Shedding of somatic angiotensin-converting enzyme (ACE) is inefficient compared with testis ACE despite cleavage at identical stalk sites

Zenda L. WOODMAN*, Sylvester Y. OPPONG†, Sarah COOK†, Nigel M. HOOPER†, Sylva L. U. SCHWAGER*, Wolf F. BRANDT‡, Mario R. W. EHLERS* and Edward D. STURROCK*§[1]

*Department of Medical Biochemistry, University of Cape Town, Rondebosch 7700, South Africa, †School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K., ‡Department of Biochemistry, University of Cape Town, Rondebosch 7700, South Africa, and §MRC Liver Research Centre, University of Cape Town, Observatory 7925, South Africa

The somatic and testis isoforms of angiotensin-converting enzyme (ACE) are both C-terminally anchored ectoproteins that are shed by an unidentified secretase. Although testis and somatic ACE both share the same stalk and membrane domains the latter was reported to be shed inefficiently compared with testis ACE, and this was ascribed to cleavage at an alternative site [Beldent, Michaud, Bonnefoy, Chauvet and Corvol (1995) J. Biol. Chem. **270**, 28962–28969]. These differences constitute a useful model system of the regulation and substrate preferences of the ACE secretase, and hence we investigated this further. In transfected Chinese hamster ovary cells, human somatic ACE (hsACE) was indeed shed less efficiently than human testis ACE, and shedding of somatic ACE responded poorly to phorbol ester activation. However, using several analytical techniques, we found no evidence that the somatic ACE cleavage site differed from that characterized in testis ACE. First, anti-peptide antibodies raised to specific sequences on either side of the reported cleavage site (Arg^{1137}/Leu^{1138}) clearly recognized soluble porcine somatic ACE, indicating that cleavage was C-terminal to Arg^{1137}. Second, a competitive ELISA gave superimposable curves for porcine plasma ACE, secretase-cleaved porcine somatic ACE (eACE), and trypsin-cleaved ACE, suggesting similar C-terminal sequences. Third, mass-spectral analyses of digests of released soluble hsACE or of eACE enabled precise assignments of the C-termini, in each case to Arg^{1203}. These data indicated that soluble human and porcine somatic ACE, whether generated *in vivo* or *in vitro*, have C-termini consistent with cleavage at a single site, the Arg^{1203}/Ser^{1204} bond, identical with the Arg^{627}/Ser^{628} site in testis ACE. In conclusion, the inefficient release of somatic ACE is not due to cleavage at an alternative stalk site, but instead supports the hypothesis that the testis ACE ectodomain contains a motif that activates shedding, which is occluded by the additional domain found in somatic ACE.

Key words: juxtamembrane, metalloprotease, phorbol ester, secretase.

INTRODUCTION

Angiotensin-converting enzyme (ACE) is a Zn-metalloendopeptidase that processes the vasoactive peptides angiotensin I and bradykinin. The somatic isoform is expressed widely at surface–fluid interfaces and plays an important role in blood pressure maintenance [1,2]. The testis isoform is limited to spermatozoa and is essential for male fertility [3,4]. Somatic ACE has a tandem repeat structure of two homologous domains, of which the second (C-terminal) domain is identical with the single domain of testis ACE, except for a unique, 36-residue, serine- and threonine-rich sequence at the N-terminus of testis ACE [5,6]. Both isoenzymes are transcribed from a single ACE gene at tissue-specific initiation sites [7].

Somatic and testis ACE are both type I transmembrane (TM) ectoproteins that share identical stalk, TM and cytoplasmic domains [5,6]. Both isoenzymes are members of a growing family of membrane proteins that are released ('shed') as soluble proteins after a specific proteolytic cleavage in the juxtamembrane stalk [8–10], catalysed by a class of proteases variously referred to as membrane-protein-solubilizing proteases, sheddases or secretases [11,12]. Tumour necrosis factor-α (TNF-α) convertase, a disintegrin Zn-metalloprotease, was the first sheddase to be isolated and characterized [13,14]. The ACE secretase appears to be similar to, but is distinct from, TNF-α convertase [15–17]. Although readily detected *in vivo*, the functions of soluble ACE are unknown. Most or all of the established physiological functions of ACE can be ascribed to the membrane-anchored form [1,18]. However, shedding of ACE is likely to be tightly regulated, as has been shown for other shed membrane proteins, such as TNF-α, L-selectin and the amyloid precursor protein [19].

