Modulation of Juxtamembrane Cleavage ("Shedding") of Angiotensin-Converting Enzyme by Stalk Glycosylation: Evidence for an Alternative Shedding Protease†

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ABSTRACT: The role of juxtamembrane stalk glycosylation in modulating stalk cleavage and shedding of membrane proteins remains unresolved, despite reports that proteins expressed in glycosylation-deficient cells undergo accelerated proteolysis. We have constructed stalk glycosylation mutants of angiotensin-converting enzyme (ACE), a type I ectoprotein that is vigorously shed when expressed in Chinese hamster ovary cells. Surprisingly, stalk glycosylation did not significantly inhibit release. Introduction of an *N*-linked glycan directly adjacent to the native stalk cleavage site resulted in a 13-residue, proximal displacement of the cleavage site, from the Arg-626/Ser-627 to the Phe-640/Leu-641 bond. Substitution of the wild-type stalk with a Ser-/Thr-rich sequence known to be heavily *O*-glycosylated produced a mutant (ACE-JGL) in which this chimeric stalk was partially *O*-glycosylated; incomplete glycosylation may have been due to membrane proximity. Relative to levels of cell-associated ACE-JGL, rates of basal, unstimulated release of ACE-JGL were enhanced compared with wild-type ACE. ACE-JGL was cleaved at an Ala/Thr bond, 14 residues from the membrane. Notably, phorbol ester stimulation and TAPI (a peptide hydroxamate) inhibition of release—universal characteristics of regulated ectodomain shedding—were significantly blunted for ACE-JGL, as was a formerly undescribed transient stimulation of ACE release by 3,4-dichloroisocoumarin. These data indicate that (1) stalk glycosylation modulates but does not inhibit ectodomain shedding; and (2) a Ser-/Thr-rich, *O*-glycosylated stalk directs cleavage, at least in part, by an alternative shedding protease, which may resemble an activity recently described in TNF-α convertase null cells [Buxbaum, J. D., et al. (1998) *J. Biol. Chem. 273*, 27765−27767].

Solubilization (or "shedding") of the extracellular domains of transmembrane proteins by a specific proteolytic cleavage in the juxtamembrane stalk region adjacent to the membrane anchor is a well-recognized phenomenon. Numerous membrane proteins that are members of diverse functional classes are susceptible to this type of posttranslational processing, which is apparently a universal characteristic of eukaryotic cells (*1*−*3*). Despite the ubiquity of membrane protein shedding, the majority of transmembrane (TM)[1] proteins are, nonetheless, resistant to proteolytic release, which suggests that this is a specific, regulated process catalyzed by specialized proteases (*4*). Evidence supporting this notion includes the following: (a) shedding is stimulated by phorbol esters and other cellular activators (*5*); (b) mutant Chinese hamster ovary (CHO) cells selected for a loss of shedding phenotype are defective for a common shedding machinery (*6*); (c) shed membrane proteins have topological features that appear to confer susceptibility to cleavage in their stalk sequences (*7*); (d) shed membrane proteins may also contain as yet undefined recognition motifs in their extracellular (EC) domains that signal stalk cleavage (*8*); and (e) candidate shedding proteases [sheddases, or membrane-protein-solubilizing proteases (MPSPs)] are unusual disintegrin Zn-metalloproteases (*9*−*12*).

However, details at the molecular level that can provide a basis for understanding the selectivity of the shedding

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[1] Abbreviations: ACE, angiotensin-converting enzyme; ACE-JGL, ACE-2JGL, and ACE-Δ6JM, juxtamembrane mutants containing glycosylated stalks; WT-ACE, wild-type ACE; ACEΔ36N-WT, WT-ACE with deletion of 36 N-terminal residues; CHO, Chinese hamster ovary; DCI, 3,4-dichloroisocoumarin; EC, extracellular; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; HPLC, high-performance liquid chromatography; LDL-R, low-density lipoprotein receptor; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MPSP, membrane-protein-solubilizing protease; phorbol ester, phorbol 12,13-dibutyrate; TAPI, TNF-α protease inhibitor; TM, transmembrane; TNF-α, tumor necrosis factor-α.

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apparatus remain largely undefined. For example, although various studies have shown that an unstructured or extended stalk of minimum length is required for MPSP cleavage (reviewed in ref *7*), we have shown recently that even a disulfide-bridged domain inserted into the stalk sequence of a membrane protein is cleaved (release, however, is prevented by residual disulfide tethering to the membrane anchor) (*13*). An alternative to a compactly folded stalk that may protect many membrane proteins is stalk glycosylation. This concept is based on work performed with CHO cell mutants defective in protein glycosylation, which indicated that incompletely glycosylated membrane proteins, such as the low-density lipoprotein receptor (LDL-R), are rapidly shed into the culture medium, whereas the fully glycosylated proteins expressed in wild-type cells are resistant to proteolysis (*14*, *15*). Moreover, shedding of the transferrin receptor is partly inhibited by a single *O*-glycosylated Thr residue located four residues distal to the stalk cleavage site (*16*, *17*).

In the present study, we tested this notion by constructing mutant angiotensin-converting enzyme (ACE) proteins in which the stalk region was either modified by incorporation of an *N*-glycosylation site or replaced with an *O*-glycosylated, Ser-/Thr-rich sequence. Surprisingly, proteolytic cleavage and release of these mutants was not significantly impaired compared to the wild-type protein. However, the presence of an *N*-linked glycan resulted in a 13-residue displacement of the cleavage site, whereas the *O*-glycosylated, Ser-/Thr-rich stalk altered the cleavage characteristics. Induction of release by phorbol ester and inhibition of release by the peptide hydroxamate TAPI—typical characteristics of regulated shedding—were blunted, which point to the presence of a second, "nonclassical" MPSP or shedding apparatus.

MATERIALS AND METHODS

Construction of Expression Vectors and CHO Cell Transfections. Vectors encoding the ACE mutants ACE-JGL, ACE-2JGL, ACEΔ36N-WT, and ACE-Δ6JM (Figure 1) were constructed based on the expression plasmids pLEN-ACE-JMΔ24 (*7*) and pLEN-ACEΔ36N (*18*). pLEN-ACE-2JGL was assembled by a two-stage PCR strategy similar to that used to generate the construct pLEN-ACE-JMLDL, previously (*7*). The sequence encoding Val-3 to Ala-27 from the N terminus of human testis ACE [numbering with respect to the presumed N terminus of the mature protein; (*19*)] was amplified using suitable primers. The PCR product was ligated into pLEN-ACE-JMΔ24 digested with *Eco*RI at a unique site (*7*), to produce pLEN-ACE-2JGL 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 the Ser-/Thr-rich N-terminal sequence (Val-3 to Ala-27) of ACE; ACE-2JGL retains the same Ser-/Thr-rich sequence in its native position at the N terminus (Figure 1). The derivative plasmid pLEN-ACE-JGL was constructed by substituting the 3′ half of the ACE cDNA in pLEN-ACE-2JGL [between the unique restriction sites *Nhe*I and *Cla*I; (*20*)] for the corresponding region in pLEN-ACEΔ36N (*18*). The encoded ACE-JGL is identical to ACE-2JGL but lacks the Ser-/Thr-rich sequence at the N terminus (Figure 1). pLEN-ACEΔ36N-WT was constructed by substituting the *Nhe*I/*Cla*I fragment from the wild-type ACE cDNA (*20*) for the corresponding region in pLEN-ACEΔ36N; the resulting mutant, ACEΔ36N-WT, contains the wild-type ACE stalk but lacks the N-terminal Ser-/Thr-rich sequence (Figure 1) [the original ACEΔ36N mutant was similar but was truncated after the stalk and lacked the TM and cytoplasmic domains; (*18*)]. Finally, ACE-Δ6JM was constructed by a deletion mutagenesis strategy, exactly as described (*7*); the sequence encoding Trp-621 to Ala-626, which lies immediately upstream of the native cleavage site (Arg-627/Ser-628) in ACE, was deleted (Figure 1).

