 3-7 for 5 minutes. Dishes 2, 4, 5, 6 and 7 were chased for 20 minutes in the presence (lanes 2, 5 an 7) or absence of ATP (lanes 4 and 6). Dishes were cooled to 4°C, alkylated with NEM, lysed and LDL receptor was immunoprecipitated using IgG-C7 (lanes 1, 2, 6 and 7) or IgG-HL1 (lanes 3, 4 and 5). Samples were split and analysed by electrophoresis under reduced (panel A) or non-reduced conditions (panel B). During the chase, ATP was depleted using azide (10mM) and 2-deoxy-D-glucose (20mM).



BFA in pulse	-	+	-	+	-	+
chase+BFA(h)	0	0	0	4	0	2
chase-BFA(h)	0	0	0	0	0	2
DTT	- + +	- + +	-	- + +	-	- + +



BFA in pulse	-	+	-	+	-	+
chase+BFA(h)	0	0	0	4	0	2
chase-BFA(h)	0	0	0	0	0	2
DTT	- +	- +	-	- +	-	- +

Figure 4.9 Formation of disulfide bonds by the LDL receptor in the presence of brefeldin A.

Panel A: Dishes were pulsed for 45 minutes with 50µCi/ml Tran[^{35}S]methionine. BFA (5µg/ml) was included in the pulse in dishes 4, 5, 6, 8, 9, 10, 12, 13 and 14. After the pulse, dishes 8-10 and 12-14 were chased in the continued presence of BFA. After 2 hours, dishes 12-14 were washed and chased in the absence of BFA for a further 2 hours. DTT (5mM) was added for the final 5 minutes of the pulse (dishes 2, 3, 5 and 6) or the chase period (dishes 9, 10, 13 and 14). Dishes 3, 6, 10 and 14 were washed to remove the DTT and incubated at 37°C in fresh chase medium for a further 10 minutes. Dishes were cooled to 4°C, alkylated and lysed in detergent. LDL receptor was immunoprecipitated with IgG-C7 and immunoprecipitates were split for analysis by electrophoresis under reduced (panel A) or non-reduced conditions (panel B).
Panel B: DTT+ dishes correspond to lanes 3, 6, 10 and 14 of panel A which were chased for 10 minutes after DTT-treatment. Samples corresponding to lanes 2, 5, 9 and 13 of panel A were not included in panel B.

(Figure 4.9A, lanes 2, 5 and 9), were washed and chased in fresh medium. Within 10 minutes, all the glycosylation-intermediates reformed disulfide bonds and were recognised by IgG-C7 (Figure 4.9A, lanes 3, 6 and 10). The structures of the refolded glycosylation-intermediates also were examined by electrophoresis under non-reduced conditions (Figure 4.9B). This showed that the refolded forms of the LDL receptor (lanes 2, 4 and 7) achieved their expected electrophoretic mobility as was obtained prior to reduction by DTT (lanes 1, 3 and 6). Thus the extent of LDL receptor glycosylation had no detectable influence on the formation of disulfide bonds.

4.3. Discussion

The major findings of these experiments are as follows: 1) folding of the LDL receptor involves disulfide bond formation and requires metabolic energy; 2) the disulfide bonds of the folded precursor LDL receptor remain sensitive to reduction by DTT and are not irreversibly hidden in the hydrophobic core during folding in the ER; 3) the LDL receptor is able to fold and form disulfide bonds post-translationally in the ER; 4) Ca^{2+} is required for the folding of the precursor LDL receptor in a manner different to its role in ligand and antibody binding to the mature LDL receptor; 5) folding and disulfide bond formation are not affected by the glycosylation of the LDL receptor.

The disulfide bonds of the LDL receptor cross-link the cysteine-rich domains into compact, robust structures which are able to endure repeated, acid-induced conformational changes during endocytosis and recycling (Goldstein et al., 1985). In the studies presented in this thesis it was shown that the mobility of the DTT-treated mature form of the LDL receptor was not altered under non-reduced electrophoretic conditions. Thus, most of the mature LDL receptor's disulfide bonds are evidently within the folded structure of the LDL receptor and are largely inaccessible to DTT. Some of the disulfide bonds were reduced by DTT, which impaired the ability of the LDL receptor to bind several different ligands, to varying degrees, with LDL binding being more severely impaired than ßVLDL binding, and with C7 binding being the least impaired. This indicated that some of the disulfide bonds in several of the ligand binding repeats were reduced, and correlates with the different number of repeats (and thus the number of disulfide bonds) normally required to bind each ligand (Russell et al., 1989).

Analysis of the reduction of the LDL receptor precursor in vitro showed that its disulfide bonds were not inherently accessible to DTT. The reduction of the disulfide bonds of influenza virus haemagglutinin and vesicular stomatitis virus G protein also were promoted in the ER, and were dependent on ATP (Tatu et al., 1993). One hypothesis is that a chaperone is contributing to the reduction of the hidden disulfides of partially folded protein in the ER. This action would facilitate the shuffling of disulfide bonds during the folding of a complex protein such as the LDL receptor. A candidate chaperone is protein disulfide isomerase which facilitates disulfide bond interchange, and which promotes disulfide bond reduction under reducing conditions (Creighton et al., 1980; Kaji and Lodish, 1993). One possibility is that the reduction of accessible disulfide bonds of the LDL receptor promotes its association with protein disulfide isomerase and which, in turn, facilitates extensive reduction and unfolding.

The specific chaperone protein(s) necessary for LDL receptor folding has not yet been identified. Calnexin is a major chaperone associated with the folding of membrane glycoproteins, associating with partially folded proteins and contributing to their retention in the ER (Rajagopalan et al., 1994; Jackson et al., 1994). As described in chapter 5, less than a few percent of the ER-pool of LDL receptor was detected in association with calnexin, which may imply that other chaperone proteins are needed for LDL receptor folding. Calnexin is unlikely also to be the chaperone responsible for mediating the DTT-induced reduction of the precursor LDL receptor in the ER. Proteins associate with calnexin through their N-linked oligosaccharide chains, but the binding is limited to proteins that have monoglucosylated core chains (Hammond et al., 1994; Ou et al., 1993; Hebert et al., 1995; Helenius, 1994, Ware et al., 1995). Thus the extent of chain-trimming regulates protein interaction with this chaperone. After a prolonged exposure to brefeldin A, the LDL receptor was processed beyond this form (was resistant to endoglycosidase H), and yet it was reduced by DTT. The interaction of the LDL receptor with calnexin is considered in detail in chapter 5.

The results showed that the reduction of the LDL receptor and its consequent unfolding, were reversed following DTT removal. Translation of the peptide chain occurred while cysteines remained reduced. When DTT was removed, disulfide bonds formed within minutes, and the refolded LDL receptor was transported rapidly from the ER. Disulfide bond formation was normal despite having been delayed by the

presence of the reducing agent. The folding machinery thus is able to prevent inappropriate disulfide bond formation or to facilitate recovery from inappropriate bonding. These findings indicate that the normal folding of the cysteine-rich repeats of the LDL receptor does not necessarily proceed vectorally from the N- to the C-terminus. Folding is dictated by the sequence of amino acids and is not constrained by limitations imposed by a co-translational folding process. Such mechanisms could add enormously to the repertoire of possible folded-conformations for a given protein. Post-translational protein folding is not always successful, or equivalent to the co-translational formation of disulfide bonds. A beta-lactamase fusion protein did not regain enzymatic activity when it refolded after reduction, though it was transported from the ER (Simonen et al., 1994). Similarly, reduced plasminogen activator was over-glycosylated when the disulfide bonds were formed post-translationally (Allen et al., 1995).

The formation of new disulfide bonds was impeded when cellular energy was depleted, resulting in the formation of partially folded LDL receptor. This was in contrast to the ability of the first and the second repeats of the ligand binding domain to fold spontaneously, as isolated peptides, in vitro (Bieri et al., 1995b; Bieri et al., 1995a; Daly et al., 1995). These findings point to the need for chaperone proteins in LDL receptor folding in vivo: energy is required by chaperones during the association-dissociation cycle with their folding-substrates (Gething and Sambrook, 1992; Hendershot et al., 1995). When energy was depleted, for example, influenza virus haemagglutinin protein and the vesicular stomatitis virus G protein were trapped as large protein aggregates containing abnormal, intermolecular disulfide bonds (Braakman et al., 1992a; de Silva et al., 1993). When energy was restored, monomeric influenza haemagglutinin was rescued and proceeded to fold normally (Braakman et al., 1992a). Transient aggregates were also detected during the folding of vesicular stomatitis virus G protein under energy-rich conditions (de Silva et al., 1993). In this thesis, neither protein aggregates, nor intermediates with partially formed disulfide bonds, were detected during normal LDL receptor folding or following recovery from DTT-treatment. Possibly this was due to the antibodies used not being able to quantitatively detect partially-folded or reduced LDL receptor. Thus, the poor recovery of immunoprecipitable LDL receptor obtained might be accounted for by the trapping of misfolded LDL receptor into aggregates during the initial phase of folding after DTT

was removed, and oxidising conditions were being re-established in the ER, particularly when metabolic energy was limiting. Aggregation might also have occurred when disulfide bonds were formed in the absence of Ca^{2+}, which also led to a poor recovery of immunoprecipitable LDL receptor (see below). The disulfide bonds of pre-existing, folded, precursor LDL receptor were not affected by depleting metabolic energy, in contrast to the altered structure reported for the influenza virus haemagglutinin protein and the vesicular stomatitis virus G protein when energy was depleted in the ER (Braakman et al., 1992a; de Silva et al., 1993). The transport of the LDL receptor from the ER was impaired, but this was not an suitable index of LDL receptor folding as energy-depletion prevents both the formation of coated transport vesicles and their mechanism of fusion with a subsequent acceptor compartment (Balch et al., 1986). Energy-depletion may have affected other mechanisms, apart from chaperone activity, which were necessary for LDL receptor folding. In this regard, energy was required for the assembly of MHC class 1 antigens in vitro (Levy et al., 1991). Interestingly, protein disulfide isomerase does not require ATP to promote disulfide bond formation in vitro (Wetterau et al., 1991). The re-establishment of an oxidative redox potential in the ER also did not appear to be critically energy-dependent, as disulfide bonds did form, albeit abnormally, when energy was depleted (Braakman et al., 1992a).

Ca^{2+} was shown to be essential for the formation of the correct disulfide bonds of the LDL receptor in the ER. This may be due to Ca^{2+} binding either to the LDL receptor itself, or to another protein required for receptor folding, such as one of the Ca^{2+}-binding chaperones, protein disulfide isomerase, calnexin, calreticulin, immunoglobulin heavy chain binding protein (BiP) and GRP94. In the LDL receptor, Ca^{2+} probably binds to the cysteine-rich repeats of the ligand-binding domain and possibly also to the cysteine-rich repeats of the EGF precursor-homology domain (Schneider, 1989; Stenflo et al., 1988). Ca^{2+} has been shown to bind to the first repeat of the ligand binding domain which contains the epitope for the Ca^{2+}-dependent antibody, IgG-C7 (van Driel et al., 1987b; Daly et al., 1995), and Ca^{2+} is required for the LDL receptor to bind lipoprotein ligands (Goldstein and Brown, 1977). Ca^{2+} manipulation enabled reversible binding of IgG-C7 to the mature LDL receptor, whereas antibody binding to the Ca^{2+}-depleted precursor LDL receptor was not restored by Ca^{2+} replacement. This indicates that the Ca^{2+}-requirements for the structure of the precursor LDL receptor were different to those of the mature LDL receptor. As shown by non-reduced electrophoresis, Ca^{2+}-

depletion disturbed the disulfide bond structure of the precursor but not of the mature LDL receptor. The precursor LDL receptor did remain partially folded and thus only some of the disulfide bonds were dependent on Ca^{2+}. The disulfide bonds of the first repeat of the ligand binding domain were not altered by Ca^{2+}-depletion when studied as an isolated peptide (Daly et al., 1995). However, the present data showed a clear Ca^{2+}-requirement for LDL receptor folding in vivo.

Precursor LDL receptor was not transported from the ER when Ca^{2+} was depleted. Ca^{2+} levels may affect several different processes involved in regulating the transport of protein from the ER. Initially it was reported that high levels of ER-Ca^{2+} were necessary to retain proteins in the ER (Sambrook, 1990). Ca^{2+} depletion had caused secretion of resident proteins from the ER (Booth and Koch, 1989) and a yeast mutant with low levels of ER-Ca^{2+} was unable to retain misfolded proteins within the ER (Rudolph et al., 1989). Subsequently it was recognised that the effects of Ca^{2+}-depletion were selective, with certain secretory proteins exiting the ER while others were retained. Ca^{2+}-depletion caused resident proteins to accumulate in the ER, partly due to their synthesis being induced (Dorner et al., 1990; Lodish and Kong, 1990; Kozutsumi et al., 1988). Thus, depletion of Ca^{2+} caused the T-cell receptor-alpha chain to dissociate from BiP and be secreted, while BiP remained in the ER (Suzuki et al., 1991). By contrast, the addition of Ca^{2+} stimulates the dissociation of proteins from other ER-chaperones, including GRP94, protein disulfide isomerase, ERp72, calreticulin and p50 (Nigam et al., 1994). These effects are consistent with a role for Ca^{2+} in aiding certain proteins to fold and to associate with chaperones, with incompletely-folded protein being retained and possibly degraded, in the ER (Wikstrom and Lodish, 1993; Lodish et al., 1992; Wileman et al., 1991). The fate of the LDL receptor has not been explored during prolonged Ca^{2+}-depletion.

Since both N- and O-linked sugar chains are added to the LDL receptor in the ER (Cummings et al., 1983), the influence of glycosylation on disulfide bond formation was determined in this protein. It has been suggested that the complex, multi-step processing of sugar chains may regulate aspects of protein folding within the ER, having effects which are transient and different to the function of mature sugar chains on folded protein at the cell surface (Helenius, 1994). There are also indications that the presence of abnormal sugars might be more disruptive to protein function than the

complete absence of the sugar chains (Helenius, 1994). Preventing N-linked glycosylation with tunicamycin did not impede LDL receptor transport from the ER, or presumably protein folding (Filipovic, 1989, Fourie, Coetzee and Van der Westhuyzen, unpublished results), whereas interruption of the trimming of N-linked chains with castanospermine, possibly did reduce receptor transport to the cell surface (Edwards et al., 1989). Similarly, deletion of the clustered O-linked sugar domain did not impair receptor function in fibroblasts (Davis et al., 1986a), though it does cause a mild form of familial hypercholesterolaemia through a subtle disturbance of receptor function (Koivisto et al., 1993). Thus, the finding that unfolded LDL receptors with mature oligosaccharide chains were able to reform disulfide bonds normally is important, as it shows that (i) these large, negatively-charged chains do not interfere with disulfide bond formation and (ii) precursor oligosaccharide chains are not necessary for LDL receptor folding, for example neither directly by affecting conformation nor by acting as a tag for association with chaperones such as calnexin (discussed above). These findings also suggest that altered disulfide bonds are not likely to be the cause of the reduced binding affinity reported for both mature LDL receptor lacking dispersed O-linked chains (Yoshimura et al., 1987) and mature LDL receptor without N-linked chains (Filipovic, 1989).