Testis ACE is released after cleavage at an Arg-Ser bond 24 residues proximal to the TM domain [20,21]. In contrast, soluble somatic ACE was reported to be generated by a cleavage at an Arg-Leu bond 100 residues proximal to the TM domain [22]. Moreover, somatic ACE was shown to be shed much less efficiently than testis ACE, which has been interpreted to mean that the second, N-terminal domain of somatic ACE modulates the activity of the secretase and directs it to an alternative cleavage site [23]. Ectodomain-mediated modulation of secretase activity is supported by data indicating that the ACE secret-

Abbreviations used: ACE, angiotensin-converting enzyme; CHO, Chinese hamster ovary; eACE, endogenous secretase-cleaved hydrophilic form of ACE; mACE, detergent-solubilized amphipathic form of ACE; pACE, hydrophilic form of ACE purified from plasma; hsACE, human somatic ACE; tACE, trypsin-cleaved hydrophilic form of ACE; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; TM, transmembrane; TNF-α, tumour necrosis factor-α.

[1] To whom correspondence should be addressed (e-mail sturrock@curie.uct.ac.za).

ase has a remarkably low stalk-sequence specificity [24,25] and that the ACE ectodomain promotes cleavage of stalks from shedding-resistant proteins [26]. However, the reported somatic ACE cleavage site [22] is not consistent with data indicating that the size of the residual anchoring domain is 6.9 kDa [15], or that deletion of 47 residues proximal to the TM domain in testis ACE renders it catalytically inactive [21]. Testis ACE is identical with the C-terminal half of somatic ACE, which contributes 80–90 % of the catalytic activity of the somatic enzyme [18,27].

By using site-specific antibodies we show in the present study that the secretase-released form of somatic ACE is cleaved C-terminal to the previously reported cleavage site [22]. The precise juxtamembrane cleavage site in released somatic ACE was determined by matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS, and we find that it is, in fact, identical with the Arg-Ser cleavage site in testis ACE. We also find, as reported previously by Beldent et al. [23], that somatic ACE expressed in Chinese hamster ovary (CHO) cells is released 3–10-fold slower than testis ACE, but this cannot be ascribed to an alternative cleavage site.

MATERIALS AND METHODS

Expression of human somatic ACE (hsACE) in CHO Cells

The hsACE cDNA (a gift from Dr Pierre Corvol, INSERM U36, College de France, Chaire de Medecine Experimentale, 3 rue d'Ulm, 75005 Paris, France) was subcloned into pBluescript and then digested with *Bam*HI and *Nhe*I to release a 3086-bp fragment that was ligated into similarly digested plasmid pLEN-ACEVII [28]. The new construct, pLEN-hsACE, contained the somatic ACE cDNA together with promoter, enhancer, termination and splicing elements previously optimized for expression of the human testis ACE cDNA in CHO cells [28]. CHO K1 cells were stably co-transfected with pLEN-hsACE and pSV2-Neo by procedures described previously [28].

Assay of somatic ACE activity and kinetics of release

Membrane-bound and soluble somatic ACE activity were assayed in detergent extracts and conditioned medium, respectively, of transfected cells using the substrate hippuryl-L-histidyl-L-leucine (Hip-His-Leu), as described in [21]. Kinetic analyses of rates of accumulation of soluble activity and changes in membrane-bound activity were performed by time-course studies, as described in [21]. Studies were performed in the presence and absence of 1 μM phorbol 12,13-dibutyrate, a phorbol ester.

