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A Point Mutation in the Juxtamembrane Stalk of Human Angiotensin I-converting Enzyme Invokes the Action of a Distinct Secretase*

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Angiotensin I-converting enzyme (ACE) is one of a number of integral membrane proteins that is proteolytically shed from the cell surface by a zinc metallosecretase. Mutagenesis of Asn^{631} to Gln in the juxtamembrane stalk region of ACE resulted in more efficient secretion of the mutant protein (ACE_{NQ}) as determined by pulse-chase analysis. In contrast to the wild-type ACE, the cleavage of ACE_{NQ} was not blocked by the metallosecretase inhibitor batimastat but by the serine protease inhibitor, 1,3-dichloroisocoumarin. Incubation of the cells at 15 °C revealed that ACE_{NQ} was cleaved in the endoplasmic reticulum, and mass spectrometric analysis of the secreted form of the protein indicated that it had been cleaved at the Asn^{635}–Ser^{636} bond, three residues N-terminal to the normal secretase cleavage site at Arg^{638}–Ser^{639}. These data clearly show that a point mutation in the juxtamembrane region of an integral membrane protein can invoke the action of a mechanistically and spatially distinct secretase. In light of this observation, previous data on the effect of mutations in the juxtamembrane stalk of shed proteins being accommodated by a single secretase having a relaxed specificity need to be re-evaluated.

Angiotensin I-converting enzyme (ACE,[1] EC 3.4.15.1) plays a key role in the control of blood pressure and fluid and electrolyte homeostasis (1). It exists primarily as a type I integral membrane glycoprotein, although a soluble form is present under normal conditions in blood plasma and other body fluids and is derived from the membrane-bound form through proteolytic cleavage in the juxtamembrane stalk region (2). The secretase responsible for the cleavage and secretion of ACE is a zinc metalloproteinase located at the cell surface (3–6). It is inhibited by hydroxamate-based compounds such as batimastat and displays a remarkably similar inhibition profile to that of the α-secretase that cleaves the amyloid precursor protein (7, 8). The precise site of cleavage in ACE has been determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry following enzymic fragmentation of the purified protein (9). This analysis revealed that the soluble somatic forms of human and porcine ACE were cleaved between Arg^{1203} and Ser^{1204}, some 27 residues on the extracellular side of the transmembrane domain.

ACE is just one of several proteins that are post-translationally shed from the membrane through the action of secretases (also called sheddases or convertases) (10–12). Other proteins proteolytically cleaved from the membrane include tumor necrosis factor-α, transforming growth factor-α, amyloid precursor protein, and L-selectin. The secretases that cleave and release such proteins from the membrane have several properties in common including up-regulation by phorbol esters and inhibition by hydroxamate-based zinc metalloproteinase inhibitors. Numerous studies have investigated the sequence/structural requirements for recognition of an integral membrane protein by its cognate secretase (for example see Refs. 13–18). This usually has been investigated by mutating residues in the extracellular juxtamembrane stalk region and monitoring their effect on secretion of the expressed protein. Such studies, in which numerous mutations failed to abrogate cleavage and secretion of the membrane protein, have concluded that for each substrate protein there is a single secretase with a relaxed sequence specificity and that the critical parameter for cleavage efficiency is the relative conformation (possibly α-helical) of the stalk region. However, an alternative explanation for these observations is that the mutations in the juxtamembrane stalk invoked the action of other distinct secretases. The involvement of multiple secretases was suggested by Zhong *et al.* (19) when numerous point mutations in the stalk region of the amyloid precursor protein resulted in that protein being cleaved at multiple sites, although there was no experimental evidence (inhibition profile, cellular location) to support this suggestion.

Here we report that a single point mutation in the juxtamembrane stalk region of ACE invokes the action of a mechanistically and spatially distinct secretase. Asn^{631} in the single domain form of ACE (20) (corresponding to Asn^{1196} in human somatic ACE), 7 residues N-terminal to the normal secretase cleavage site, was mutated to Gln (Fig. 1). The mutant protein (ACE_{NQ}) was transport-competent and enzymatically active,

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[1] The abbreviations used are: ACE, angiotensin I-converting enzyme; ACE_{NQ}, ACE mutant protein; ER, endoplasmic reticulum; DCI, 1,3-dichloroisocoumarin; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; wt, wild type; Endo, endo-β-*N*-acetylglucosaminidase.