CHO-K1 cells were grown, maintained, and cotransfected with each of the plasmids pLEN-ACE-JGL, -ACE-2JGL, -ACEΔ36N-WT, or -ACE-Δ6JM together with pSV2NEO, by methods detailed previously (*7*).

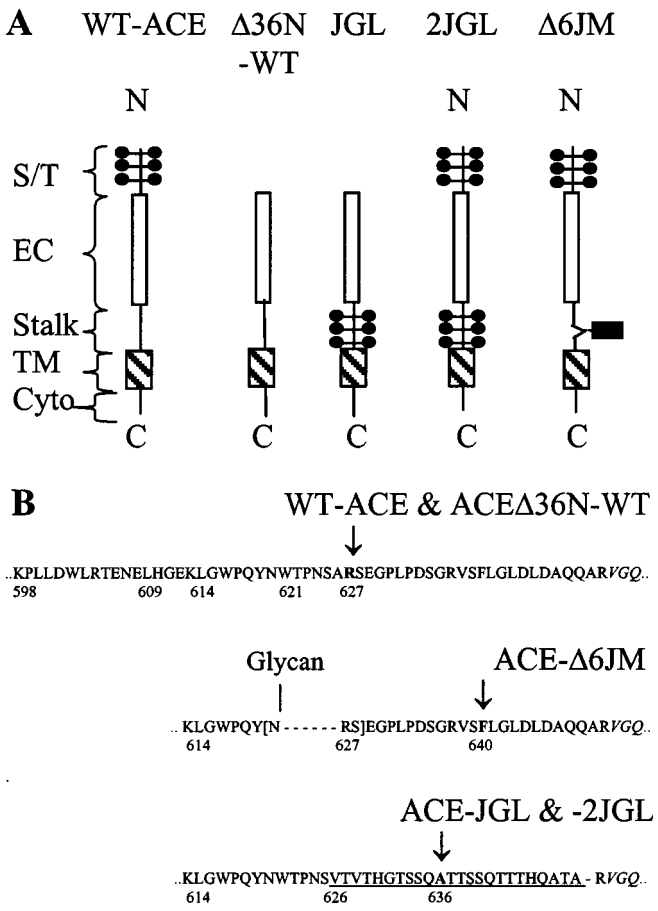

FIGURE 1: Design of ACE mutants that contain glycosylated stalks. (A) Schematic of wild-type and juxtamembrane mutants (not to scale). Wild-type (WT) ACE is a type I ectoprotein that comprises an N-terminal, Ser-/Thr-rich (S/T) domain that is extensively *O*-glycosylated (ball-and-sticks); a large extracellular (EC) domain (open rectangle); a juxtamembrane stalk, estimated to be approximately 30 residues in length; and transmembrane (TM, hatched box) and cytoplasmic (Cyto) domains. ACEΔ36N-WT is identical to WT-ACE but lacks the N-terminal S/T domain. In ACE-JGL the native stalk was replaced with the N-terminal S/T sequence, and the latter was deleted from the N terminus. ACE-2JGL is identical to ACE-JGL but retains the S/T sequence at the N terminus. ACE-Δ6JM contains a 6-residue deletion in the native stalk, creating a new *N*-glycosylation site (filled rectangle). (B) Wild-type and mutant stalk sequences. For clarity, wild-type numbering of residues is retained throughout, irrespective of deletions; the inserted sequence (underlined) in ACE-JGL and -2JGL is numbered consecutively from Ser-625 onward. Stalk cleavage sites are indicated by arrows; the C termini of the released ectodomians are in boldface type. The start of the TM domain at right (VGQ) is in italics. The 6-residue deletion (dashes) in ACE-ΔJM creates a new *N*-linked site (NRS, in brackets).

Determination of Juxtamembrane Cleavage Sites in Released Mutant ACE Proteins. After selection for stable transfectants, soluble ACE proteins were isolated from the conditioned media of CHO cells and purified to electrophoretic homogeneity by affinity chromatography on a Sepharose-28-lisinopril affinity resin (*19*, *20*). Purified soluble (released) ACE-JGL, ACE-2JGL, ACE-Δ6JM, and ACEΔ36N-WT proteins were then reduced, chemically or enzymatically cleaved, and fractionated by reversed-phase high-performance liquid chromatography (HPLC), and eluted fractions were analyzed by limited N-terminal sequencing and by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, by methods detailed elsewhere (*7*, *13*), except as noted. Enzymatic digestions were performed both with endoproteinase Lys-C (*13*) and with endoproteinase Glu-C, in both cases at 37 °C for 18 h (1 μg of protease per 1 nmol of ACE). The HPLC-purified, C-terminal, Lys-C peptide of ACE-Δ6JM (0.1 nmol) was deglycosylated with 1 unit of *N*-glycosidase F (Boehringer Mannheim) in 20 mM NaH_2PO_4, pH 8.0, 25 mM EDTA, for 2 h at 37 °C, and then N-terminally sequenced before mass spectral analysis. Mass spectra were usually generated from a sinapinic acid matrix (10 mg/mL) using a Perceptive Voyager Elite Biospectrometry Workstation.

Carbohydrate Analysis of ACE-JGL. Quantitative carbohydrate analyses were performed on purified soluble ACE-JGL, WT-ACE, and ACEΔ36N by HPLC of protein hydrolysates derivatized with 1-phenyl-3-methyl-5-pyrazolone for reducing sugars, as described (*18*).

Assay of ACE Activity and Kinetics of Mutant ACE Release. Membrane-bound and soluble ACE activities were assayed in detergent extracts or conditioned media, 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 (released) activity and changes in membrane-bound activity were performed by time-course studies (*7*, *13*, *21*), in the presence and absence of 1 μM phorbol 12,13-dibutyrate, 0.1−100 μM TAPI (peptide hydroxamate, defined in ref *22*; a gift from Roy A. Black, Immunex Corp.), or 200 μM 3,4-dichloroisocoumarin (DCI). The amphipathic characteristics of ACE-JGL and ACEΔ36N-WT protein released from transfected cells in the presence of DCI were determined by Triton X-114 phase separation by methods described previously (*23*).

