Proteolytic Release of Membrane-Bound Angiotensin-Converting Enzyme: Role of the Juxtamembrane Stalk Sequence†

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ABSTRACT: Many structurally and functionally diverse membrane proteins are solubilized by a specific proteolytic cleavage in the stalk sequence adjacent to the membrane anchor, with release of the extracellular domain. Examples are the amyloid precursor protein, membrane-bound growth factors, and angiotensin-converting enzyme (ACE). The identities and characteristics of the responsible proteases remain elusive. We have studied this process in Chinese hamster ovary (CHO) cells stably expressing wild-type ACE (WT-ACE; human testis isozyme) or one of four juxtamembrane (stalk) mutants containing either deletions of 17, 24, and 47 residues (ACE-JMΔ17, -JMΔ24, and -JMΔ47, respectively) or a substitution of 26 stalk residues with a 20-residue sequence from the stalk of the low-density lipoprotein receptor (ACE-JMLDL). The C termini of released, soluble WT-ACE and ACE-JMΔ17 and -JMLDL were determined by MALDI-TOF mass spectrometry analyses of C-terminal peptides generated by CNBr cleavage. Observed masses of 4264 (WT-ACE) and 4269 (ACE- JMΔ17) are in good agreement with an expected mass of 4262 for the C-terminal CNBr peptide ending at Arg-627, indicating cleavage at the Arg-627/Ser-628 bond in both WT-ACE and ACE-JMΔ17, at distances of 24 and 10 residues from the membrane, respectively. Data for ACE-JMΔ24 are also consistent with cleavage at or near Arg-627. For ACE-JMLDL, in which the native cleavage site is absent, observed masses of 4372 and 4542 are in close agreement with expected masses of 4371 and 4542 for peptides ending at Ala-628 and Gly-630, respectively, indicating cleavages at 17 or 15 residues from the membrane. These data indicate that the membrane-protein-solubilizing protease (MPSP) in CHO cells is not constrained by a particular cleavage site motif or by a specific distance from the membrane but instead may position itself with respect to the putative proximal, folded extracellular domain adjacent to the stalk. Nevertheless, cleavage at a distance of 10 residues from the membrane is more favorable, as ACE-JMΔ17 is cleaved 12-fold faster than WT-ACE. In contrast, ACE-JMΔ24 is released 17-fold slower, suggesting that a minimum distance from the membrane must be preserved. This is supported by results with the ACE-JMΔ47 mutant, which is membrane-bound but not cleaved, likely because the entire stalk has been deleted. Finally, soluble full-length (anchor-plus) WT-ACE is not cleaved when incubated with various CHO cell fractions or intact CHO cells. On the basis of these and other data, we propose that the CHO cell MPSP that solubilizes ACE (1) only cleaves proteins embedded in a membrane; (2) requires an accessible stalk and cleaves at a minimum distance from both the membrane and proximal extracellular domain; (3) positions itself primarily with respect to the proximal extracellular domain; and (4) may have a weak preference for cleavage at Arg/Lys-X bonds.

Numerous membrane-anchored proteins are solubilized by limited proteolysis with release of their extracellular domains. These proteins include cytokine receptors, membrane-bound growth factors, cell adhesion molecules, and ectoenzymes [for reviews see Massagué (1990), Ehlers and Riordan (1991), and Bazil (1995)]. One of the most celebrated examples is the amyloid precursor protein (APP),[1] which undergoes proteolytic release of this kind but which is also subject to alternative proteolytic processing events that generate the β-amyloid peptide implicated in Alzheimer's disease (Haass & Selkoe, 1993). The generality of this

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[1] Abbreviations: ACE, angiotensin-converting enzyme; WT-ACE, wild-type ACE; ACE-JMΔ17, -JMΔ24, and -JMΔ47, juxtamembrane mutants with deletions of 17, 24, and 47 residues, respectively; ACE-Δanchor, soluble ACE mutant with deleted transmembrane and cytoplasmic domains; RL-ACE, rabbit lung ACE; APP, amyloid precursor protein; CHO, Chinese hamster ovary; EGF, epidermal growth factor; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; His-Leu, L-histidyl-L-leucine; KL-1 and -2, kit ligands 1 and 2; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MPSP, membrane-protein-solubilizing protease; nt, nucleotide(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; p55 and p75 TNF-R, 55- and 75-kDa tumor necrosis factor receptor; phorbol ester, phorbol 12,13-dibutyrate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SFM, serum-free medium; TGF-α, transforming growth factor-α; TM, transmembrane; TNF-α, tumor necrosis factor-α.

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phenomenon argues strongly that the release of membrane proteins by proteolytic cleavage is of considerable physiological significance and likely subserves a number of functions that depend on the nature of the membrane protein concerned. For example, in the case of receptors and cell adhesion molecules, release will lead to an abrogation of signaling and adhesion events by altering the cell-surface phenotype and by generating soluble antagonists [for examples see Ehlers and Riordan (1991) and Bazil (1995)]. In contrast, the release of membrane-anchored growth factors will lead to a switch from localized to regional or systemic actions (Massagué, 1990).

The proteases responsible for this releasing activity remain unidentified. A common theme that has emerged is that in every case where this has been studied the release mechanism is inducible [reviewed in Arribas and Massagué (1995) and Bazil (1995)]. Release is markedly stimulated by a variety of agonists, including activators of protein kinase C, calcium ionophores, and unidentified serum factors (Pandiella & Massagué, 1991; Ehlers et al., 1995), and, in the case of receptors or adhesion molecules by ligation, by antibodies or ligands (Bazil, 1995). A second characteristic that is apparently common to these proteases is that they are membrane-bound enzymes, although their precise location—post-Golgi, cell surface, or endocytic—is unclear (Sisodia, 1992; Roberts et al., 1994; Kahn et al., 1994; Bosenberg et al., 1993; Ramchandran et al., 1994).

Despite these common features, other evidence suggests that there is considerable heterogeneity among releasing proteases [which we have termed membrane-protein-solubilizing proteases (MPSPs) (Ehlers & Riordan, 1991)]. This conclusion is based on the observations, first, that there is a wide diversity of cleavage site sequences, and second, that various MPSPs have differing sensitivities to protease inhibitors. Regarding the former, at least two types of motifs have been identified. Among membrane-bound growth factors, exemplified by transforming growth factor-α (TGF-α) and the Kit ligand KL-1, cleavage occurs within strings of small apolar residues, which is typical of elastase-like proteases (Pandiella et al., 1992); this type of cleavage site is also found in the 75-kDa tumor necrosis factor receptor (p75 TNF-R) (Brakebusch et al., 1994). In contrast, there is a group of membrane proteins in which cleavage does not occur within a defined sequence motif but rather appears to depend on conformational or topological features (such as distance from the transmembrane domain), as in APP (Sisodia, 1992), p55 TNF-R (Brakebusch et al., 1994), and L-selectin (Chen et al., 1995; Migaki et al., 1995).

Similar heterogeneity exists with respect to the sensitivity of MPSPs to protease inhibitors. The proteases that cleave at elastase-like sites in TGF-α and KL-1 are sensitive to general serine protease inhibitors as well as to specific elastase and chymotrypsin inhibitors (Pandiella et al., 1992). Metalloproteases are implicated in the release of membrane-bound TNF (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994), p75 TNF-R (Crowe et al., 1995), and APP (Roberts et al., 1994). In contrast, the proteases responsible for solubilizing angiotensin-converting enzyme (ACE) (Ramchandran et al., 1994; Ehlers et al., 1995), L-selectin (Bazil & Strominger, 1994; Kahn et al., 1994), and the transferrin receptor (Rutledge et al., 1994) are not inhibited by a wide range of inhibitors added to intact cells. On the basis of these data it has been argued that multiple proteases are involved in the cleavage and release of membrane proteins (Zhong et al., 1994; Bazil, 1995).

This apparent diversity notwithstanding, all MPSPs are regulated, membrane-bound proteases (as discussed above), and data have been presented recently that indicate that the regulated cleavage and secretion of membrane proteins are mediated by a common machinery that responds to various agonists and which activates a variety of "effector" proteases (MPSPs) (Arribas & Massagué, 1995). The identities of the components of this machinery remain unknown, and many of its characteristics are unclear. In an effort to address some of these questions, we have examined the proteolytic cleavage and release of membrane-bound ACE, both wild-type and juxtamembrane (stalk) mutants, stably expressed in Chinese hamster ovary (CHO) cells. We show that the cleavage of membrane-bound ACE is not constrained by a defined distance from the membrane or by a specific cleavage site motif. Instead, we propose that the cleavage of ACE is primarily determined by the distance to the proximal extracellular domain. Our data, in the context of other published reports, suggest that cleavage by the CHO cell MPSP is topologically constrained to an "open" stalk region with minimum distances with respect to both the transmembrane (TM) domain and the first proximal extracellular domain and that this protease has a weak preference for cleavage after Arg or Lys residues.