In summary, in the ER, the disulfide bonds of the LDL receptor were unstable and remained sensitive to the redox potential and the concentration of Ca^{2+}, but not to metabolic energy. Reduced disulfide bonds were able to reform reversibly in an energy-dependent manner following redox changes, but not following Ca^{2+} depletion. The glycosylation of the LDL receptor did not influence disulfide bond formation in this protein.

Chapter 5

The association between the chaperone, calnexin, and the LDL receptor

5.1. Introduction

Chaperone proteins are present at high concentrations in the ER and contribute to the folding of membrane and secreted proteins. Chaperones function either through catalytic effects, such as the isomerisation of disulfide bonds by PDI, or they bind partially-folded proteins and promote their efficient folding by stabilising folding intermediates and by preventing their aggregation (Gething and Sambrook, 1992; Hartl and Martin, 1995; Hartl, 1995; Hartl et al., 1994). Chaperone-binding also serves to retain malfolded proteins in the ER. In this section, the role of the chaperone, calnexin, is considered in relation to the folding of the LDL receptor.

Calnexin was originally identified as one of several phosphoproteins radiolabelled by [γ^{32}P]GTP in canine pancreatic rough microsomes (Wada et al., 1991). It was later recognised to be identical to an 88 kDa protein which associated with class 1 major histocompatibility (MHC) molecules during their assembly in the ER, and a 90 kDa protein that associated with incompletely assembled forms of the T-cell receptor, class 1 MHC molecules and the B-cell antigen receptor (Degen and Williams, 1991; Ahluwalia et al., 1992; Galvin et al., 1992; Hochstenbach et al., 1992). Calnexin is a type 1 transmembrane protein of 573 amino acids (Bergeron et al., 1994). Its luminal domain has sequence homology with calreticulin, the major calcium binding protein in the ER (Michalak et al., 1992), and does not contain motifs for N-linked glycosylation (in mammals). The cytoplasmic domain contains an ER-retention motif (RKPRRE) and a phosphorylated serine residue (function unknown).

Three lines of evidence indicate calnexin's role in protein folding and transport (Bergeron et al., 1994). First, pulse-chase experiments show that many newly-synthesised proteins associate transiently with calnexin and that the kinetics of association correlate with protein folding, and in many cases, the rate of transport from the ER (Ou et al., 1993; Kim and Arvan, 1995; David et al., 1993; Galvin et al., 1992; Hammond and Helenius, 1994; Hammond et al., 1994; Tatu et al., 1995). Transferrin folding-intermediates bind to calnexin during disulfide bonds formation, while the more mature ER-forms are not bound (Kim and Arvan, 1995). Second, incompletely assembled and unfolded proteins associate with calnexin for prolonged periods (Kim and Arvan, 1995; Degen et al., 1992; David et al., 1993; Loo and Clarke, 1994; Pind et al., 1994; Gelman et al., 1995; Le et al., 1994; Schreiber et al., 1994; Lenter and

Vestweber, 1994; Ou et al., 1993; Wada et al., 1994). This is illustrated by the assembly of class 1 MHC molecules, where calnexin shows prolonged binding to incompletely assembled subunits and the duration of binding correlates with the different rates of secretion of allotypes from the ER (Degen et al., 1992). Both the wild type and mutant forms of the cystic fibrosis conductance transmembrane regulator and α1-antitrypsin interact transiently with calnexin, but the common mutant form of cystic fibrosis conductance transmembrane regulator (ΔF508) is unable to dissociate from calnexin and is trapped in the ER apparently until it is degraded (Pind et al., 1994). Similarly, malfolding induced by the proline analogue, azetidine-2-carboxylic acid, prolongs association with calnexin (Ou et al., 1993). Third, the involvement of calnexin in the quality control mechanism which retains malfolded proteins in the ER, is demonstrated by the finding that truncated forms of calnexin that are unable to be retained in the ER, redistribute associated proteins to the Golgi and the cell surface (Rajagopalan et al., 1994). Conversely, in cells from Drosophila melanogaster, the rapid transport of class 1 molecules from the ER, is retarded by the co-expression of calnexin, again clearly indicating its critical role in modulating transport from the ER (Jackson et al., 1994).

Calnexin substrates includes soluble and membrane proteins, but are restricted, with few exeptions, to glycoproteins (Ou et al., 1993; Ware et al., 1995). For glycoproteins, the binding is specific for N-linked oligosaccharide chains that are partially trimmed to contain core monoglucosylated chains (incomplete folding alone is not sufficient for binding) (Hammond et al., 1994; Hebert et al., 1995; Helenius, 1994). Further processing of the N-linked chains prevents binding to calnexin. The conformation of the folding protein determines its suitability as a substrate for the interplay between glucosidase II, UDP-Glc:glycoprotein glucosyltransferase and calnexin, which in turn modulates further folding and transport from the ER (Sousa and Parodi, 1995; Labriola et al., 1995). The role of the N-linked chains of the target protein is to modulate the initial binding of calnexin to its substrate; once bound, the sugar chains are not required for continued association (Ware et al., 1995; Zhang et al., 1995). Apart from the effects of glycosylation, substrate binding requires calcium and ATP, though it is not clear whether the ATP requirement is due to direct or indirect effects, as calnexin does not contain an obvious nucleotide binding site (Wada et al., 1994).

The specific chaperone protein(s) necessary for LDL receptor folding has not yet been identified. A protein with the molecular mass of BiP (a major chaperone for secreted proteins) was co-immunoprecipitated with LDL receptors containing defects in their binding domain (Esser and Russell, 1988), but was not characterised further. As a membrane glycoprotein which contains N-linked oligosaccharides, the LDL receptor may be suitable substrate for binding to calnexin. The role of calnexin in the folding of the LDL receptor was assessed. In addition, the association between calnexin and mutant forms of the LDL receptor was studied. The mutant LDL receptors used were the 3 common mutant alleles identified as causing FH in the Afrikaner population in South Africa (see chapter 6) (Rubinsztein et al., 1994). These LDL receptors contain missense mutations in repeat 4 (FH Afrikaner-3) and repeat 5 (FH Afrikaner-1) of the ligand binding domain, and in the domain with homology to the EGF precursor (FH Afrikaner-2). The possibility that calnexin might be involved in the retarded transport of these mutant LDL receptors from the ER was investigated.

5.2. Results

5.2.1. Detection of calnexin in HepG2 and CHO cells by immunoblotting

Calnexin was identified in cultured cells by western blotting using a polyclonal antibody directed at the cytoplasmic domain (residues 555-573) (Figure 5.1). In both cell-lines tested, CHO cells (lane 1) and HepG2 cells (lanes 2 and 3), a single protein band was recognised with the expected molecular weight of calnexin (90kDa) which indicated that the polyclonal antibody did not cross-react with other proteins. A subtle difference in the electrophoretic mobility of calnexin was noted in the HepG2 cells compared to the CHO cells, but was not further characterised.

5.2.2. Immunoprecipitation of calnexin and associated proteins

To detect calnexin either with or without its associated proteins, cells were biosynthetically labelled with [^{35}S] methionine and were lysed either under non-denaturing or denaturing conditions. Under denaturing conditions (boiling in SDS), a protein band corresponding to the molecular weight of calnexin was immunoprecipitated (Figure 5.2, lane 1). The anti-calnexin antibody also detected albumin which was used as a conjugate during the production of the polyclonal

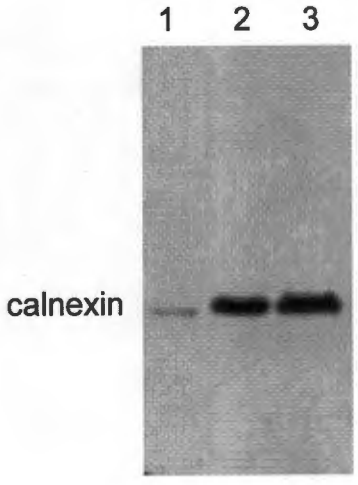


Figure 5.1 Detection of the calnexin by immunoblotting.

Proteins derived from CHO (lane 1) and HepG2 (lanes 2 and 3) cells were subject to SDS polyacrylamide gel electrophoresis under non-denaturing conditions and transferred to nitrocellulose membranes, as described in section 2.2.9. Cell protein loaded: lane 1, 43µg; lane 2, 114µg; lane 3, 228µg. Calnexin was identified with a rabbit polyclonal antibody directed against residues 555-573 of calnexin. The calnexin-antibody complex was detected with ^{125}I-labelled goat anti-mouse antibody ($1.5x10^6$cpm/ml) and autoradiography. The membrane was exposed to Kodak XAR5 film for 17 hours at -70°C. In comparison to molecular weight standards, calnexin migrated as a protein of approximately 90kDa, similar to phosphorylase B (97kDa).

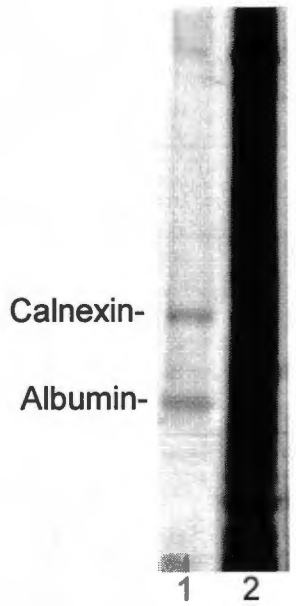


Figure 5.2 Immunoprecipitation of calnexin and associated proteins

After labelling HepG2 cells with TRAN[^{35}S]methionine for 20 minutes, calnexin was immunoprecipitated under denaturing (lane 1) or non-denaturing conditions (lane 2). Denaturing conditions involved lysing and boiling cells in the presence of SDS in order to elute proteins from calnexin. Non-denaturing conditions involved cell lysis in the presence of cholate, without boiling, as described under section 2.2.8. Immunoprecipitated proteins were subject to SDS polyacrylamide gel electrophoresis and detected by fluorography.

Albumin was detected since it was used as a conjugate during the production of the anti-calnexin antibody in rabbits.

antibody in rabbits. Under non-denaturing conditions (lysis in cholate), many newly-synthesised proteins were co-immunoprecipitated with calnexin (lane 2). These proteins were eluted from calnexin by boiling in SDS, but no LDL receptor was observed amongst them following immunoprecipitation with the antibody, IgG-C7.(not shown). It was subsequently determined that the IgG-C7 epitope, in the 1st cysteine-rich repeat of the binding domain of the LDL receptor, was destroyed during elution from calnexin (by being boiled in SDS). The antibody, IgG-HL1, which is able to recognise the denatured LDL receptor (Figure 5.3), was used in subsequent experiments. Its epitope lies in the linker region between the 4th and 5th binding repeats of the ligand binding domain. Unexpectedly, despite using IgG-HL1, less than 1% of the LDL receptor in the ER was detected among the proteins eluted from calnexin in either HepG2 or CHO cells (results not shown).

The duration of the labelling-period was varied in order to detect a transient calnexin-LDL receptor complex. However, using CHO or HepG2 cells, similar results were obtained when the labelling-period (using 200μCi/ml [^{35}S]methionine) was varied between 10 and 30 minutes (results not shown). Shorter pulse-periods did not adequately label the LDL receptor.

α1-Antitrypsin was selected as a positive control in order to confirm that the technique used to immunoprecipitate calnexin was adequate to retrieve the associated proteins. α1-Antitrypsin has been identified previously as a calnexin-substrate in HepG2 cells (Ou et al., 1993; Le et al., 1994). In these cells, after 10 minutes of labelling with [^{35}S]methionine, α1-antitrypsin was detected easily which indicated that its expression was far greater than the expression of the LDL receptor (Figure 5.4, lane 1). However, as shown in Figure 5.4 (lanes 1 and 3), less than 1% of the ER-pool of α1-antitrypsin co-immunoprecipitated with calnexin. Similar results were obtained in three separate experiments. This was in contrast to the published report that about 25% of α1-antitrypsin binds to calnexin (Ou et al., 1993). Importantly, although the fraction of α1-antitrypsin that was bound to calnexin was extremely low, the specificity of this interaction was demonstrated by: 1) it was only the ER-form of α1-antitrypsin that bound calnexin (as expected, the Golgi-processed form of α1-antitrypsin did not bind calnexin)(compare lanes 2 and 4); and 2) the fraction of newly-synthesised α1-

antitrypsin, that was bound to calnexin, as expected, declined during a 20 minute chase (lanes 3 and 4).

The poor recovery of calnexin-associated protein may have been due to the incomplete immunoprecipitation of calnexin. In order to improve the immunoprecipitation of calnexin, 2 polyclonal antibodies (raised against different peptides of calnexin) were used simultaneously in the same immunoprecipitation reaction. This did improve the immunoprecipitation of calnexin under denaturing (Figure 5.5, lane 1) and non-denaturing conditions (lane 2) (The comparisons to the individual use of the antibodies are not shown). However, the enhanced avidity of this interaction did not significantly improve the yield of α1-antitrypsin retrieved after elution from calnexin (Figure 5.5, lanes 3 and 4). In these experiments controls were performed to confirm that the antibody used (against α1-antitrypsin) was not affected by boiling nor by SDS (results not shown).

The extent to which calnexin was recovered in a single round of immunoprecipitation was determined. Sequential rounds of calnexin immunoprecipitation were performed on the same sample of cell lysate. The second round of immunoprecipitation recovered less than 10% of the calnexin recovered in the first round (results not shown). Thus, about 90% of the calnexin in the lysate was recovered during the first round of immunoprecipitation.

