Phorbol Ester-Induced Juxtamembrane Cleavage of Angiotensin-Converting Enzyme Is Not Inhibited by a Stalk Containing Intrachain Disulfides†

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Received May 27, 1998; Revised Manuscript Received August 10, 1998

ABSTRACT: Specialized proteases, referred to as sheddases, secretases, or membrane-protein-solubilizing proteases (MPSPs), solubilize the extracellular domains of diverse membrane proteins by catalyzing a specific cleavage in the juxtamembrane stalk regions of such proteins. A representative MPSP (tumor necrosis factor-α convertase) was cloned recently and shown to be a disintegrin metalloprotease that is inhibited by peptide hydroxamates including the compound TAPI. Substrate determinants that specify cleavage by MPSPs remain incompletely characterized, but may include the physicochemical properties of the stalk or unidentified recognition motifs in the stalk or the extracellular domain. We constructed a mutant angiotensin-converting enzyme (ACE) in which the stalk has been replaced with an epidermal growth factor (EGF)-like domain (ACE-JMEGF), to test the hypothesis that MPSP cleavage requires an open, comparatively unfolded or extended stalk. Wild-type ACE is a type I transmembrane (TM) ectoprotein that is efficiently solubilized by a typical MPSP activity. We found that ACE-JMEGF was solubilized inefficiently and accumulated in a cell-associated form on transfected Chinese hamster ovary (CHO) cells; cleavage was stimulated by phorbol ester and inhibited by TAPI, features typical of MPSP activity. Determination of the C-terminus of soluble ACE-JMEGF revealed that, surprisingly, cleavage occurred at a Gly-Phe bond between the fifth and sixth cysteines within the third disulfide loop of the EGF-like domain. Reduction of intact CHO cells with tributylphosphine resulted in the rapid release of ACE-JMEGF (but not wild-type ACE) into the medium, suggesting that a proportion of membrane-bound ACE-JMEGF is cleaved but remains cell-associated via disulfide tethering. The mechanism for the release of ACE-JMEGF in the absence of chemical reduction is unclear. We conclude that the presence of a compact, disulfide-bridged domain does not per se inhibit cleavage by an MPSP activity, but ectodomain release is prevented by disulfide tethering to the TM domain.

The regulated proteolytic release of membrane protein ectodomains by a specific cleavage in the juxtamembrane stalk region appears to be a universal characteristic of eukaryotic cells. This process, often referred to as "shedding", affects multiple classes of integral membrane proteins and serves diverse biological roles, including modulation of ligand−receptor interactions and cell adhesion, and delivery of enzymes and receptors to distant sites (for review, see refs *1*−*4*). Several characteristics of regulated ectodomain release are now well-established, including induction of shedding by phorbol esters, calcium ionophores, and unidentified serum factors; inhibition of the shedding protease by hydroxamate-based metalloprotease inhibitors; localization of the shedding event at the cell surface; and structural requirements of the juxtamembrane stalk that determine cleavage efficiency (*5*−*7*). Recently, a candidate "sheddase" (i.e., a membrane-protein-solubilizing protease, MPSP[1]) that solubilizes membrane-bound pro-tumor necrosis factor-α (TNF-α) was isolated and cloned (*8, 9*); sequence analysis revealed it to be a disintegrin metalloprotease. It remains to be established whether the TNF-α-converting enzyme is a general MPSP or one of a family of related, hydroxamate-

† Supported by grants from the Volkswagen Foundation, Roche Research Foundation, and the South African Medical Research Council (M.R.W.E.) and a grant from the Foundation for Research Development (W.F.B.).

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[1] Abbreviations: ACE, angiotensin-converting enzyme; WT-ACE, wild-type ACE; ACE-JMEGF and ACE-JMΔ17, juxtamembrane mutants with a substitution of the wild-type stalk with an EGF-like domain or with a deletion of 17 residues, respectively; CHO, Chinese hamster ovary; EC, extracellular; EGF, epidermal growth factor; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MPSP, membrane-protein-solubilizing protease; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; p55 and p75 TNF-R, 55-kDa and 75-kDa tumor necrosis factor receptor; phorbol ester, phorbol-12,13-dibutyrate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAPI, TNF-α protease inhibitor; TGF-α, transforming growth factor-α; TM, transmembrane; TNF-α, tumor necrosis factor-α.

10.1021/bi981260k CCC: $15.00
Published on Web 10/10/1998

inhibitable proteases. A second disintegrin metalloprotease, the product of the *Drosophila kuz* gene, has also been shown recently to cleave the Notch receptor, likely at a juxtamembrane site (*10*).

Of considerable interest are the structural determinants in target membrane proteins that confer susceptibility to cleavage by an MPSP. Clearly, not all cell surface proteins are shed; for instance, the entire family of integrins, the P- and E-selectins (in contrast to L-selectin), and the epidermal growth factor (EGF) receptor are not shed (*11*−*13*). However, the reasons for this are not clear, particularly as MPSPs do not appear to be sequence-specific (reviewed in refs *7*). One possibility is that susceptible membrane proteins contain recognition motifs that are critical for MPSP activation. One such motif was thought to comprise a cytoplasmic C-terminal valine residue in pro-transforming growth factor α (TGF-α) (*14*), but it has since been established that the C-terminal valine is required for maturation and cell-surface expression of TGF-α and not for MPSP shedding (*15*). Indeed, cytoplasmic domains appear to be dispensable for MPSP-dependent release in all membrane proteins in which this has been examined (*13, 16*−*18*).