MATERIALS AND METHODS

Construction of Expression Vectors and CHO Cell Transfections. Expression vectors encoding mutant human testis ACE proteins with deletions of 17, 24, and 47 juxtamembranous residues (Figure 1), termed pLEN-ACE-JMΔ17, -JMΔ24, and -JMΔ47, respectively, are based on the vector pLEN-ACEVII (Ehlers et al., 1991a,b) and were constructed by deletion mutagenesis using the polymerase chain reaction (PCR) as follows. For pLEN-ACE-JM17, advantage was taken of the fact that there is a unique *Not*I site at nucleotide (nt) 2019 in the ACE cDNA (Ehlers et al., 1989) which extends to the codon for Arg-637, 14 residues proximal to the start of the TM domain (Figure 1). [In the original publications the start of the TM domain was given as Trp-655 (Soubrier et al., 1988; Ehlers et al., 1989), but this predicted a TM domain only 17 residues in length, which is unusually short. We now assign the start to Val-652 (Figure 1).] The sequence coding for residues Trp-655 to Ser-701 (the C terminus) was amplified with introduction of a 5′ *Not*I site and ligated into pLEN-ACEVII cut with *Not*I and *Cla*I, resulting in the deletion of nt 2027−2077, encoding Val-638 to Glu-654 (Figure 1).

pLEN-ACE-JMΔ24 and -JMΔ47 were constructed by a two-stage PCR procedure. For pLEN-ACE-JMΔ24, nt 1854−1996 and nt 2069−2216 of the full-length ACE cDNA (Ehlers et al., 1989) were amplified with pairs of primers designed to generate overlapping hybrid sequences and to introduce an *Eco*RI site at nt 1984 (see below). The products were reamplified with the outside primers to generate a recombinant sequence from which nt 1998−2068 [encoding Ser-628 to Arg-651 (Figure 1)] are deleted, and this product was ligated into pLEN-ACEVII digested with *Bcl*I and *Cla*I. pLEN-ACE-JMΔ47 was constructed in the same manner except that nt 1928−2068 [encoding Thr-605 to Arg-651 (Figure 1)] are deleted. A similar two-stage PCR strategy

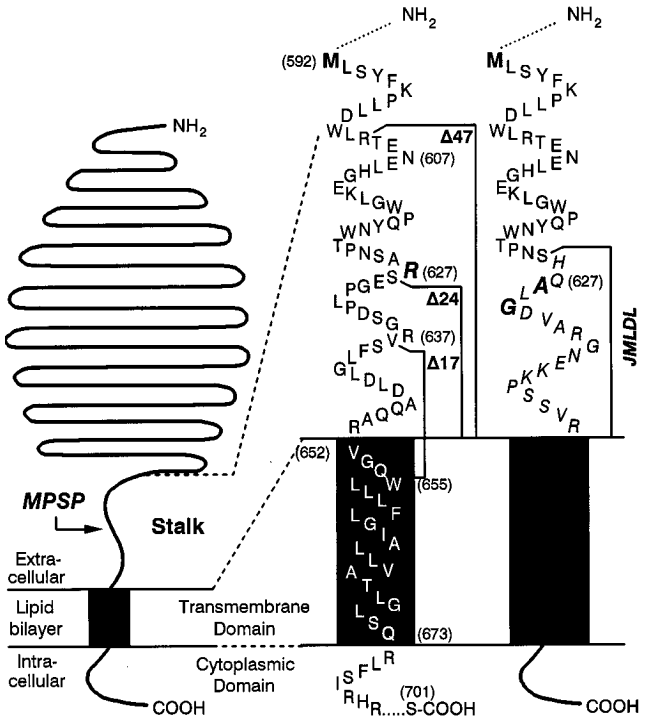

FIGURE 1: Schematic representation of membrane-bound ACE and of wild-type and mutant stalk sequences. ACE is a type I transmembrane ectoprotein, and the connection of the N-terminal extracellular domain and the transmembrane domain is through a putative juxtamembrane "stalk" region. The dimension of this stalk is speculative. Proteolytic release of the extracellular domain occurs via cleavage by a membrane-protein-solubilizing protease (MPSP) in the stalk region. An expanded view of the ACE transmembrane and stalk domains is shown to the right of the general scheme. The juxtamembrane mutants ACE-JMΔ17, -JMΔ24, and -JMΔ47 have 17, 24, and 47 residues deleted, respectively, from the stalk, as indicated. Note that in ACE-JMΔ17, 14 residues are deleted from the stalk and three residues are deleted from the TM domain, based on the original prediction of the TM domain dimensions (Ehlers et al., 1989), now revised (see text). The numbers in parentheses refer to the amino acid sequence positions in human testis ACE (Ehlers et al., 1989). The ultimate methionine residue, M-592 is shown in large bold type. The sequence on the extreme right is that of the chimeric mutant ACE-JMLDL, in which Ala-626 to Arg-651 of the ACE stalk have been replaced with His-748 to Arg-767 from the LDL-R stalk (in italics) (Yamamato et al., 1984); note that numbering is consecutive with respect to the ACE sequence. The determined C termini of the released, soluble proteins are shown in large, bold italics: Arg-627 for WT-ACE and ACE-JMΔ17, and Ala-628 and Gly-630 for ACE-JMLDL.

was used to construct pLEN-ACE-JMLDL, which codes for a mutant ACE in which the wild-type stalk is replaced by the stalk from the low-density lipoprotein receptor (LDL-R). Through use of the unique *Eco*RI site engineered into pLEN-ACE-JMΔ24, the sequence encoding Ala-626 to Arg-651 (nt 1991−2068) is replaced with the sequence encoding His-748 to Arg-767 of LDL-R (Yamamoto et al., 1984) (Figure 1). All constructs were verified by restriction mapping and sequencing of PCR-amplified fragments. CHO K1 cells were co-transfected with 10 μg of each of the constructs and 2 μg of pSV2NEO, as detailed previously (Ehlers et al., 1991b).

Analysis of Recombinant Proteins. After selection for stable transfectants, mutant soluble and membrane-bound ACE proteins were isolated from the conditioned media and detergent extracts of CHO cells, respectively, and purified to electrophoretic homogeneity by affinity chromatography on a Sepharose-28-lisinopril affinity resin (Ehlers et al., 1989, 1991b). Purified recombinant proteins were analyzed by SDS−PAGE, amino acid analysis, and Triton X-114 phase separation (Ehlers et al., 1991a). Specific activities of the mutant proteins were determined by hydrolysis of 2-furanacryloyl-L-phenylalanylglycylglycine under standard conditions (Ehlers et al., 1991b) with protein quantitation by amino acid analysis.

Determination of the Juxtamembrane Cleavage Site in Released ACE. Released (soluble) wild-type and mutant ACE were isolated from the conditioned media of transfected CHO cells as described. Purified proteins (generally 0.5−2.0 mg) were subjected to CNBr cleavage by standard procedures (Gross, 1967). CNBr cleavage peptides were prefractionated by chromatography on a Sephadex G-50 column developed in 0.1% trifluoroacetic acid (TFA). Eluted fractions were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and then fractionated further by reverse phase high-performance liquid chromatography (HPLC) on a C_4 column developed with a linear gradient of 0.1% TFA to 80% CH_3CN/0.1% TFA. HPLC fractions were subjected to N-terminal peptide sequencing by automated Edman degradation (five cycles) on a gas−liquid−solid phase sequencer, as described (Brandt et al., 1984). The C-terminal CNBr peptides identified in this manner were then analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, either on a Voyager-Elite Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) using a nitrogen laser at 337 nm and an α-cyano-4-hydroxycinnamic acid matrix or on a Kratos Kompact MALDI 3 (Manchester, U.K.).

Assay of Membrane-Bound and Soluble ACE Activity. CHO cells were grown to early confluence in standard media (50% Ham's F-12/50% DME medium supplemented with 20 mM Hepes, pH 7.3, 20 mM L-glutamine) containing 2% fetal calf serum (heated to 70 °C for 15 min before use) and 80 μM $ZnCl_2$. Cell-associated (membrane-bound) ACE activity of transfectants expressing mutant or wild-type ACE was assayed directly by addition of serum-free medium (SFM) (Ham's F-12, 20 mM Hepes, pH 7.3) containing 5 mM hippuryl-L-histidyl-L-leucine, as described (Ehlers et al., 1991a). In parallel, the soluble ACE activity in the saved, conditioned media was also estimated.