Cognisant of the low level of expression of both calnexin and the LDL receptor in transfected CHO cells (relative to the level of calnexin and α1-antitrypsin in HepG2 cells), the association between calnexin and mutant forms of the LDL receptor was studied (Figure 5.6 and Figure 5.7). The mutant LDL receptors used were the 3 common alleles which cause FH in the Afrikaner population in South Africa (see chapter 6). These LDL receptors contain missense mutations in repeat 4 (FH Afrikaner-3) and repeat 5 (FH Afrikaner-1) of the ligand binding domain, and in the domain with homology to the EGF precursor (FH Afrikaner-2). These mutations produce receptor molecules which are transported slowly from the ER. The aim was to determine whether calnexin was involved in this process. The results of two separate experiments are presented (Figure 5.6 and Figure 5.7). CHO cells expressing the wild type (lanes 1 and 2) or the mutant LDL receptors (lanes 3-8) were pulse-labelled with

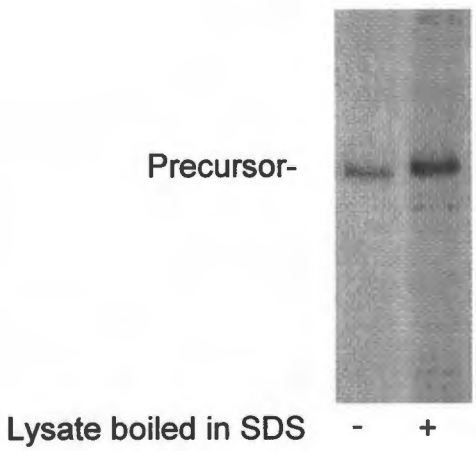


Figure 5.3 The sensitivity of the LDL receptor epitope for IgG-HL1 to SDS and boiling

After being biosynthetically labelled with TRAN[^{35}S]methionine for 30 minutes, CHO cells were lysed in the presence of 1% Triton X-100. In lane 1, the LDL receptor was immunoprecipitated from the post-nuclear supernatant (12 000g for 10 minutes). In lane 2, the post-nuclear supernatant was boiled for 3 minutes in the presence of 1% SDS and then diluted 20-fold by the addition of 1% Triton X-100 lysis buffer, prior to the immunoprecipitation of the LDL receptor with IgG-HL1. Immunoprecipitated protein was analysed by SDS polyacrylamide gel electrophoresis and detected by fluorography.

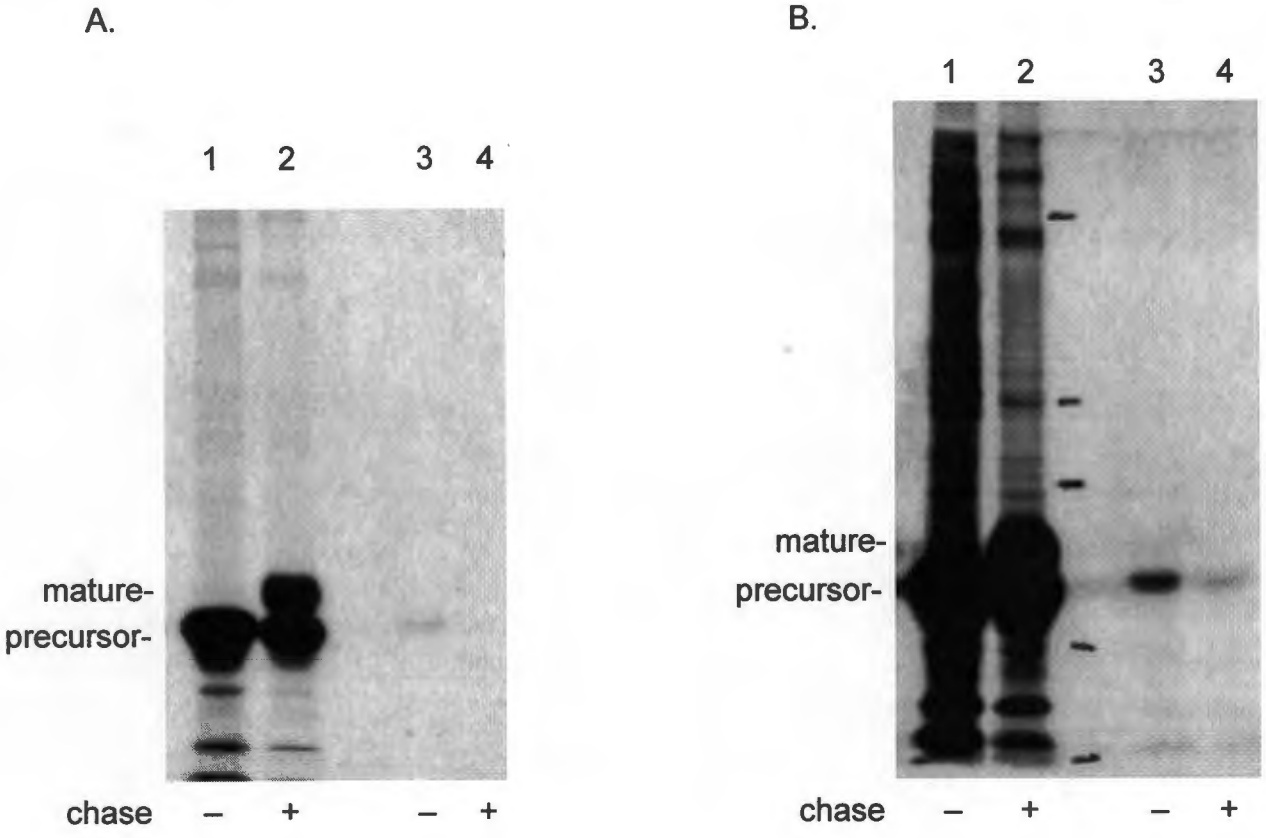


Figure 5.4 Immunoprecipitation of calnexin and α1-antitrypsin.

HepG2 cells were biosynthetically labelled with 200μCi/ml TRAN[^{35}S]methionine for 10 minutes and chased (lanes 2 and 4) for 20 minutes in the presence of unlabelled methionine. Cells were lysed and calnexin was immunoprecipitated under non-denaturing conditions, as described in section 2.2.8. α1-Antitrypsin then was immunoprecipitated from the proteins which did not bind to calnexin (lanes 1 and 2) or amongst the proteins eluted from immunoprecipitated calnexin (lanes 3 and 4). Immunoprecipitated protein was analysed by SDS polyacrylamide gel electrophoresis and detected by fluorography. The precursor and the mature forms of α1-antitrypsin are indicated. Panel A is a 6 hour exposure and panel B is a 72 hour exposure of the same gel.

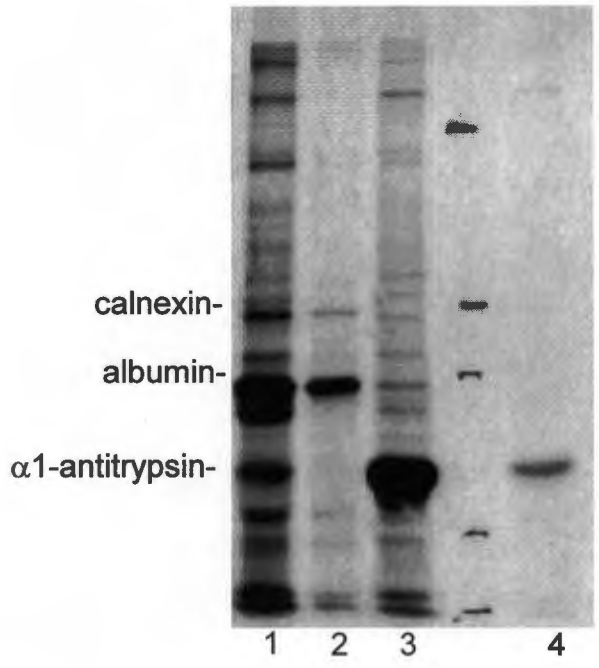


Figure 5.5 The immunoprecipitation of calnexin with a mixture of 2 polyclonal antibodies.

After being biosynthetically labelled with 200μCi/ml TRAN[^{35}S]methionine for 30 minutes, HepG2 cells were lysed and calnexin was immunoprecipitated under denaturing (lane 2) or non-denaturing conditions (lane 1, 3 and 4), as described in section 2.2.8. A mixture of the polyclonal antibodies directed against 2 different epitopes of calnexin were used to enhance the avidity during immunoprecipitation. Lanes 3 and 4: α1-Antitrypsin then was immunoprecipitated from the proteins which did not bind to calnexin (lanes 3) or amongst the proteins eluted from immunoprecipitated calnexin (lanes 4). Immunoprecipitated protein was analysed by SDS polyacrylamide gel electrophoresis and detected by fluorography.

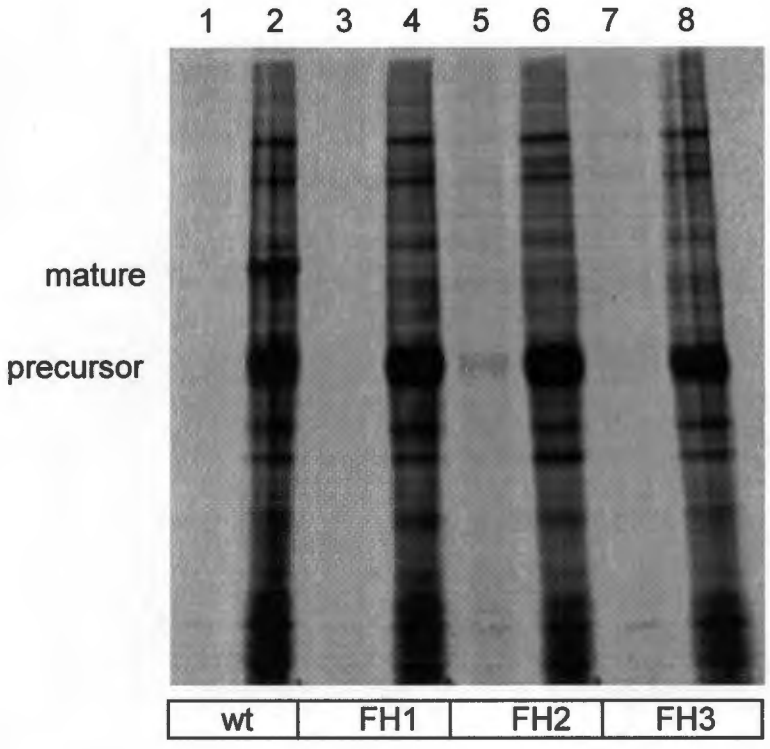


Figure 5.6 Immunoprecipitation of calnexin and the LDL receptor.

CHO cells expressing the wild type LDL receptor (wt), FH Afrikaner-1 (FH1), FH Afrikaner-2 (FH2) and FH Afrikaner-3 (FH3) mutant LDL receptors were pulsed for 30 minutes with 150μCi/ml [^{35}S]methionine. Cells were lysed and calnexin was immunoprecipitated under non-reducing conditions, as described in section 2.2.8. The LDL receptor then was immunoprecipitated, with IgG-HL1, from the proteins which did not bind to calnexin (lanes 2, 4, 6 and 8) or amongst the proteins eluted from immunoprecipitated calnexin (lanes 1, 3, 5 and 7). The LDL receptor was analysed by SDS polyacrylamide gel electrophoresis and fluorography. The positions of the mature and the precursor forms of the LDL receptor are indicated. The gel was overexposed (for 48 hours) to Kodak Biomax film in order to detect the small degree of association between the LDL receptor and calnexin.

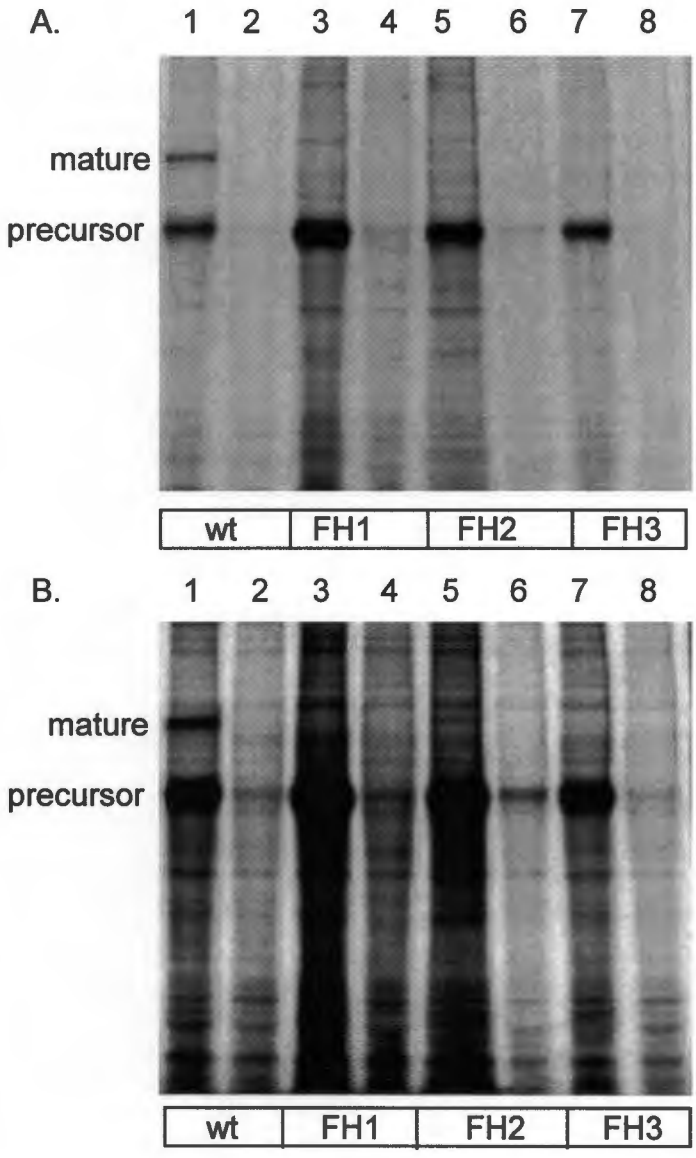


Figure 5.7 Immunoprecipitation of calnexin and the LDL receptor.