Instead, a recognition motif may reside in the extracellular (EC) domain, either proximal or distal to the stalk cleavage site. Data obtained with the release of wild-type and stalk-mutated testis angiotensin-converting enzyme (ACE) expressed in Chinese hamster ovary (CHO) cells suggested that the MPSP positioned itself with respect to the proximal EC domain (*7*); this model implies the existence of a recognition motif in this domain. This notion is supported by a recent study indicating that the EC domain of testis ACE enables the regulated cleavage of the juxtamembrane region of CD4, a protein not usually susceptible to MPSP cleavage (*18*). Moreover, the structure of the TNF-α-converting enzyme catalytic domain suggested an arrangement in which the folded compact cone of trimeric pro-TNF-α may be specifically recognized, with positioning of the stalk scissile bond within the catalytic cleft at a distance of 8 residues from the TNF-α cone and 20 residues from the membrane (*19*). This arrangement is remarkably similar to the positioning and recognition requirements inferred for the stalk processing of membrane-bound ACE (*7, 18*).

An alternative or additional mechanism controlling the susceptibility to MPSP cleavage is the steric accessibility of the juxtamembrane stalk. Numerous studies have indicated an apparent requirement for an unhindered stalk of minimum length for efficient MPSP cleavage (ref *7*, and refs cited therein). This is an attractive concept because noncleavable proteins such as integrin β subunits and P- and E-selectins contain proximal, cysteine-rich EC domains that are immediately adjacent to the membrane with minimal or no stalks (*12, 20*). To test this, we constructed a testis ACE mutant in which the wild-type stalk, including the native cleavage site, was replaced with a 37-residue epidermal growth factor (EGF)-homology domain, and we stably expressed the mutant in CHO cells. Compared to wild-type ACE and the ACE-JMΔ17 deletion mutant (*7*), the ACE-JMEGF mutant was released poorly and accumulated on the cell surface; however, release was induced by phorbol ester and inhibited by TAPI. Surprisingly, cleavage occurred at a Gly-Phe bond located between the fifth and sixth cysteines in the EGF-homology domain, 11 residues proximal to the transmembrane (TM) domain. Hence, poor release of ACE-JMEGF was not due to steric hindrance of the phorbol ester-induced, TAPI-inhibitable MPSP, but to residual disulfide tethering of the ectodomain to the membrane-embedded C-terminal stump.

MATERIALS AND METHODS

Construction of Expression Vector and CHO Cell Transfection. Construction of pLEN-ACE-JMEGF, which codes for a mutant ACE in which the wild-type stalk is replaced by an EGF homology domain, was achieved with a two-stage PCR strategy similar to that used previously for pLEN-ACE-JMLDL (*7*). The sequence encoding Glu-296 to Glu-332 of the low-density lipoprotein receptor (LDL-R), which represents the first of three EGF-like units in the EGF precursor homology domain of LDL-R (*21*), was amplified using suitable primers. The PCR product was ligated into pLEN-ACE-JMΔ24 (*7*) digested with *Eco*RI at a unique site (nucleotide 1984), to produce pLEN-ACE-JMEGF in which the sequence encoding Ala-626 to Ala-650 (comprising the wild-type ACE stalk and the native MPSP cleavage site at Arg-627/Ser-628) was replaced with a sequence encoding a 37-residue EGF homology domain. CHO-K1 cells were grown and transfected with 10 μg of pLEN-ACE-JMEGF and 2 μg of pSV2NEO by methods detailed previously (*7, 22*).

Determination of the Juxtamembrane Cleavage Site in Released ACE-JMEGF. After selection for stable transfectants, soluble and membrane-bound ACE-JMEGF's were isolated from the conditioned media and detergent extracts, respectively, of CHO cells, and purified to electrophoretic homogeneity by affinity chromatography on a Sepharose-28-lisinopril affinity resin (*22, 23*). Purified soluble (released) ACE-JMEGF (1 nmol) was subjected to reduction and *S*-β-4-pyridylethylation of cysteine residues, as detailed elsewhere (*24*), and digested at 37 °C with endoproteinase Lys-C (1 μg) for 18 h. The digest was fractionated by reversed-phase high-performance liquid chromatography (HPLC), and eluted fractions were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Reflex II time-of-flight mass spectrometer (Bruker, Bremen, Germany) using a nitrogen laser at 337 nm and a 2,5-dihydroxybenzoic acid matrix, by methods described previously (*7*). The identity of the C-terminal peptide was confirmed by N-terminal peptide sequencing by automated Edman degradation (5 cycles).

Assay of ACE Activity and Kinetics of ACE Release. Membrane-bound and soluble ACE activities were assayed in detergent extracts or conditioned medium, respectively, of transfected cells using the substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu), as described (*7*). Kinetic analyses of rates of accumulation of soluble activity and changes in membrane-bound activity were performed by time-course studies as described previously (*7, 25*). Studies were performed in the presence and absence of 1 μM phorbol 12,13-dibutyrate, or in the presence of 0.01−100 μM TAPI (for "TNF-α protease inhibitor"; also referred to as compound 2, *N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2′naphthyl)-alanyl-L-alanine 2-aminoethyl amide), a hydroxamate-based inhibitor of the pro-TNF-α-converting metalloprotease (*26*).