Kinetics of ACE Release. CHO cells stably expressing ACE-JMΔ17 and ACE-JMΔ24 were subjected to a kinetic analysis of ACE release by determining the rate of accumulation of soluble activity and accompanying changes in membrane-bound activity, exactly as described for WT-ACE (Ehlers et al., 1995). These studies were performed in the presence and absence of 1 μM phorbol 12,13-dibutyrate.

Activity of CHO Cell MPSP toward Anchor-Plus ACE in Solution. Un-transfected CHO cells, maintained in serum-free medium for 48 h, were homogenzied in PBS, sonicated, and detergent-extracted in 1% Triton X-100. Various CHO cell fractions (10−90 μL, representing ~3×10^6 cells) were incubated with 8 μg of full-length, "anchor-plus" rabbit lung ACE [RL-ACE, purified as described (Pantoliano et al., 1984)] in 50 mM Tris, pH 7.5, 0.15 M NaCl, at 37 °C for 2 h. All samples were then subjected to Triton X-114 phase separation, and the detergent-rich and -poor phases were assayed for ACE activity by the Hip-His-Leu assay (Ehlers et al., 1991a). In addition, un-transfected CHO cells were

Table 1: Cell-Associated Expression and Release of Wild-Type ACE and Juxtamembrane Mutants of ACE in CHO Cells[a]

recombinant ACE	ACE activity (milliunits per 2.4 × 10^6 cells)		ratio of soluble to cell-associated activity
	cell-associated	soluble	
un-transfected	0	0	–
ACE-Δanchor	0.02	24.1	1205
WT-ACE	14.6	70.5	4.8
ACE-JMΔ17	9.6	203	21.1
ACE-JMΔ24	10.2	6.5	0.6
ACE-JMΔ47	0	0	–
ACE-JMLDL	1.9	37.4	19.7

[a] Un-transfected CHO cells and CHO cells stably transfected with expression vectors coding for WT-ACE, ACE-Δanchor, and ACE-JMΔ17, -JMΔ24, -JMΔ47, and -JMLDL were grown to confluence in 12-well plates and induced with Zn^{2+} to achieve maximal ACE expression. After 48 h, media were assayed with the substrate Hip-His-Leu to estimate total soluble activity, and total cell-associated activity was determined by incubating washed cells with the same substrate.

grown to confluence, washed with SFM, and then re-fed with 0.4 mL SFM containing 16 μg of RL-ACE and incubated at 37 °C. Samples (25 μL) were withdrawn at 30 min, 2 h, 6 h, and 22 h and subjected to phase separation as above.

RESULTS

Expression of Juxtamembrane Mutants of ACE in CHO Cells. The cell-associated expression (which is presumed to reflect predominantly cell-surface expression) and extent of release of the four juxtamembrane mutants are compared to those of WT-ACE and ACE-Δanchor [a mutant lacking the TM and cytoplasmic domains (Ehlers et al., 1991a)] in Table 1. ACE-Δanchor is found almost exclusively in the cell medium, whereas full-length WT-ACE is both bound to the cell surface and released into the medium (Ehlers et al., 1991a). It is notable that similar amounts of WT-ACE, ACE-JMΔ17, and ACE-JMΔ24 are present on the cell surface but that the extent of release, expressed as the ratio of soluble to membrane-bound activity, differs by 35-fold. Compared to WT-ACE, this ratio is ~4-fold greater for ACE-JMΔ17 and ~8-fold smaller for ACE-JMΔ24 (Table 1), suggesting a difference in the rate of release of these mutants compared to the wild-type protein. Cell-surface expression of ACE-JMLDL is only 13% of WT-ACE levels, but the soluble activity is 53% of WT-ACE, giving a release ratio that is similar to that of ACE-JMΔ17. ACE-JMΔ47 could not be detected by enzymatic activity. By Western blotting it is shown to be present in the CHO cell lysate (Figure 2) but not in the medium; as expected, cell-associated ACE-JMΔ47 appears to have a lower molecular weight than WT-ACE.

Analysis of Recombinant Proteins. ACE-JMΔ17, ACE-JMΔ24, and ACE-JMLDL were purified to electrophoretic homogeneity from both conditioned media and detergent-extracted cell pellets. On SDS−PAGE, like WT-ACE (Ehlers et al., 1991a,b), they run as broad smears (Figure 3), likely due to variable glycosylation by CHO cells (Ehlers et al., 1992), which masks the size difference between membrane-bound and soluble ACE. The amino acid compositions of ACE-JMΔ17 and -JMΔ24 and that of WT-ACE (Table 2) are within experimental limits of the predicted integer values. The soluble forms differ from the membrane-bound forms by a decrease of Glx, His, Arg, Leu, and Phe,

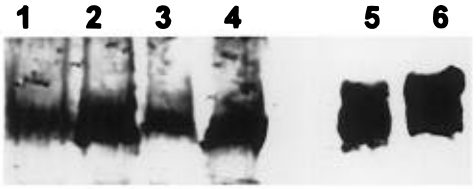

FIGURE 2: Western blot of lysates of CHO cells expressing WT-ACE (lane 1), ACE- JMΔ17 (lane 2), ACE-JMΔ24 (lane 3), and ACE-JMΔ47 (lane 4). Transfected cells (between 1 × 10^6 and 5 × 10^6 cells) were scraped into PBS, solubilized in SDS−PAGE loading buffer, boiled for 5 min, and electrophoresed on a 10%−20% gradient SDS−PAGE gel. As a control, 1 μg each of purified soluble WT-ACE and ACE-JMΔ17 were run in lanes 5 and 6, respectively. The gel was electroblotted and immunostained with rabbit anti-human kidney ACE antiserum as described (Ehlers et al., 1991b).

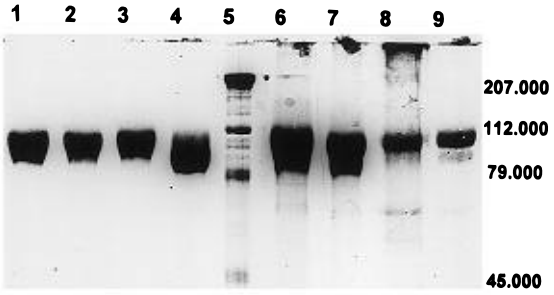

FIGURE 3: Purified wild-type and mutant ACE proteins. Recombinant proteins were isolated from the conditioned media (soluble ACE) and detergent-extracted cell lysates (membrane-bound ACE) of transfected CHO cells, electrophoresed on a 10% SDS−polyacrylamide gel (~5 μg per lane), and stained with Coomassie Brilliant Blue. Lanes 1−4 (soluble) and 6−9 (membrane-bound) are WT-ACE, ACE-JMΔ17, ACE-JMLDL, and ACE-JMΔ24, respectively; lane 5, molecular weight markers.

the prominent residues in the juxtamembrane, transmembrane, and cytoplasmic domains. The soluble proteins partitioned predominantly (>98%) into the detergent-poor phase on Triton X-114 phase separation, whereas the membrane-bound proteins partitioned favorably (~40%) into the detergent-rich phase (Table 3). Taken together, these data indicate that the soluble forms of ACE-JMΔ17 and ACE-JMΔ24 arise from the membrane-bound forms by proteolytic cleavage with removal of the hydrophobic transmembrane domain and the cytoplasmic domain, as was inferred previously for WT-ACE (Ehlers et al., 1991a). Except for ACE-JMΔ47, both the soluble and membrane-bound forms of the deletion mutants and of WT-ACE have specific activities of 16 ± 1 units/nmol (toward the substrate 2-furanacryloyl-L-PheGlyGly).