CHO cells expressing the wild type LDL receptor (wt), FH Afrikaner-1 (FH1), FH Afrikaner-2 (FH2) and FH Afrikaner-3 (FH3) mutant LDL receptors were pulsed for 30 minutes with 150µCi/ml [^{35}S]methionine. Cells were lysed and calnexin was immunoprecipitated under non-reducing conditions, as described in section 2.2.8. The LDL receptor then was immunoprecipitated, with IgG-HL1, from the proteins which did not bind to calnexin (lanes 1, 3, 5 and 7) or amongst the proteins eluted from immunoprecipitated calnexin (lanes 2, 4, 6 and 8). The LDL receptor was analysed by SDS polyacrylamide gel electrophoresis and fluorography. The positions of the mature and the precursor forms of the LDL receptor are indicated. Panel A: The gel was exposed for 36 hours to Kodak Biomax film.
Panel B: The gel was overexposed (for 8 days) in order to detect the small degree of association between the LDL receptor and calnexin (lanes 2, 4, 6 and 8).

[^{35}S]methionine and calnexin was immunoprecipitated. LDL receptors were recovered by immunoprecipitation from the co-immunoprecipitated proteins which were eluted from calnexin, and compared to the LDL receptors recovered from the proteins which did not bind calnexin. Similar results were obtained in both experiments. The results showed that the overall level of association of all these mutant LDL receptors with calnexin was very poor - the overwhelming proportion of the LDL receptors recovered were in the fractions that were not co-immunoprecipitated with calnexin (Figure 5.6, lanes 2, 4, 6 and 8; Figure 5.7, lanes 1, 3, 5 and 7). Thus, the mutant LDL receptors which were slowly transported from the ER, were not retained in the ER in a calnexin-LDL receptor complex. Total LDL receptor was immunoprecipitated from control dishes (without calnexin immunoprecipitation) and found to be quantitatively equivalent to the pool that was not bound to calnexin (results not shown). Thus, LDL receptor was not lost during the immunoprecipitation procedure. Carryover of signal between adjacent lanes during electrophoresis did not occur, as indicated by the absence of mature wild type LDL receptor in the calnexin-associated lanes (Figure 5.6, lane 1; Figure 5.7, lane 2).

Interestingly, the FH Afrikaner-2 mutant bound calnexin to a greater degree in comparison to the other mutants and the wild type LDL receptor (Figure 5.6, lane 5; Figure 5.7, lanes 6). This finding was consistent in the 2 separate experiments. The possible role of LDL receptor conformation in determining its association with calnexin also was assessed by reducing the disulfide bonds of the wild type LDL receptor by the addition of DTT to intact cells (see chapter 4 for a detailed discussion of this technique). This unfolding did not enhance LDL receptor binding to calnexin (results not shown).

A limitation of the pulse-labelling technique was that the [^{35}S]methionine label was used in trace quantities and possibly was not incorporated into every protein molecule synthesised during the labelling period. The high cost of the [^{35}S]methionine label also limited the size of the dishes (hence the number of cells) and concentration of label used. An alternative strategy was to use enhanced chemiluminescence immunoblotting (instead of immunoprecipitation) to detect the LDL receptor amongst the proteins eluted from immunoprecipitated calnexin. The advantages were that a blotting method would detect the entire pool of LDL receptors in the ER (not only the labelled fraction) and

could be performed on a larger number of cells. Several methods for the immunoblotting of the LDL receptor have been described which have the sensitivity to detect the pool of mature LDL receptor in the recycling endocytic pathway (ligand and immunoblotting) (Daniel et al., 1983; Beisiegel et al., 1982). However, these methods do not have the sensitivity to detect the small pool of LDL receptor present within the ER, let alone the fraction possibly bound to calnexin. Once the relative antibody concentrations were optimised (not shown), enhanced chemiluminescence immunoblotting blotting was able to detect the precursor LDL receptor in the ER of cells derived from a 60mm dish (Figure 5.8, compare lanes 1 and 2). The absence of signal in cells which were not transfected (lane 3) indicated that the bands detected (lanes 1 and 2) were derived from the human LDL receptor. Promising preliminary results show that the precursor forms of two slowly-processed (class 2), mutant LDL receptors (lanes 4 and 5) were detected more readily than the wild type LDL receptor (lanes 1 and 2), due to the enlarged pool of these mutant LDL receptors in the ER. The absence of mature LDL receptor in the mutant with a substitution (to alanine) of the 3rd cysteine in the 5th binding repeat (lane 4), contrasts with the FH Afrikaner-1 mutant, where significant mature LDL receptor was detected (lane 5). Interestingly, a protein band with a molecular weight intermediate between the precursor and the mature LDL receptor was detected in those lanes where mature LDL receptor was detected (lanes 1, 2 and 5). This form either may be a degradation fragment derived from the mature LDL receptor, or else may derive from the incomplete processing of the LDL receptor in the Golgi apparatus during transport to the cell surface.

This immunoblotting technique will allow for a definitive experiment be carried out aimed at determining the actual relative amounts of mutant LDL receptor associated with calnexin in the ER, independently of pulse-labelling techniques. This experiment had yet to be performed at the time of the writing of this thesis.

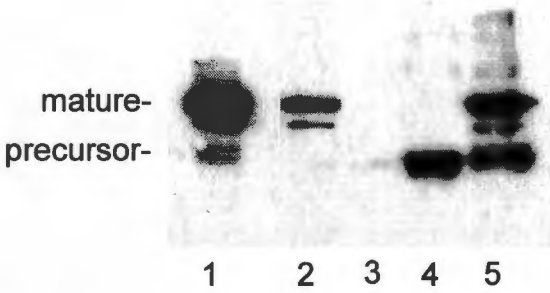


Figure 5.8 Detection of the LDL receptor by enhanced chemiluminescence western blotting.

Proteins derived from CHO-ldlA7 cells were subject to SDS polyacrylamide gel electrophoresis under non-denaturing conditions and transferred to nitrocellulose membranes, as described in section 2.2.7. Protein corresponding to confluent 60mm dish was loaded in each lane. The LDL receptor was identified with IgG-HL1 and detected with ECL immunoblotting using a horseradish peroxidase-coupled secondary antibody. An exposure of 5 minutes was used in lane 1; an 8 second exposure was used in lanes 2-5. The CHO cells were transfected with the wild type LDL receptor (lanes 1 and 2), the mutant LDL receptor with a substitution (to alanine) of the 3rd cysteine of the 5th binding repeat (lane 4), and the FH Afrikaner-1 LDL receptor (lane 5). The CHO cells in lane 3 did not express the human LDL receptor (were not transfected).

5.3. Discussion

In summary, only a small fraction of the newly-synthesised LDL receptors was found to be associated with calnexin in the ER. These results were inconclusive in indicating a role for calnexin in the folding of the LDL receptor. The interaction between calnexin and the LDL receptor was not increased when transport was retarded by the FH Afrikaner-1 mutation, or when disulfide bonds were reduced by DTT. Thus the retention of all class 2 LDL receptors did not occur in a complex with calnexin. The FH Afrikaner-2 mutant demonstrated an increased association with calnexin compared to other forms of the LDL receptor studied, but this association could not account directly for the large accumulation of LDL receptor in the ER.

Transferrin and α1-antitrypsin were used as positive controls in order to confirm that the immunoprecipitation of calnexin was adequate. The apparent poor binding of these proteins to calnexin is unexplained, and reason for caution in interpreting the results for the involvement of calnexin with the LDL receptor. In other studies, the maximal degree of association of newly-synthesised α1-antitrypsin (Ou et al., 1993; Le et al., 1994) and transferrin (Ou et al., 1993) with calnexin was about 30%, which is equivalent to the degree of association reported for several other proteins: major histocompatibility complex class 1 heavy chain (Jackson et al., 1994; Degen and Williams, 1991), thyroglobulin (Kim and Arvan, 1995), vesicular stomatitis virus G protein (Hammond and Helenius, 1994), C3 (Ou et al., 1993) and cystic fibrosis transmembrane conductance regulator (Pind et al., 1994). Glycoprotein80 (Wada et al., 1994) and ß1 integrin (Lenter and Vestweber, 1994) show an even greater (about 60%) association with calnexin. These findings might suggest that the methods used in this thesis were not adequate to immunoprecipitate calnexin and then detect the associated proteins. However, the anti-calnexin antibodies used in this thesis were obtained from the same source as in several of these studies (Wada et al., 1994; Le et al., 1994; Ou et al., 1993; Hammond et al., 1994), and were shown to effectively immunoblot and immunoprecipitate calnexin. Similarly, immunoprecipitation of the LDL receptor (using IgG-HL1), α1-antitrypsin and transferrin were not affected by the conditions (boiling in SDS) used to elute proteins from calnexin. During the immunoprecipitation of calnexin, neither Ca^{2+} nor ATP were depleted, both of which are reported to dissociate proteins from calnexin (Wada et al., 1994). Since Ca^{2+} is required for the folding of the LDL receptor (see chapter 4), experiments were

performed in the presence and absence of added Ca^{2+} - this did not affect calnexin-association (results not shown).

The finding that the significant accumulation of a LDL receptor-calnexin complex could not be demonstrated certainly did not exclude calnexin's possible role in the folding of the mutant forms of the LDL receptor. Mutant proteins may fold along independent paths compared to their wild type counterparts, and interact with a different set of chaperones, although this has not been reported. Certainly, the kinetics of folding of mutant proteins (even along the same folding paths) may be sufficiently different to reveal transient interactions not detected during rapid wild type folding (Le et al., 1994; Ou et al., 1993; Pind et al., 1994; Loo and Clarke, 1994). Similarly, the reduction of disulfide bonds by DTT-treatment enhances the association of thyroglobulin with calnexin (Kim and Arvan, 1995), though this was not the case for the LDL receptor. The manner in which mutations disrupt the folding of the LDL receptor is considered further in chapter 6. LDL receptor mutants are described to fold rapidly to an abnormal conformation in which they persist until their exit from the ER. Results presented here (chapter 5) indicate that despite their abnormal conformation, the retained LDL receptors do not persist in a complex with calnexin in the ER.

The actual role of calnexin in folding has not been determined. Calnexin does not possess catalytic activity (such as protein disulfide isomerase) and may promote folding by preventing the aggregation of partially-folded proteins. In chapter 4, the influence of glycosylation on the folding of the LDL receptor, is discussed. These findings show that the extent of processing of the glycosylation chains of the LDL receptor, does not affect its folding, as assessed by the formation of disulfide bonds. The implication is that calnexin is not critically involved in these processes, as the highly-processed sugar chains of the LDL receptor would prevent its interaction with calnexin (Helenius, 1994). Similarly, unpublished results (Fourie, Coetzee and Van der Westhuyzen) show that tunicamycin-treatment, which prevents N-linked glycosylation (and hence protein association with calnexin) (Ou et al., 1993), did not enhance the rate of transport of the slowly processed, FH-Afrikaner-2 LDL receptor (Fourie et al., 1988) from the ER. Thus neither direct evidence (association with calnexin) nor indirect evidence (manipulating glycosylation) implicates a role for calnexin in the retention of mutant forms of the LDL receptor in the ER.

The apparent limited extent of the association between the LDL receptor and calnexin, does not preclude that a brief interaction may be critical for the folding of the LDL receptor. Indeed, vesicular stomatitis virus G protein associated with BiP and calnexin briefly, and in sequence, during its folding (Hammond and Helenius, 1994). Such transient binding, especially at an early stage in the folding of the LDL receptor, would be difficult to detect without using systems with a much higher level of protein expression than used in this thesis. Viral systems have the advantage that viral proteins alone are synthesised while the synthesis of endogenous proteins are suppressed (Hammond and Helenius, 1994). Another approach that may be useful in the context of brief interactions would be the use of dysfunctional forms of calnexin (Rajagopalan et al., 1994). Such experiments require the calnexin-mutants to exert dominant negative effects, which may not occur considering that several chaperones aid the folding of a given protein (Langer et al., 1992; Kim and Arvan, 1995; Kuznetsov et al., 1994; Kahn-Perles et al., 1994; Hammond and Helenius, 1994). It is not known whether different chaperones are able to substitute for each other, which could complicate interpretation of results.

The results presented using the enhanced chemiluminescence western blotting technique are preliminary. They simply demonstrate that the technique is sensitive enough to detect the precursor LDL receptor in the ER, and could be an alternative to the sequential immunoprecipitation technique. Unfortunately, use of this technique does not bypass the problems, referred to above, concerning the overall poor association of protein with calnexin.

Chapter 6

Disulfide bond formation in mutant LDL receptors

6.1. Introduction

In this chapter the folding and the transport of mutant LDL receptors, from the ER, is considered. The transport of the LDL receptor from the ER is reviewed in section 1.5 and the folding of the LDL receptor is reviewed in section 1.8.2.

The manner in which mutations affect the folding of the LDL receptor molecule has not been determined. Presumably mutations alter the folding of the LDL receptor and cause its retention to be mediated through a chaperone quality-control mechanism in the ER. Structural studies would show definitively how mutations alter the structure of the LDL receptor. However, these studies have been prevented by the difficulty of crystallising membrane proteins such as the LDL receptor. Insights have been obtained by the functional consequences (effects on binding, recycling and stability) of the various mutations and have been used to assign functional roles to the various domains and subdomains of the LDL receptor (Hobbs et al., 1992; Hobbs et al., 1990). Within the binding domain, a myriad of mutations are described to reduce LDL receptor transport from the ER and binding activity on the cells surface (Hobbs et al., 1992). Other phenotypes are apparent which vary between the different mutant alleles. Differences are observed between the rates of LDL receptor transport from the ER, the apparent stability of the LDL receptor in the ER and on the cell surface, and the apparent mobility assessed by SDS polyacrylamide gel electrophoresis. The structural basis for these phenotypes has not been determined.

Many questions can be phrased concerning the folding of the different ligand binding repeats: are they equivalent?; do they fold in sequence?; are all of the repeats similar in structure?; are all the disulfide bonds necessary for folding?; do missense mutations affect the disulfide bonds?; are the phenotypes (transport rate, stability and mobility on gels) influenced by which disulfide bonds are disrupted by mutations?