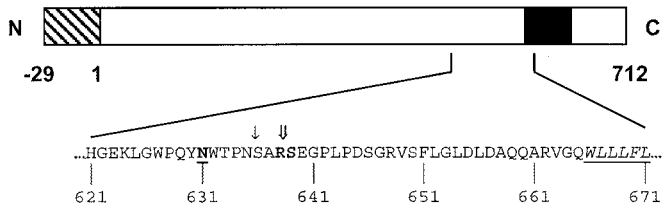

FIG. 1. **Schematic diagram of angiotensin I-converting enzyme.** The single domain form of human ACE has an N-terminal signal peptide (*diagonally hatched box*) and a C-terminal transmembrane domain (*black box*) followed by a short cytoplasmic tail (20). The amino acid sequence of the juxtamembrane stalk region is shown and numbered below the sequence according to this single domain form of ACE. The batimastat-sensitive secretase cleavage site (⇓) and the DCI-sensitive cleavage site (↓) are indicated, and part of the hydrophobic transmembrane domain is in *italics* and *underlined*. The Asn residue (631) that was mutated to Gln is in *bold* and *underlined*.

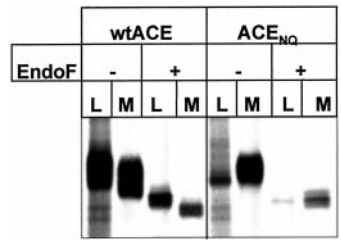

FIG. 2. **Secretion of ACE from IMR-32 cells.** IMR-32 cells were transfected with either wtACE or ACE$_{NQ}$ cDNA and biosynthetically labeled for 6 h with [^{35}S]Met. ACE was then immunoprecipitated from the cell lysate (*L*) or medium (*M*) with the anti-ACE antibody, and the immunoprecipitate was incubated in the absence (−) or presence (+) of Endo F for 2 h at 37 °C. The samples were analyzed on a 6% polyacrylamide SDS gel and visualized by fluorography.

indicating that the mutation has no general effects on the folding of ACE. Pulse-chase analysis revealed that ACE$_{NQ}$ was more readily cleaved and secreted into the medium than the wild-type protein. Mass spectrometric analysis of the secreted protein and temperature block and inhibitor studies indicated that ACE$_{NQ}$ was being cleaved between Asn^{635} and Ser^{636} in the endoplasmic reticulum (ER) by a serine protease and not between Arg^{638} and Ser^{639} at the cell surface by the batimastat-sensitive metallosecretase. In light of this observation, the conclusions of earlier studies investigating the sequence requirements of various secretases through analysis of the effect of mutations on the secretion of the substrate protein require re-evaluation and should not be interpreted solely in the context of a single protease activity having a relaxed specificity.

EXPERIMENTAL PROCEDURES

Construction of ACE$_{NQ}$—The expression vector pECE containing a C-terminal fragment of human ACE in which the N-terminal signal peptide was fused with the C-terminal domain (pECE hACE) (20) was used. In this construct the membrane-proximal stalk region, transmembrane, and cytosolic domains are identical to those in human somatic ACE. Asn^{631} (Fig. 1) in this construct, which corresponds to Asn^{1196} in human somatic ACE, was the template for oligonucleotide-directed mutagenesis with the Quick Change™ *in vitro* mutagenesis system (Stratagene) using the following oligonucleotides: ACE$_{NQ}$up (5′-TGG CCG CAG TAC CAA TGG ACG CCG AAC-3′) and ACE$_{NQ}$do (5′-GTT CGG CGT CCA TTG GTA CTG CGG CCA-3′). The mutation of T to C was confirmed by sequencing, and the plasmid obtained was denoted pECE hACE$_{NQ}$.

Cell Culture—The neuronal cell line IMR-32 (21) was cultured in Dulbecco's modified Eagle's medium/Ham's F-12 supplemented with 10% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 mg/ml), and 2 mM glutamate (all from Life Technologies, Inc.). Cells were maintained at 37 °C in 5% CO_2 in air, grown to 70% confluence in 25-cm^2 flasks, washed once with Opti-MEM, and then transiently transfected using LipofectAMINE and 8 μg of DNA diluted in 2 ml of Opti-MEM. After 5 h, 3 ml of growth medium was added to the flasks, and the cells were incubated overnight. The LipofectAMINE/medium mix was then replaced with fresh growth medium. After another 24 h the cells were washed with Opti-MEM and incubated with either batimastat (provided by Dr. G. Christie, SmithKline Beecham Pharmaceuticals, Harlow, U.K.) or 1,3-dichloroisocoumarin (DCI, Sigma) for 7 h. The medium was then harvested and centrifuged at 1000 × *g* to remove cell debris. Cells were then washed in phosphate-buffered saline (PBS), scraped into PBS, centrifuged at 1000 × *g* for 5 min, and resuspended in PBS. After sonication and centrifugation at 5000 × *g* for 20 min to remove nuclei, the cell membranes were pelleted by centrifugation at 100,000 × *g* for 90 min.