Determination of the Juxtamembrane Cleavage Site in Released ACE. CNBr cleavage of soluble, wild-type, human testis ACE is predicted to yield 18 peptides. The C-terminal CNBr peptide—the C terminus of which is the actual site of juxtamembrane cleavage that solubilizes the membrane-bound protein—is expected to be maximally 59 residues in length (if the cleavage is at the junction of the stalk and TM domains) and probably shorter (Figure 1). Initial fractionation by HPLC on a C_4 column yielded seven major fractions (Figure 4), each of which was analyzed by automated N-terminal sequencing. These analyses revealed that the C-terminal CNBr peptide, starting with the sequence LSYFK, which is the sequence immediately after the ultimate Met residue in the protein (Figure 1), was located in fraction 3 (Figure 4). Improved purification was achieved by prefrac-

Table 2: Amino Acid Compositions of Soluble and Membrane-Bound Forms of Wild-Type and Juxtamembrane Deletion Mutants of ACE[a]

	wild-type ACE		ACE-JMΔ17		ACE-JMΔ24	
amino acid	soluble	membrane-bound	soluble	membrane-bound	soluble	membrane-bound
Asx	62.1 (62)	64.5 (64)	60.7 (61)	62.1 (62)	65.9 (59)	60.3 (61)
Glx[b]	82.8 (80)	88.8 (87)	82.7 (79)	84.9 (84)	76.7 (78)	81.8 (84)
Ser	41.6 (43)	48.3 (49)	40.2 (40)	48.2 (48)	38.1 (39)	43.0 (46)
Gly	34.3 (33)	41.6 (39)	32.7 (31)	41.5 (37)	34.0 (31)	42.2 (38)
His	22.8 (23)	26.3 (28)	22.7 (23)	25.5 (28)	22.9 (23)	24.4 (28)
Arg	28.7 (28)	33.5 (34)	26.8 (26)	33.7 (33)	26.3 (26)	32.0 (32)
Thr	41.2 (42)	42.4 (43)	40.3 (42)	42.4 (43)	38.4 (41)	40.8 (43)
Ala	50.8 (49)	57.7 (53)	50.2 (48)	57.0 (51)	50.5 (48)	56.8 (51)
Pro	36.4 (36)	38.4 (37)	33.0 (34)	38.3 (37)	31.1 (32)	34.8 (35)
Tyr	25.2 (26)	26.1 (26)	24.2 (26)	25.8 (26)	23.9 (25)	25.0 (26)
Val	28.7 (31)	32.4 (34)	27.7 (30)	31.8 (32)	27.9 (32)	32.1 (33)
Met	16.4 (17)	16.6 (17)	15.7 (17)	16.3 (17)	14.8 (17)	15.8 (17)
Ile	23.3 (25)	26.0 (27)	23.2 (25)	27.0 (27)	22.8 (25)	25.5 (27)
Leu	64.6 (63)	74.7 (77)	60.1 (62)	72.9 (74)	61.4 (62)	70.5 (73)
Phe	24.3 (25)	28.2 (29)	24.1 (25)	26.5 (28)	23.9 (25)	27.1 (28)
Lys	31.4 (30)	32.1 (30)	32.6 (30)	33.1 (30)	31.7 (30)	32.0 (30)

[a] Samples were analyzed as described (Strydom et al., 1986). Results are the average of four to six analyses, expressed as residues per molecule. Integer values based on predicted sequences are in parentheses; the values for the soluble forms were calculated for proteins lacking the transmembrane and cytoplasmic domains. [b] Underlined are residues expected to differ between the soluble and membrane-bound forms, on the basis of the prevalence of these residues in the juxtamembrane, transmembrane, and cytoplasmic domains.

Table 3: Triton X-114 Phase Separation of Soluble and Membrane-Bound Forms of Wild-Type and Juxtamembrane Deletion Mutants of ACE[a]

recombinant ACE	enzyme activity in detergent phase, % of total activity
WT-ACE	
soluble	1.2
membrane-bound	43
ACE-JMΔ17	
soluble	1.1
membrane-bound	39
ACE-JMΔ24	
soluble	0.9
membrane-bound	41

[a] Recombinant ACE, purified from the media (soluble) and detergent-extracted cell pellets (membrane-bound) of CHO cells stably expressing WT-ACE and the juxtamembrane mutants ACE-JMΔ17 and -JMΔ24, was subjected to phase separation in Triton X-114, as described (Ehlers et al., 1991a). The detergent-rich and detergent-poor phases were assayed in triplicate with the ACE substrate Hip-His-Leu. Activity recovered in the detergent-rich phase is expressed as a percentage of the total starting activity and is the mean for three phase separations.

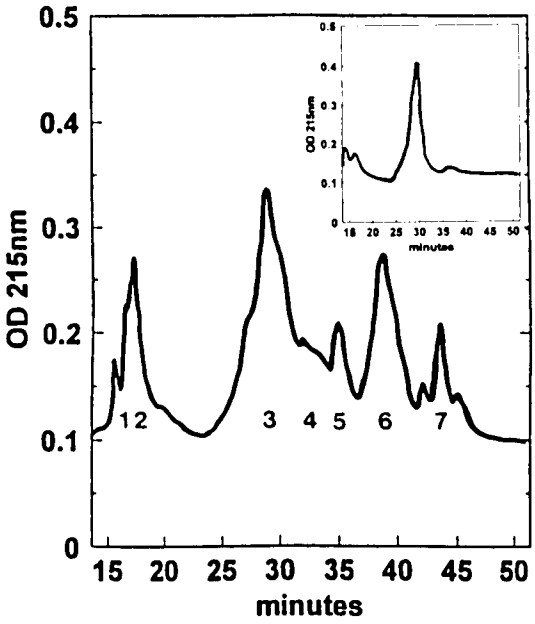

FIGURE 4: HPLC elution profile of CNBr-digested WT-ACE. Approximately 1 mg of released, soluble WT-ACE, purified from conditioned media of transfected CHO cells, was digested with CNBr and fractionated on a C_4 reverse phase column developed with a linear gradient of 30%−60% CH_3CN in 0.1% TFA. Fractions 1−7 were analyzed by Edman degradation. Inset, elution profile after initial Sephadex G-50 prefractionation of the CNBr digest.

tionation of the CNBr digest on a Sephadex G-50 column, and subsequent fractionation of the Sephadex column eluates on the C_4 HPLC column was as before. This enabled isolation of a near-homogeneous peak, which by N-terminal sequencing was found to comprise the C-terminal peptide with negligible contaminants. Analyses by MALDI-TOF mass spectrometry revealed $[M + H]^+$ ions at *m/z* of 4262 and 4264, respectively, for fractions obtained without or with prefractionation (Table 4). These masses are in very close agreement with a theoretical mass of 4262 for a C-terminal CNBr peptide ending at Arg-627 [numbering as described in Ehlers et al. (1989)]. This represents a cleavage site between Arg-627 and Ser-628, which is 24 residues proximal to the TM domain (Figure 1).

Released, soluble protein of the ACE-JMΔ17 mutant was similarly analyzed. The HPLC elution profile was virtually identical, and the C-terminal CNBr peptide starting with the sequence LSYFK was identified. Analysis of this fraction by mass spectrometry also revealed a major ion at *m/z* 4269 (Table 4), which indicates an identical cleavage site between Arg-627 and Ser-628. The yield of purified soluble protein of the ACE-JMΔ24 mutant was too low for a complete analysis. The HPLC elution profile of CNBr-digested protein was virtually identical to that of WT-ACE and ACE-JMΔ17, and the C-terminal peptide starting with LSYFK was identified, but it was not analyzed by mass spectrometry. However, the data suggest that the cleavage site is close to or identical to that found in WT-ACE and ACE-Δ17. In contrast, a complete analysis was possible for ACE-JMLDL (Table 4). Mass determinations of the C-terminal CNBr peptide (again starting with LSYFK) revealed molecular ions at *m/z* 4372.1 and *m/z* 4542, which are in close agreement with calculated masses of 4370.8 and 4541, respectively, for C-terminal peptides ending at Ala-628 and Gly-630 (num-