Metabolic Labeling and Pulse-Chase Experiments. Stably transfected cells were grown to confluence in standard complete media (50% Ham's F-12/50% DME medium supplemented with 20 mM Hepes, pH 7.3, 20 mM L-glutamine, 2% fetal calf serum, and 80 μM $ZnCl_2$) (*22*, *27*). The cells were then incubated for 30 min in cysteine- and methionine-free minimum essential Eagle medium containing 2% dialyzed fetal calf serum, followed by incubation in the same medium containing 50 μCi/mL [^{35}S] methionine-cysteine for a further 30 min at 37 °C. The cells were chased in complete medium, with or without phorbol ester, for up to 24 h. At the indicated time points, conditioned media were harvested and cells were lysed in 50 mM Tris, pH 8.0, 1% Triton X-100. Soluble and cell-associated ACE proteins were specifically precipitated from the media and cell lysate samples, respectively, by a technique of "affinity precipitation" using lisinopril-agarose beads, as follows. After a 10-min centrifugation in a microfuge, samples (~0.3 mL) were mixed with ~40 μL of a slurry (50% v/v) of Sepharose-28-lisinopril affinity resin (*23*) in 20 mM Hepes, pH 7.5, 0.3 M NaCl, and incubated with continuous agitation at room temperature for 30 min. The affinity beads were then washed in the same buffer three times, mixed with 50 μL of 2× sodium dodecyl sulfate (SDS) sample buffer, and analyzed by SDS−10% polyacrylamide gel electrophoresis (PAGE) followed by autoradiography.

Reduction of Cell-Associated ACE-JMEGF. Membrane-bound ACE-JMEGF was purified from detergent extracts of CHO cell lysates and then reduced in 1% β-mercaptoethanol at 100 °C for 5 min. The reduced protein was analyzed by SDS−PAGE. In separate experiments, cell-associated ACE-JMEGF was reduced in situ by the direct addition of tributylphosphine (*28*) to the media of transfected cells. ACE-JMEGF- and WT-ACE-expressing cells were grown to confluence in complete medium, washed, and refed with complete medium containing 0.0625%−1.0% tributylphosphine, in the absence or presence of 100 μM TAPI. After a 2-h incubation, the conditioned media were assayed for ACE activity.

RESULTS

Expression of ACE-JMEGF in CHO Cells and Kinetics of Release. Several independent transfections of CHO cells with pLEN-ACE-JMEGF yielded a number of stable lines, each exhibiting a similar pattern. The extent of release of ACE-JMEGF and the accumulation of soluble protein in the conditioned media over a 24-h period were poor (Figure 1); in comparison to wild-type ACE (WT-ACE) the levels of soluble activity were 2−3-fold lower, a pattern previously seen with the stalk deletion mutant ACE-JMΔ24 (*7*). However, in contrast to the latter, the levels of membrane-bound (cell-associated) ACE-JMEGF activity were high, approximately 2-fold higher than the WT-ACE levels, suggesting that the rate of release was reduced. This difference from the wild-type protein was further examined by treatment of the transfected cells with phorbol ester, which typically leads to a marked enhancement of WT-ACE release with a concomitant reduction in the levels of membrane-bound protein (*7*, *25*). Phorbol ester stimulation resulted in a reproducible, 3-fold increase in the initial rate (first 60 min) of release of ACE-JMEGF, compared to a 5-fold increase of WT-ACE release (Figure 1). However, compared to the 2.5-fold decrease in membrane-bound WT-ACE (maximal at 2 h), there was either no change or only a modest change in membrane-bound ACE-JMEGF levels (Figure 1).

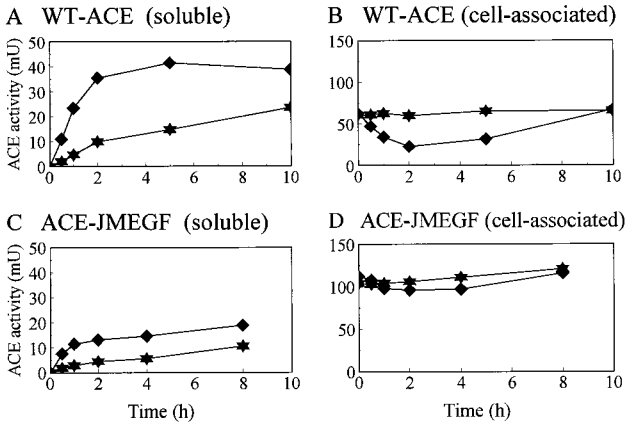

FIGURE 1: Kinetics of solubilization (A, C) and changes in membrane-bound levels (B, D) of wild-type ACE (A, B) and the stalk mutant ACE-JMEGF (C, D) expressed in CHO cells. Stably transfected cells were grown in the presence (diamonds) or absence (stars) of 1 μM phorbol-12,13-dibutyrate. Media samples (soluble activity) and detergent lysates (cell-associated activity) were collected at the indicated time points and assayed with the ACE substrate Hip-His-Leu. ACE-JMEGF results are from one of four independent, stably transfected cell lines that were analyzed; all cell lines exhibited the same trends and absolute values varied by <20%. Wild-type ACE results are similar to those reported previously (*7*, *25*). Each time point was performed in triplicate, and data varied <10%.

Inspection of the release curves of ACE-JMEGF revealed that they followed the typical pseudo-first-order kinetics previously described for WT-ACE and ACE-JMΔ17 and -JMΔ24, which have been interpreted to reflect a combination of substrate (membrane-bound protein) depletion and a transient burst of stimulated release; this burst is induced by both phorbol ester and simple media change (*7*, *25*). The pattern of expression and the kinetic data gave the appearance that membrane-bound ACE-JMEGF was relatively resistant to release, with resultant accumulation of the cell-associated form; however, the release mechanism had the characteristics previously described for WT-ACE, notably enhancement by phorbol ester.