Metabolic Labeling and Phase Separation in Triton X-114—Transiently transfected IMR-32 cells were labeled 48–60 h post-transfection with 80 μCi of [^{35}S]Met in Met-free Dulbecco's modified Eagle's medium containing 2% fetal calf serum, 50 units/ml penicillin, and 50 mg/ml streptomycin (denoted Met-free medium). In pulse-chase experiments, labeling was performed for 1 h at 37 °C followed by a chase with nonlabeled Met for different periods of time. The labeled cells were rinsed twice with PBS prior to harvesting. Cells were subjected to phase separation in Triton X-114 essentially as described by Wilson *et al*. (22).

Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis—Cells were solubilized with 1 ml/dish cold lysis buffer (25 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.5% Triton X-100, and 0.5% sodium deoxycholate), and the cell extracts were centrifuged at 10,000 × *g* for 15 min to remove nuclei and debris. Thereafter, the supernatants were incubated with the rabbit anti-ACE polyclonal antibody (RP183) (23) and precipitated with protein A-Sepharose. After immunoprecipitation, the protein A-Sepharose beads were washed three times with washing buffer A (0.5% Triton X-100 and 0.05% sodium deoxycholate in PBS) and three times with washing buffer B (500 mM NaCl, 10 mM EDTA, and 0.5% Triton X-100 in 125 mM Tris/HCl, pH 8.0) prior to analysis of the samples by SDS-polyacrylamide gel electrophoresis and fluorography as described previously (24). The digestion of ^{35}S-labeled immunoprecipitates with endo-β-*N*-acetylglucosaminidase (Endo) H and Endo F/glycopeptidase F was performed as previously described (24).

Activity Assays—ACE activity in the medium and membrane samples was determined by incubation with 5 mM benzoyl-Gly-His-Leu in 0.1 M Tris/HCl, pH 8.3, 0.3 M NaCl, and 10 μM $ZnCl_2$ at 37 °C. The released benzoyl-Gly was separated from the substrate and quantitated by reverse phase HPLC as described previously (25).

Isolation and Mass Spectral Analysis of Secreted ACE—The medium was collected from IMR-32 cells transiently transfected with either pECE hACE or pECE hACE$_{NQ}$, centrifuged at 3000 × *g* for 5 min and then concentrated in a stirred ultrafiltration cell (Amicon) using a 10-kDa cut-off membrane. The samples were then dialyzed overnight against 10 mM Hepes/NaOH, 0.3 M KCl, and 0.1 mM $ZnCl_2$, pH 7.5. ACE was then isolated by affinity chromatography on lisinopril-Sepharose as described previously (26). The enzyme was eluted from the affinity column with 0.1 M sodium borate, pH 9.5, dialyzed, and concentrated using Centricon 10-kDa cut-off filters (Vivascience, Cambridge, U.K.). Purified secreted wtACE and ACE$_{NQ}$ proteins were reduced and protected with vinyl pyridine prior to digestion with endoproteinase Lys-C. The total digest was analyzed directly by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, or the digest was first fractionated by HPLC and the C-terminal peptide was identified by automated N-terminal peptide sequencing, before mass spectral analysis (27, 28).

RESULTS

ACE$_{NQ}$ Is Secreted from the IMR-32 Cells—IMR-32 cells, which previously have been shown to cleave and release ACE in a batimastat-sensitive manner (8), were transfected with cDNA encoding either wtACE or ACE$_{NQ}$. Both constructs were expressed in the cells as determined by metabolic labeling with [^{35}S]Met followed by immunoprecipitation from the cell lysate with the anti-ACE antibody (Fig. 2). wtACE was present in the medium, consistent with its release from the cell surface by the batimastat-sensitive secretase (Fig. 2). ACE$_{NQ}$ was also detected in the medium from the cells, and indeed the relative ratio of ACE protein in the medium compared with the cell lysate appeared greater for the mutant as compared with the wild-type protein. Deglycosylation with Endo F revealed that both wtACE and ACE$_{NQ}$ were *N*-glycosylated (Fig. 2). Deglycosylation revealed that there was a distinct difference in size between wtACE in the cell lysate as compared with that secreted into the medium, consistent with removal of the C-

