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Deletion of the cytoplasmic domain increases basal shedding of angiotensin-converting enzyme☆

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Abstract

Ectodomain shedding generates soluble isoforms of cell-surface proteins, including angiotensin-converting enzyme (ACE). Increasing evidence suggests that the juxtamembrane stalk of ACE, where proteolytic cleavage-release occurs, is not the major site of sheddase recognition. The role of the cytoplasmic domain has not been completely defined. We deleted the cytoplasmic domain of human testis ACE and found that this truncation mutant (ACE-ΔCYT) was shed constitutively from the surface of transfected CHO-K1 cells. Phorbol ester treatment produced only a slight increase in shedding of ACE-ΔCYT, unlike the marked stimulation seen with wild-type ACE. However, for both wild-type ACE and ACE-ΔCYT, shedding was inhibited by the peptide hydroxamate TAPI and the major cleavage site was identical, indicating the involvement of similar or identical sheddases. Cytochalasin D markedly increased the basal shedding of wild-type ACE but had little effect on the shedding of ACE-ΔCYT. These data suggest that the cytoplasmic domain of ACE interacts with the actin cytoskeleton and that this interaction is a negative regulator of ectodomain shedding.

Keywords: ACE; Ectodomain shedding; Cytoplasmic domain; Actin cytoskeleton; Cytochalasin D; CHO

The cytoplasmic domains of ectoproteins have been shown to play important roles in targeting, ligand binding, and signal transduction [1,2]. Cytoplasmic domains may also play a role in the process of ectodomain shedding of various membrane-bound proteins, including ectoenzymes, adhesion proteins, and receptors. For example, a basic amino acid in the cytoplasmic domain of the amyloid precursor protein (Arg747) is essential for α-secretase cleavage of the ectodomain [3]. However, in other cases, deletion or modification of the cytoplasmic domain did not inhibit ectodomain shedding [4–7].

Shedding of the membrane-bound somatic and testis isoforms of ACE occurs through a regulated proteolytic cleavage event in the extracellular juxtamembrane 'stalk' region at the Arg627/Ser628 bond [8]. Numerous deletion and insertion mutations in the juxtamembrane stalk region of ACE failed to abolish shedding, indicating that this is not the site of sheddase recognition. Instead, the sheddase recognition domain must lie either in the cytoplasmic domain or in the extracellular ectodomain. Sadhukhan et al. [9] truncated the entire cytoplasmic domain of rabbit testis ACE and found that the shedding of this mutant from the surface of HeLa cells was markedly increased. Interestingly, these authors found that removal of the sequence QRLFSIRYQS was

☆ *Abbreviations:* wtACE, wild-type human testis angiotensin I-converting enzyme; CHO, Chinese hamster ovary; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; PDBu, phorbol 12,13-dibutyrate; TAPI, TNF-α protease inhibitor; BSA, bovine serum albumin; PFA, paraformaldehyde; SDS–PAGE; sodium dodecyl sulphate–polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; APP, amyloid precursor protein; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser-desorption–ionisation time-of-flight.

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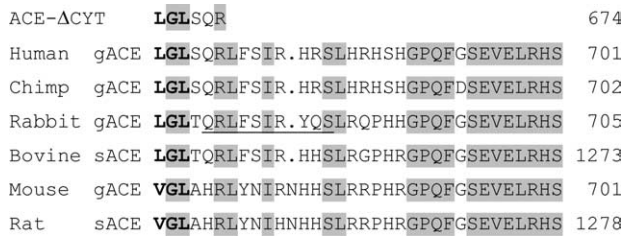

Fig. 1. Alignment of mammalian ACE cytoplasmic domains The cytoplasmic domains of chimpanzee, rabbit, and mouse germinal ACE (gACE), and bovine and rat somatic ACE (sACE), are aligned with that of human testis (germinal) ACE. Conserved residues are highlighted; the C-terminal end of the transmembrane region is in bold. Residues 29–20 from the C-terminus of rabbit testis ACE are underlined. The alignment was performed using Lynnon BioSoft's DNAMAN program.

sufficient to produce constitutive shedding (Fig. 1) [9]. Thus, the ACE cytoplasmic tail is not necessary for sheddase recognition but may instead be involved in the modulation of ACE shedding. To investigate this further, we have truncated the cytoplasmic domain of human testis ACE and found that this mutant also exhibited a marked increase in basal shedding from the surface of transfected CHO cells, with cleavage occurring at the same R627/S628 site. Furthermore, this increase in basal shedding was similar to that seen for wild-type ACE in the presence of cytochalasin D, suggesting that the inhibitory effect on sheddases was actin dependent.