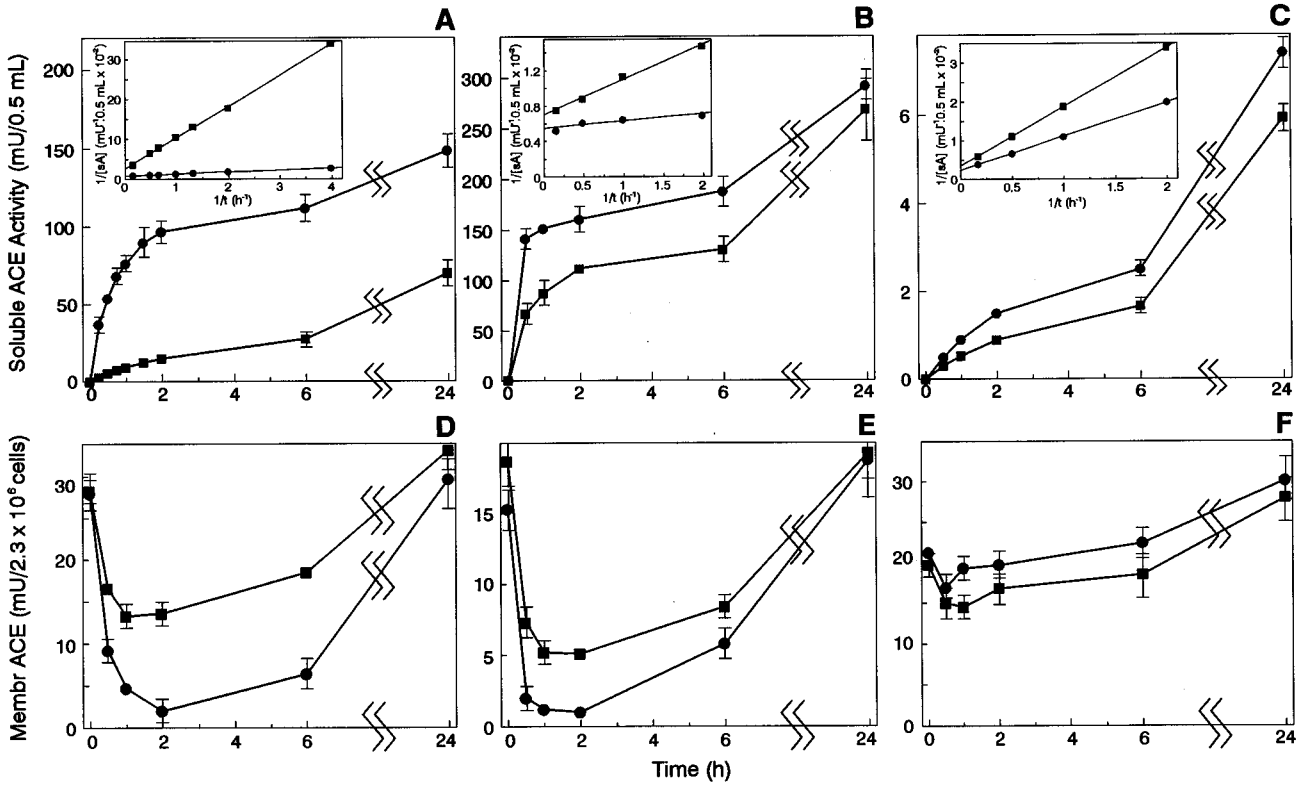

FIGURE 5: Kinetics of solubilization (A−C) and changes in membrane-bound levels (D−F) of wild-type ACE (A, D) and deletion mutants [ACE-JMΔ17 (B, E); -JMΔ24 (C, F)] expressed in CHO cells. CHO cells stably expressing each of the recombinant proteins were grown to confluence, induced with Zn^{2+} for 24 h, and then washed and re-fed with fresh, indentical media, either with (circles) or without (squares) 1 μM phorbol -12,13-dibutyrate (time zero). Media were sampled at the indicated time points (20 μL out of 0.5 mL) and assayed for ACE activity by hydrolysis of Hip-His-Leu; in separate wells, cells were washed (2 × 1 mL of PBS per well) and lysed in 50 mM Tris, pH 8.0, 1% Triton X-100 (0.2 mL per well), and the lysates were assayed in the same manner. Data are the means ± SD for three separate experiments; data for WT-ACE are taken from Ehlers et al. (1995). Insets (A−C): double-reciprocal plot of the data, i.e., 1/[sA] (where sA is soluble ACE) versus 1/*t*; the *x*- and *y*-intercepts give $-1/t_{1/2}$ and $1/[sA]_{max}$, respectively (Ehlers et al., 1995).

Table 4: Mass Analyses of Purified and Partially Purified CNBr C-terminal Peptides Obtained from Soluble ACE Proteins[a]

ACE proteins	N-terminal sequence[d]	mass obsd[f]	mass exp	deduced C terminus
WT-ACE[b]	LSYFK[e]	4262	4262	Arg-627
	FKEAD		2917	(F-316 to M-340)[g]
	WAQTW		2411	(W-279 to M-305)[g]
WT-ACE[c]	LSYFK[e]	4264	4262	Arg-627
ACE-JMΔ17[c]	LSYFK[e]	4269	4262	Arg-627
ACE-JMLDL[b]	LSYFK[e]	4372	4371	Ala-628
		4542	4541	Gly-630
	QYKDL	6042	6043	(Q-393 to M-448)[g]

[a] All mass values are protonated average molecular weights isotopically averaged *m*/*z*, calculated for C-terminal homoserine lactone. Soluble wild-type and mutant ACE proteins were purified, CNBr-digested, and fractionated, either [b] without or [c] with Sephadex G-50 prefractionation, as described in Figure 4. [d] In every case, fraction 3 (Figure 4) contained the [e]C-terminal peptide, as determined by N-terminal sequencing. [f] Mass observed by MALDI-TOF mass spectrometry of [c]pure or [b]partially purified HPLC fractions. [g] Internal peptides. Abbreviations: obsd, observed; exp, expected.

bering with respect to the chimeric ACE-JMLDL mutant, see Figure 1).

Kinetics of Release of Juxtamembrane Deletion Mutants of ACE. We have shown previously (Ehlers et al., 1995) that the release of WT-ACE from resting, unstimulated cells is slow and approximately linear but that following washing and re-feeding of the cells is a transient stimulation (or "burst") of the rate of release (Figure 5A). This results in a rapid, nonlinear accumulation of soluble ACE in the medium and a concomitant fall in the level of membrane-bound ACE (Figure 5D). The kinetics of release during this burst period (i.e., the rate of appearance of soluble ACE) are first order, due to depletion of membrane-bound ACE (Ehlers et al., 1995). The rate of release of WT-ACE is further enhanced by phorbol ester (Figure 5A). The kinetics of release of ACE-JMΔ17 and ACE-JMΔ24 into the media of cells stably expressing these mutants are qualitatively similar but quantitatively different from WT-ACE and from each other (Figure 5). Kinetic constants for the release of WT-ACE, ACE-JMΔ17, and ACE-JMΔ24 from transfected CHO cells stimulated by a media change or with phorbol ester are given in Table 5. The first-order rate constant, *k*, and the half-time, $t_{1/2}$, describe the rate of conversion of membrane-bound ACE (mACE) to soluble ACE and the time required to generate 50% of maximal soluble ACE, respectively. These constants assume first-order conditions, which are only approximated in the first 2−6 h of the time course (Ehlers et al., 1995). In fact, the process of conversion of membrane-bound to soluble ACE is complex and depends on [mACE], the rate of de novo synthesis of mACE, the susceptibility to cleavage by the MPSP, and the duration of the media change- or phorbol-induced stimulation (Ehlers et al., 1995). A more direct estimate of the differences in the processing of these recombinant proteins is simply the rate of production of soluble protein in the first 30 min of the time course (Table 5), which reflects the capacity of each cell line to generate soluble ACE. Analyzed in this manner, soluble ACE-JMΔ17 is produced 12-fold faster than soluble WT-ACE in the first

Table 5: Initial Rates of Solubilization and Kinetic Constants for the Stimulated Release of Membrane-Bound ACE in CHO Cells[a]

form of ACE expressed in CHO cells	first-order rate constant k ($\times 10^{-1}$ min^{-1}) from ln([sA]$_\infty$−[sA]) vs t plots	first-order rate constant k ($\times 10^{-1}$ min^{-1}) from $t_{1/2}$	"half-time" $t_{1/2}$ (min)[b]	initial rates (milliunits min^{-1} $\times 10^{-1}$)[c]
WT-ACE[d]				
media[e]	0.038	0.040	174 ± 12	1.9
phorbol	0.12	0.17	40 ± 6	22.8
ACE-JMΔ17				
media	0.10	0.22	31 ± 4	22.5
phorbol	nd	0.63	11 ± 4	47.3
ACE-JMΔ24				
media	0.022	0.022	318 ± 30	0.11
phorbol	0.034	0.032	222 ± 24	0.17

[a] Derived from graphical analyses of data presented in Figure 5, as described (Ehlers et al., 1995). [b] Values for $t_{1/2}$ represent means ± SD for three to six separate experiments. [c] Derived from 30-min time point (Figure 5). [d] Values for WT-ACE are taken from Ehlers et al. (1995). [e] Abbreviations: media, media change-stimulated release; phorbol, phorbol ester (12,13-dibutyrate)-stimulated release; nd, not determined.

30 min, and soluble ACE-JMΔ24 is produced 17-fold slower (Table 5).

The initial release curve for ACE-JMΔ17 after a media change is hyperbolic (Figure 5B), and the double reciprocal plot is linear (Figure 5B, inset), with the value of the x-intercept, $t_{1/2} = 31 \pm 4$ min (compared to 2.9 h for WT-ACE; Ehlers et al., 1995). Phorbol ester stimulation results in a further increase in the rate of release of ACE-JMΔ17, although this increase is only about 2-fold in the first 30 min versus 12-fold for WT-ACE (Table 5). The phorbol ester-stimulated time course gives a $t_{1/2}$ value of 11 ± 4 min. As noted for WT-ACE, total membrane-bound ACE-JMΔ17 decreases to about 27% of the initial value after washing and re-feeding the cells and to 6% in phorbol ester-stimulated cells, and in both cases slowly returns to the initial levels by 24 h (Figure 5E).