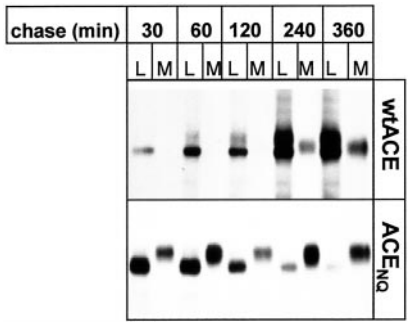

FIG. 3. **Transport kinetics of ACE.** After transfection with either wtACE or ACE_{NQ} cDNA, IMR-32 cells were biosynthetically labeled with [^{35}S]Met for 30 min and chased with cold medium as indicated. ACE was then immunoprecipitated from the cell lysate (*L*) or medium (*M*) with the anti-ACE antibody, and the immunoprecipitated samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

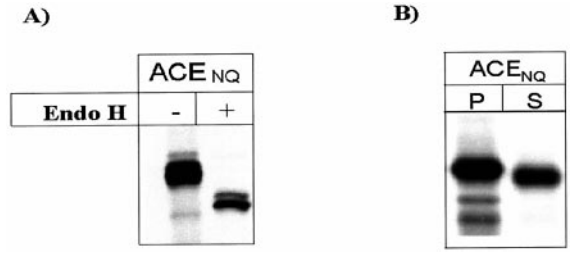

FIG. 4. **Analysis of the cellular compartment of ACE_{NQ} cleavage.** After transfection with ACE_{NQ} cDNA, IMR-32 cells were biosynthetically labeled with [^{35}S]Met at 15 °C for 4 h. *A*, cells were lysed in lysis buffer, and ACE was immunoprecipitated with the anti-ACE antibody prior to incubation in the absence (−) or presence (+) of Endo H for 2 h at 37 °C. *B*, the cells were lysed in the presence of Triton X-114 followed by centrifugation at 13,000 × *g*. Supernatant (*S*) and pellet (*P*) fractions were immunoprecipitated separately with the anti-ACE antibody and analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

TABLE I

Effect of inhibitors on the shedding of ACE and ACE_{NQ}

IMR-32 cells transfected with either wtACE or ACE_{NQ} cDNA were incubated for 7 h in the absence of inhibitor or in the presence of either 20 μM batimastat or 100 μM DCI. The medium was then harvested, and membranes were prepared as described under "Experimental Procedures," and the samples assayed for ACE activity with benzoyl-Gly-His-Leu as substrate. Results are the mean ± S.E. of three experiments.

Sample	Inhibitor	Specific activity	
		Membranes	Medium
		nmol/benzoyl-Gly/min/mg of protein	
wtACE	None	174.2 ± 34.1	184.8 ± 15.5
	Batimastat	248.2 ± 14.5	34.9 ± 4.4
	DCI	32.4 ± 2.2	273.6 ± 12.3
ACE_{NQ}	None	0.0	299.2 ± 8.5
	Batimastat	0.0	284.0 ± 16.9
	DCI	171.9 ± 6.8	73.9 ± 12.0

terminal transmembrane and cytosolic domains of the protein by the secretase (9). Interestingly, such a size difference was not apparent between the cell lysate and medium samples of ACE_{NQ} (Fig. 2).

ACE_{NQ} Is Secreted into the Medium Faster than Wild-type ACE—The rate at which ACE_{NQ} was secreted into the medium compared with that of wild-type ACE was assessed by pulse-chase labeling (Fig. 3). wtACE appeared in the medium after 4 h of chase, consistent with previous reports using other cell lines (27, 29). At 6 h of chase a considerable amount of wtACE was still present in the cell lysate. In contrast, ACE_{NQ} was detected in the medium from the cells after 30 min of chase. After 4 h of chase the majority of ACE_{NQ} had been secreted from the cell and was no longer present in the lysate sample. The apparent larger size of the medium form of the mutant protein compared with that in the lysate is because of complex glycosylation of the protein as it traffics along the secretory pathway. The ACE_{NQ} detected in the cell lysate is mannose-rich glycosylated as assessed by its complete sensitivity toward Endo H, whereas the ACE_{NQ} species secreted into the medium was Endo H-resistant, indicating that it has acquired complex-type glycans in the Golgi apparatus (data not shown).

ACE_{NQ} Is Not Cleaved by a Batimastat-sensitive Secretase—The ability of the secretase inhibitor batimastat to block the release of wtACE and ACE_{NQ} from the IMR-32 cells was assessed (Table I). As compared with untreated cells, batimastat significantly inhibited (81%) the release of wtACE into the medium with a concomitant increase in the amount of activity detected in the membrane fraction, consistent with previous results (8). Surprisingly, no ACE activity could be detected in the membranes from the cells expressing ACE_{NQ}, and batimastat failed to significantly inhibit the release of this mutant form of the protein into the medium. No ACE activity was detected in the total cellular membrane fraction from the untreated cells expressing ACE_{NQ} even after an eight-times longer incubation period with the substrate benzoyl-Gly-His-Leu (data not shown). Incubation of the cells expressing ACE_{NQ} with the serine protease inhibitor DCI reduced the secretion of the protein into the medium by 75% with a concomitant increase in activity detected in the membrane sample. Interestingly, DCI seemed to stimulate the release of wtACE into the cell medium with a concomitant decrease in the membrane fraction. This is consistent with a previous observation (27), although the mechanism responsible for this increased shedding is not known.