Metabolic labelling and pulse–chase experiments

Transfected cells were pulsed with [^{35}S]methionine/cysteine for 30 min and chased in complete medium (50 % Ham's medium F-12/50 % Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, pH 7.3, 20 mM L-glutamine, 2 % fetal calf serum and 40 μM Zn_2Cl_2) for up to 24 h. Supernatant and cell lysate fractions were then affinity-precipitated with lisinopril–Sepharose beads, exactly as described in [24]. Affinity-precipitates were resolved by SDS/PAGE and analysed by autoradiography.

Purification of ACE

Soluble (released) recombinant somatic ACE was purified from the conditioned media of transfected CHO cells by affinity chromatography on lisinopril–Sepharose, exactly as described for recombinant testis ACE [28]. Soluble seminal plasma ACE was purified from pooled human semen samples obtained from the Andrology Clinic, Groote Schuur Hospital (Cape Town, South Africa). Semen was centrifuged at 2000 ***g*** for 10 min and the pelleted spermatozoa were retained. The seminal plasma was dialysed against 20 mM Hepes (pH 7.5), 0.5 M NaCl and 0.1 % Triton X-100 and then centrifuged at 14000 ***g*** for 10 min. The supernatant was subsequently applied to a Sephadex G-50 column in the same buffer. Eluted fractions positive for ACE activity were then purified on lisinopril–Sepharose.

Porcine somatic ACE was purified from plasma (pACE) or kidney cortex by affinity chromatography on lisinopril–Sepharose as described previously [10,29]. ACE was solubilized from kidney membranes by one of three methods: (a) with 20 % (v/v) Triton X-100 (7:1 detergent:protein ratio) in the presence of 10 mM EDTA for 1 h at 4 °C, for isolation of the amphipathic form of ACE (mACE); (b) with trypsin [1:10 (w/w) trypsin:protein ratio] for 1 h at 37 °C, for isolation of the trypsin-cleaved hydrophilic form of ACE (tACE); or (c) by incubation of the membranes on their own at 37 °C for 1 h, which generates the endogenous secretase-cleaved hydrophilic form of ACE (eACE). All forms of ACE were purified to apparent homogeneity as assessed by SDS/PAGE.

Antibody production

Peptides CDIYQSKEAG-amide and CAMKLGFS-amide, corresponding to amino acids 1126–1135 and 1141–1147, respectively, on either side of the proposed cleavage site in hsACE [22], were coupled via an N-terminal cysteine to keyhole-limpet haemocyanin [30]. mACE (1.6 mg) labelled with 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine (0.5 mCi) [15] was combined with unlabelled mACE (25 mg) and incubated with 50 units of trypsin–agarose in 20 mM Tris/HCl (pH 7.5) and 0.1 % Triton X-100 for 44 h at 37 °C. The trypsin–agarose was removed by centrifugation at 3000 ***g*** for 1 min, and undigested mACE and the cleaved ectodomain were removed from the cleaved hydrophobic anchoring domain by chromatography on lisinopril–Sepharose. The hydrophobic anchoring domain was further purified by hydrophobic interaction chromatography on alkyl-Superose and phenyl-Superose (Amersham Pharmacia Biotech, Uppsala, Sweden) following the manufacturer's recommended procedures. New Zealand White rabbits were immunized with either the conjugated peptides or the purified hydrophobic anchoring domain (the latter generating antiserum RP165) according to standard protocols [30]. The IgG fraction of the antiserum was isolated by chromatography on Protein G–Sepharose.

SDS/PAGE and Western-blot analysis

Proteins were resolved by SDS/PAGE on 7–17 % or 5–20 % polyacrylamide gradient gels and analysed by Western-blotting, as described in [29], using either a polyclonal antiserum (RP183) raised against porcine kidney ACE ectodomain [31], at a dilution of 1:4000, or the anti-peptide antibodies at a dilution of 1:1000.

ACE competitive ELISA

A competitive ELISA was developed for ACE by a modification of methods described elsewhere [32–34]. Ninety-six-well microtitre plates were coated with affinity-purified porcine kidney ACE (100 ng of protein/well), blocked with 1 % (w/v) BSA, and then incubated overnight at 4 °C with a mixture (previously incubated at 37 °C for 6 h) of either antiserum RP183 (anti-porcine kidney ACE ectodomain [31]) or antiserum RP165 (see above) and one of the following: mACE; eACE; pACE; or tACE. After washing, wells were incubated with biotinylated anti-rabbit IgG, detected with streptavidin–horseradish peroxi-

dase and developed with the substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). The absorbance was measured at 405 nm in an Anthos 2001 micro-titre plate reader.