Materials and methods

Construction of mutant ACE vectors and CHO cell transfections. The ACE-ΔCYT mutant was constructed in a single PCR step that introduced a stop codon directly downstream of Arg674 (thereby C-terminally truncating human testis ACE by 27 residues) by procedures similar to those described previously [10]. The expression vector pLEN-ACE-ΔCYT was stably co-transfected with pSV2NEO into CHO-K1 cells using the calcium phosphate method, as described elsewhere [11].

ACE shedding kinetics. Stably transfected CHO-K1 cells were grown to confluence in six-well plates. Shedding-kinetics studies were carried out as described previously [12], in the presence of 1 μM phorbol 12,13-dibutyrate (PDBu), 10 μM TNF-α protease inhibitor (TAPI [13]), or 2 μM cytochalasin D.

Western blot analysis. Western blotting was performed using a rabbit polyclonal anti-human kidney ACE antibody R147, as described [10].

Confocal immunofluorescence microscopy. Transfected CHO-K1 cells were seeded onto glass coverslips in 12-well dishes, cultured to 40% confluence, cold washed with PBS, and fixed for 5 min using ice-cold 3% paraformaldehyde (PFA). Extracellular ACE was probed using a rabbit polyclonal antibody and FITC-conjugated goat anti-rabbit IgG antibody, after which the coverslips were mounted and viewed under a scanning confocal microscope, as described [14].

Cleavage site determination. Identification of the stalk cleavage site was determined by MALDI-TOF mass spectrometry after limited proteolysis of the soluble ACE-ΔCYT purified from conditioned media, exactly as described [12].

Hydrophilicity determination. Triton X-114 phase separation was used to determine the relative hydrophilicity of the cellular (membrane-bound) and soluble (shed) forms of ACE-ΔCYT and wtACE. ACE-expressing CHO-K1 cells were grown to confluence in six-well plates and shedding was either stimulated with 1 μM PDBu or inhibited with 10 μM TAPI. After 4 h, the medium and cell-extract samples were each separated into lipophilic and hydrophilic fractions using Triton X-114 phase separation and assayed for ACE activity using the HHL assay, as described previously [10].

Results and discussion

Alignment of testis ACE from several mammalian species revealed almost total conservation of the C-terminal 13 residues of ACE (Fig. 1), suggesting a role in ACE function, processing, or shedding. However, Sadhukhan et al. [9] previously reported that rabbit testis ACE truncation mutants lacking 8 and 19 residues spanning the C-terminal 13 residues exhibited plasma membrane transport and shedding rates similar to the wild-type protein. The truncation of 10 additional residues (underlined in Fig. 1) resulted in an increase in the rate of shedding, indicating that a putative shedding-inhibitory motif may be encoded by this membrane-proximal decapeptide [9]. To investigate further the role of the cytoplasmic domain of human testis ACE in ectodomain shedding, we truncated 27 residues from the C terminus, retaining only the charged SQR sequence (Fig. 1), expressed this mutant in CHO-K1 cells, and examined the rate of ectodomain cleavage-secretion.

Expression of ACE-ΔCYT in CHO cells

The ACE-ΔCYT mutant was stably expressed in CHO-K1 cells and the cells were assayed for cell-associated ACE activity using HHL as substrate. Cellular ACE activity was found to be almost 16-fold lower than in cells expressing full-length ACE.

Western blot analysis of ACE-ΔCYT showed a clear separation between the precursor and mature forms (Fig. 2A), with sizes similar to that of wtACE (~90 and ~100 kDa for the precursor and mature forms, respectively). The expected change in mass in the ACE-ΔCYT mutant of 3.2 kDa was not evident on the blot. The intensity of the band corresponding to the mature form in cell extracts was significantly reduced after 4 h of phorbol ester stimulation, with a concomitant accumulation of soluble ACE-ΔCYT in the medium (Fig. 2).

Confocal microscopy was performed on fixed, unpermeabilised CHO cells expressing ACE-ΔCYT (Fig. 2B). This showed strong cell-surface labelling, indicating that the mutant protein was processed to the cell surface in significant quantities, similar to full-length ACE (Fig. 2C).