Metabolic Labeling and Pulse−Chase Experiments. Stably transfected cells were pulsed for 30 min with [^{35}S]methionine−cysteine and chased for up to 24 h, and cell lysates and conditioned media were analyzed at the indicated time points by the method of "affinity-precipitation", exactly as described (*13*). Pulse−chase experiments were performed in the presence or absence of phorbol ester, TAPI, and DCI.

RESULTS

Expression of ACE-JGL, ACE-2JGL, ACE-Δ6JM, and ACEΔ36N-WT in CHO Cells and Kinetics of Release. The mutant ACE-JGL was designed to test the effect of stalk *O*-glycosylation on the proteolytic release of membrane proteins. To achieve this, a Ser-/Thr-rich sequence known to be heavily *O*-glycosylated within the testis ACE protein (*18*) was used to substitute for the native stalk sequence in ACE, to give ACE-JGL; at the same time, this Ser-Thr-rich sequence was deleted from its usual position at the N terminus of the protein (Figure 1), to eliminate the theoretical possibility that the presence of two heavily *O*-glycosylated sequences in the same protein could overload the host cell glycosylation machinery. As a control, however, ACE-2JGL was constructed, in which the Ser-/Thr-rich sequence was present within its native position at the N terminus and also within the stalk (Figure 1). A further control was ACEΔ36N-WT, in which the native stalk was intact but the Ser-/Thr-rich sequence has been deleted from the N terminus (Figure 1); we have used ACEΔ36N-WT as the "wild-type" control for ACE-JGL in most of the experiments described here, in addition to WT-ACE. ACE-Δ6JM was a second stalk glycosylation mutant, in which an *N*-glycosylation site (Asn-Arg-Ser) was incorporated across the native cleavage site in the wild-type stalk (Figure 1). The *N*-glycosylation site was created by deleting residues Trp-621 to Ala-626, thereby bringing Asn-620 adjacent to Arg-627−Ser-628.

Several independent transfections of CHO cells with pLEN-ACE-JGL, -ACEΔ36N-WT, and -ACE-Δ6JM were performed, with selection of several stable lines, which yielded consistent results for each mutant. Initial analysis revealed that at confluence, levels of soluble ACE-JGL, ACEΔ36N-WT, and WT-ACE activity varied by less than 2-fold, whereas levels of cell-associated ACE-JGL were up to 5-fold lower than those of wild-type ACE. Time-course experiments revealed that the unstimulated rate of release of ACE-JGL was indeed approximately equal to that of ACEΔ36N-WT, whereas the cell-associated level at the beginning of the time course was 4−5-fold lower (Figure 2). The kinetics of release of ACE-2JGL (see Figure 1) were essentially identical to those of ACE-JGL, although expression levels of ACE-2JGL were low (not shown). A significant difference in release rates was observed in the presence of phorbol ester. As is typical for WT-ACE and most ACE mutants (*7*, *13*), phorbol ester enhanced the rate of release of ACEΔ36N-WT, by 3−4-fold in the initial period (2−4 h). In contrast, the rate of release of ACE-JGL was enhanced on average by only 1.5-fold (we found that the phorbol stimulation for ACE-JGL was variable, ranging from no enhancement to a maximum of a 2-fold increase in release rates) (Figure 2). Total cell-associated ACEΔ36N-WT decreased approximately 2−3-fold, which was consistent with the enhanced rate of release after phorbol stimulation, and is a pattern typical for WT-ACE (*7*, *13*). As expected from the blunted phorbol response, levels of cell-associated ACE-JGL decreased by only 1.5-fold or less compared to levels in the absence of phorbol ester (Figure 2).

The differences between ACE-JGL and wild-type ACE were analyzed quantitatively in Table 1 by calculation of the ratios of soluble to cell-associated ACE activity (data taken from Figure 2). There was an up to 16-fold higher ratio of soluble to cell-associated ACE-JGL compared with the wild-type controls under unstimulated conditions. This difference was erased during treatment with phorbol ester, indicating the enormous enhancement of release of wild-type ACE induced by phorbol ester compared to the much more modest effects on the release of ACE-JGL (Table 1). These results suggested that the rate of release of ACE-JGL was near maximum under unstimulated conditions and that

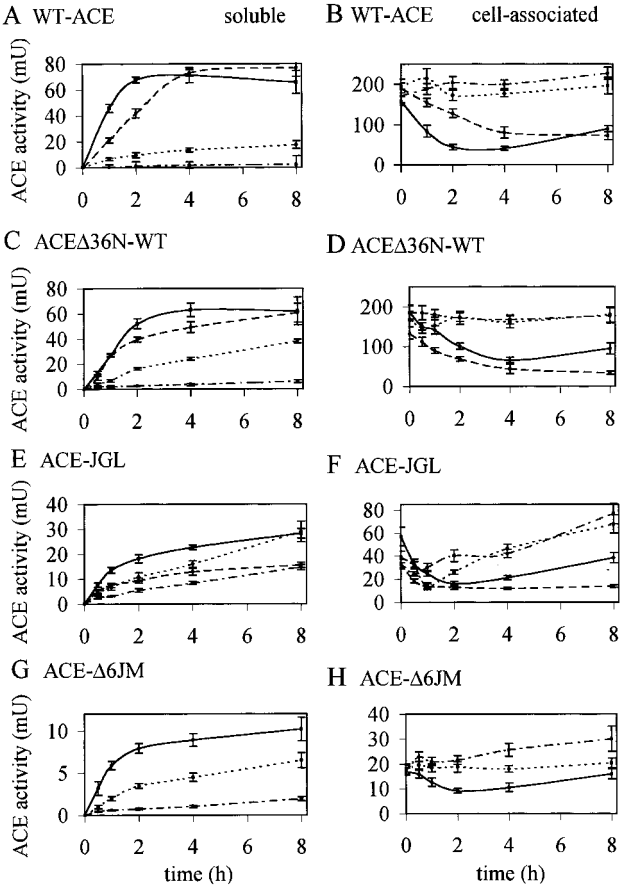

FIGURE 2: Kinetics of solubilization (shedding) (A, C, E, and G) and changes in cell-associated levels (B, D, F, and H) of wild-type ACE (WT-ACE and ACEΔ36N-WT) and the stalk mutants ACE-JGL and ACE-Δ6JM expressed in CHO cells. Stably transfected cells were grown in complete medium (dotted line) or in medium supplemented with 1 μM phorbol 12,13-dibutyrate (solid line), 200 μM 3,4-dichloroisocoumarin (dashed line), or 10 μM TAPI (dashed and dotted line). 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. The results are means and standard errors drawn from 6−8 separate experiments per mutant, each experiment performed in duplicate.

the addition of phorbol ester produced only a modest additional rate enhancement.

A second characteristic of the proteolytic release of membrane-bound WT-ACE and most stalk mutants is inhibition of release by the peptide hydroxamate TAPI (*13*). Addition of 10 μM TAPI resulted in inhibition of the unstimulated release of ACEΔ36N-WT to 20% of control levels at 2 h, whereas ACE-JGL release under identical conditions was consistently inhibited only to 40% of control levels (Figure 2). The difference between ACEΔ36N-WT and ACE-JGL was particularly striking when the extent of inhibition of release by TAPI was compared to maximal release rates achieved in the presence of phorbol ester (Figure 2), although in these experiments TAPI and phorbol ester were not added simultaneously to the transfected cells.