Determination of the juxtamembrane cleavage site in soluble somatic ACE

Recombinant soluble somatic ACE purified from the conditioned medium of transfected cells was reduced and protected with vinyl pyridine, followed by hydrolysis with endoproteinase Lys-C. The total Lys-C digest was either analysed directly by MALDI–TOF MS, or the digest was first fractionated by HPLC and the C-terminal peptide was identified by automated N-terminal peptide sequencing, before MS analysis [21,24,25]. Soluble seminal plasma ACE and porcine ACE were analysed in the same manner, as was soluble testis ACE [21], which served as an internal control for these experiments.

RESULTS

Expression of somatic ACE in CHO cells and kinetics of release

Several stably transfected lines of CHO cells were established, and a high-expressing clone was generated by selection after limiting dilution. Each of the lines analysed, as well as the derived clone, exhibited a similar pattern of expression. A kinetic analysis of the release of somatic ACE into the medium revealed that in comparison with testis ACE, levels of soluble somatic ACE were 2–3-fold lower at all time points (Figure 1A). This difference became even more pronounced (10-fold difference) in the presence of phorbol 12,13-dibutyrate, which characteristically leads to a 3–4-fold enhancement of the release of testis ACE at the early time points (Figure 1C); see also [21,35]. In contrast, enhancement of somatic ACE release was only approx. 1.5-fold (Figure 1A). Consistent with this reduced phorbol ester response and generally inefficient release, levels of cell-associated somatic ACE did not change significantly over the time course, unlike the typical pronounced decline in cell-associated testis ACE levels after phorbol 12,13-dibutyrate treatment (Figures 1B and 1D).

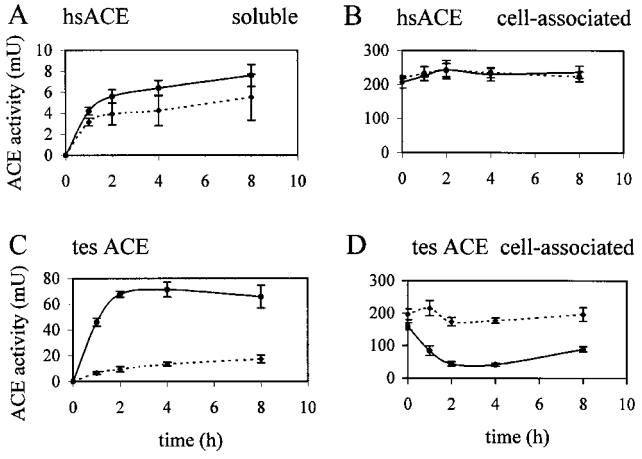

Figure 1 Kinetics of solubilization (shedding) (A and C) and changes in cell-associated levels (B and D) of hsACE and testis ACE (tes ACE), respectively, expressed in CHO cells

Stably transfected cells were grown in complete medium. 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 solid lines represent experiments carried out in the presence of phorbol and the hashed lines represent experiments carried out in the absence of phorbol. The results are means and standard errors drawn from four separate experiments, each experiment performed in duplicate. mU, m-units.

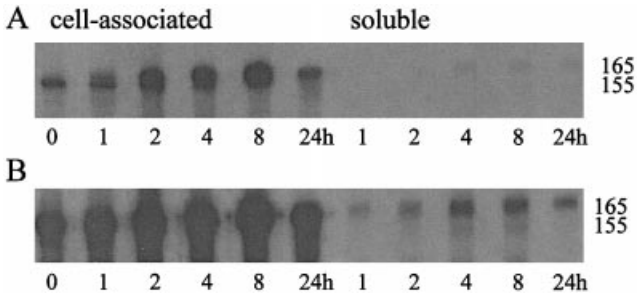

Figure 2 Metabolic labelling and pulse–chase analysis of the biosynthesis and release (solubilization) of wild-type hsACE

Stably transfected CHO cells were grown to confluence, pulsed for 30 min in medium containing 50 μCi of [^{35}S]methionine and [^{35}S]cysteine, washed, re-fed in complete medium, and chased for up to 24 h. At the indicated times, cell lysate and media samples were affinity-precipitated using lisinopril–Sepharose, subjected to SDS/PAGE, and autoradiographed for 1 week (**A**) and 3 weeks (**B**). Estimated molecular masses (in kDa) of the major bands are indicated on the right.