Inhibition of Phorbol Ester-Induced Release of ACE Proteins by TAPI. Hydroxamate-based inhibitors of matrix metalloproteases have been shown to inhibit the release of a variety of membrane proteins expressed in CHO cells (*5*), and TAPI inhibited the shedding of rabbit testis ACE expressed in mouse C127 cells (*29*). We examined the effect of TAPI on the rate of solubilization of membrane-bound WT-ACE, ACE-JMEGF, and ACE-JMΔ17 [a rapidly released stalk-deletion mutant (*7*)], all stably expressed in CHO cells; experiments were performed in the presence and absence of phorbol ester.

Direct comparisons of WT-ACE, ACE-JMEGF, and ACE-JMΔ17 revealed that, in each case, the generation of soluble protein was inhibited by TAPI in a dose-dependent manner with an IC_{50} of ~10 μM, in the presence and absence of phorbol ester (Figure 2). In the presence of phorbol ester, the addition of TAPI resulted in a dose-dependent increase in levels of membrane-bound WT-ACE and ACE-JMΔ17 (Figure 2) indicating that TAPI inhibition led to an accumulation of uncleaved, cell-associated protein; a small but

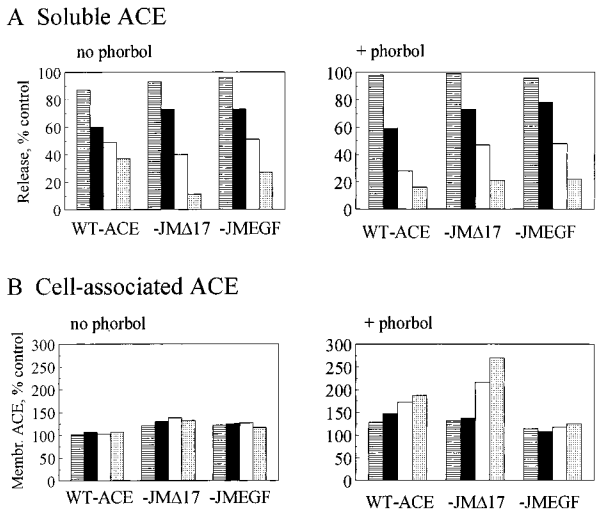

FIGURE 2: Inhibition of phorbol ester-induced solubilization of ACE proteins by the hydroxamate metalloprotease inhibitor TAPI. CHO cells stably expressing WT-ACE, ACE-JMΔ17, and ACE-JMEGF were grown to confluence, washed, and refed with media containing 0.07, 0.7, 7.0, or 70 μM TAPI (hatched, solid, open, and stippled bars, respectively), in the absence (left panels) or presence (right panels) of 1 μM phorbol ester. After a 4-h incubation, media samples (A, soluble activity) and detergent lysates (B, cell-associated activity) were assayed with the substrate Hip-His-Leu. Results are expressed as a percentage relative to the control (zero TAPI), taken as 100% in each case (not shown). Data are from one of two experiments with essentially identical results; each concentration was performed in triplicate, and data varied by <10%.

consistent increase in membrane-bound ACE-JMEGF (25%) was detected at the highest concentration of TAPI (70 μM) (Figure 2). These effects were not the result of a nonspecific cytotoxic effect of TAPI. At the highest concentration tested (70 μM), TAPI did not affect cell viability after 4 h, as assessed by Trypan blue exclusion (>99% viable).

Metabolic Labeling and Pulse-Chase Analysis of ACE Release. The kinetic data based on changes in ACE activity in the medium and in the cell lysate fractions indicated that the ACE-JMEGF mutant accumulated to high levels in a membrane-bound form, and that it was released (shed) at a reduced rate (compared to WT-ACE), albeit in a typical phorbol ester- and TAPI-dependent manner. To investigate this further and to exclude the possibility that the differences in kinetics of ACE-JMEGF release compared to WT-ACE release were due to differences in intracellular processing and maturation rather than due to differences in ectodomain cleavage, we performed metabolic labeling studies. Labeled ACE proteins were visualized by a novel technique we call affinity precipitation, in which ACE was specifically precipitated by binding to the tight-binding, active site-directed inhibitor lisinopril immobilized on agarose beads. Cells were pulsed for 30 min and then chased at intervals up to 10 h.

Analysis of affinity-precipitated cell lysates of WT-ACE-expressing cells revealed a 90-kDa protein at the early time points (between 0 and 2 h) and a larger, 105-kDa protein that appeared at 30 min and persisted up to 24 h in the absence of phorbol ester (Figure 3A). Affinity precipitation of the media revealed only the larger, 105-kDa protein, which was visible after a 2-h chase and then grew in intensity up to 24 h. In the presence of phorbol ester the same species were seen, but in affinity precipitates of cell lysates, the 105-kDa protein declined much more rapidly from 2 h onward,