To achieve a more quantitative analysis of the apparent differences in the responses of ACEΔ36N-WT and ACE-JGL to TAPI, we performed dose response experiments. These clearly revealed that in the presence or absence of phorbol ester the IC_{50} for the inhibition of release of ACEΔ36N-WT by TAPI was ≤ 1 μM (Figure 3), which was very similar to that estimated previously for WT-ACE (*13*).

Table 1: Ratios of Soluble to Cell-Associated ACE Activity of Stalk Mutants Expressed in CHO Cells[a]

treatment	soluble/cell-associated activity			
	WT-ACE	ACEΔ36N-WT	ACE-JGL	ACE-Δ6JM
control				
1 h	0.03	0.04	0.48	0.05
4 h	0.08	0.15	0.43	0.19
phorbol				
1 h	0.55	0.51	0.54	0.46
4 h	1.74	0.95	1.07	0.84
TAPI				
1 h	0.002	0.01	0.10	0.03
4 h	0.01	0.02	0.20	0.04
DCI				
1 h	0.14	0.30	0.54	nd[b]
4 h	0.91	1.12	1.07	nd

[a] Ratios were calculated from the mean values for the 1-h and 4-h time points of the kinetic experiments shown in Figure 2. [b] Not determined.

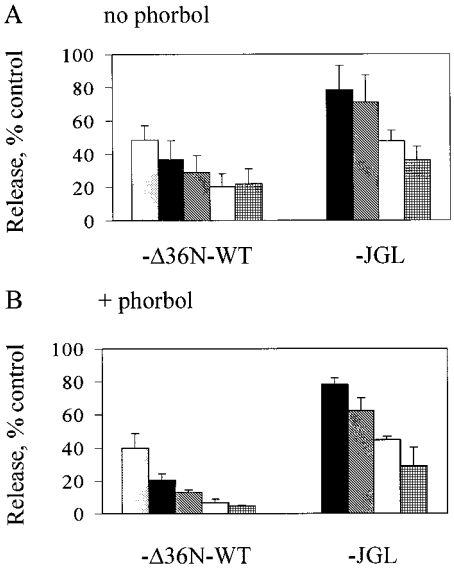

FIGURE 3: Inhibition of solubilization (shedding) of wild-type and mutant ACE by TAPI in the absence (A) or presence (B) of 1 μM phorbol ester. CHO cells stably expressing ACEΔ36N-WT and ACE-JGL were grown in medium containing 1.0, 5.0, 10, 50, or 100 μM TAPI (stippled, solid, hatched, open, and cross-hatched bars, respectively). After a 4-h incubation, media samples were assayed with the substrate Hip-His-Leu. Results are expressed as a percentage relative to control (zero TAPI), taken as 100% in each case (not shown), and are means plus standard errors of three experiments, each performed in duplicate.

In contrast, the IC_{50} for inhibition of ACE-JGL release in the presence or absence of phorbol ester was ≥ 10 μM; similar results were obtained for ACE-2JGL (not shown). This difference was also clearly evident from the ratios of soluble to cell-associated ACE levels, which were ≥ 10-fold higher for ACE-JGL compared with wild-type ACE during TAPI inhibition (Table 1). Thus, susceptibility to inhibition of release by TAPI was at least an order of magnitude lower for ACE-JGL compared to wild-type ACE.

The results with TAPI prompted us to examine the response to other protease inhibitors. We showed previously that the release of WT-ACE was insensitive to a broad range of class-specific protease inhibitors, except for a paradoxical enhancement of release by the serine protease inhibitor 3,4-dichloroisocoumarin (DCI) (*21*). A kinetic analysis of the response to DCI revealed that both ACEΔ36N-WT and WT-

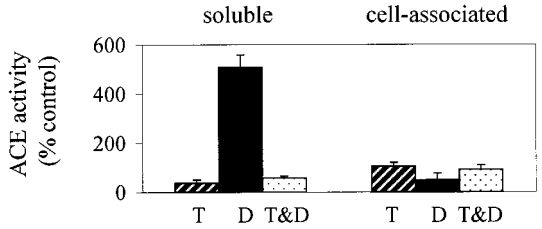

FIGURE 4: Effects of TAPI and DCI on levels of soluble and cell-associated wild-type ACE. CHO cells stably expressing WT-ACE were incubated for 5 h in medium containing no additives, 5 μM TAPI (hatched bars), 200 μM DCI (solid bars), or 5 μM TAPI plus 200 μM DCI (stippled bars). Samples of media (soluble ACE) or cell lysates (cell-associated ACE) were assayed with the substrate Hip-His-Leu. Results are expressed as a percentage relative to control (no treatment), taken as 100% in each case (not shown), and are means plus standard errors of two independent experiments, each consisting of 2−3 separate plate wells per variable.

ACE exhibited a burst of release of soluble activity into the media that was quantitatively and qualitatively similar to the apparent first-order release induced by phorbol ester (Figure 2). This effect was highly reproducible and was consistently observed in separate experiments. Notably, the DCI-induced burst of WT-ACE release was completely inhibited by TAPI (Figure 4), suggesting that DCI represents a novel activator for the common, TAPI-inhibitable MPSP in CHO cells. Concomitant with the enhanced release rate, DCI induced a decline in levels of cell-associated ACEΔ36N-WT and WT-ACE that was equal in magnitude to that induced by phorbol ester (Figure 2); the DCI-induced decline was abrogated by simultaneous addition of TAPI (Figure 4). However, a key difference between the phorbol- versus the DCI-induced decline was that the former reached a nadir at 4 h and then recovered [returning to base line levels by ∼20 h; (*7*)], whereas the latter declined and did not recover (Figure 2). Unlike wild-type ACE, the release of ACE-JGL was not enhanced by DCI; instead, in some experiments release appeared to be decreased to a small extent (Figure 2). Despite an absence of release stimulation, cell-associated levels of ACE-JGL were reduced by DCI in a pattern similar to that seen with wild-type ACE (Figure 2).

In contrast to the *O*-glycosylation mutant ACE-JGL, the *N*-glycosylation mutant ACE-Δ6JM demonstrated release kinetics that were more similar to those of WT-ACE (Figure 2). Although expression levels were consistently low, the ratios of the levels of soluble to cell-associated activity were approximately the same for ACE-Δ6JM compared to wild-type ACE (Table 1). Moreover, ACE-Δ6JM release was enhanced 2-fold by phorbol ester, and the release was strongly inhibited by TAPI (Figure 2); the effect of DCI was not examined.

In summary, in terms of several key parameters, the characteristics of release of ACE-JGL differed from those of wild-type ACE. Notably, compared to the latter, ACE-JGL release showed a blunted or absent phorbol response, reduced inhibition by TAPI, and absence of a DCI-induced burst. In contrast, the release kinetics of ACE-Δ6JM were broadly similar to those of WT-ACE.