Metabolic labelling and pulse–chase analysis of somatic ACE release

The kinetic data of changes in soluble and cell-associated levels of somatic ACE activity were supported by the results of pulse–chase experiments. Affinity precipitates of cell lysates of transfected cells revealed intensely labelled bands at 155 and 165 kDa (Figure 2), which presumably reflected a precursor–product relationship, analogous to the 90 and 105 kDa testis ACE proteins [24]. The 165 kDa band appeared at 1 h after the chase, and the smaller 155 kDa band was no longer detected at 24 h (Figure 2), a pattern identical with that reported previously by Beldent et al. [23]. In contrast with the cell lysates, affinity precipitates of the conditioned media revealed only traces of soluble somatic ACE, which became visible at 2 h after the chase (Figure 2). The presence of phorbol 12,13-dibutyrate did not significantly alter the pulse–chase results, except that a faint trace of soluble somatic ACE could be detected earlier, at 1 h (results not shown).

The metabolic labelling results were consistent with the kinetic experiments based on ACE activity, indicating that somatic ACE accumulated to high levels in cell lysates, was released (shed) inefficiently, and release was stimulated only modestly by phorbol 12,13-dibutyrate. This suggested that, in contrast to testis ACE, somatic ACE was a poor substrate for the CHO cell secretase.

Immunocharacterization of membrane-bound and soluble porcine ACE

The amphipathic form of ACE containing the membrane-anchoring domain was purified from porcine kidney cortex, following its solubilization from the membrane, with Triton X-100 in the presence of EDTA (to inhibit the endogenous secretase activity). mACE partitioned predominantly ($> 60\,\%$) into the detergent-rich phase on phase separation in Triton X-114 and reconstituted into liposomes (results not shown), indicating that it retained the hydrophobic membrane-anchoring domain [29]. Hydrophilic forms of ACE lacking the membrane-anchoring domain were released from the membrane either by treatment with trypsin or by the action of the endogenous secretase. These