As noted, the rate of release of ACE-JMΔ24 is much slower than that of WT-ACE, with levels of soluble activity about 17-fold less in the first 30 min (Table 5). The release curve also shows an initial hyperbolic response after stimulation and a steady, linear background rate in resting cells (Figure 5C); a double-reciprocal plot of the data for the first 6 h is linear, with $t_{1/2} = 5.3 \pm 0.5$ h. Phorbol ester stimulation results in only a modest additional increase in the rate of release, about 1.5-fold in the first 30 min, and the $t_{1/2}$ is reduced to 3.7 ± 0.4 h (Table 5). The levels of membrane-bound ACE-JMΔ24 show a small but consistent decrease in the first 30−60 min; however, there is no significant difference between the levels in the presence or absence of phorbol ester (Figure 5F).

Activity of CHO Cell MPSP toward Full-Length (Anchor-Plus) ACE in Solution. Various CHO cell fractions were incubated with full-length (anchor-plus) ACE, and the extent of conversion of anchor-plus to anchor-minus ACE was assayed by Triton X-114 phase separation. Despite extended incubations (up to 2 h), no conversion of anchor-plus to anchor-minus ACE could be detected, unlike a positive control in which RL-ACE was incubated with trypsin, which is known to solubilize ACE and other membrane proteins (Ehlers et al., 1991a). Moreover, when RL-ACE was added directly to the media of intact, living CHO cells and incubated for up to 22 h, no cleavage of the membrane anchor of RL-ACE could be detected. Taken together, these data indicate that soluble anchor-plus ACE, i.e., ACE containing its membrane-spanning domain but in solution as opposed to anchored in a membrane, is not susceptible to cleavage by the CHO cell MPSP.

DISCUSSION

Membrane-anchored human ACE expressed in CHO cells is solubilized by a vigorous MPSP activity (Ehlers et al., 1991a, 1995; Wei et al., 1991), thereby providing a model system that may assist in resolving the problem of the cleavage site specificity of MPSPs. It is at present unclear whether specificity at the cleavage site is conferred by amino acid sequence or by topological parameters. We have addressed this problem by constructing four mutants of ACE with deletions or substitutions in the juxtamembrane region and by analyzing the rates of release of these mutants with respect to the wild-type protein. Moreover, we have identified the juxtamembrane cleavage site in the wild-type protein and in two of the mutants.

The mutant with the largest deletion, ACE-JMΔ47 (Figure 1), is catalytically inactive, and it is not detectable in the medium. Presumably the 47-residue juxtamembranous deletion extends beyond the stalk in the ACE protein and significantly disturbs the bulk of the enzyme, rendering it inactive. This would also imply that deletion of the entire stalk has resulted in fusion of the extracellular domain of the protein directly to the transmembrane domain. If the mutant is expressed at the cell surface (for which we have no direct data), then failure to detect soluble protein indicates that cleavage by the MPSP requires an accessible stalk; this question is discussed further, below.

The mutants ACE-JMΔ17, ACE-JMΔ24, and ACE-JMLDL, unlike ACE-Δ47, are catalytically active. The soluble isoforms of the two deletion mutants do not segregate into the detergent-rich phase after Triton X-114 phase separation (Table 3) and have amino acid compositions consistent with loss of the transmembrane and cytoplasmic domains (Table 2), likely as a result of a proteolytic cleavage in the stalk region.

Direct evidence for proteolytic release is provided by the determination of the C terminus of released, soluble ACE. WT-ACE and ACE-JMΔ17 both have Arg-627 as their C-terminal residue, as found by mass spectrometry of the C-terminal CNBr cleavage peptides (Table 4). This establishes the bond between Arg-627 and Ser-628 as the major cleavage site, which in WT-ACE represents a distance of 24 residues from the membrane (Figure 1). It is possible that there are additional minor cleavage sites that we have not detected or that the major cleavage site is C-terminal to Arg-627 and that one or more residues are removed by a carboxypeptidase activity in the CHO cell conditioned media. Significantly, the cleavage site determined here for membrane-bound human testis ACE expressed in CHO cells is identical to that determined for rabbit testis ACE expressed in mouse C127 cells by Ramchandran et al. (1994). These authors located the site by performing a combination of N-terminal sequencing of the residual membrane anchor and carboxypeptidase Y digestion of the soluble protein. There was some evidence, although not conclusive, for a minor cleavage after Arg-673 (in the rabbit sequence, equivalent to Arg-637 in

the human sequence) (Ramchandran et al., 1994). In contrast to these data, Beldent et al. (1993) have reported a cleavage site at a distance of 89 residues from the membrane for human somatic ACE[2] expressed in CHO cells, between Arg-1137 and Leu-1138 (which corresponds to Arg-561 and Leu-562 in testis ACE). This site was determined by carboxypeptidase Y and P digestion of soluble ACE; no attempts were made to analyze C-terminal peptides after fragmentation of the soluble protein or by N-terminal sequencing of the residual membrane anchor. In view of the data presented here and those reported by Ramchandran et al. (1994), and our finding that ACE-JMΔ47 is neither active nor secreted, it seems unlikely that cleavage at Arg-1137/Leu-1138 (which represents an 89-residue juxtamembrane deletion) would be the primary cleavage site.

Cleavage of WT-ACE at a distance of 24 residues from the membrane is unexpected, because the cleavage of APP, L-selectin, and p55 TNF-R occurs at distances of 12, 11, and 10 residues from the membrane, respectively (Esch et al., 1990; Kahn et al., 1994; Brakebusch et al., 1994). Like ACE, these proteins are type I transmembrane proteins. Moreover, construction of APP mutants with deletions involving the cleavage site results in cleavage at a new site that is also 12 residues from the membrane (Maruyama et al., 1991), and mutants with four- or six-residue deletions proximal to the native site are cleaved in both cases at two sites three and four residues N-terminal to the native site, preserving a distance of between nine and thirteen residues from the membrane (Zhong et al., 1994) (see Figure 7). From these data it can be inferred that cleavage in these proteins is constrained to a distance of nine to thirteen residues from the membrane, and the expectation was that a similar pattern might pertain for ACE.

However, whereas the mutant protein ACE-JMΔ17, with a 14-residue deletion in the proximal stalk, is cleaved at a distance of 10 residues from the membrane, wild-type ACE is cleaved at the identical site but at a distance of 24 residues. One explanation for these data is that the CHO cell MPSP acting on ACE is sequence-specific rather than distance-specific. An alternative explanation is that cleavage is constrained either by a minimum distance from the membrane or by a minimum distance from the first folded extracellular domain, or both, and that the Arg-627/Ser-628 bond is the most convenient site that satisfies these criteria, in both WT-ACE and ACE-JMΔ17. Support for the second alternative derives from an analysis of the ACE-JMLDL chimeric mutant, in which 26 residues of the ACE stalk, including the native cleavage site, are replaced by 20 residues immediately proximal to the TM domain in the stalk of LDL-R (Figure 1). This mutant is readily cleaved at a rate that appears to be comparable to the ACE-JMΔ17 mutant (Table 1), indicating that the Arg-627/Ser-628 cleavage site is not essential. Soluble ACE-JMLDL has two C termini, Ala-628 and Gly-630 (Table 4), indicating either two primary cleavage sites, at the Ala-628/Leu-629 and Gly-630/Asp-631 bonds (Figure 1), or primary cleavage at the Gly-630/Asp-631 bond with secondary carboxypeptidase processing. In either event, the MPSP has ignored the Arg-635/Gly-636 bond, which more closely resembles the native site and is at a distance of 10 residues from the membrane (Figure 1), cleaving instead at a distance of 15 and/or 17 residues. Taken together, these data establish that neither a specific sequence at the cleavage site nor a defined distance from the membrane is the primary determinant for cleavage by the CHO cell MPSP. Instead, these data are consistent with the notion that the MPSP is principally constrained by a minimum distance or interval from both the first, proximal extracellular domain and the TM domain.