Determination of Juxtamembrane Cleavage Sites in Released ACE Mutants. Juxtamembrane cleavage sites were determined by fragmenting the released proteins, fractionating and identifying the C-terminal peptides, and determining the masses of the peptides by MALDI-TOF mass spectrometry (*7*, *13*). Several separate batches of ACE-JGL, derived from independent, stably transfected CHO cell lines, were fragmented by three different procedures, and each indicated the same cleavage site after mass spectral analysis. CNBr cleavage followed by HPLC fractionation and N-terminal sequencing led to identification of a peptide starting with LSYFK, which is the sequence immediately after the last methionine residue before the transmembrane domain, hence comprising the C-terminal peptide in a stalk-cleaved, released protein (*7*). Mass spectral analysis revealed a strong peak at *m*/*z* 5132.5 (Table 2), which likely represented the formylated peptide Lys-593−Ala-636 [*m*/*z* (5104.5 + 28); calculated unformylated mass, 5103.6]. The C terminus at Ala-636 was confirmed by endoproteinase Lys-C and Glu-C digestions, HPLC fractionations, and mass spectra, which revealed peptides at *m*/*z* 2532.5 and 2661.4, respectively, corresponding to the peptides Leu-614−Ala-636 (calculated, 2532.7) and Lys-613−Ala-636 (2660.9) (Table 2).

Analysis of a fractionated, endoproteinase Lys-C digest of soluble ACE-2JGL also revealed the Leu-614−Ala-636 peptide at *m*/*z* 2531.2 (Table 2), further strengthening the conclusion that Ala-636 was the C terminus in released, soluble ACE mutants that contained the Ser-/Thr-rich stalk sequence (Figure 1). In contrast, digestion and analysis of soluble ACEΔ36N-WT revealed masses consistent with a C terminus at Arg-627, identical to that previously determined for the cleaved native stalk in WT-ACE (*7*). Mass spectra of fractionated endoproteinase Lys-C and Glu-C digests indicated peaks at *m*/*z* 1689.9 and 2255.2, respectively, corresponding to peptides Leu-614−Arg-627 (1690.8) and Leu-609−Arg-627 (2255.5).

Mass spectral analysis of a fractionated, endoproteinase Lys-C digest of soluble ACE-Δ6JM revealed a strong peak at *m*/*z* 4131.3, which was inconsistent with calculated masses for any unglycosylated peptides. However, subtraction of the mass for peptide Leu-614−Phe-640 (2363.6; see Figure 1) gave a mass of 1767.7, which was in agreement with the calculated mass of 1767.8 for a fucosylated biantennary oligosaccharide ($Hex_5HexNAc_4DeoxyHex_1$), a common *N*-linked glycan (*24*), suggesting that the C-terminal Lys-C peptide was glycosylated. This was strengthened by the detection of a minor peak at *m*/*z* 4421.3, consistent with the addition of a sialic acid residue (4131.3 + 290), and by the N-terminal peptide sequence LGWPQY?RSEG determined for the HPLC-purified C-terminal peptide, which suggested that Asn-620 (Figure 1) was glycosylated. To test this, the C-terminal, Lys-C peptide of ACE-Δ6JM was deglycosylated with *N*-glycosidase F and then reanalyzed by N-terminal sequencing and mass spectrometry. As expected, Edman degradation now gave the sequence LGWPQYD, indicating that deglycosylation had converted Asn-620 to an Asp residue. Moreover, a peak at *m*/*z* 2363.4 could now be detected, in excellent agreement with the calculated mass (2363.6) for the C-terminal peptide ending at Phe-640.

In summary, the mass spectral data are consistent with a cleavage site at the Ala-636/Thr-637 bond in the stalks of both ACE-JGL and ACE-2JGL, and with the native cleavage site at the Arg-627/Ser-628 bond in ACEΔ36N-WT and WT-ACE. Our ability to detect the C-terminal peptides after chemical or enzymatic digestion of both ACE-JGL and ACE-2JGL indicates that in a fraction of these mutants this part of the Ser-/Thr-rich stalk (Val-626 to Ala-636) was not

Table 2: Mass Spectral Analysis of C-Terminal Peptides Generated from Soluble (Shed) Wild-Type and Stalk-Mutant ACE Proteins[a]

peptide (residue no.)	ACEΔ36N-WT	ACE-JGL	ACE-2JGL	ACE-Δ6JM
Lys-C				
598−613	1950.3 (1951.2)	1951.3 (1951.2)	1951.6 (1951.2)	1950.8 (1951.2)
614−627	1689.6 (1690.8)			
614−636		2532.5 (2532.7)	2531.2 (2532.7)	
614−640[b]				4131.3 (4131.4)
614−640[c]				2363.4 (2363.6)
Glu-C				
613−627	1819.9 (1819.0)			
609−627	2255.2 (2255.5)			
613−636		2661.4 (2660.9)		
CNBr				
593−636		5104.5[d] (5103.6)		

[a] Soluble (shed) ACE proteins were purified from the conditioned media of transfected cells, digested with endoproteinase Lys-C or Glu-C, or CNBr, fractionated by HPLC, and analyzed by MALDI-TOF mass spectrometry. Shown are the masses of the penultimate and ultimate C-terminal peptides. The latter are peptides ending in residue number 627 (ACEΔ36N-WT), 636 (ACE-JGL and −2JGL), and 640 (ACE-Δ6JM); these were the only peptides identified that did not end in lysine, glutamate, or homoserine lactone (see Figure 1). All mass values are calculated for protonated isotopically averaged molecular weights *m/z*. In parentheses are expected masses. [b] Glycosylated peptide. [c] Peptide after deglycosylation with *N*-glycosidase F. [d] Observed mass calculated after subtraction of mass representing formylation (5132.8 − 28).

Table 3: Carbohydrate Analysis of Recombinant Wild-Type and Mutant ACE Proteins[a]

carbohydrate	recombinant ACE proteins		
	WT-ACE	ACE-JGL	ACEΔ36N
NAc-galactosamine	6.2	1.2	0
NAc-glucosamine	17.5	16.3	11.7
galactose	23.4	13.5	7.3
mannose	12.6	10.1	7.9
fucose	5.0	4.3	2.7
xylose	2.1	1.0	1.2
ribose	0.4	0.3	0
glucose	6.5	2.4	3.1

[a] Soluble (released) recombinant ACE proteins purified from the conditioned media of transfected CHO cells were acid-hydrolyzed, derivatized with 1-phenyl-3-methyl-5-pyrazolone, and then analyzed by HPLC, as described (*18*). Values are the means of three determinations.

O-glycosylated. ACE-Δ6JM was cleaved at the Phe-640/Leu-641 bond, 13 residues C-terminal to the wild-type cleavage site, apparently due to the presence of an *N*-linked glycan immediately adjacent to the native cleavage site.