ACE_{NQ} Is Cleaved in the ER—The failure to detect ACE_{NQ} enzymic activity in the total cellular membrane fraction (Table I) suggested that this mutant form of ACE may be cleaved soon after synthesis in the ER. To investigate this, IMR-32 cells expressing ACE_{NQ} were incubated at 15 °C to prevent transport of the proteins beyond the ER (Fig. 4*A*). ACE_{NQ} was susceptible to deglycosylation with Endo H, indicating that it was in the high mannose form. Phase separation in Triton X-114 was used to determine whether ACE_{NQ} retained in the ER by the temperature block lacked the hydrophobic membrane-anchoring domain (Fig. 4*B*). Although a significant amount of newly synthesized ACE_{NQ} was detected in the detergent-rich pellet, possibly because the 15 °C treatment impaired the activity of the DCI-sensitive protease, a significant amount of a smaller form was also present in the detergent-poor supernatant, consistent with removal of the C-terminal membrane-anchoring and cytosolic domains by an activity within the ER.

ACE_{NQ} Is Cleaved at a Different Bond in the Juxtamembrane Stalk to wtACE—The cleaved forms of both wtACE and ACE_{NQ} were purified from the conditioned medium of transfected cells by chromatography on lisinopril-Sepharose (25). The purified proteins were digested with endoproteinase Lys-C and subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Mass spectrometric analysis of wtACE secreted from the IMR-32 cells revealed a peak at *m/z* 1690.8 (Table II), identical to the calculated *m/z* for the peptide Leu^{625}–Arg^{638}. This is consistent with the normal secretase cleavage site between Arg^{638} and Ser^{639} and is in agreement with the site of cleavage seen in ACE secreted from other cells and in serum (9). In contrast, analysis of the HPLC fractionated Lys-C digest of ACE_{NQ} revealed a major peak at *m/z* 1406.3 (Table II), which likely represented the peptide Leu^{625}–Asn^{635} (*m/z* 1406.3, calculated *m/z* 1390.5: the increase in mass

TABLE II
Observed [M + H$^+$] ions of Lys-C peptides in wtACE and ACE$_{NQ}$

Peptide no.	Amino acid residue	Mass M + H$^+$ (calculated)	ACE$_{NQ}$ mass M + H$^+$ (observed)	wtACE mass M + H$^+$ (observed)
9–10	129–148	2345.6	2344.8	
10	130–148	2217.4	2215.9	2215.6
12	186–199	1868.0	1866.9	1867.7
13	200–210	1264.5	1263.8	
15	319–328	1307.6	1306.7	
21	407–436	3075.5	3074.6	3074.7
22	437–460	2695.0	2695.1	2696.4
24	466–489	2946.4	2946.1	
25	490–502	1766.9	1766.2	1766.2
			1782.6[a]	
			1798.6[a]	1799.9[b]
29	568–578	1176.4	1175.6	
31	609–624	1951.1	1951.4	1950.9
32	625–632	1078.2	1076.95	
32	625–635	1390.5	1406.3[a,b]	
			1422.3[a,b]	
32	625–638	1690.8		1690.81

[a] Mass increase due to the oxidation of Trp.
[b] The C-terminal peptide was also identified by partial N-terminal sequencing after HPLC fractionation of a Lys-C digest.

is caused by the oxidation of Trp^{632}). The identity of this peptide was confirmed by partial N-terminal sequencing. Thus, the major site of cleavage was at the Asn^{635}–Ser^{636} bond, three residues on the N-terminal side of the normal Arg^{638} and Ser^{639} cleavage site (see Fig. 1). There was also evidence for secondary trimming of the C terminus of the soluble form of ACE$_{NQ}$ to Trp^{632}.