A comparative analysis of all known cleavage sites of membrane proteins solubilized by MPSP activity lends support to the view that cleavage is constrained by the proximity of both the membrane and the first folded extracellular domain, which together constitute the boundaries of the stalk region. Inspection of the sequences aligned in Figure 6 indicates that the distance of the cleavage site from the transmembrane domain is highly variable, ranging from eight residues (p55 TNF-R, IL-6 receptor, and TGF-α) to 36 residues (rat β4 heregulin). In those cases where a defined, proximal extracellular domain can be identified, such as the second short consensus repeat in L-selectin (Kahn et al., 1994), the fourth cysteine-rich repeat domain in p55 TNF-R (Nophar et al., 1990), and the EGF structural motif in the membrane-bound, EGF-like growth factors (Holmes et al., 1992), the distance from this domain to the cleavage site varies from three residues (amphiregulin) to 11 residues (EGF), with most in the order of six to eight residues. The start of the first, proximal extracellular domain has not been identified in ACE or APP. Mutational analyses of APP, L-selectin, and p55 TNF-R appear to indicate that the distance from the membrane is more important in determining the cleavage site. On the other hand, sequence analysis of the cleavage sites of multiple isoforms of membrane-bound heregulins indicates that despite considerable variation in the length of the stalk, the cleavage sites are always five to eight residues from the proximal extracellular domain (Figure 6).

Our data suggest that cleavage of ACE is similar to the heregulins, in that the MPSP positions itself primarily with respect to the proximal extracellular domain. Shortening of the stalk in ACE-JMΔ17 by deletion of 14 residues between the cleavage site and the membrane therefore does not alter the cleavage site, provided, presumably, that a minimum distance to the membrane is preserved. From the data in Figure 6 it can be concluded that release of a membrane protein by an MPSP requires an open or accessible stalk region of at least 11 residues in length, allowing a minimum distance of three residues from the proximal extracellular domain and eight residues from the membrane. From this follows the prediction that membrane proteins with stalks less than 11 residues in length are poor MPSP substrates, a prediction that is borne out by P- and E-selectins and by integrin β subunits, which are poorly cleaved and which have stalk lengths of eight, seven, and five residues, respectively (Kahn et al., 1994; Kishimoto et al., 1987). Moreover, this conclusion is strongly supported by mutational studies with L-selectin, in which deletion of four residues from the 15-residue stalk strongly diminishes cleavage and deletion of five, seven, or eight residues abolishes cleavage (Migaki et

[2] ACE is expressed as two isozymes, somatic ACE and testis ACE, that arise by transcription of the same gene from two distinct promoters selected in a tissue-specific manner. The somatic isoform consists of two, homologous domains arranged in tandem; the C-terminal domain, which includes the stalk, transmembrane, and cytoplasmic regions, is identical in sequence to the entire testis enzyme, except for a short, 36-residue extension at the N terminus of, and unique to, testis ACE [reviewed in Ehlers et al. (1992)].

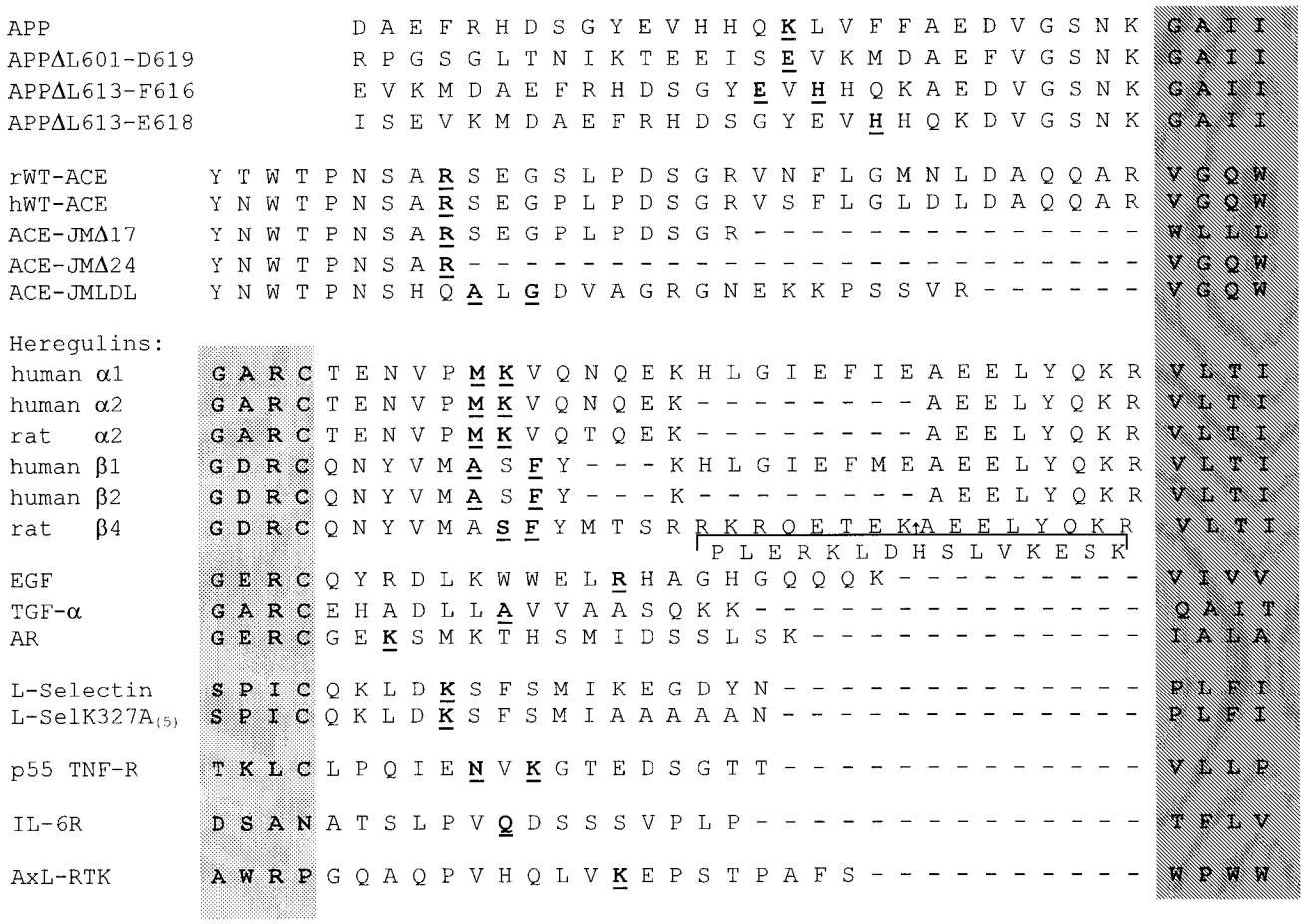

FIGURE 6: Cleavage sites in membrane proteins solubilized by an MPSP activity. Juxtamembrane (stalk) sequences are aligned with respect to either the transmembrane domain (hatched box on right) or the proximal extracellular domain, where known (stippled box on left). Gaps or deletions are represented by dashes, inserted to maintain maximal alignment; the unusually long stalk of the rat β4 heregulin is accommodated as a bracketed insertion. Cleavage sites are indicated by bold, underlined N-terminal residues at the scissile bond (P_1 residues). Abbreviations not defined elsewhere (and references): APPΔL601-D619, APP mutant with deletion from Leu-601 to Asp-619 [numbering based on sequence reported by Kang et al. (1987)] (Maruyama et al., 1991); APPΔL613-F616 and APPΔL613-E618, APP mutant with deletions from Leu-613 to Phe-616 or Glu-618 (Zhong et al., 1994); rWT-ACE, rabbit wild-type ACE (Ramchandran et al., 1994); hWT-ACE, human WT-ACE (this study); heregulin growth factors (Lu et al., 1995); AR, amphiregulin; EGF, TGF-α, and AR (Holmes et al., 1992); L-selectin (Kahn et al., 1994); L-SelK327A$_{(5)}$, L-selectin mutant with substitution of Lys-327 to Tyr-331 with five Ala residues (Migaki et al., 1995); IL-6R, interleukin-6 receptor (Müllberg et al., 1994); Axl-RTK, Axl receptor tyrosine kinase, cleavage site is inferred only (O'Bryan et al., 1995). Identified proximal extracellular domains (references as above): EGF-like growth factors, including heregulins, EGF, TGF-α, and AR, all contain an EGF-like structural motif; L-selectin, last short consensus repeat domain; p55 TNF-R, fourth cysteine-rich repeat domain (Nophar et al., 1990); IL-6R, N-linked glycosylation site; Axl-RTK, second fibronectin type III domain.

al., 1995; Chen et al., 1995). Similarly, in p55 TNF-R deletion of five residues, either including the cleavage site or located C-terminal to it, from the 16-residue stalk abolishes cleavage (Brakebusch et al., 1994).