Carbohydrate Analysis of Released, Soluble ACE-JGL. A quantitative carbohydrate analysis of ACE-JGL revealed that levels of most saccharides were similar to those of WT-ACE, whereas galactose levels were reduced by 42% and *N*-acetylgalactosamine (GalNAc) by 79% (Table 3). Galactose and GalNAc are considered to be constituents of the common region of mucin-like *O*-linked oligosaccharide chains (*25*), and in the ACEΔ36N mutant (which completely lacks the Ser-/Thr-rich sequence), GalNAc was consistently undetectable and galactose was reduced by 69% [Table 3; (*18*)]. These results indicated that the N-terminal half of the Ser-/Thr-rich stalk sequence that was retained on the shed ACE-JGL was *O*-glycosylated, at least in part. This was also evident from the electrophoretic mobility of ACE-JGL on SDS−PAGE, which was similar to that of WT-ACE but clearly less than that of ACEΔ36N (Figure 5). Full-length, membrane-anchored ACE-JGL could not be purified in sufficient quantity for analysis.

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

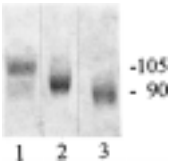

FIGURE 5: SDS−PAGE of WT-ACE (lane 1), ACE-JGL (lane 2), and ACE-Δ36N (lane 3), purified as the soluble (shed) forms from the conditioned media of transfected cells. Proteins (∼5 μg per lane) were electrophoresed on a 10% SDS−polyacrylamide gel and stained with Coomassie Brilliant Blue. Molecular mass estimates (in kDa) are indicated at the right.

ACE-JGL was released (shed) at a high rate relative to the cell-associated levels when compared to wild-type ACE, but that the phorbol response was blunted. Pulse−chase analysis revealed that the ACE-JGL protein in the cell lysate fraction matured from a 90-kDa to a 105-kDa species, and that the latter was released as a soluble protein into the supernatant fraction (Figure 6A); this sequence of events was also observed for WT-ACE [Figure 6A; (*13*)] and the wild-type control ACEΔ36N-WT (not shown). However, a significant difference between ACE-JGL and wild-type ACE was that levels of mature, cell-associated ACE-JGL were comparatively low at all time points and that soluble ACE-JGL appeared early and increased rapidly, suggesting that ACE-JGL was shed efficiently as soon as it reached the cell surface, consistent with the kinetic data. Moreover, phorbol ester did not significantly enhance release into the supernatant, unlike its effect on WT-ACE [Figure 6A; (*13*)], presumably because of a relative lack of cell-associated ACE-JGL that could serve as a substrate for release. In contrast to ACE-JGL, the levels of soluble ACE-Δ6JM were lower, but the patterns of biosynthesis and release and the response to phorbol ester were similar to those observed for WT-ACE (Figure 6B).

We also examined the differential response to protease inhibitors. TAPI markedly reduced the appearance of soluble wild-type ACE, whereas, as anticipated from the kinetic data, the effect of TAPI on the generation of soluble ACE-JGL was modest (Figure 6A). By pulse−chase labeling, DCI profoundly inhibited the production of soluble WT-ACE, which was surprising, since DCI induced a transient burst of ACE release as detected by an increase of soluble ACE

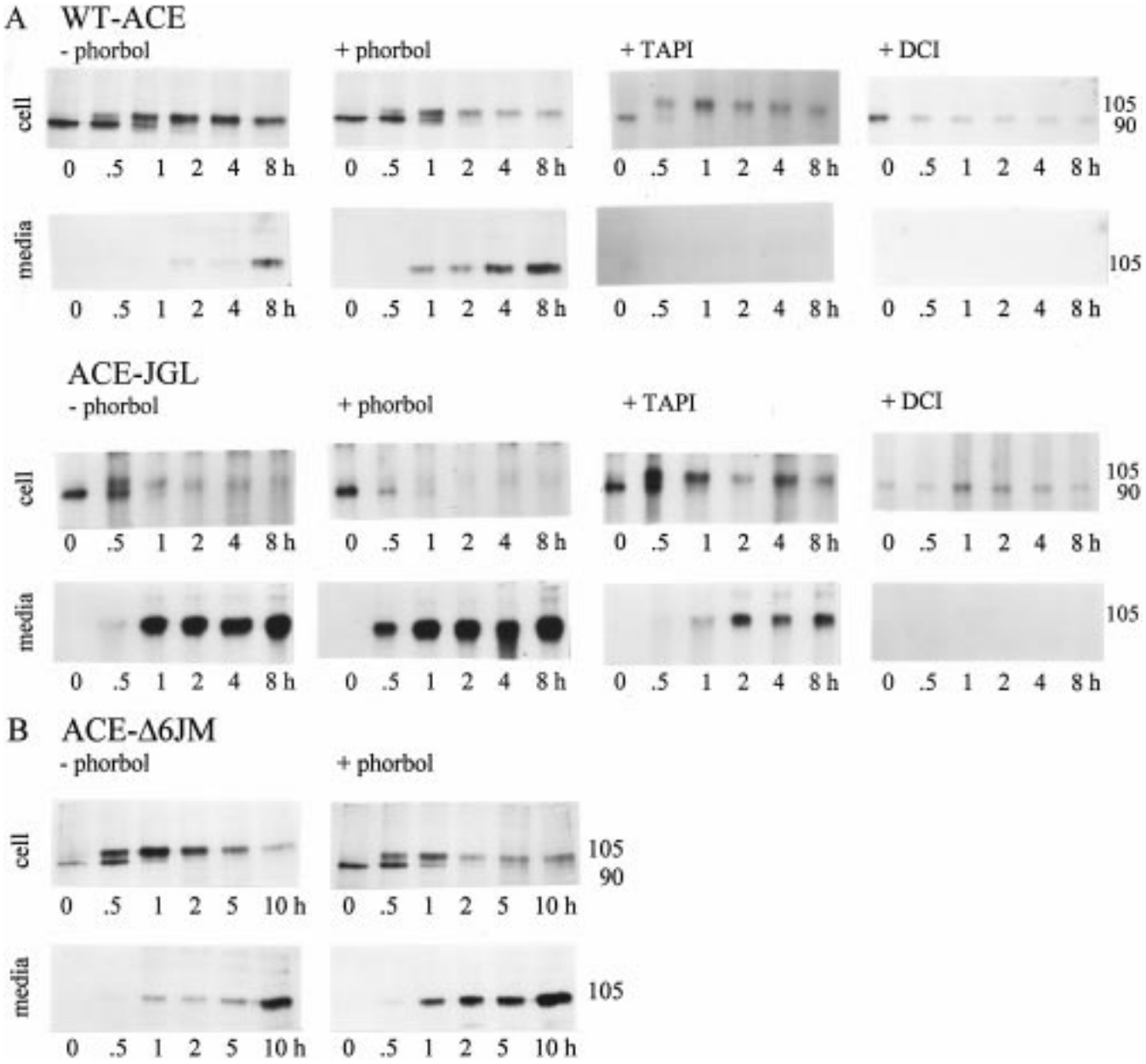

FIGURE 6: Metabolic labeling and pulse−chase analysis of the biosynthesis and release of WT-ACE and ACE-JGL (A) and ACE-Δ6JM (B). Stably transfected CHO cells were grown to confluence, pulsed for 30 min in medium containing [^{35}S]methionine and [^{35}S]cysteine, washed, refed in complete medium containing no additives (extreme left panels), 1 μM phorbol ester (middle-left panels), 10 μM TAPI (middle-right panels), or 200 μM DCI (extreme right panels), and chased for up to 10 h; in (B), cells were grown in the absence or presence of phorbol ester (left and right panels, respectively) only. At the indicated times, cell lysate and media samples were affinity-precipitated with lisinopril-28-Sepharose, subjected to SDS−PAGE, and autoradiographed. Estimated molecular masses (in kDa) of the major bands are indicated on the right.

activity. Unlike the effect of TAPI, DCI also inhibited maturation of the lower to the higher molecular weight WT-ACE species (Figure 6A). Similar results were observed for ACE-JGL (Figure 6A).