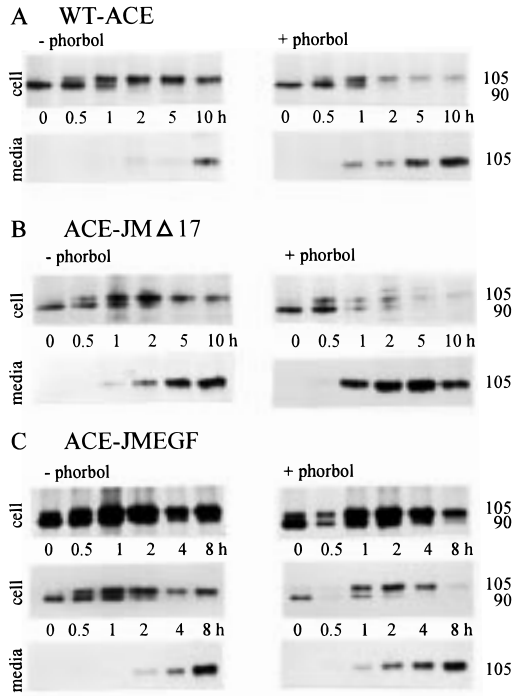

FIGURE 3: Metabolic labeling and pulse-chase analysis of the biosynthesis and release (solubilization) of WT-ACE (A), ACE-JMΔ17 (B), and ACE-JMEGF (C). Stably transfected CHO cells were grown to confluence, pulsed for 30 min in medium containing 50 μCi [^{35}S]-methionine and [^{35}S]-cysteine, washed, refed in complete medium with (right panels) or without (left panels) 1 μM phorbol ester, and chased for up to 10 h. At the indicated times, cell lysate and media samples were affinity-precipitated with lisinopril-28-Sepharose, subjected to SDS−PAGE, and autoradiographed. Estimated molecular weights (in kDa) of the major bands are indicated on the right.

whereas in the media a much stronger band at 105 kDa was observed, which was already detected at 1 h (Figure 3A). These results are consistent with the interpretation that, in the cell lysates, the smaller 90-kDa protein is an immature form that is processed to a mature, 105-kDa form, which in turn is cleaved to release a soluble, 105-kDa protein into the media. [Close inspection of the mature cell lysate protein and the media protein revealed that, when run side by side, the latter sometimes appeared smaller, at 100 kDa (not shown). However, as reported previously (*7*), we did not consistently observe a difference in electrophoretic mobility between the cell-associated and soluble proteins, likely due to heavy glycosylation.] Phorbol ester stimulates the conversion of the mature cell-associated protein into the soluble protein, but does not significantly alter the rate of maturation of the cell-associated protein. Our data are qualitatively and quantitatively similar to those reported previously for rabbit testis ACE expressed in mouse C127 cells (*30*) and for a testis isozyme-like C-terminal domain construct of human somatic ACE expressed in CHO cells (*31*).

Pulse-chase analysis of the rapidly shed ACE stalk mutant ACE-JMΔ17 (*7*) gave results that were qualitatively similar to those for WT-ACE, particularly in terms of the presumed precursor−product relationship between the immature, 90-kDa protein and the mature, 105-kDa protein (Figure 3B). As expected from kinetic studies based on levels of ACE activity (*7*), however, the intensity of the soluble protein in the medium was greater at all time points, in the presence and absence of phorbol ester, and the cell-associated, mature

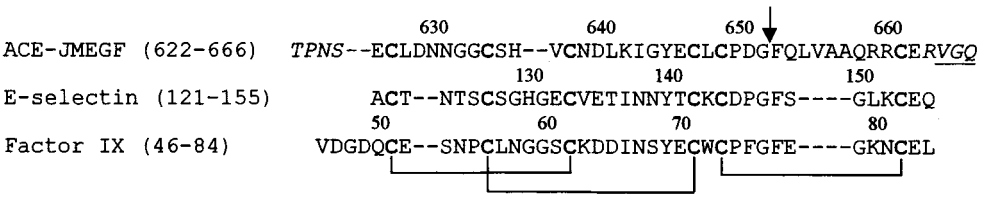

FIGURE 4: Sequence of the EGF-like domain in ACE-JMEGF and alignment with related domains in E-selectin and factor IX. The most N-terminal EGF-like unit (Glu-296 to Glu-332) in the EGF precursor homology domain of LDL-R (*21*) was inserted into ACE-JMEGF, replacing the wild-type stalk (Ala-626 to Ala-650); numbering is consecutive with respect to the testis ACE sequence (*7*), and flanking ACE sequences, including the start of the TM domain (underlined), are in italics. The MPSP cleavage site in ACE-JMEGF is indicated by an arrow. The factor IX EGF-like domain corresponds to Val-46 to Leu-84 in factor IX (*34*) and is numbered accordingly; similarly, the E-selectin EGF-like domain corresponds to Ala-121 to Gln-155 in E-selectin (*36*). The conserved cysteine residues are in bold, and the disulfide bonding pattern is indicated.

protein declined more rapidly, compared to WT-ACE (Figure 3A, and B). These data are consistent with the previous conclusion that the ACE-JMΔ17 mutant is a more favorable substrate for the CHO cell MPSP than WT-ACE (*7*).

Pulse-chase analysis of ACE-JMEGF revealed some interesting differences from both WT-ACE and ACE-JMΔ17. Affinity precipitates of cell lysates in the absence of phorbol ester showed strong bands at the 90-kDa and 105-kDa positions at all time points following a standard autoradiography exposure; even after a short exposure, mature protein was visible already at time 0 of the chase (Figure 3C). In the presence of phorbol ester, there was a more evident decline in the mature protein by 8 h of the chase. In marked contrast to the high levels of cell-associated, mature protein at all time points, the amounts of soluble protein in the media were comparatively modest, although the rate of appearance of protein in the media appeared to parallel that seen for WT-ACE (Figure 3, A and C). We interpret these data to mean that the ACE-JMEGF mutant accumulates to high levels on the cell surface, partly because cleavage and release are comparatively inefficient.