DISCUSSION

The Asn to Gln point mutation in the juxtamembrane stalk of ACE clearly results in more efficient cleavage and secretion of the protein. At first sight this could be attributed to the mutation making ACE$_{NQ}$ a better substrate for the batimastat-sensitive cell surface metallosecretase. However, closer inspection clearly shows that the shedding of ACE$_{NQ}$ is caused by the action of a spatially and mechanistically distinct secretase. The serine protease cleaving ACE$_{NQ}$ is acting in the ER and cleaves ACE at a different bond in the stalk region to the normal secretase. Interestingly a mutant form of ACE, ACE-JGL, in which the stalk region had been replaced with a Ser/Thr-rich sequence that was partially *O*-glycosylated, was observed to be secreted from Chinese hamster ovary cells more rapidly than wtACE, and its shedding was not blocked by the hydroxamic acid-based compound TAPI (27). Similar to ACE$_{NQ}$, the shedding of ACE-JGL was blocked by DCI; however, it was not determined whether cleavage of this mutant occurred intracellularly. The major serine proteases in the secretory pathway are the family of proprotein convertases, including furin, which are involved in the proteolytic processing of a variety of secreted and membrane-bound proteins (30). However, this family of proteases shows a specificity for cleaving after dibasic or monobasic sequences, and the cleavage site in ACE$_{NQ}$ does not fit this specificity (see Fig. 1). Also, the active forms of the proprotein convertases are located primarily in the trans-Golgi network rather than in the ER.

The effect of the Asn^{631} to Gln mutation invoking a distinct secretase is in contrast to a recent report in which a different point mutation in the juxtamembrane stalk of somatic ACE, associated with a variation in the levels of soluble ACE in plasma, resulted in enhanced cleavage by a metallosecretase (31). In that case mutation of Pro^{1199} in human somatic ACE (equivalent to Pro^{634} in the ACE construct used in the present study; see Fig. 1) to Leu resulted in more efficient cleavage/secretion of the mutant protein. Although the cleavage of the P1199L mutant was blocked by the hydroxamate-based metallosecretase inhibitor compound 3, the precise site of cleavage was not determined. Those authors, Eyries *et al.* (31), proposed that a local conformational modification caused by the Pro to Leu mutation leads to better accessibility of the stalk region to the normal ACE secretase, resulting in the enhancement of the cleavage/secretion process. Secondary structure predictions of wild-type ACE using the on-line JPred server indicated that the region from Leu^{620} to Asp^{657} is predicted to be a flexible loop, bounded by an α-helix on the N-terminal side and the transmembrane α-helix on the C-terminal side. The mutation of Asn^{631} to Gln had no affect on the secondary structure prediction, suggesting that the observed difference in cleavage of ACE$_{NQ}$ is not caused by a dramatic change in secondary structure. However, it is possible that the mutation promotes binding to the serine protease either directly (Gln is a larger side chain and may be able to make more favorable contacts with the enzyme) or indirectly (by allowing the loop to adopt a more favorable conformation for binding).

The residue mutated in the present study, Asn^{631}, lies in a potential *N*-glycosylation sequon (Asn-Trp-Thr-Pro), raising the possibility that this residue is glycosylated such that its mutation to Gln prevents glycosylation and exposes a site, that is normally obscured by the glycan chain, to cleavage by a serine protease within the ER. However, this explanation seems unlikely on four counts. 1) Trp in the middle position of the *N*-glycosylation sequon has been shown to severely reduce the efficiency of glycosylation of the preceding Asn, whereas a Pro following the sequon prevents *N*-glycosylation altogether (32, 33). 2) Consistent with this, there is no evidence for glycosylation of this Asn residue in the mature form of either human somatic or testicular ACE (Refs. 27 and 34 and Table II). 3) In rabbit ACE this potential glycosylation site is absent, yet the enzyme is cleaved by a hydroxamic acid-sensitive metallosecretase (6). 4) Attempts to mimic the effect of the Asn to Gln mutation in wild-type ACE by incubation of cells with tunicamycin, which blocks formation of the core dolichol-linked oligosaccharide thus preventing *N*-glycosylation from taking place, failed to lead to secretion of wtACE in a batimastat-insensitive manner (data not shown).

An interesting feature of ACE$_{NQ}$ is the dramatic increase in its transport kinetics and maturation rate along the secretory pathway as compared with the membrane-bound wild-type protein. In fact, attainment of complex glycans on ACE$_{NQ}$ occurs at a rate that is almost three orders of magnitude greater than that of wtACE. In general it is unlikely that nonanchoring of a protein leads *per se* to a higher efficiency in its intracellular transport relative to its anchored counterpart. This view is supported by several examples of proteins from which the transmembrane domains have been eliminated. Membrane-bound intestinal sucrase-isomaltase, for instance, exhibits similar transport kinetics as its soluble anchorless isoform (35). Likewise, deletion of the transmembrane domain in the neurotrophin receptor has no implications on the intracellular transport of the mutant protein (36). Furthermore, there is no general rule that anchorless proteins are transported more rapidly along the secretory pathway than membrane-bound ones. Indeed, with certain proteins the reverse seems to be the case. For example, deletion of the membrane-anchoring domain from the murine prion protein results in the secreted form of the protein reaching the cell surface at a 4-fold slower rate than the wild-type protein (37), and the soluble form of the trypanosome variant surface glycoprotein is also trafficked at a slower rate than the membrane-bound form (38).