These results suggested that DCI rapidly inhibited the maturation of newly synthesized ACE, thus leading to a sustained suppression of cell-associated ACE levels which did not recover (Figure 6A). The initial burst of WT-ACE release observed in the kinetic analysis of soluble ACE activity presumably represented shedding of a preexisting pool of cell-surface protein. This was not observed with ACE-JGL because, in part, the steady-state levels on the cell surface were low (Figure 6A). The mechanism of the DCI-induced transient burst of WT-ACE release is unknown, but like phorbol ester, DCI activated a TAPI-inhibitable shedding machinery (Figure 4). The DCI effect was not due to a nonspecific disruption of the cell membranes, because the released, soluble protein after DCI treatment was hydrophilic whereas the cell-associated protein was amphipathic, as assessed by Triton X-114 phase separation (data not shown). Also, the transfected CHO cells remained viable by Trypan blue exclusion over a period of 8 h in 200 μM DCI.

DISCUSSION

Release or shedding of membrane proteins is apparently a universal characteristic of eukaryotic cells (*1*−*3*). However, shedding is nevertheless restricted to a limited subset of membrane proteins, which are usually type I, type II, or GPI-

anchored membrane proteins (*1*, *4*, *13*). In principle, susceptibility to cleavage by an MPSP may be governed by accessibility to a cleavable stalk or by a requirement for the presence of a recognition motif. Evidence for both possibilities has been presented (*7*, *8*). A number of studies have suggested that release of membrane proteins requires the presence of a juxtamembrane stalk ≥11 residues in length (reviewed in ref *7*), but the exact dimensions and characteristics of the stalk that confer susceptibility to cleavage remain uncertain (*13*).

"Stalks" are poorly characterized structural elements that may be best defined as intervening segments between the proximal, folded EC domain and the TM domain. Three-dimensional structure determinations for the ectodomains of CD8 (*26*) and CD2 (*27*) have indicated that the juxtamembrane stalks in these proteins adopt extended conformations, although the structure of the 48-residue CD8 stalk is comparatively disordered, possibly due to variable orientations relative to the EC domain (*26*). The function of such extended but disordered stalk sequences is unclear; they may simply provide a convenient spacer or filler that allows the folded EC domain freedom of movement and orientation with respect to the membrane. Alternatively, stalks may function predominantly as points of access for MPSPs.

In a number of instances, stalks have been shown, or have been assumed, to be extensively *O*-glycosylated in regions with high abundance of serine, threonine, and proline residues; examples include LDL-R (*28*), the sucrase−isomaltase complex (*29*), nerve growth factor receptor (*30*), p75 TNF receptor (*31*), and decay-accelerating factor (*32*). Although an argument has been made that stalk *O*-glycosylation protects membrane proteins against ectodomain shedding (*14*, *15*), the evidence for this is contradictory. For instance, contrary to earlier conclusions, wild-type LDL-R expressed in CHO cells is readily shed in a phorbol ester-inducible, TAPI-inhibitable manner (M. Begg and D. R. van der Westhuyzen, personal communication); similar results have been obtained for the shedding of p75 TNF receptor in T lymphocytes (*33*). Moreover, soluble forms of nerve growth factor receptor are generated by a presumed proteolytic release mechanism in cell culture and in vivo (*34*, *35*). On the other hand, elimination of potential *O*-glycosylation sites in the stalks of the transferrin and neurotrophin receptors resulted in stalk cleavage and release of soluble ectodomains (*16*, *36*). Similarly, when the Ser-/Thr-rich region of the LDL-R stalk was brought immediately adjacent to the TM domain by deletion of 18 membrane-proximal residues, phorbol ester-activated shedding was completely inhibited (M. Begg and D. R. van der Westhuyzen, personal communication). Hence, the true significance of stalk *O*-glycosylation with regard to susceptibility to cleavage by an MPSP has remained uncertain.

We examined the effects of inserting into the ACE stalk a Ser-/Thr-rich sequence that specifies *O*-glycosylation. Carbohydrate analysis of the resulting mutant, ACE-JGL, purified as the soluble (shed) form from conditioned media of transfected cells, revealed that the inserted sequence was *O*-glycosylated, but the extent of glycosylation was less than half that found for the same sequence in its native position at the N terminus of the protein. Likely explanations for this discrepancy are the following: (a) the analysis was performed on the released ACE-JGL, which contained only 11 of the 24 residues of the Ser-/Thr-rich sequence; (b) glycosylation was incomplete, as we were able to detect unglycosylated forms of the C-terminal peptide containing part of the inserted sequence; and (c) membrane proximity may constrain the efficiency of *O*-glycosyltransferases. *N*-Glycosyltransferases have been shown to require a minimum spacer distance of 14−15 residues from the membrane before oligosaccharide addition occurs (*37*), which may also apply to *O*-glycosyltransferases. However, in light of the results obtained with the neurotrophin receptor (*36*) and LDL-R, discussed above, membrane proximity does not appear to be an absolute constraint for *O*-glycosyltransferases. Clearly, additional data are required.

The cleavage site determined for ACE-JGL and ACE-2JGL was at the Ala-636/Thr-637 bond, 14 residues from the TM domain (Figure 1). The dipeptide Gln-635−Ala-636 may constitute a window between two potential *O*-glycosylated clusters, namely, Thr-632−Ser-634 and Thr-637−Thr-644 (Figure 1), which have high probabilities for *O*-glycosylation, as predicted by the algorithm NetOGlyc 2.0 (*38*). However, the specific glycosylation status of each of these residues was unknown, and it must be noted that only the unglycosylated fraction of the peptide Leu-614−Ala-636 could be identified by mass spectrometry. Hence, we have no direct evidence that Ala-636/Thr-637 was also the cleavage site in the fully glycosylated mutant stalk. The CHO cell MPSP exhibits considerable flexibility in terms of positioning of the cleavage site with respect to the membrane. In WT-ACE, the native cleavage site is 24 residues from the membrane, but we have shown that this distance can be shortened considerably, down to 10 residues depending on the stalk mutation, with preserved or even enhanced cleavage efficiency (*7*). In ACE-JGL, the position of the cleavage site may have been dictated by a combination of (a) a minimum 8−10-residue spacing from the membrane; (b) selection of Ala as the P1 residue, which is not uncommon in cleaved stalks (*7*); and (c) a minimum 2−3-residue spacing from an upstream steric hindrance, such as, presumably, an *O*-linked oligosaccharide (*7*) (Figure 1).