Determination of the Juxtamembrane Cleavage Site in Released ACE-JMEGF. Denatured, reduced, and vinylpyridine-protected soluble ACE-JMEGF was digested with endoproteinase Lys-C and fractionated by HPLC, and peptides were analyzed by MALDI-TOF mass spectrometry (Table 1). The spectra revealed, inter alia, $[M + H]^+$ ions at *m*/*z* of 3580.1 and 1279.6, which corresponded closely to the theoretical masses of the peptides LGWQYNWTPNSEBLDNNGGBSHVBNDLK (3579.57) and IGYEBLBPDG (1279.55) (where B represents *S*-*β*-4-pyridylethylated cysteine residues), respectively, which constitute most of the EGF homology domain. IGYEBLBPDG was identified as the C-terminal peptide because it was the only peptide that did not contain a C-terminal lysine, and no other peptides C-terminal to it in the ACE-JMEGF sequence were detected in the mass spectra, including in a spectrum of the total endoproteinase Lys-C digest in which most of the theoretical peptides were identified (Table 1). Moreover, the IGYEBLBPDG peptide corresponded to a single peak on the HPLC chromatogram; this fraction gave a single mass on mass spectral analysis, and N-terminal sequence analysis (5 cycles) of this fraction gave the sequence IGYEB. Hence, we conclude that the MPSP cleavage in the juxtamembrane region in ACE-JMEGF is between Gly-652 and Phe-653, 11 residues proximal to the start of the TM domain (Figure 4). Remarkably, this cleavage is between the fifth and sixth cysteine residues of the EGF homology domain.

Table 1: Observed $[M + H]^+$ Ions of C-Terminal ACE-JMEGF Peptides Generated by Endoproteinase Lys-C Digestion[a]

predicted Lys-C peptide (residue no.)	peptide number	calculated *m*/*z*	
		expected	observed
369−395	19	3339.6	3339.7
396−425	20	3073.6	3073.3
426−449	21	2693.3	2692.9
450−454	22	577.3	na[b]
455−478	23	2944.6	2944.3
479−491	24	1765.9	1766.4
492−511	25	2251.1	2251.0
512−549	26	4491.3	na[b]
550−556	27	961.5	961.5[c]
557−567	28	1175.6	1175.8[c]
568−597	29	3358.6	nf[d]
598−613	30	1950.0	1950.5
614−642	31	3579.6	3580.1
643−652	32	1279.6	1279.6[c]

[a] Soluble (released) ACE-JMEGF was purified from the conditioned medium of transfected CHO cells and digested with endoproteinase Lys-C. The total digest was analyzed directly by MALDI-TOF mass spectrometry or after prior fractionation by reverse-phase HPLC. Only peptides identified in the C-terminal half of the protein are shown (peptides 19−32 as predicted from the primary structure). All mass values are calculated for protonated monoisotopic molecular weights *m*/*z*. Peptide 32 (residues 643−652, see Figure 4) was the only peptide identified that did not end in a lysine. No peptides C-terminal to peptide 32 were found. The identities of peptides 31 and 32 were confirmed by N-terminal sequencing. [b] Masses <900 or >4000 were not analyzed (na). [c] HPLC-purified peptides. [d] Not found.

Reduction of Cell-Associated ACE-JMEGF. As the disulfide-bonding pattern in EGF homology domains is Cys 1−3, 2−4, and 5−6 (*32*) (see Figure 4), we wondered whether the soluble ACE-JMEGF was generated by cleavage of a population of cell-surface protein with incomplete or aberrant EGF disulfide bonding, or whether cleavage of intact, disulfide-bonded ACE-JMEGF was occurring but remained occult because of residual disulfide tethering to the membrane-attached stalk. To test this we performed two experiments. First, membrane-bound ACE-JMEGF was purified from detergent extracts of cell lysates, reduced with *β*-mercaptoethanol, and resolved by SDS−PAGE. Analysis of the gel revealed a band at ∼7.3 kDa that appeared after treatment with *β*-mercaptoethanol but that was not evident without reduction (not shown); its relative migration agreed closely with an expected size of 7150 Da for the C-terminal fragment after cleavage at the Gly-652/Phe-653 bond.

Second, CHO cells stably expressing ACE-JMEGF were grown to confluence, and then treated for 30 min with the reducing agent tributylphosphine. This resulted in a striking, dose-dependent increase in the levels of soluble ACE-