In order to assess the requirements for disulfide bonds in the folding of the 5th binding repeat, cysteine residues of that repeat were individually substituted with alanine. Six mutant LDL receptors were constructed and the phenotypes of the expressed proteins were assessed in transfected CHO cells. The 5th ligand binding repeat was selected as it is unique (compared to the other binding repeats) in being required for both LDL and βVLDL binding to the LDL receptor (Esser et al., 1988; Russell et al., 1989). It is

also the repeat affected by the FH Afrikaner-1 mutation (Asp206Glu) which results in the intriguing phenomenon of the expression of 2 surface forms of the same mutant LDL receptor (Fourie et al., 1992; Fourie et al., 1988). One form binds lipoproteins with normal high affinity while the other does not. The possibility that these alternative stable structures involve the formation of different patterns of disulfide bonds was assessed by electrophoresis under non-reduced conditions. The disulfide bonds of the other 2 common mutant alleles causing FH in the Afrikaner population of South Africa (Rubinsztein et al., 1994) also were examined. These LDL receptors contain missense mutations in repeat 4 of the ligand binding domain (FH Afrikaner-3, Asp154Asn) (Graadt van Roggen et al., 1995), and in the domain with homology to the EGF precursor (FH Afrikaner-2, Val408Met) (Leitersdorf et al., 1989; Fourie et al., 1988). The use of electrophoresis under non-reduced conditions is discussed in chapter 4 where the formation of the disulfide bonds in the wild type LDL receptor is considered.

In the course of studying mutations affecting the oligomerisation of the LDL receptor, it was noted that alterations to the cytoplasmic tail retards the LDL receptor's rate of processing from the ER (Davies, Graadt van Roggen, van der Westhuyzen, unpublished results). The possibility that these mutations (Phe807Ala, Stop792 or Stop812) affect the formation of disulfide bonds in the LDL receptor was examined.

6.2. Results

6.2.1. Phenotypic characterisation of mutant LDL receptors with cysteine substitutions in the 5th binding repeat

Site-directed mutagenesis was performed to individually substitute the cysteine residues in the 5th binding repeat by alanine, as described under methods. 6 mutant LDL receptors were constructed, and designated mutant 1-6, dependent on the cysteine residue mutated: mutant 1, Cys176Ala; mutant 2, Cys183Ala; mutant 3, Cys188Ala; mutant 4, Cys195Ala; mutant 5, Cys201Ala; mutant 6, Cys210Ala. Briefly, after subcloning a DNA fragment corresponding to the 5th repeat of the LDL receptor into bacteriophage M13, oligonucleotide-directed mutagenesis was performed according to the method of Kunkel (Kunkel et al., 1987). The plasmid, pLDLR2, was re-assembled with the mutated DNA fragment and the mutations were confirmed by DNA sequencing. The mutants were stably-expressed in CHO cells and selected by resistance to the antibiotic, geneticin (G418). Colonies of thriving cells were isolated

with cloning rings and cloned by limiting dilution. Clones were expanded into cell lines and the expression of the LDL receptor was confirmed by immunoprecipitation. The results presented for each mutant was representative of experiments performed on at least 2 separate clones.

For each mutant with one altered disulfide bond, a precursor was observed that had an apparent molecular weight that was equivalent to the wild type LDL receptor (120 kDa) as determined by SDS polyacrylamide gel electrophoresis under reducing conditions (Figure 6.1A). Hence the core sugar chains of the precursor were not altered. When electophoresed under non-reducing conditions (Figure 6.1B), the altered disulfide bonded structure of the mutant LDL receptors (lanes 2-7) was evident from their enhanced mobility compared to the wild type LDL receptor (lane 1). Subtle differences were noted in the mobilities of the different mutant LDL receptors under non-reduced conditions, indicating slight differences in the structure dependent on which disulfide bonds were altered (Figure 6.1C). Resolution was not improved by altering the electrophoretic gel density (over a range from 4% to 11%) in uniform or gradient gels (results not show).

When pulse-labelled or then chased or 3 hours, no significant loss of precursor signal was observed (Figure 6.2A). Mature LDL receptors were not detected in any of the mutants. This indicated that alterations to any of the disulfide bonds of the 5th ligand binding repeat impaired LDL receptor transport from the ER. When the chase period was extended to 13 hours, each mutant was processed through to mature forms to some extent, though the relative yields of precursor and mature LDL receptor were quite different after this time for each of the different mutants (Figure 6.2B). Mutants 4, 5 and 6 had more mature than precursor, mutants 1 and 2 equivalent amounts, and mutant 3 had more precursor than mature LDL receptor. For each of the mutant LDL receptors, the ratio of precursor to mature forms remaining at the end of the 13 hour chase period was a function of the rates of LDL receptor processing through the ER and LDL receptor stability both in the ER and the post-ER compartments, including potentially the recycling endocytic pathway.

Given that little loss of precursor LDL receptor occurred during a 3 hours chase (Figure 6.2A), it seemed that there was a time lag of more than 5 hours (2 hours of pulse + 3

hours of chase) before transport to the cell surface occurred. Since it was after this period that the LDL receptor was unstable (Figure 6.2B), the most likely explanation is that the mutants were unstable once they had departed from the ER. Mutant 3 was more stable than the others, possibly due to an extremely slow rate of transport from the ER.

Another feature of mutant 3 was that the precursor form did not alter in molecular weight during the 3 hour (or 13 hour) chase period (compared to the pulsed forms) (Figure 6.2A and B), unlike all the other mutants which had a slightly retarded electrophoretic mobility, under reduced conditions, after the chase period. This slight change in molecular weight was probably due to enhanced processing of the sugar chains. It was not determined whether this resulted from the addition of more chains or the extension of existing chains of the precursor LDL receptor.

Despite mature LDL receptor being noted in all 6 mutants in pulse-chase experiments (after a 13 hour chase), in all cases the cell-surface binding of ^{125}I-labelled IgG-C7 antibody (performed at 4°C) was less than 15% of the binding measured for the wild type LDL receptors (results not shown). One caution in the interpretation of this result is that it does not take into account the differences in the level of LDL receptor expression in the different transfected cell lines. Biosynthetic experiments with [^{35}S]methionine indicated that the level of expression of all of the mutants except mutants 1 and 6 were greater than that of the wild type LDL receptor. Lipoprotein binding studies were not performed due to the low number of surface LDL receptors, and since the disruption of cysteine residues in the 5th binding repeat of the LDL receptor would be expected to severely impair lipoprotein binding (Esser and Russell, 1988; Russell et al., 1989).

In summary, disruption of any of the disulfide bonds of the 5th ligand binding repeat of the LDL receptor severely retarded its transport from the ER. The mutant LDL receptors were unstable after having exited from the ER and were reduced in number at the cell surface in comparison to the wild type LDL receptors. Differences were noted in the stabilities of the different mutants and in the ratio of precursor to mature LDL receptors remaining after a 13 hour chase period. The individual mutation of disulfide bonds in a localised region of the LDL receptor resulted in subtle differences

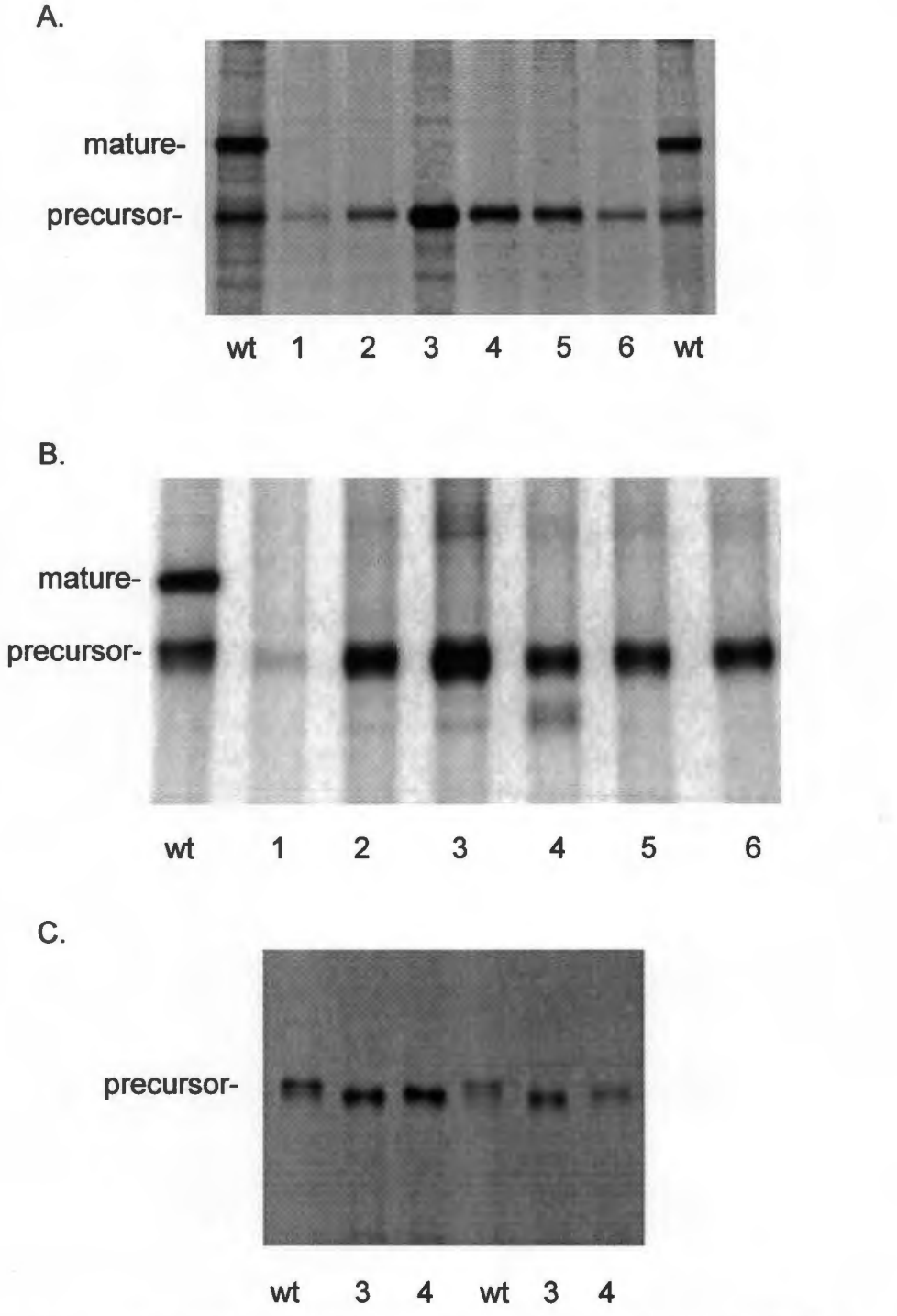
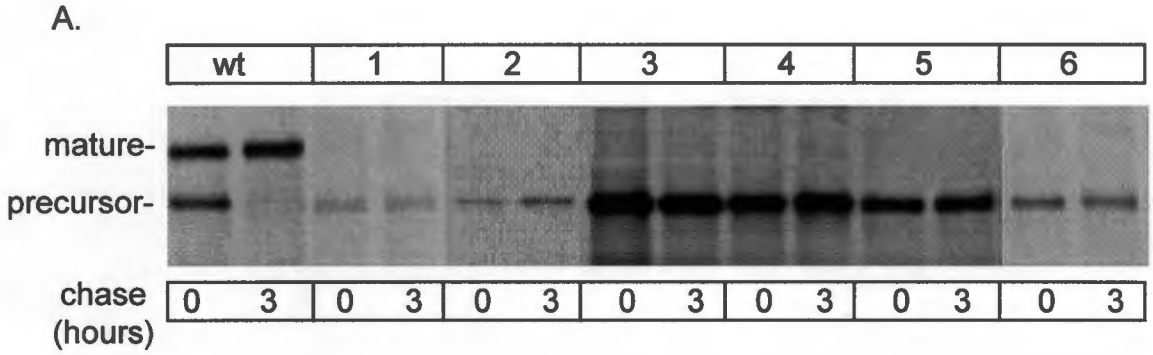


Figure 6.1 Expression of LDL receptors with cysteine substitutions in the 5th ligand binding repeat.

CHO cells expressing mutant LDL receptors with cysteine substitutions in the 5th ligand binding repeat were pulse-labelled for 1 hour with 100µCi/ml Tran[^{35}S]methionine. The LDL receptor was immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis under reducing (panel A) or non-reducing conditions (panel B and C). Immunoprecipitated protein was detected by fluorography. The labels indicate the wild type LDL receptor (wt) and the cysteine residue which was substituted in the mutant LDL receptors: 1, Cys176Ala; 2, Cys183Ala; 3, Cys188Ala; 4, Cys195Ala; 5, Cys201Ala; 6, Cys210Ala.



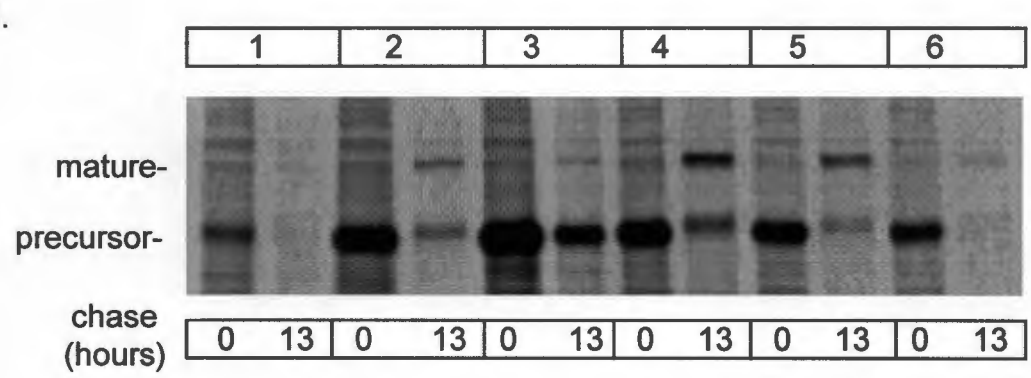


Figure 6.2 Processing of LDL receptors with cysteine substitutions in the 5th ligand binding repeat.

CHO cells expressing mutant LDL receptors with cysteine substitutions in the 5th ligand binding repeat were pulse-labelled for 2 hours with 100μCi/ml Tran[^{35}S]methionine and then chased for 3 hours (panel A) or 13 hours in the presence of unlabelled methionine. The LDL receptor was immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis under reducing conditions. Immunoprecipitated protein was detected by fluorography. The labels indicate the wild type LDL receptor (wt) and the cysteine residue which was substituted in the mutant LDL receptors: 1, Cys176Ala; 2, Cys183Ala; 3, Cys188Ala; 4, Cys195Ala; 5, Cys201Ala; 6, Cys210Ala.

in the phenotype of the expressed proteins. Paired phenotypes resulting from the substitution of cysteine residues involved in the same disulfide bond were not detected.