In contrast to stalk length and accessibility, the amino acid sequence at the cleavage site appears to be less important. Nevertheless, inspection of Figure 6 reveals that there does appear to be a preference for cleavage following Lys or Arg residues, although this is weak. Extensive analysis of site-specific mutations at Lys-16 in APP [numbering with respect to the N terminus of the β-amyloid peptide, see Kang et al. (1987) and Esch et al. (1990)] revealed that most amino acids are tolerated in this position (Sisodia, 1992; Zhong et al., 1994). In L-selectin, deletion of Lys-321 or substitution with Ala, Glu, or Pro did not abolish cleavage (Migaki et al., 1995; Chen et al., 1995); however, it is evident that the Lys is preferred, since the various alterations resulted in a 70%−80% reduction in the rate of release (Chen et al., 1995). Substitution of Lys-174 in p55 TNF-R (the Lys-174/Gly-175 bond may represent the primary cleavage site) with Ala, Glu, or Thr has no effect on cleavage, but substitution with Pro abolishes release (Brakebusch et al., 1994). We find that the ACE-JMLDL mutant is readily cleaved after Ala-628 and/or Gly-630 in spite of the presence of an Arg at position 635.

The kinetics of release of the juxtamembrane deletion mutants of ACE can be interpreted on the basis of the prediction that an 11-residue stalk is the minimum required for efficient cleavage by an MPSP. The ACE-JMΔ47 mutant is not cleaved and released in soluble form, presumably because the entire stalk has been deleted, as discussed earlier. The ACE-JMΔ24 mutant is a poor substrate for the MPSP, presumably because the stalk has been shortened considerably, although not abolished as in ACE-JMΔ47. The 24-residue deletion extends from the membrane to the native cleavage site, and Arg-627 is fused with Val-652. From our data it is likely that the cleavage is at or close to Arg-627 (see Results). This suggests that the distance to the proximal extracellular domain is maintained as it is in WT-ACE and ACE-JMΔ17 and that this distance is the most critical parameter. Cleavage of ACE-JMΔ24 is poor because an adequate distance between the cleavage site and the mem-

brane is not achieved. The dimensions of the stalk in ACE are not known but can be inferred on the basis of these results to be greater than 24 but less than 47 residues. Given that the average distance from the proximal extracellular domain to the MPSP cleavage site is six residues (Figure 6), a reasonable estimate for the length of the ACE stalk is 30 residues, extending from Thr-622 to Arg-651 (Figure 1).

In contrast to ACE-JMΔ24, ACE-JMΔ17 is cleaved more rapidly than WT-ACE. The distance from the cleavage site to the presumptive proximal extracellular domain is maintained, but the distance to the membrane is shortened from 24 to 10 residues. It can be speculated that this shorter interval is more favorable (this is the distance determined in APP, L-selectin, and p55 TNF-R) but that in WT-ACE the longer distance to the membrane is maintained because the interval between the cleavage site and the proximal extracellular domain is more critical.

MPSPs appear to require the substrate protein to be embedded in a membrane. Sisodia (1992) found that α-secretase does not cleave APP mutants in which the entire cytoplasmic domain and 13 or 18 residues of the transmembrane domain are deleted, despite retention of the native cleavage site and an unaltered stalk. Similarly, we find that full-length ACE in solution is not cleaved when incubated with live, intact cells or with various CHO cell fractions. Presumably this indicates that an otherwise suitable membrane protein must be anchored in a membrane before it is recognized as a substrate by the MPSP, which itself is membrane-associated (Sisodia, 1992; Sahasrabudhe et al., 1992; Roberts et al., 1994; Bosenberg et al., 1993; Oppong & Hooper, 1993; Ramchandran et al., 1994; Ramchandran & Sen, 1995). One explanation for this is that the MPSP, analogous to signal peptidases, recognizes a structural or topological motif that includes positioning of the cleavable peptide with respect to a membrane. Alternatively, the stalk becomes inaccessible when the protein is removed from the membrane (Cappelluti et al., 1993).

The subcellular localization of MPSPs may also have a bearing on their sensitivity to protease inhibitors. In a number of instances, MPSPs were found to be resistant to a wide range of inhibitors (Bazil & Strominger, 1994; Kahn et al., 1994; Rutledge et al., 1994; Ramchandran et al., 1994; Müllberg et al., 1994; Ehlers et al., 1995), which may be due to restricted access to the protease. Many MPSPs are metalloproteases, but they are often resistant to standard inhibitors unless appropriately pretreated or unless treated with certain hydroxamate-based inhibitors. For instance, the release of rabbit ACE in transfected mouse C127 cells is not inhibited by a cocktail of inhibitors added to intact cells (Ramchandran et al., 1994), but cleavage of ACE can be blocked in a cell-free membrane preparation (Oppong & Hooper, 1993; Ramchandran & Sen, 1995). Similarly, cleavage of APP can be significantly blocked with metalloprotease inhibitors only in membrane fractions that have been prewashed with detergent, suggesting that there is significant steric hindrance to access of the inhibitors (Roberts et al., 1994). Peptide hydroxamate inhibitors of metalloproteases inhibit the release of membrane-bound TNF-α (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994), p75 TNF-R (Crowe et al., 1995), and the release of rabbit ACE by intact cells (Ramchandran & Sen, 1995). Some membrane-bound growth factors, namely TGF-α and the kit ligands KL-1 and KL-2, are released by an elastase-like serine protease (Pandiella et al., 1992; Cappelluti et al., 1993). Cleavage occurs at elastase-like sites, and, unlike most membrane proteins, cleavage depends on the presence of a cytoplasmic C-terminal valine (Bosenberg et al., 1992).

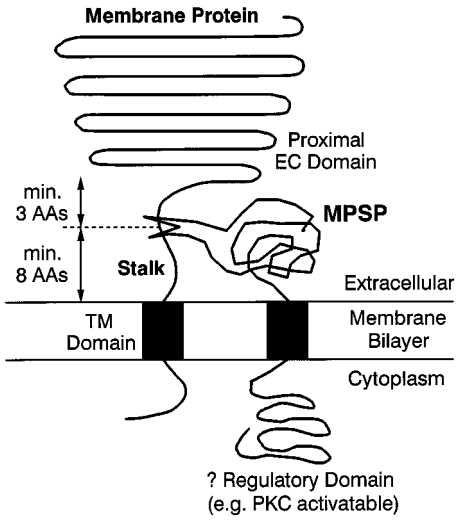

FIGURE 7: Hypothetical model for the CHO cell MPSP. This model is based on the present study and on published reports, as detailed in the text. The salient features are as follows: (1) Susceptible membrane proteins must be anchored in a membrane, usually the plasma membrane. (2) The MPSP is also membrane-anchored, and it is likely but not proven that the substrate membrane protein and MPSP must be co-resident in the same membrane. (3) Cleavage requires an open, accessible stalk of minimum length, predicted to be 11 residues. (4) Cleavage occurs at a minimum (min.) distance of three amino acid (AA) residues from the proximal extracellular (EC) domain and eight AA residues from the transmembrane (TM) domain. (5) The MPSP is subject to regulation, which includes direct or indirect activation by protein kinase C (PKC).

On the basis of the present data, we propose that there are two classes of MPSP. One class (referred to as type I and depicted in Figure 7) consists of metalloproteases that have strict topological requirements for cleavage within an open stalk of minimum length and which have a weak preference for cleavage after Lys or Arg; such proteases are involved in the cleavage of APP, L-selectin, p55 TNF-R, ACE, and presumably many other membrane proteins. A second class (type II) consists of serine proteases with an elastase-like sequence specificity and a dependence on a cytoplasmic C-terminal valine; known substrates are presently limited to TGF-α, KL-1, and perhaps CSF-1. Both classes are membrane-associated proteases that are inducible by a variety of agonists, most notably activators of protein kinase C (Arribas & Massagué, 1995; Bazil, 1995). Moreover, both classes of MPSPs may be separate effector arms of a common (shared) machinery for releasing cell-surface proteins. Recently, mutagenized CHO cell clones have been selected that have lost the capacity for releasing TGF-α, APP, and various unidentified, endogenous CHO cell membrane proteins (Arribas & Massagué, 1995). Since release of APP and TGF-α is by the putative type I and type II MPSPs, respectively, these data argue strongly in favor of a common, "core" machinery that is shared by eukaryotic cells across the phylogenetic spectrum [from yeasts (Zhong et al., 1994) and insect cells (Lowery et al., 1991) to mammalian cells (Arribas & Massagué, 1995)]. This membrane-protein-solubilizing machinery is activated by both protein kinase C-dependent and -independent mechanisms, and it includes at least two, and possibly more, effector proteases, or MPSPs.

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