In ACE-Δ6JM, a 6-residue "upstream" deletion conveniently created an *N*-linked site across the native cleavage site in the wild-type stalk. We found that Asn-626 was indeed glycosylated and that this presumably precluded the MPSP from cleaving at the native Arg-627/Ser-628 site, cleaving instead 13 residues more proximal to the membrane, at Phe-640/Leu-641. Cleavage after Phe-640 was not a consequence of the 6-residue deletion per se, since an 11-residue deletion in the same position, which did not generate an *N*-linked site, resulted in cleavage at the native site (after Arg-627; unpublished results). However, it remains possible that the 6-residue deletion or the presence of the *N*-glycan has created an altered stalk sequence favored by an alternative shedding protease that was also stimulated by phorbol ester and inhibited by TAPI. Nevertheless, the ACE-Δ6JM mutant further demonstrated the remarkable flexibility of TAPI-sensitive shedding proteases. Within the broad constraints discussed above, virtually any stalk appears to be susceptible to cleavage (see also ref *13*). Cleavage directly next to a glycosylated residue likely is blocked, but this only induces the protease (or proteases) to reposition itself, provided an accessible stalk sequence of sufficient length remains.

In contrast to ACE-Δ6JM, the partially *O*-glycosylated, Ser-/Thr-rich sequence in the juxtamembrane stalk clearly modulated the cleavage and release of ACE-JGL. Unlike the wild-type control, ACE-JGL was shed rapidly from the cell surface, leading to low steady-state levels of the membrane-bound protein. This in turn resulted in a blunted response to phorbol ester stimulation and the absence of a DCI-induced burst of release, both of which are characteristic of WT-ACE and presumably require a reservoir of membrane-bound protein as substrate for the MPSP ("sheddase"). We cannot exclude the possibility that the altered characteristics of ACE-JGL cleavage were the result of the unusual Ser-/Thr-rich primary structure of the stalk rather than stalk glycosylation. However, in view of the high probability that at least some of the residues were *O*-glycosylated (as predicted by NetOGlyc 2.0), it seems likely that *O*-glycosylation played a role.

The release-enhancing effect of phorbol ester is well characterized (*4*), but the burst of release stimulated by DCI has not been described for proteins other than ACE. We found that the rate of release of WT-ACE and several stalk mutants, but not of ACE-JGL, was consistently enhanced severalfold by DCI; kinetically this effect resembled the phorbol effect, when rates of accumulation of soluble ACE activity in the media were measured. However, mechanistically the phorbol ester and DCI effects were distinct, as the latter produced a long-term depression in cell-associated activity that resulted from a block in ACE maturation, as revealed by pulse−chase labeling. Hence, the burst of WT-ACE shedding induced by DCI was generated exclusively from a pool of preexisting cell-surface protein. Moreover, the DCI-induced burst of ACE release was completely blocked by TAPI, indicating that, like phorbol ester, DCI activates the general, TAPI-inhibitable MPSP. The speed of the shedding burst renders a role for DCI in inhibiting a putative MPSP-degrading serine protease unlikely; moreover, recent data indicate that cell-surface levels of TNF-α convertase, a candidate MPSP, are stable under basal conditions (*39*). Previous work has established that DCI inhibits membrane protein maturation and, indirectly, shedding (*4*). These studies revealed that DCI did not inhibit the phorbol-stimulated shedding of membrane proteins, but a DCI-induced acute release of preexisting cell-surface proteins was not described (*4*). The DCI effect may provide an additional tool in dissecting the cellular shedding apparatus.

The reduced response to phorbol ester may indicate that the release machinery involved in shedding ACE-JGL differed from that for WT-ACE (and ACE-Δ6JM). This was supported by the 10-fold reduction in sensitivity to TAPI-mediated inhibition. Phorbol ester stimulation and TAPI inhibition of membrane protein release are considered key characteristics of a presumed general shedding machinery that has been widely characterized (*4*, *13*, *40*, *41*). It is possible that the reduced response to phorbol ester was simply a reflection of the low level of membrane-bound ACE-JGL available for accelerated shedding; this, in turn, was due to the high rate of basal shedding, which may indicate that ACE-JGL was a better substrate for the conventional MPSP. However, this explanation cannot account for the significantly reduced sensitivity to TAPI. In our studies, we have not observed similar changes in sensitivity to phorbol ester or TAPI in over 10 different stalk mutants of ACE, which include ACE-Δ6JM as well as other deletion, substitution, and chimeric mutants [(*7*, *13*); unpublished results]. Hence, cleavage and release of ACE-JGL may be mediated in part by a "non-classical" MPSP. The identity of such a protease is unknown.

TAPI-inhibited, phorbol-enhanced release of WT-ACE is presumably the result of a protease that is similar to the TNF-α convertase, which is a disintegrin Zn-metalloprotease, although recent reports have suggested that "ACE secretase" and TNF-α convertase are distinct (*42*), and ACE shedding is not impaired in TNF-α knockout cells (*43*). TNF-α convertase mediates the regulated shedding of several other membrane protein ectodomains, including amyloid precursor protein and L-selectin (*44*, *45*), but in knockout cells a fraction of ectodomain shedding still occurs, which is partially inhibited by TAPI but unresponsive to phorbol ester stimulation (*44*, *45*), characteristics that are consistent with the mode of release observed here for ACE-JGL. The function and properties of this alternative MPSP are unknown, but work with mutants such as ACE-JGL may offer some insights.

The significance of the results with ACE-JGL and ACE-Δ6JM is that stalk glycosylation modulates membrane protein release in a manner that is more subtle than formerly appreciated. Glycosylation redirects stalk cleavage to alternative sites, and cleavage efficiency likely depends on the length of the remaining exposed stalk. In addition, *O*-glycosylation may shift cleavage from the regulated, TAPI-inducible pathway to an alternative pathway with unknown properties.

A further conclusion that can be drawn from this work is that the Ser-/Thr-rich sequence at the N terminus of WT-ACE is not a recognition motif for the CHO cell MPSP. This sequence is unique to testis ACE, and no comparable sequence is present in somatic ACE (*18*). Compared to testis ACE, somatic ACE is shed severalfold less efficiently by transfected CHO cells [(*46*); Z. L. Woodman, M. R. W. Ehlers, and E. D. Sturrock, unpublished results], although both proteins share the same cytoplasmic, TM, stalk, and proximal EC domains. The EC domain of testis ACE has been suggested to contain a recognition motif for the MPSP (*8*), but based on the identical patterns of release of ACEΔ36N-WT and WT-ACE, the unique N-terminal Ser-/Thr-rich sequence in testis ACE is clearly not acting as such a recognition motif. The existence of a motif elsewhere in the EC domain of testis ACE, and which is occluded by the N-terminal domain in somatic ACE, remains a possibility.

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