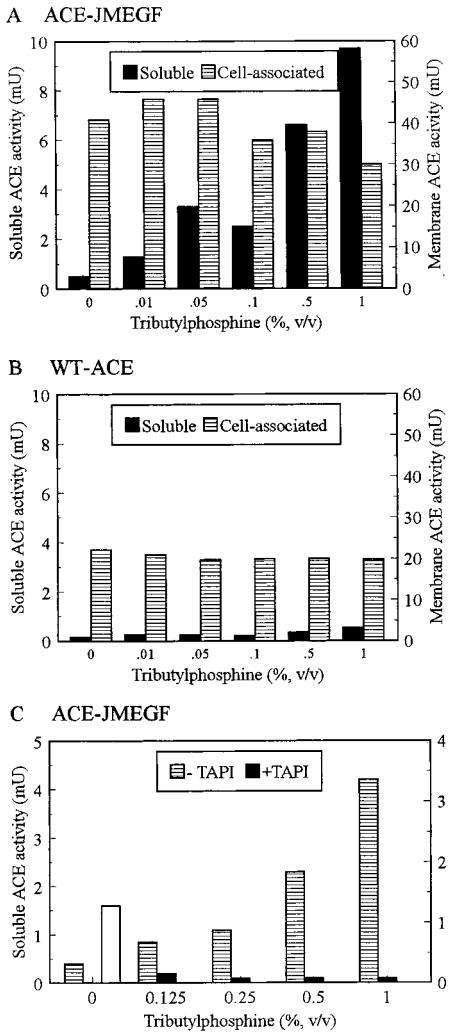

FIGURE 5: Treatment of CHO cells expressing ACE-JMEGF with the reducing agent tributylphosphine. Transfected cells expressing ACE-JMEGF (A) or WT-ACE (B) were grown to confluence, washed, and incubated in PBS containing up to 1% tributylphosphine for 30 min. Media samples (solid) and cell lysates (hatched) were assayed with the ACE substrate Hip-His-Leu. (C) Cells expressing ACE-JMEGF were grown to confluence, washed, incubated in PBS containing 0.25% tributylphosphine for 10 min, washed, and refed in complete medium containing up to 1% tributylphosphine, with or without 100 μM TAPI (solid and hatched bars, respectively). All cells were incubated for 2 h; those cells exposed to TAPI were also treated with 1 μM phorbol ester after the first 30 min of incubation, as were control cells that were incubated in medium only without TAPI or tributylphosphine (open bar). In all cases, media samples were assayed with Hip-His-Leu. Results in (A) are from one of three experiments that gave similar results; experiments in (B) and (C) were performed once. In all cases, each concentration of tributylphosphine was performed in triplicate and data varied by <10%.

JMEGF activity in the conditioned media (up to a 10-fold increase at 1% tributylphosphine), and a concomitant, modest decrease in the levels of cell-associated ACE-JMEGF activity (up to 35%) (Figure 5A). Similar treatment of CHO cells expressing WT-ACE had virtually no effect (Figure 5B), indicating that the effect of tributylphosphine was not the result of a general activation of MPSP activity. [Although tributylphosphine inhibited the ACE enzyme activity itself, this was only significant (≥50%) after a 4 h incubation in ≥0.5% tributylphosphine (not shown).] The increased soluble ACE-JMEGF activity derived from a fraction of membrane-bound protein that had undergone proteolytic (i.e., MPSP) cleavage (although the ACE remained cell-associated prior to reduction), because the release of this activity could be completely abolished by 100 μM TAPI, even in the presence of phorbol ester (Figure 5C). In this experiment, cells were first treated with 0.25% tributylphosphine to release precleaved protein, washed, and then incubated for 2 h with tributylphosphine in the presence or absence of TAPI (Figure 5C).

We conclude from these experiments that a significant fraction of membrane-bound ACE-JMEGF is cleaved by the CHO cell MPSP, apparently at the Gly-652/Phe-653 bond between disulfide-bridged cysteines 5 and 6. The cleaved protein accumulates on the cell surface because it remains disulfide-linked to the residual stalk and TM domains. In situ reduction results in the release of this precleaved protein into the medium. It is unclear why soluble ACE-JMEGF can be detected in the media in the absence of reduction. It is possible that a fraction of full-length ACE-JMEGF contains incompletely folded EGF-like domains such that the sixth cysteine is not involved in a disulfide bond. This fraction of ACE-JMEGF would then be released into the medium directly following MPSP cleavage.

DISCUSSION

Although numerous and diverse membrane proteins are released by a specific juxtamembrane cleavage (*1*), many membrane proteins are not. Indeed, in CHO cells it has been estimated that only 2%−4% of resident cell surface proteins are shed by regulated cleavage (*5*). A complete inventory of cleaved versus uncleaved membrane proteins is not available, but of the latter we are aware of some notable examples, which include the entire family of integrin cell adhesion proteins, EGF receptors, and P- and E-selectins. The last two are particularly interesting, because the third member of the selectin family, L-selectin, is a well-known example of a shed membrane protein (*12, 16, 33*). A possible explanation (*7*) for the difference in cleavage susceptibility between L-selectin and P- and E-selectin is stalk length: L-selectin contains a 15-residue stalk, whereas P- and E-selectin contain 8- and 7-residue stalks, respectively; similarly, integrin subunits contain a 5-residue stalk. We have argued, on the basis of numerous studies, that stalk lengths of less than 11 residues result in inefficient or no stalk cleavage by MPSPs, due to presumed steric requirements for stalk access (*7*).

In membrane proteins that are cleaved by an MPSP, stalk sequences of at least 11 residues are interposed between the TM domain and a proximal EC domain that is frequently cysteine-rich and presumed to be multiply disulfide-bonded, such as in the EGF-like family of membrane-bound growth factors, p55 TNF receptor, and L-selectin (*7*). In cases where the proximal, cysteine-rich EC domain is brought closer to the TM domain, as is the case in P- and E-selectin or in stalk deletion mutants of L-selectin and p55 TNF receptor, MPSP cleavage is reduced or absent (*12, 13, 33*). This led us to speculate that a disulfide-bonded domain close to or physically joined to the TM domain would be particularly unfavorable for MPSP cleavage and that this is the explanation for the observed resistance to cleavage by P- and E-selectin and integrin subunits; the latter contain a disulfide-

bonded cysteine residue that is within 5 residues of the TM domain. To test this, we constructed ACE-JMEGF, in which the cleavable stalk of testis ACE, an efficiently shed membrane protein, was replaced by an EGF homology domain, and the mutant was stably expressed in CHO cells.

Analysis of the distribution of the ACE-JMEGF protein between membrane-bound and soluble forms, both by assays for ACE activity and by metabolic labeling and affinity precipitation, revealed that the ACE-JMEGF mutant accumulated to high levels on the cell surface but that release of a soluble form into the conditioned medium was comparatively inefficient. This was evident when ACE-JMEGF was compared to WT-ACE and to the rapidly shed stalk-deletion mutant ACE-JMΔ17. These results were consistent with the prediction that a stalk composed of a well-ordered, disulfide-bonded domain is relatively resistant to cleavage by the CHO cell MPSP and inhibits shedding of the ectodomain.