6.2.2. Assessment by non-reduced electrophoresis of the disulfide bonds of FH-Afrikaner LDL receptors

Three founder mutations cause FH in the South African Afrikaner population (Rubinsztein et al., 1994). These mutations are located in the 4th binding repeat (FH Afrikaner-3, Asp154Asn) (Graadt van Roggen et al., 1995), 5th binding repeat (FH Afrikaner-1, Asp206Glu) and in the domain with homology to the EGF precursor (FH Afrikaner-2, Val408Met) (Fourie et al., 1992; Leitersdorf et al., 1989; Fourie et al., 1988). Although none of these missense mutations involve cysteine residues, experiments were performed to determine whether they affected the formation of disulfide bonds. None of these mutations affected the mobility of the mutant proteins when electrophoresed under reduced conditions (Figure 6.3A, lanes 2-4)., indicating that their molecular weights were equivalent to the wild type LDL receptor (lane 1). Under non-reduced conditions, the mobility of the FH Afrikaner-1 mutant was increased compared to the wild type LDL receptor, indicating that the disulfide bond structure was altered by the amino acid substitution (Figure 6.3B, lanes 1 and 2). This enhanced mobility was similar to that of the 6 mutants with altered disulfide bonds in the 5th repeat (see Figure 6.1B). The FH Afrikaner-2 and FH Afrikaner-3 LDL receptors had mobilities equivalent to the wild type LDL receptor (Figure 6.3B, lanes 1, 3 and 4).

The mature forms of the FH Afrikaner-1 and FH Afrikaner-3 LDL receptors also were compared and found to have an electrophoretic mobility equivalent to the wild type mature LDL receptor under reduced (results not shown) and non-reduced conditions (Figure 6.4, compare lanes 2, 3 and 4). The mature LDL receptor with the FH Afrikaner-1 mutation was of particular interest as it reportedly exists in 2 conformations at the cell surface (Fourie et al., 1992). However only a single mature form was identified (compare lanes 2 and 3).

6.2.3. Assessment by non-reduced electrophoresis of the disulfide bonds of LDL receptors with cytoplasmic tail mutations

In the course of studying mutations affecting the internalisation of the LDL receptor, it was noted that alterations to the cytoplasmic tail retarded their rate of processing from

the ER. 3 different mutations were assessed: Phe807Ala, and mutants with stop codons at positions 792 (Stop792) or 812 (Stop812) (van Driel et al., 1987a). The electrophoretic mobility of the Phe807Ala was equivalent to that of the wild type LDL receptor under either reduced (Figure 6.3A, lanes 1 and 5) or non-reduced conditions (Figure 6.3B, lanes 5 and 6). The truncated mutants, Stop792 and Stop812, as expected, had an enhanced mobility compared to the wild type LDL receptor under reduced conditions (Figure 6.3A, lanes 1, 6 and 7). Under non-reduced conditions, it was difficult to assess the folding of the truncated mutants from their gel mobility (Figure 6.3B, lanes 7 and 8) as the truncation directly affected their mobility. The relative differences between their mobilities and the wild type LDL receptor obtained under reduced conditions (Figure 6.3A, compare lanes 1, 6 and 7) were maintained under non-reduced conditions (Figure 6.3B, lanes 6, 7 and 8). The diffuseness of the bands corresponding to the mutant LDL receptor forms (lanes 5, 7 and 8) were equivalent to the wild type LDL receptor (lane 6). Thus alterations to the cytoplasmic tail did not affect the disulfide bonded structure of the LDL receptor as assessed by electrophoresis under non-reduced conditions.

6.2.4. The formation of disulfide bonds by mutant LDL receptors

The ability of the mutant LDL receptors with cysteine substitutions in the 5th binding repeat to reform disulfide bonds was assessed after they were reduced with DTT (Figure 6.5). The methods used were the same as described for the wild type LDL receptor in chapter 4. The DTT-sensitivities of the cysteine-mutants were not different to that of the wild type LDL receptor (results not shown). After washing, the reduced LDL receptors were chased in the presence of fresh medium to enable disulfide bonds to reform. Within 10 minutes, all of the cysteine-mutants refolded to the conformation, on non-reduced gels, that they had prior to reduction (Figure 6.5). These slowly processed mutants thus folded rapidly to an altered conformation from which they were processed to the mature form at a retarded rate compared to the wild type LDL receptor.

The ability of DTT to reduce the FH Afrikaner-1 mutant LDL receptor was assessed (Figure 6.6). When DTT was added after normal protein synthesis, the DTT-sensitivity of the FH Afrikaner-1 LDL receptor (panel B) was equivalent to that of the wild type LDL receptor (panel A). DTT (0.5mM) induced a similar covalent change in the FH

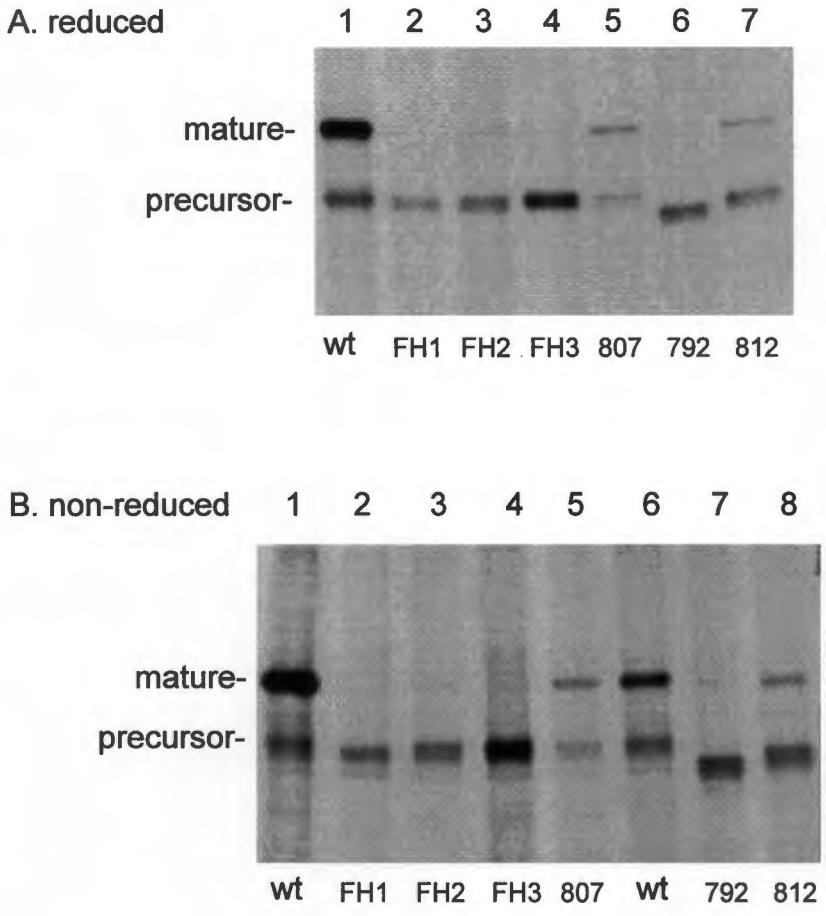


Figure 6.3 Expression of FH Afrikaner mutant LDL receptors and LDL receptors with mutations within the cytoplasmic tail.

CHO cells expressing the wild type LDL receptor (wt), FH Afrikaner mutant LDL receptors (FH Afrikaner-1 (FH1), FH Afrikaner-2 (FH2) and FH Afrikaner-3 (FH3)), and LDL receptors with mutations within the cytoplasmic tail (Tyr807Cys (807), Stop792 (792)and Stop812 (812)) were pulse-labelled for 1 hour with 50μCi/ml Tran[^{35}S]methionine and the LDL receptors were immunoprecipitated with IgG-HL1. Immunoprecipitates were analysed by SDS polyacrylamide gel electrophoresis under reducing (panel A) or non-reducing conditions (panel B) and were detected by fluorography.

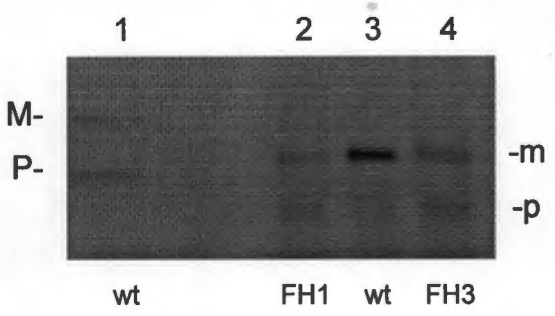


Figure 6.4 Electrophoretic mobility of the FH Afrikaner-1 and FH Afrikaner-3 LDL receptors.

CHO cells expressing the wild type (wt), FH Afrikaner-1 (FH1) and FH Afrikaner-3 LDL receptors (FH3) were pulse-labelled with Tran[^{35}S]methionine and immunoprecipitated with IgG-C7. Immunoprecipitates were analysed by electrophoresis under reducing (lane 1) or non-reducing conditions (lanes 2-4) and fluorography. The positions of the precursor (p, P) and the mature (m, M) LDL receptor are indicated under reducing (P, M) and non-reducing conditions (p, m).

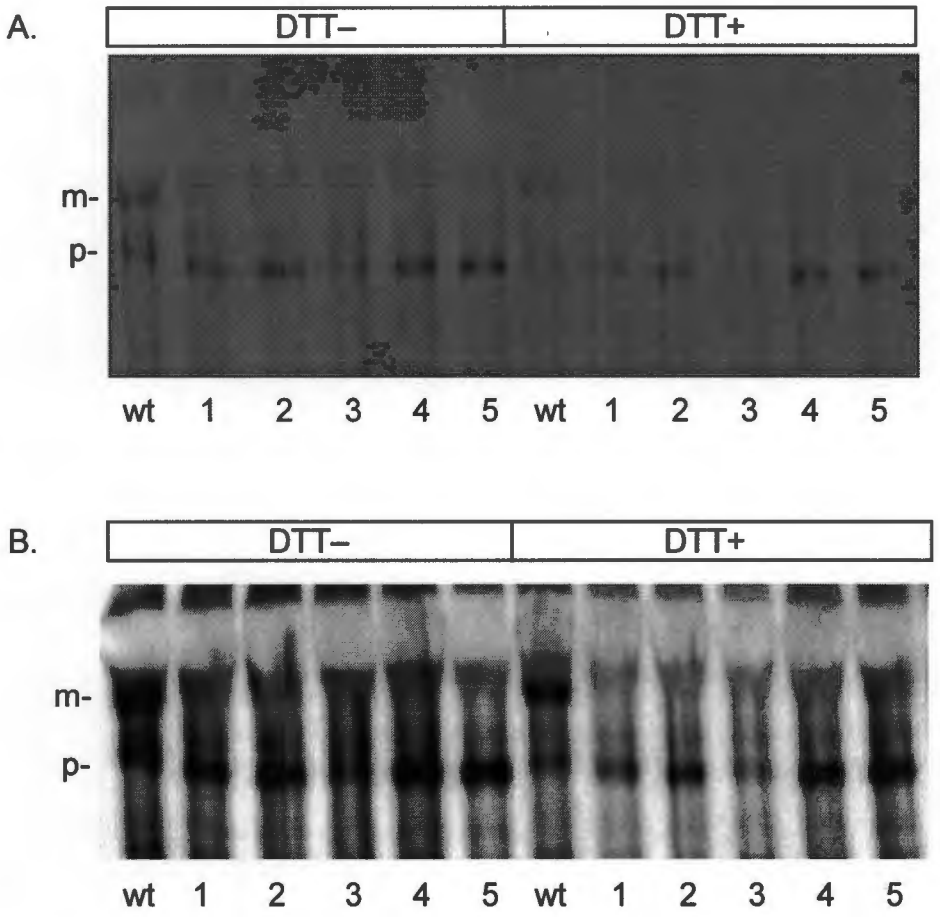


Figure 6.5 Post-translational folding of LDL receptors with cysteine substitutions in the 5th ligand binding repeat.

CHO cells expressing mutant LDL receptors with cysteine substitutions in the 5th ligand binding repeat were pulse-labelled for 1 hour with 100µCi/ml Tran[^{35}S]methionine. The DTT– set was alkylated and lysed at 4°C, while the DTT+ set was treated with 5mM DTT for 5 minutes at 37°C, then washed and chased in the presence of fresh medium for a further 10 minutes. The cells then were alkylated and lysed at 4°C. LDL receptor was immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis under non-reducing conditions and fluorography. The labels indicate the wild type LDL receptor (wt) and the cysteine residue which was substituted in the mutant LDL receptors: 1, Cys176Ala; 2, Cys183Ala; 3, Cys188Ala; 4, Cys195Ala; 5, Cys201Ala. Panel A is a 18 hour exposure and panel B is a 72 hour exposure of the same gel.

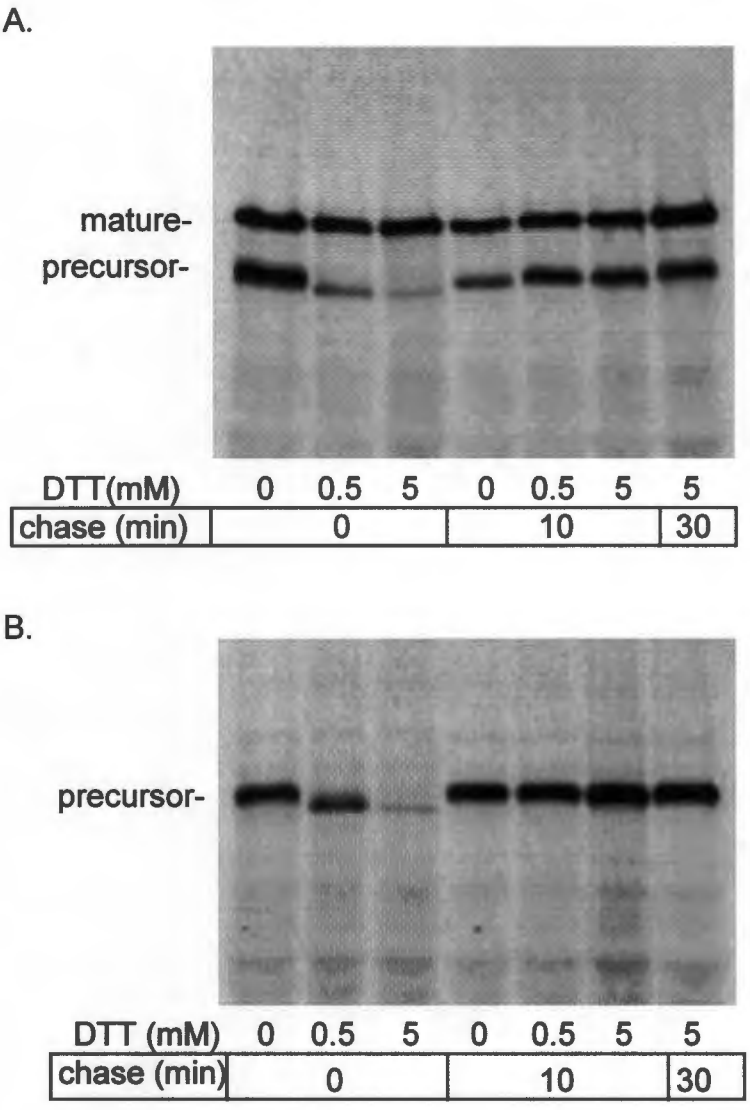


Figure 6.6 Reduction and post-translational folding of the wild type and the FH Afrikaner-1 LDL receptors.