The main rate-limiting step in the transport of membrane and secretory proteins from the ER to the Golgi apparatus is their acquisition to a correct folding and transport-competent

quaternary structure in the ER (for a review see Ref. 39). In this respect, oligomerization in the ER constitutes for many proteins a crucial criterion before they exit this organelle. This event has been shown to involve the transmembrane domains (40–45) (for a review see Ref. 46). Wild-type membrane-bound ACE does not dimerize in the ER (47), thus excluding a possible influence of dimerization and the transmembrane domain on its transport kinetics as compared with soluble ACE. One may speculate that the delayed intracellular transport of membrane-bound ACE *versus* ACE_{NQ} is caused by the existence of a structural motif in the cytosolic tail that promotes an interaction of ACE with soluble and/or membrane-bound factors in the cytosol and thus regulates its transport kinetics. As such, cleavage of ACE_{NQ} in the ER eliminates this motif and results in a more efficiently transported protein.

In conclusion, in previous studies the results of mutations at or near the secretase cleavage site in a membrane protein have usually been interpreted in the context of a single protease activity having a relaxed sequence specificity. However, the results of the present study reveal another explanation: such mutations may invoke the action of other distinct proteases. In the light of this and in the absence of such additional data, the conclusions of earlier studies investigating the sequence requirements of various secretases through analysis of the effect of mutations on the secretion of the substrate protein must be interpreted with caution and require re-evaluation.