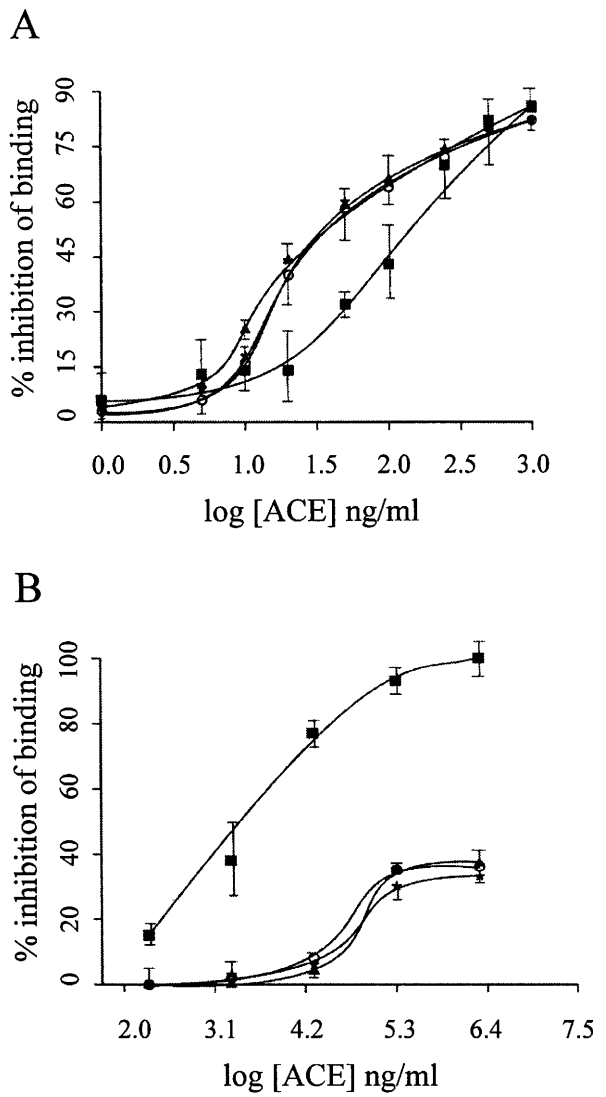

Figure 3 Competitive ELISA of differently solubilized forms of somatic ACE

Affinity-purified porcine kidney ACE was added to each well of a 96-well microtitre plate (100 ng of protein/well). A polyclonal antiserum (RP183) raised to the ectodomain of affinity-purified porcine kidney ACE (**A**) or an antiserum (RP165) to the C-terminal hydrophobic anchor (**B**) was incubated with the indicated amounts of purified mACE, eACE, pACE or tACE for 6 h at 37 °C. Following centrifugation for 10 min at 8800 ***g***, triplicate aliquots of the supernatant were transferred to the coated microtitre plate wells and the ELISA was developed as described in the Materials and methods section. ■, mACE; ★, eACE; ▲, tACE; ○, pACE. The results are the mean ± S.E. ($n = 4$).

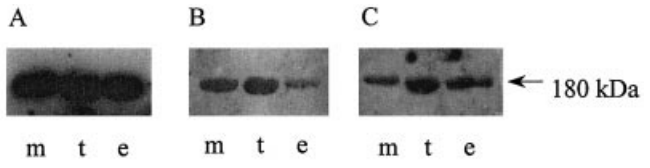

Figure 4 Immunoblot of differently solubilized forms of ACE

ACE was purified from porcine kidney by affinity chromatography on lisinopril–Sepharose after solubilization from the membrane by Triton X-100 in the presence of EDTA (mACE; lane m), trypsin (tACE; lane t), or by the endogenous secretase (eACE; lane e). Samples (10 μg) were electrophoresed on SDS gels and immunoblotted using the anti-ACE ectodomain antiserum RP183 (**A**), or the anti-peptide antisera anti-CAMKLGFS (**B**) or anti-CDIYQSKEAG (**C**).

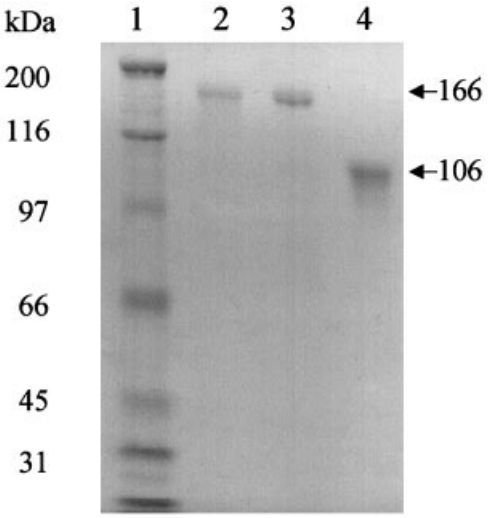

Figure 5 Purified hsACE and testis ACE

Recombinant proteins were isolated from the conditioned media (released soluble ACE) of CHO cells and seminal ACE from human seminal plasma, electrophoresed on a SDS gel, and stained with Coomassie Brilliant Blue. Lane 1, marker; lane 2, seminal plasma ACE; lane 3, recombinant somatic ACE; and lane 4, wild-type testis ACE.

hydrophilic forms of ACE partitioned predominantly (> 96 %) into the detergent-poor phase and failed to reconstitute into liposomes, indicating that they had been cleaved in the juxtamembrane stalk region, as reported previously [29].