ACE-ΔCYT-expressing cells were tested for their ability to shed ACE into the culture medium over 4 h using HHL as substrate (Fig. 3). Surprisingly, medium with no supplements (control) resulted in the release of

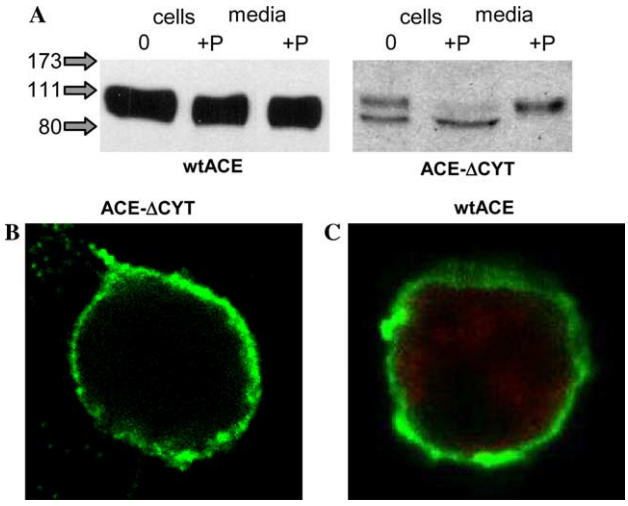

Fig. 2. ACE-ΔCYT expression in CHO-K1 cells. (A) Western blot analysis was performed on cell lysates from CHO-K1 cells stably transfected with either full-length ACE (wtACE) or the truncation mutant ACE-ΔCYT. Cells were grown to confluence and lysed at zero time (0) or after 4 h of 1 μM PDBu treatment (+P). Extracellular ACE expression of ACE-ΔCYT (B) and wtACE (C) is shown with confocal microscopy of fixed, unpermeabilised CHO cells probed with an anti-ACE polyclonal antibody and a FITC-labelled anti-rabbit secondary antibody.

58.6% of the total ACE activity from the cells. This was increased to 70.1% on cellular stimulation with phorbol ester, but this activation was insignificant compared to the ninefold increase for full-length ACE. TAPI (TNFα convertase inhibitor) was, however, able to inhibit 60% of this release, indicating that a proteolytic activity was responsible for most of the release of ACE-ΔCYT into the medium.

ACE-ΔCYT was membrane bound

TAPI inhibition did not prevent the release of 25.2% of total ACE-ΔCYT over 4 h, unlike wtACE, in which all but 0.6% release was inhibited by TAPI (Fig. 3). This raised the possibility that the ACE-ΔCYT was produced intracellularly as a soluble form that accounted for 25%

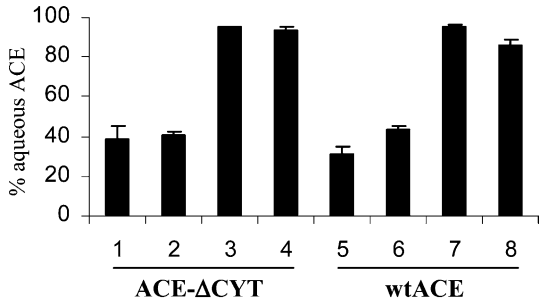

Fig. 4. Hydrophilicity of soluble ACE-ΔCYT in conditioned media. phase separation was performed on cell lysates or media from CHO cells expressing either ACE-ΔCYT (lanes 1–4) or wtACE (lanes 5–8), and the ACE activity in the aqueous and detergent phases was measured using HHL as substrate. Results are expressed as the percent ACE activity in the aqueous phase, in cell lysates at zero-time (lanes 1 and 5) and after 4 h of phorbol ester treatment (lanes 2 and 6), and in media after 4 h of phorbol activation (lanes 3 and 7) or 4 h of TAPI inhibition (lanes 4 and 8) (±SD, $n = 2$).

of the total cell-associated ACE. Triton X-114 extraction was used to determine the relative lipophilicity of the soluble and cell-associated proteins (Fig. 4). The cell-associated ACE-ΔCYT mutant partitioned into the aqueous and lipid phases in the same ratio (40% aqueous:60% hydrophobic) as seen for wtACE (Fig. 4), indicating that the mutant had the same amphipathic nature as wtACE. Furthermore, the soluble form of ACE-ΔCYT released after 4 h of phorbol ester treatment exhibited the same hydrophilicity as soluble wtACE (~90%), indicating that it no longer contained the hydrophobic region and must have been proteolytically cleaved to appear in the medium. Interestingly, the ~25% shed protein that was released into the medium in the presence of TAPI did not contain a hydrophobic anchor and thus was likely also the result of proteolytic cleavage-release from the plasma membrane of cells (Fig. 4).