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REFERENCES

1. Ehlers, M. R. W., and Riordan, J. F. (1989) *Biochemistry* **28,** 5311–5318
2. Hooper, N. M. (1991) *Int. J. Biochem.* **23,** 641–647
3. Oppong, S. Y., and Hooper, N. M. (1993) *Biochem. J.* **292,** 597–603
4. Ramchandran, R., Sen, G. C., Misono, K., and Sen, I. (1994) *J. Biol. Chem.* **269,** 2125–2130
5. Beldent, V., Michaud, A., Bonnefoy, C., Chauvet, M.-T., and Corvol, P. (1995) *J. Biol. Chem.* **270,** 28962–28969
6. Ramchandran, R., and Sen, I. (1995) *Biochemistry* **34,** 12645–12652
7. Parvathy, S., Oppong, S. Y., Karran, E. H., Buckle, D. R., Turner, A. J., and Hooper, N. M. (1997) *Biochem. J.* **327,** 37–43
8. Parvathy, S., Hussain, I., Karran, E. H., Turner, A. J., and Hooper, N. M. (1998) *Biochemistry* **37,** 1680–1685
9. Woodman, Z. L., Oppong, S. Y., Cook, S., Hooper, N. M., Schwager, S. L. U., Brandt, W. F., Ehlers, M. R. W., and Sturrock, E. D. (2000) *Biochem. J.* **347,** 711–718
10. Hooper, N. M., Karran, E. H., and Turner, A. J. (1997) *Biochem. J.* **321,** 265–279
11. Schlondorff, J., and Blobel, C. P. (1999) *J. Cell Sci.* **112,** 3603–3617
12. Primakoff, P., and Myles, D. G. (2000) *Trends Genet.* **16,** 83–87
13. Maruyama, K., Kametani, F., Usami, M., Yamao-Harigaya, W., and Tanaka, K. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1670–1676
14. Sisodia, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6075–6079
15. Deng, P., Rettenmier, C. W., and Pattengale, P. K. (1996) *J. Biol. Chem.* **271,** 16338–16343
16. Ehlers, M. R. W., Schwager, S. L. U., Scholle, R. R., Manji, G. A., Brandt, W. F., and Riordan, J. F. (1996) *Biochemistry* **35,** 9549–9559
17. Deng, P., Wang, Y.-L., Haga, Y., and Pattengale, P. K. (1998) *Biochemistry* **37,** 17898–17904
18. Althoff, K., Reddy, P., Voltz, N., Rose-John, S., and Mullberg, J. (2000) *Eur. J. Biochem.* **267,** 2624–2631
19. Zhong, Z., Higaki, J., Murakami, K., Wang, Y., Catalano, R., Quon, D., and Cordell, B. (1994) *J. Biol. Chem.* **269,** 627–632
20. Wei, L., Alhenc-Gelas, F., Corvol, P., and Clauser, E. (1991) *J. Biol. Chem.* **266,** 9002–9008
21. Christie, G., Markwell, R. E., Gray, C. W., Smith, L., Godfrey, F., Mansfield, F., Wadsworth, H., King, R., McLaughlin, M., Cooper, D. G., Ward, R. V., Howlett, D. R., Hartmann, T., Lichtenthaler, S. F., Beyreuther, K., Underwood, J., Gribble, S. K., Cappai, R., Masters, C. L., Tamaoka, A., Gardner, R. L., Rivett, A. J., Karran, E. H., and Allsop, D. (1999) *J. Neurochem.* **73,** 195–204
22. Wilson, J. M., Whitney, J. A., and Neutra, M. R. (1987) *J. Cell Biol.* **105,** 691–703
23. Williams, T. A., Barnes, K., Kenny, A. J., Turner, A. J., and Hooper, N. M. (1992) *Biochem. J.* **288,** 875–881
24. Naim, H. Y., Sterchi, E. E., and Lentze, M. J. (1987) *Biochem. J.* **241,** 427–434
25. Hooper, N. M., and Turner, A. J. (1987) *Biochem. J.* **241,** 625–633
26. Hooper, N. M., Keen, J., Pappin, D. J. C., and Turner, A. J. (1987) *Biochem. J.* **247,** 85–93
27. Schwager, S. L. U., Chubb, A. J., Scholle, R. R., Brandt, W. F., Mentele, R., Riordan, J. F., Sturrock, E. D., and Ehlers, M. R. W. (1999) *Biochemistry* **38,** 10388–10397
28. Schwager, S. L. U., Chubb, A. J., Scholle, R. R., Brandt, W. F., Eckerskorn, C., Sturrock, E. D., and Ehlers, M. R. W. (1998) *Biochemistry* **37,** 15449–15456
29. Sen, I., Samanta, H., Livingston, W., and Sen, G. C. (1991) *J. Biol. Chem.* **266,** 21985–21990
30. Seidah, N. G., Day, R., Marcinkiewicz, M., and Chretien, M. (1998) *Ann. N. Y. Acad. Sci.* **839,** 9–24
31. Eyries, M., Michaud, A., Deinum, J., Agrapart, M., Chomilier, J., Kramers, C., and Soubrier, F. (2001) *J. Biol. Chem.* **276,** 5525–5532
32. Kaplan, H. A., Welply, J. K., and Lennarz, W. J. (1987) *Biochim. Biophys. Acta* **906,** 161–173
33. Mellquist, J. L., Kasturi, L., Spitalnik, S. L., and Shakin-Eshleman, S. H. (1998) *Biochemistry* **37,** 6833–6937
34. Yu, C. X., Sturrock, E. D., Wu, Z., Biemann, K., Ehlers, M. R. W., and Riordan, J. F. (1997) *J. Biol. Chem.* **272,** 3511–3519
35. Jacob, R., Alfalah, M., Grunberg, J., Obendorf, M., and Naim, H. Y. (2000) *J. Biol. Chem.* **275,** 6566–6572
36. Yeaman, C., Le Gall, A. H., Baldwin, A. N., Monlauzeur, L., Le Bivic, A., and Rodriguez-Boulan, E. (1997) *J. Cell Biol.* **139,** 929–940
37. Walmsley, A. R., Zeng, F., and Hooper, N. M. (2001) *EMBO J.* **20,** 703–712
38. McDowell, M. A., Ransom, D. M., and Bangs, J. D. (1998) *Biochem. J.* **335,** 681–689
39. Hammond, C., and Helenius, A. (1995) *Curr. Opin. Cell Biol.* **7,** 523–529
40. Doyle, C., Sambrook, J., and Gething, M. J. (1986) *J. Cell Biol.* **103,** 1193–1204
41. Gething, M. J., McCammon, K., and Sambrook, J. (1986) *Cell* **46,** 939–950
42. Roth, M. G., Doyle, C., Sambrook, J., and Gething, M. J. (1986) *J. Cell Biol.* **102,** 1271–1283
43. Boulay, F., Doms, R. W., Webster, R. G., and Helenius, A. (1988) *J. Cell Biol.* **106,** 629–639
44. Copeland, C. S., Zimmer, K. P., Wagner, K. R., Healey, G. A., Mellman, I., and Helenius, A. (1988) *Cell* **53,** 197–209
45. Naim, H. Y., and Naim, H. (1996) *Eur. J. Cell Biol.* **70,** 198–208
46. Ellgaard, L., Molinari, M., and Helenius, A. (1999) *Science* **286,** 1882–1888
47. Naim, H. Y. (1992) *Biochem. J.* **286,** 451–457

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