The hydrophobic anchoring domain in purified mACE was labelled with 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine and then cleaved from the protein with trypsin [15]. Following removal of the ectodomain of ACE using lisinopril–Sepharose chromatography, the membrane-anchoring domain was purified and used to generate a polyclonal antiserum (RP165). The ability of RP165 to recognize the differently solubilized forms of porcine kidney ACE (mACE, eACE and tACE) and pACE was assessed in a competitive ELISA. The binding of RP165 to immobilized ACE was inhibited in a dose-dependent manner only by mACE, rising to a maximum of 100 % (Figure 3B). The hydrophilic forms of ACE (eACE, tACE and pACE) only partially inhibited the binding of RP165, with a maximum inhibition of binding of 35 % (Figure 3B). The lack of inhibition of binding of RP165 by eACE, tACE and pACE confirmed that these forms lacked the hydrophobic anchoring domain. In addition the superimposable inhibition curves suggested that they were all cleaved at a similar point in the juxtamembrane stalk region. In contrast, the binding of a polyclonal antiserum (RP183) raised against the ectodomain of ACE [31] to immobilized ACE was inhibited in a dose-dependent manner by all three solubilized forms of kidney ACE and by pACE, rising to a maximum of 85 % (Figure 3A). The amphipathic mACE showed a curve slightly displaced to the right (Figure 3A), possibly due to the hydrophobic anchoring domain causing aggregation of the protein.

Antibodies were raised against peptides CDIYQSKEAG-amide and CAMKLGFS-amide, which correspond to amino acids 1126–1135 and 1141–1147, respectively, on either side of the proposed cleavage site in hsACE [22]. These sequences are highly conserved in all ACE species that have been cloned. The

Table 1 Observed $[M+H]^+$ ions of C-terminal somatic ACE peptides generated by endoproteinase Lys-C digestion

Soluble (released) somatic ACE was purified from the conditioned medium of transfected CHO cells (recombinant hsACE), from human seminal plasma (semACE), and from porcine kidney after solubilization by the endogenous secretase (eACE) or trypsin (tACE). In each case, proteins were digested with endoproteinase Lys-C and the total digests were analysed directly by MALDI–TOF MS. Only peptides identified in the C-terminal half of the protein up to and including the peptide 1133–1143, which contained a previously reported alternative cleavage site [23], are shown (see Figure 6). All values are calculated for protonated average molecular mass *m/z*. Peptides 1190–1203 and 1190–1202 were the only peptides identified not ending in a lysine; no peptides C-terminal to these peptides were found.

		Observed *m/z*			
Peptide (residue no.)	Expected *m/z*	hsACE	semACE	eACE	tACE
1133–1143	1176.4	1175.6	1175.5	nf*	nf
1144–1173	3360.9	glyc†	glyc†	nf	nf
1174–1189	1951.2	1950.1	1951.6	nf	nf
1190–1203	1690.8	1691.1	–	1689.9‡	1691.4‡
1190–1202	1533.7	–	1533.9	–	–

* Not found.

† Glycosylated peptide.

‡ C-terminal peptides of porcine somatic ACE eluted on reverse-phase HPLC with the identical retention time as the C-terminal peptide of testis ACE.

amphipathic mACE, that retains the membrane-anchoring domain, and the two hydrophilic forms of ACE (tACE and eACE) were subjected to immunoblot analysis with the anti-peptide antibodies. All three forms of ACE were recognized by an antiserum raised against the ectodomain of porcine kidney ACE (RP183), and by the two anti-peptide antibodies (Figure 4). Prior incubation of the anti-peptide antibodies with their respective peptide abolished the recognition (results not shown). If either of the two hydrophilic forms of ACE had been cleaved at the reported cleavage site [22], they should not have been recognized by the anti-CAMKLGFS-amide antibody, whose sequence does not appear elsewhere in somatic ACE. Thus, both the trypsin- and secretase-cleaved forms of somatic ACE appear to have been cleaved C-terminal to the reported cleavage site [22].