CHO cells expressing either the wild type LDL receptor (panel A) or the FH Afrikaner-1 LDL receptor (panel B) were pulsed with 100μCi/ml Tran[^{35}S]methionine for 45 minutes and then treated with the indicated concentrations of DTT for 5 minutes at 37°C. Cells were washed and chased in the presence of fresh medium for 0 minutes, 10 minutes or 30 minutes, as indicated. The LDL receptor was immunoprecipitated with IgG-C7 and analysed by SDS polyacrylamide gel electrophoresis under reducing conditions. Immunoprecipitated protein was detected by fluorography. The positions of the mature and the precursor forms of the LDL receptor are indicated.

Afrikaner-1 LDL receptor as in the wild type LDL receptor (panel A and B, lane 2). The altered structure of the FH Afrikaner-1 LDL receptor thus did not render the protein more unstable to reduction by DTT as compared to the wild type LDL receptor. After DTT treatment, the FH Afrikaner-1 LDL receptor refolded with 100% efficiency and reformed the IgG-C7 epitope within 10 minutes (Figure 6.6B, compare lanes 4, 5 and 6). After refolding, the FH Afrikaner-1 LDL receptor remained retarded in transport from the ER (lane 7). Thus the post-translational folding of the mutant LDL receptor was indistinguishable from its folding prior to the addition of DTT.

When DTT was added during the pulse-period (Figure 6.7), translation of the FH Afrikaner-1 LDL receptor continued as was shown for the wild type LDL receptor in chapter 4 (Figure 4.6). However, under these circumstances, the amount of LDL receptor recovered was impaired (Figure 6.7A, lanes 1 and 2). After DTT was removed, disulfide bonded forms of the FH Afrikaner-1 LDL receptor were detected within 1 minute (Figure 6.7B, lane 6). This refolding occurred at a similar rate as was seen for the wild type LDL receptor in Figure 4.6. Whereas the mature form of the wild type LDL receptor was detected within 30 minutes after removal of DTT, the FH Afrikaner-1 LDL receptor was transported slowly (over hours) from the ER (Figure 6.7C). Thus after having been treated with DTT, the quality control mechanism of the ER was still able to distinguish between folded and malfolded proteins.

At no point during the chase period was a precursor form of the FH Afrikaner-1 LDL receptor detected which had the electrophoretic mobility of the wild type precursor under non-reduced conditions (Figure 6.7C and results not shown). Throughout its residence in the ER, the FH Afrikaner-1 precursor maintained the abnormal electrophoretic mobility seen 10 minutes after the removal of DTT (Figure 6.7C). Thus, after an initial rapid period of folding, the FH Afrikaner-1 LDL receptor remained in an abnormal conformation in the ER.

6.3. Discussion

In this section, cysteine residues were substituted within the 5th binding repeat and the protein phenotypes of the mutant LDL receptors were determined. Substitution of any of the 6 cysteine residues altered the disulfide bond structure of the LDL receptor (assessed by mobility on non-reduced gels) and delayed processing from the ER. All

of the mutants were unstable and seemed to be processed at different rates. Very low levels of mature LDL receptor were expressed at the cell surface. These results indicate that all 6 cysteine residues are required for the folding of the 5th binding repeat, and are involved in disulfide bonds. The instability of the mutant LDL receptors prevented a precise measurement of the processing rates and similarly, the low level of mature LDL receptors prevented an assessment of their degradation rates. LDL receptor turnover seemed to occur after exit from the ER as the precursors were stable in this compartment, over 3 hours. During this period, a slight increase in apparent molecular weight of the mutants was noted, which possibly was due to enhanced processing of the sugar chains beyond that of the wild type LDL receptor. Mutant 3 remained at the same molecular weight throughout its residence in the ER and was distinguished by being markedly more stable than the other mutants while in the ER.

The unique phenotype of mutant 3 was important in negating the hypothesis that either cysteine residue of a disulfide bond may be substituted with the same effect. According to this hypothesis, for the 6 cysteine residues substituted, 3 phenotypes might be obtained, each corresponding to the disulfide bond that was disrupted. In this manner, it was anticipated that the pattern of disulfide bonding might be determined. These arguments have been used to determine the disulfide bonding of the Mr 46 000 mannose 6-phosphate receptor; clearly such evidence is indirect, but has been termed 'internally consistent' and is compelling (Wendland et al., 1991). A weakness of experiments involving amino acid substitutions is that the final effect is a combination both of the effect of the loss of the original amino acid and the possible gain of function induced by the inserted amino acid. Alanine, with its short side chain, was chosen as the substitute for cysteine in order to minimise the potential for undesired interactions. Serine is of a similar size to cysteine but was not chosen as it contains a hydroxyl group and also may be O-glycosylated. The cysteine residue substituted in mutant 1, Cys176Ala, is involved in two naturally-occurring mutations, FH Shreveport (Cys176Phe) and FH El Salvador-1 (Cys176Tyr). These substitutions also slow processing from the ER and cause LDL receptor binding activity on the cell surface to be reduced to less than 2% of the wild type activity (Hobbs et al., 1992). Apart from the unique phenotype for mutant 3, the phenotypes of the other 5 mutants could not be distinguished clearly and thus cysteine residues could not be paired for the purposes of assigning disulfide bonds.

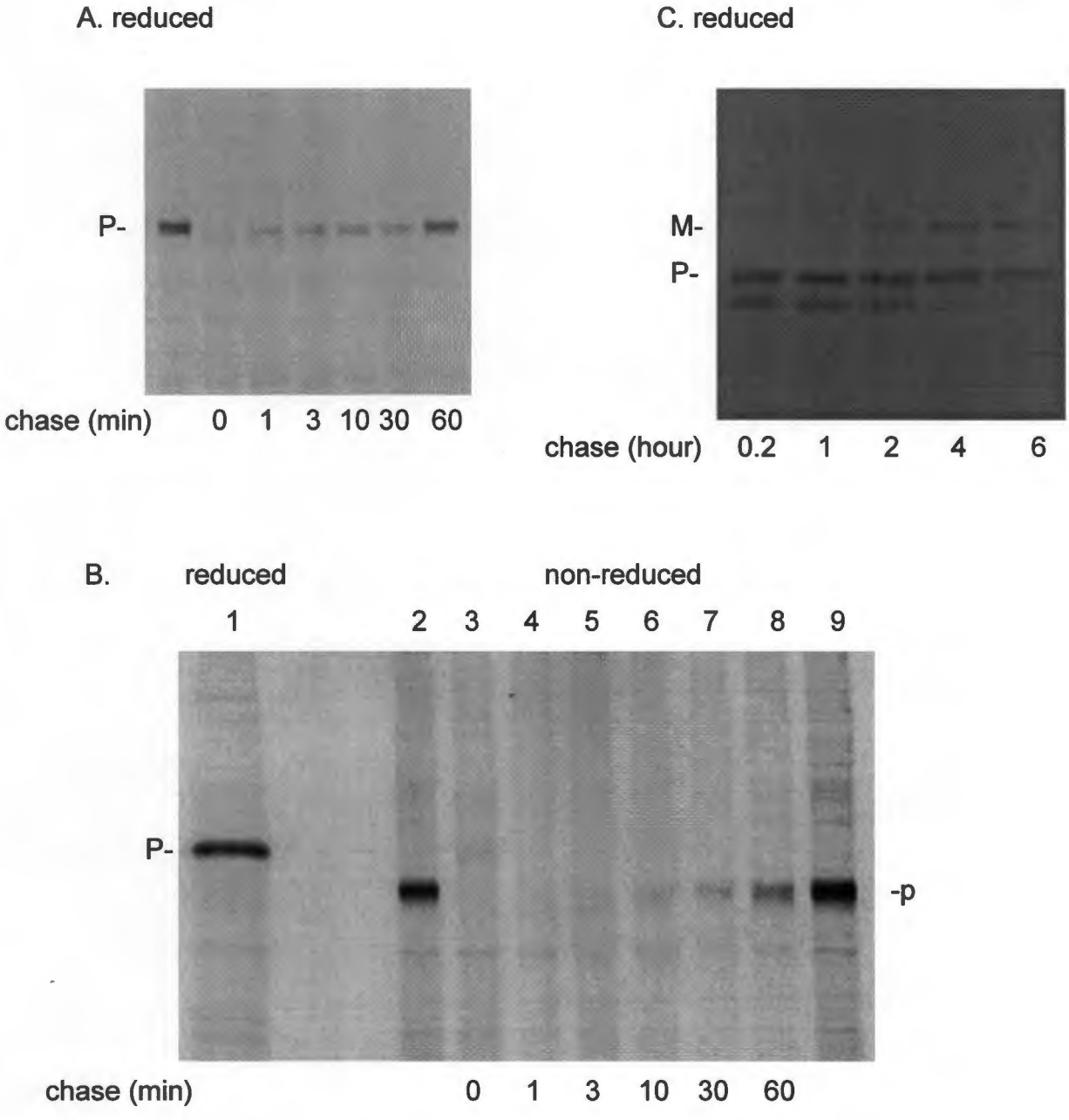


Figure 6.7 Post-translational folding of the FH Afrikaner-1 LDL receptor.

CHO cells expressing the FH Afrikaner-1 LDL receptor were pulsed for 1 hour with 100µCi/ml Tran[^{35}S]methionine. DTT (5mM) was included in the pulse-period of dishes 2-7 (panel A), dishes 3-8 (panel B) and all of the dishes (panel C). Dishes were washed and then chased in fresh medium containing cycloheximide for the times indicated. Dishes were cooled to 4°C, alkylated and lysed in detergent. LDL receptor was immunoprecipitated with IgG-HL1 and then divided for electrophoresis under reduced (panels A, all lanes, panel B, lane 1) and non-reduced conditions (panel B, lanes 2-9). The cells in panel C were labelled in a separate experiment to those in panel A and B, and were electrophoresed under reduced conditions. As described in Figure 5.1, the positions of the precursor (p, P) and the mature (m, M) LDL receptor are indicated under non-reduced (p, m) and reduced (P, M) conditions.

The absence of paired phenotypes indicated that at least some of the amino acid substitutions had effects other than simply the disruption of a single disulfide bond. One possibility is that the unpaired cysteine residue (the partner of the substituted cysteine) may have affected the formation of disulfide bonds by the other cysteine residues within the 5th repeat. This may have prevented more than one bond from forming or have altered the pattern of disulfide bond pairing.

The compact structure of the mutant LDL receptors indicated that the folding of the neighbouring cysteine-rich repeats were unlikely to have been affected by the misfolding of the 5th repeat. The overall compact structure of the mutant LDL receptors was indicated by their enhanced gel mobility compared to the wild type protein under non-reduced conditions. Conventionally, driven by hydrophobic effects, proteins fold into compact globular structures which exhibit enhanced gel-mobilities compared to their partially-unfolded counterparts. These findings suggest that the wild type disulfide bonds prevent the LDL receptor from folding into the most compact structure and maintain the 5th repeat in an extended conformation. Hence the substitution of the cysteine residues cause the LDL receptor to collapse into a more compact structure. It can be speculated that the extended conformation of the 5th binding repeat aids the spatial arrangement and the presentation of the charged residues needed for lipoprotein binding (see section 1.6).

Peptides corresponding to the first and the second ligand binding repeats fold to form the same disulfide bond pattern, with cysteine 1 and 3, 2 and 5, 4 and 6 pairing in each peptide (Bieri et al., 1995a; Bieri et al., 1995b; Daly et al., 1995). It is probable that the other repeats fold to form similar structures. For the first repeat, folding is Ca^{2+}-dependent (the first repeat binds Ca^{2+}) and forms a single stable structure with the cysteine residues positioned within the core and the charged residues generally exposed on the surface (Daly et al., 1995). The structure is tightly folded and is resistant to proteases (Bieri et al., 1995a). Folding was verified by the formation of the epitope for the conformation-specific antibody, IgG-C7 (van Driel et al., 1987b). Deletion of Asp26 and Gly27, corresponding to the FH Cape Town-1 mutant LDL receptor (Leitersdorf et al., 1988), causes the peptide corresponding to the 1st repeat to fold into an equilibrium mixture of four isomers, rather than the single structure of the

wild type repeat (personal communication, P. Kroon). Each isomer contains three disulfide bonds, but none were recognised by IgG-C7. The mutant LDL receptors studied in this chapter were not more heterogeneous than the wild type LDL receptor when assessed by non-reduced electrophoresis. This technique was able to detect, albeit to a limited degree, the subtle differences in the disulfide bonded structures of the LDL receptors with different cysteine substitutions (Figure 6.1C), which suggests that it would have been able to detect LDL receptor isomers. At the time of writing this thesis, the mobility of the FH Cape Town-1 LDL receptor had not been determined by electrophoresis under non-reduced conditions. It was anticipated that its non-reduced mobility would be affected directly by the loss of the two amino acids and could not be compared to the wild type LDL receptor. However, a comparison of the heterogeneity of the electrophoretic bands of the mutant and the wild type LDL receptor would be of interest.

Other mutagenesis studies have largely ignored cysteine residues and have assumed that they were critical for the structure of the binding domain. Esser has reported that the replacement of cysteine residues in repeat 1 (residues 6 and 18) did not affect the behaviour of the LDL receptor, though in that study COS cells were used, which process the wild type LDL receptor slowly compared to CHO cells and are not a sensitive assay for LDL receptor processing (Esser et al., 1988). The assumption that cysteine residues are critical for protein structure is not necessarily valid, even when those cysteines are involved in disulfide bonds. Recently, it was reported that substitution of the cysteine residues involved in a disulfide bond in the H2b subunit of the asialoglycoprotein receptor did not prevent proper folding of the protein, and surprisingly enhanced its transport form the ER (Yuk and Lodish, 1995).