However, although inefficient, solubilization of the ACE-JMEGF ectodomain nevertheless occurred and exhibited two typical features of MPSP cleavage in mammalian cells, namely, (a) enhancement of release rates by phorbol ester stimulation, which gave the appearance of a "burst" with apparent first-order kinetics; and (b) inhibition of release by the hydroxamate-based metalloprotease inhibitor TAPI. This led to the important question of the location of the cleavage site. Digestion of purified soluble ACE-JMEGF with endoproteinase Lys-C and analysis of fractionated peptides by MALDI-TOF mass spectrometry indicated that the C terminus of soluble ACE-JMEGF was at Gly-652. This surprising result placed the cleavage site at the Gly-652/Phe-653 bond located between the fifth and sixth cysteines in the EGF homology domain, which are thought to be disulfide linked. How could the cleaved but apparently disulfide-anchored ACE-JMEGF ectodomain appear as a soluble form in the conditioned medium? We considered two possibilities, which are not mutually exclusive: (a) a fraction of membrane-bound ACE-JMEGF proteins contains incompletely or aberrantly folded EGF homology domains with an extended juxtamembrane sequence accessible to the MPSP and no disulfide bond between cysteines 5 and 6; or (b) the MPSP is able to cleave a proportion of membrane-bound ACE-JMEGF containing fully folded juxtamembrane EGF homology domains, of which a small fraction is released as soluble protein, possibly following disulfide rearrangements. If the second possibility is true, it can be predicted that a fraction of membrane-bound ACE-JMEGF is cleaved but remains cell-associated via disulfide bonding, which can be disrupted by chemical reduction.

In vitro reduction of membrane-bound ACE-JMEGF purified from detergent-extracted cell lysates resulted in the release of a 7.3-kDa peptide that matched the size predicted (7.1 kDa) for the residual cleaved stalk and TM and cytoplasmic domains. Moreover, treatment of intact, ACE-JMEGF-expressing CHO cells with the reducing agent tributylphosphine resulted in rapid release of a large amount of soluble ACE-JMEGF. The release into the medium was blocked by the addition of TAPI, indicating that the source of soluble ACE-JMEGF after tributylphosphine reduction was proteolytically cleaved but membrane-associated protein, and not protein that was released in a nonspecific, MPSP-independent manner (e.g., as a result of tributylphosphine-induced membrane damage). Moreover, treatment of CHO cells expressing WT-ACE did not result in enhanced release, indicating that tributylphosphine did not induce a general increase in MPSP acitivity. These results suggested that a proportion of membrane-bound ACE-JMEGF was cleaved in the juxtamembrane region but remained cell-associated by a reduction-sensitive mechanism, presumably by a disulfide bridge between cysteines 5 and 6 in the EGF homology domain. The nature of the spontaneous (reduction-independent) release mechanism of a fraction of cleaved ACE-JMEGF is unknown.

All EGF-like domains for which crystal or solution structures exist have been shown to assume compact structures constrained by the three disulfide bonds, but with variability in the loops between cysteines. In particular, the N-terminal two-thirds of EGF domains, including the first two disulfide bridges, are folded into two closely packed antiparallel β sheets (e.g., see refs *34*, *35*−*37*); this fold has recently been classified as a disulfide β-cross (*38*). It is highly likely that the LDL-R EGF-like domain assumes a similar general structure, and it is significant that the MPSP does not cleave the compact β-cross fold (corresponding to Glu-626 to Leu-648 in ACE-JMEGF, Figure 4), even though this region contains a sequence, Leu-Lys-Ile, that is most similar to the sequence around the native cleavage site in WT-ACE, Ala-Arg-Ser (*7*).

Juxtamembrane cleavage of ACE-JMEGF occurred within the third disulfide loop, at a Gly-Phe bond (Figure 4). The third disulfide loop in EGF domains presents a structure that is comparatively more exposed than the β-cross structure involving the first two disulfide loops, as is clearly seen in the EGF-like domains in factor IX and E-selectin (*34*, *36*). Molecular modeling of this loop in ACE-JMEGF revealed that it folds as an extended loop with a single β turn; the MPSP cleavage site is within one of the extended arms of this loop and does not involve the β turn (K. J. Naidoo and E. D. Sturrock, unpublished results). Additional experiments are required to establish whether an extended loop is a prerequisite for cleavage by the MPSP. The recent crystal structure of the TNF-α-converting enzyme catalytic domain revealed an extended catalytic cleft that may favor an extended or unstructured stalk sequence around the scissile bond (*19*).

We conclude from these studies that the phorbol ester-stimulated, TAPI-inhibited MPSP activity in CHO cells is able to recognize and cleave a disulfide-bridged, folded domain inserted into the stalk region of a membrane protein. This result contradicts the hypothesis that a prerequisite for cleavage by an MPSP is accessibility to a stalk sequence that adopts an extended, unfolded, or disordered conformation of at least 11 amino acids in length. Disulfide bonding does not per se present a barrier to the MPSP, although it may represent a mechanism for inhibiting release of membrane proteins by preserving disulfide tethering to the membrane-anchored stalk. It is possible, therefore, that in proteins such as integrin β subunits and in P- and E-selectin, cryptic MPSP cleavage is occurring, and this may warrant investigation.

ACKNOWLEDGMENT

We are indebted to James F. Riordan for incisive comments and criticisms, which facilitated the completion of this

work, to Hans Fritz for advice and support, and to Gulam A. Manji for assistance with the CHO cell transfections. We thank Roy A. Black (Immunex Corporation) for the TAPI.

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BI981260K