ACE-ΔCYT was cleaved by the ACE sheddase

Cleavage-site determination was then performed on ACE-ΔCYT purified from the medium of transfected

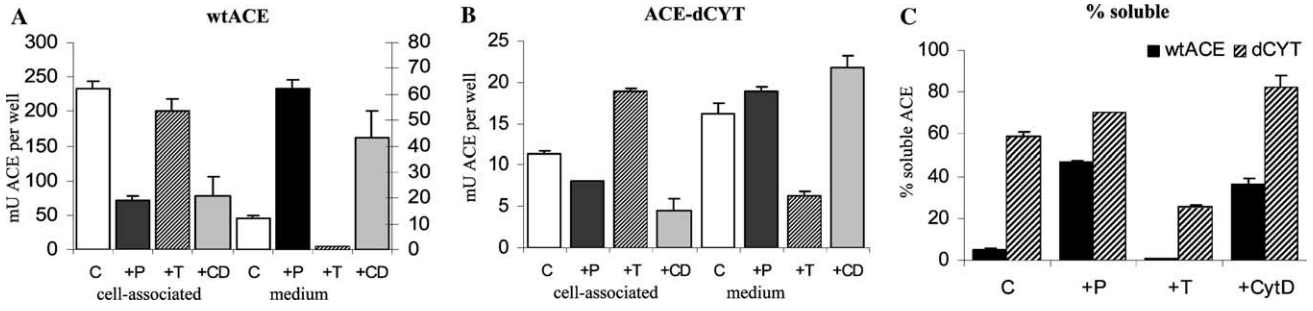

Fig. 3. Shedding of ACE-ΔCYT. cells expressing wtACE (A) or ACE-ΔCYT (B) were grown to confluence and then incubated with fresh medium supplemented with nothing (C), 1 μM PDBu (+P), 10 μM TAPI (+T), or 2 μM cytochalasin D (+CD). After 4 h, the medium and cell-associated samples were assayed for ACE activity using HHL as substrate and expressed as milliunits ACE activity per well ±SD ($n = 3$). (C) The ratio of soluble ACE activity at 4 h is shown as a percentage of total ACE activity.

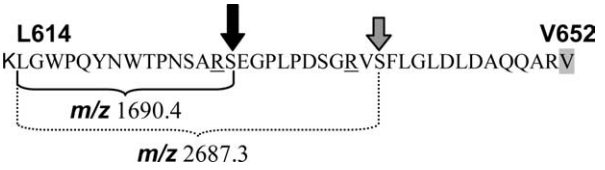

Fig. 5. Cleavage sites for ACE-ΔCYT shedding. The human testis stalk region is shown from the most C-terminal lysine (K613) to the start of the transmembrane domain (V652). The major cleavage site R627/S628 and the minor site R637/V638 are indicated by arrows; the arginines are underlined.

CHO-K1 cells. The denatured, disulphide-reduced, and vinylpyridine-protected protein was digested with endoproteinase Lys-C and subjected to MALDI-TOF mass spectrometry analysis. Numerous peaks could be assigned to ACE-ΔCYT peptides generated, but one of them, *m/z* 1690.4, related to the calculated size of the peptide L614-R627 (1690.85 Da), indicating that the ACE-ΔCYT mutant, like wtACE [12], was cleaved at the R627/S628 bond (Fig. 5). Interestingly, a minor peak at *m/z* 2687.3 corresponded to the calculated mass of the peptide L614-R637 (2686.89 Da), indicating that a secondary site (R637/V638) was also used by the sheddase. This minor peak was not seen in wtACE when analysed in parallel, although both share identical sequences.