Mutations within the cytoplasmic tail slow the processing of LDL receptors from the ER, which suggests that the cytoplasmic domain possibly could influence the folding of the intralumenal domain. These mutations did not retard the electrophoretic mobility of the LDL receptor unduly under non-reduced conditions, beyond the change accounted for by the truncation of the length of the protein. This indicates that the disulfide bond structure of the LDL receptor was not influenced by mutations within the cytoplasmic tail. Another possibility is that the cytoplasmic tail mutations impair secretion by preventing the assembly of oligomeric structures in the ER. Oligomers involving the

mature LDL receptor have been detected by cross-linking, and require amino acids 812 to 839 of the cytoplasmic tail to assemble (van Driel et al., 1987a). In that study, the involvement of the precursor LDL receptor in oligomers could not be assessed because the blotting system used to identify the components of the oligomers was not sufficiently sensitive to detect the precursor LDL receptor.

The enhanced electrophoretic mobility of the precursor LDL receptor with the FH Afrikaner-1 mutation (Asp206Glu) indicated that the disulfide bond structure of its 5th binding repeat was abnormal in a manner similar to the cysteine substitutions described above. This is the first demonstration that the disulfide bond structure of the LDL receptor is altered by a missense mutation which does not involve a cysteine residue. The finding that disulfide bonding was altered resolves the enigma posed by this extremely conservative substitution involving 2 amino acids (aspartate and glutamate) with similar properties. This change does not lead to the loss of a negative charge in the conserved serine-aspartate-glutamate triplet thought to be necessary for ligand binding to the LDL receptor (Fourie et al., 1992). The subtly difference in the length of the side-chain of the substituted amino acid is probably what interferes with the formation of disulfide bonds.

The electrophoretic mobility of the FH Afrikaner-1 was abnormal throughout its residence in the ER. It is possible that the precursor undergoes a conformational change immediately prior to exit from the ER, which may be critical for escape from the quality control apparatus, though this change was not detected. The mature form of the FH Afrikaner-1 LDL receptor did not have an altered electrophoretic mobility under non-reduced conditions, suggesting that the disulfide bonds were intact. The mature FH Afrikaner-1 population exhibits functional heterogeneity, with about 20% of the LDL receptors able to bind LDL with a normal affinity (Fourie et al., 1992); these LDL receptors must have intact disulfide bonds. The inactive population was not separated from this population by non-reduced electrophoresis, either because their disulfide bonds were not different, or because the gels were unable to resolve the differences. The latter possibility is likely in that the mature forms of the mutants with cysteine substitutions were not resolved from the mature form of the wild type LDL receptor. Neither the precursor nor the mature forms of the FH Afrikaner-1 LDL receptor were

more sensitive to DTT than the wild type LDL receptor, which indicated that the region of unfolding that was induced by the mutation, probably was localised (see below).

The FH Afrikaner-2 mutant is processed slowly from the ER and undergoes rapid degradation once it has reached the cell surface (Fourie et al., 1988). Both of these phenotypes suggest that the structure of the protein is altered, yet the electrophoretic mobility of the protein indicated that the disulfide bonds were intact. Despite the electrophoretic mobility assay not being sensitive to small alterations in LDL receptor structure (see above), the results indicate that the instability of the FH Afrikaner-2 mutant does not require extensive unfolding of the LDL receptor. Rather, a localised region of unfolding is sufficient to target the protein molecule for a rapid rate of degradation. The altered phenotype of a mutant LDL receptor with intact disulfide bonds complements the findings of Esser and Russell (Esser and Russell, 1988), who substituted free cysteine residues in the EGF precursor homology domain of truncated LDL receptors to show that unpaired cysteines were not required for slow processing from the ER.

Chapter 7

Concluding discussion

The aim of this thesis was to study the influence of post-translational events on the synthesis of the LDL receptor. Requirements for the folding of the LDL receptor were characterised and aspects of the inter-relationship between LDL receptor folding and its transport from the ER were determined. A number of novel findings have emerged from these studies. Co-translational folding was not essential for normal LDL receptor synthesis and disulfide bonds were able to form post-translationally. Folding required metabolic energy which implicated a requirement for associated chaperone protein(s). Ca^{2+} was required for LDL receptor folding in a manner that was different to its role in ligand binding to the mature LDL receptor. The glycosylation of the LDL receptor did not influence the formation of disulfide bonds. Conversely, reduction of the disulfide bonds of the LDL receptor did not irreversibly alter its glycosylation. LDL receptor transport from the ER was impaired when ATP or Ca^{2+} was depleted, when disulfide bonds were reduced and when cysteine residues were mutated in the 5th binding repeat of the ligand binding domain. All of these changes were shown to affect the folding of the LDL receptor. These findings clearly show the inter-relationship between LDL receptor folding and transport.

A missense mutation causing the conservative substitution of an aspartate residue by a glutamate residue (FH Afrikaner-1) was shown to impair the disulfide bonding of the mutant LDL receptor in the ER. By contrast, other missense mutations did not alter the formation of disulfide bonds. Similarly, mutations (truncation and missense) in the cytoplasmic tail of the LDL receptor retarded processing but did not alter the formation of disulfide bonds. The chaperone, calnexin, did not show quantitatively significant association with the LDL receptor. Together with other results, this suggested that calnexin is not critical for the folding of the wild type LDL receptor. Reduced or mutant forms of the LDL receptor were not retained in the ER in a complex with calnexin.

7.1. Relationship between folding, glycosylation and transport

The 'bulk' rate of transport from the ER is defined as the transport rate for proteins containing neither retention nor transport-enhancing signals (Wieland, 1992). This rate has been measured using tripeptides which are too small to contain signals that could affect transport. The transport rate of the wild type LDL receptor transport from the ER (half-time of processing of about 15 minutes) (Tolleshaug et al., 1982) is similar to the bulk rate, which suggests that the LDL receptor does not contain signals that influence

exit from the ER. Similarly, the findings that missense mutations at many different sites in the LDL receptor cause retarded processing (Hobbs et al., 1992; Hobbs et al., 1990) cannot be consistent with damage to a putative transport signal. By contrast, recent evidence indicates that the transport of vesicular stomatitis virus glycoprotein is enhanced by its selective concentration into transport vesicles (Balch et al., 1994).

In general, it is presumed that misfolding causes exposed hydrophobic groups to associate with resident ER proteins and retards exit from the ER (Gething and Sambrook, 1992). The areas of misfolding probably do not constitute a linear sequence of hydrophobic amino acids; rather, they are probably patches assembled from hydrophobic residues from adjacent parts of the partially folded protein. In the present study, the altered folding of LDL receptors with missense mutations was shown. When cysteine residues were mutated in the LDL receptor binding domain, misfolding was localised. Despite the disulfide bonds being affected directly, these mutants folded into compact structures which would not have been possible if unfolding was extensive. In other studies, the finding that missense mutations do not affect the binding functions of the neighbouring cysteine-rich repeats (Esser et al., 1988; Russell et al., 1989) also indicates that missense mutations disturb folding only within their respective repeat. These areas of misfolding are able to cause the retention of the LDL receptor despite most of the protein apparently having folded correctly.

Surprisingly, while the overall effect of the normal disulfide bonds is to cross-link the LDL receptor into a compact, convoluted structure, when the cysteine residues of the 5th repeat were individually mutated, or the LDL receptor was partially reduced with low doses of DTT, the LDL receptor folded into an abnormally compact structure. Normal LDL receptor folding thus does not entail the attainment of the most compact structure. These findings also suggest that the quality control mechanism in the ER recognises conformational features on the mutant (or the partially-reduced) proteins even when they are folded into compact structures. In general, protein malfolding causes hydrophobic groups to be exposed and recognised by chaperones (Gething and Sambrook, 1992; Hartl and Martin, 1995; Hartl et al., 1994). However, these features are not easily reconciled with a folded structure more compact than that achieved by the wild type protein.

Recently, it was reported that a mutant form of the H2b subunit of the asialoglycoprotein receptor proteins folds at an enhanced rate compared to the wild type protein (Yuk and Lodish, 1995). The mutant protein lacks a disulfide bond, yet it is transported more rapidly from the ER than the wild type protein. Possibly, in this example, the wild type disulfide bond complicates the folding itinerary by stabilising multiple structures, and wild type protein folding is delayed by disulfide bond shuffling. It must be remembered that the folding of proteins is not driven by criteria of rapid folding (see example above), nor by criteria of efficient folding. In fact, only a small fraction of newly-synthesised erythropoietin receptors exit the ER and are competent to bind erythropoietin at the cell surface (Hilton et al., 1995). The majority of the newly-made erythropoietin receptors are retained within the ER (and are degraded). This inefficient folding may contribute to regulating the number of active surface receptors and, in turn, the response of cells to erythropoietin. Intriguingly, a mutant form of the erythropoietin receptor was processed through the secretory pathway more efficiently than the wild type receptor, probably due to better folding (Hilton et al., 1995). Similarly, the folding of the cystic fibrosis transmembrane conductance regulator protein is inefficient, with only about 25% of the newly-synthesised molecules being detected as mature forms at the cell surface (Ward and Kopito, 1994). These findings are different to those for the wild type LDL receptor, where there is quantitative conversion from the precursor to the mature form (Tolleshaug et al., 1982). In this thesis, the folding of the wild type LDL receptor was efficient and was coupled to transport from the ER. In all instances, malfolding was associated with retarded transport from the ER. This malfolding was induced both by mutations (substitution of cysteine residues or FH Afrikaner mutant LDL receptors) and by manipulating the folding of the wild type LDL receptor (using DTT or by depleting Ca^{2+} or ATP).

The involvement of chaperone proteins in LDL receptor folding poses new challenges. It has not been determined whether chaperones are able to substitute for one another, or whether the same chaperones that are necessary for normal folding are the cause of the ER-retention of malfolded proteins. By analogy with the assembly of the transcription initiation complex, it is possible that chaperones may function as a mixture of general and specific protein factors. The definition of what qualifies as a chaperone is complicated by the inter-relationship between different processes such as glycosylation and folding (Helenius, 1994). Since certain glycosyltranferases

distinguish between the state of folding of their substrates, and modulate the activity of other proteins, such as calnexin (Helenius, 1994), these distinctions are not so clear. Similarly, a chaperone that retains a malfolded protein in the ER and thus targets it for degradation, could hardly be considered as aiding protein folding. Ultimately, the definition of a chaperone should be expanded to include those functions which are essential for 1.) the efficient translocation of proteins into the ER; 2.) protein folding and 3.) the assessment of protein competence for transport from the ER.

7.2. Therapeutic implications

Despite several human diseases having been identified to result in a phenotype of impaired transport from the ER to the cell surface (see Table 1.1), and the probability that most mutant forms of other surface molecules are similarly affected, the prospects for therapeutic intervention are probably limited. Intervention would be appropriate where the mutation does not severely impair the function of the protein apart from the delay in transport. The FH Afrikaner-1 mutant would be a suitable candidate as there is about 20% residual receptor activity (Fourie et al., 1992; Fourie et al., 1988). Considerable benefit might also be expected where retention is associated with rapid degradation within the ER. It is possible that the shorter retention time in the degradation compartment may lead to greater protein stability. Of course, once transported from the ER, the protein may exhibit instability in one of the post-ER compartments, such as at the cell surface or within the endocytic pathway. The common mutant form of the cystic fibrosis transmembrane conductance regulator (delta Tyr508) is transported from the ER at an enhanced rate when the temperature is reduced from 37°C to 24-28°C (Denning et al., 1992). Unfortunately, this mutant protein, which is functional, is unstable at the cell surface compared to the wild type protein (Ward and Kopito, 1994; Lukacs et al., 1993).

Another scenario where the manipulation of protein folding and transport from the ER may be advantageous is in the heterozygous form of familial hypercholesterolaemia. Under these circumstances, heterodimers of normal and mutant receptors may form oligomeric assemblies and may influence each other's transport. The mutant LDL receptors may cause the retention of the wild type LDL receptors and may reduce surface receptor activity beyond that caused simply by the loss of the function of the mutant allele (Schneider et al., 1983a). However, it is difficult to conceive of a

therapeutic strategy suitable for in vivo use that would be selective for the target protein (in this case, the LDL receptor). The unregulated transport from the ER of other proteins would be expected to cause undesired effects, which have not been determined. The assembly of oligomeric proteins, for example cell adhesion molecules and immune cell receptors, is dependent on the ordered processing from the ER, and would be expected to be disrupted.

7.3. Future study goals

A major goal of continued studies in this field is to define the structural basis for the phenotype of slowed processing of mutant forms of the LDL receptor. To achieve this aim, the structure of the wild type LDL receptor would need to be determined and compared with the structure of mutant forms of the protein. Presently, the crystallisation of membrane proteins such as the LDL receptor is not possible. In this regard, current attempts to express truncated soluble forms of the LDL receptor are of great interest (Dower and van der Westhuyzen, unpublished results). It is anticipated that it will be possible to crystallise the soluble LDL receptor and hence determine its three dimensional structure.

The folding of peptides corresponding to the different ligand binding repeats of the LDL receptor is also of interest (Daly et al., 1995; Bieri et al., 1995b; Bieri et al., 1995a). These studies address whether the structures of the different binding repeats are equivalent and the manner in which their folding and structures are disrupted by mutations. Despite the limitations inherent in studying the folding of peptides rather than the intact LDL receptor molecule, these studies they have the potential to address whether the repeats fold and behave as modular units. These studies could be extended to address the manner in which the arrangement of the binding repeats determines the different ligand binding specificities of the members of the LDL receptor gene family (Herz and Willnow, 1994). The structural features which underlie the ability of a single binding domain to bind disparate ligands could be addressed (Krieger and Herz, 1994). Ultimately, these studies could lead to the design of custom receptor molecules with binding specificities for defined ligands.

The identification of candidate chaperones which aid LDL receptor folding/retention is another key task. In this regard, the possible role for the chaperone, petidyl-prolyl cis-

trans isomerase, in LDL receptor folding is of interest. The function of this chaperone is possibly important for the compact folding of convoluted structures such as the binding domain of the LDL receptor (Schmid, 1993). Conveniently, the requirement for this chaperone in protein folding can be determined as it is inhibited by the immunosuppressive agent, cyclosporin (Lodish and Kong, 1991).

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