The de-regulated shedding of ACE-ΔCYT suggested that the C-terminal 27 residues of human testis ACE contain an inhibitory signal that, when removed, allowed for rapid shedding of the ACE ectodomain. This finding corroborates the work of Sadhukhan et al. [9]. A possible explanation is that ACE and/or its sheddase may be compartmentalised in the plasma membrane by an actin-binding protein, and that ACE-ΔCYT thus had greater lateral mobility for interaction with the sheddase. We investigated this possibility using the actin polymerisation inhibitor cytochalasin D.

wtACE shedding was stimulated by cytochalasin D

Treatment of CHO cells expressing full-length wtACE with 2 μM cytochalasin D resulted in a 2.5-fold increase in the release of ACE over that seen with medium alone (Fig. 6). The cytochalasin D-enhanced shedding was ~60% of the full shedding response seen with phorbol ester stimulation (Fig. 2C) and was markedly greater than that induced by medium containing no supplements. Furthermore, cytochalasin D-stimulated shedding was inhibited by TAPI, indicating that the same or similar sheddase was involved (Fig. 6). Cytochalasin D-induced shedding was not increased further using 20 μM (data not shown). In contrast to wtACE, when CHO cells expressing ACE-ΔCYT were treated with 2 μM cytochalasin D, no significant increase in the percentage soluble ACE-ΔCYT above that of unstimulated cells was seen ($P = 0.3$), indicating that the

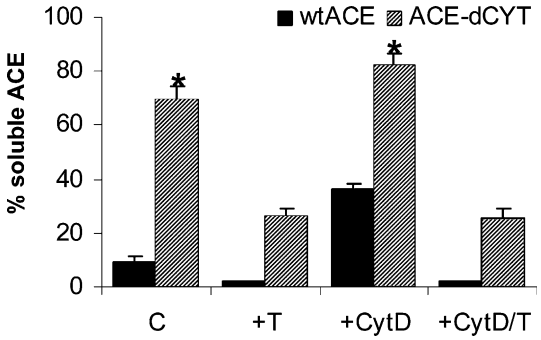

Fig. 6. Effect of cytochalasin D on ectodomain shedding of ACE. cells expressing either wtACE or ACE-ΔCYT were grown to confluence and then incubated with fresh medium (C), 10 μM TAPI (T), 2 μM cytochalasin D (CytD), or 10 μM TAPI plus 2 μM cytochalasin D (CytD/T). Soluble ACE activity is expressed as percentage of total activity (media plus cell lysate activities). Asterisk indicates $P > 0.1$.

de-regulated shedding of ACE-ΔCYT already occurred at a maximal rate without supplements (Fig. 6).

The identity of the possible actin-associated protein that interacts with the cytoplasmic domain of ACE has not been determined. However, Santhamma and Sen [15] identified two proteins bound to rabbit testis ACE, the endoplasmic reticulum chaperone BiP and ribophorin 1, a dolichyl-diphosphooligosaccharide-protein glycosyltransferase. Furthermore, the authors found that the PKC isoforms PKCι, PKCγ, PKCδ, and PKCλ were all bound to ACE, presumably to the cytoplasmic domain, whereas PKCα and PKCβ were not. Significantly, PKCγ and PKCδ dissociated from ACE after PMA stimulation, indicating that this dissociation may be involved in ACE shedding. The de-regulated shedding of the ACE-ΔCYT mutant may thus have resulted from 'constitutive' dissociation of PKCγ and PKCδ, proteins that may act as sheddase inhibitors in the normal state. Shedding may therefore be dependent on the rapid dissociation of an inhibitory shedding regulator from the cytoplasmic domain. Our data suggest that this putative regulator may also be actin-associated.

Conclusion

The present data suggest that the cytoplasmic domain of ACE is involved in modulating ectodomain shedding from the plasma membrane. The truncation mutant ACE-ΔCYT was expressed in transfected CHO cells and processed to the plasma membrane as the glycosylated mature form. Furthermore, it was proteolytically released into the medium by the same or similar sheddase that cleaves full-length ACE, as cleavage occurred predominantly at the same site (R627/S628) and was inhibited by 10 μM TAPI. Notably, shedding of ACE-ΔCYT was de-regulated, as it was not enhanced further by phorbol ester treatment. The activation of shedding

of full-length ACE by cytochalasin D indicated that the cytoplasmic domain of ACE may be bound to an actin-associated protein that constitutively inhibits shedding. Disruption of the actin skeleton by cytochalasin D, or removal of the cytoplasmic domain, thus resulted in enhanced, de-regulated shedding.

Therefore, activation of ectodomain shedding can be achieved by disrupting the association of the cytoplasmic domain of ACE with the actin cytoskeleton. The nature of the ACE–cytoskeleton interaction, and associated interactions with the ACE sheddase, remains to be elucidated.

Acknowledgments

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