Determination of the juxtamembrane cleavage site in released somatic ACE

Soluble hsACE purified from conditioned media of transfected cells (Figure 5) was digested with endoproteinase Lys-C. Mass-spectral analysis of the total digest enabled ready detection of a peptide at *m/z* 1691.1 (Table 1), which was in close agreement with the calculated mass for the peptide LGWPQYNWTPNSAR (1690.8), and which was virtually identical with the mass of *m/z* 1690.8 identified in the Lys-C digest of released testis ACE. The somatic ACE peptide ends in Arg^{1203} (Figure 6) and corresponds exactly to Arg^{627} in testis ACE, which has been determined to be the C-terminus of released testis ACE [21]. Moreover, HPLC fractionation of the Lys-C digest of somatic ACE enabled purification of a peptide, that by N-terminal sequencing (five cycles) gave the sequence LGWPQ. … Mass-spectral analysis of this fraction again revealed a single major peak at *m/z* 1691.9.

The peak at $m/z \approx 1691$ was observed consistently in separate Lys-C digests of soluble somatic ACE obtained from two independent purifications. Furthermore, in all mass-spectral analyses of total digests, a characteristic peak at m/z 1951 ± 0.5 was also detected, which corresponded to the peptide immediately N-terminal to the C-terminal peptide, with the sequence PLLDWLRTENELHGEK [5] (Figure 6). We have observed this peak in the digests of wild-type testis ACE and of all stalk mutants of testis ACE in which this sequence has not been altered [24,25]. Significantly, no peaks corresponding to peptides C-terminal to Arg^{1203} were identified in any of the digests, and neither were peaks corresponding to peptides ending in Arg^{1137} or Arg^{1227}, which have been suggested to be the C-termini in endothelial ACE and recombinant somatic ACE, respectively [22,23].

To correlate the C-terminus determined for recombinant somatic ACE released from transfected CHO cells with (a) that of a native soluble somatic ACE present in a physiological human fluid, and (b) somatic ACE from another mammalian source, we analysed seminal plasma ACE and porcine kidney ACE. The former was chosen because seminal plasma is a rich source of soluble somatic ACE, unlike blood plasma, in which the concentrations are very low [36]. As expected, soluble somatic ACE was abundant in seminal plasma and was readily purified by affinity chromatography. The seminal plasma enzyme had an identical electrophoretic mobility to recombinant somatic ACE (Figure 5). Mass-spectral analysis of a total Lys-C digest of seminal plasma ACE again revealed the characteristic peak at *m/z* 1951.6 (Table 1), corresponding to the penultimate C-terminal peptide. Instead of a peak at *m/z* 1691, however, the seminal plasma ACE digest revealed a new peak at *m/z* 1533.9, which corresponded closely to the calculated mass for the peptide LGWPQYNWTPNSA (1533.7), giving a C-terminus at Ala^{1202} instead of Arg^{1203} (Figure 6). Again, no peaks corresponding to peptides C-terminal to Ala^{1202}, or to a peptide ending in Arg^{1137}, were identified.

Porcine kidney ACE was solubilized using trypsin as well as the endogenous kidney ACE secretase in a cell-free system. For the total Lys-C digests of both trypsin- and secretase-solubilized porcine ACE, molecular ions at *m/z* 1691.4 and *m/z* 1689.9 were

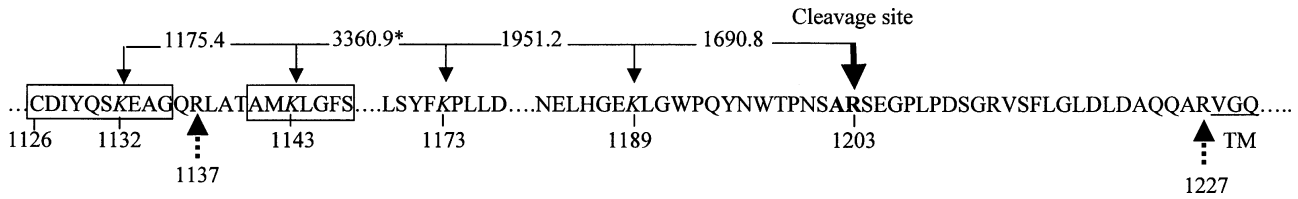

Figure 6 C-terminal Lys-C fragmentation for somatic ACE

Lys-C cleavage sites are indicated (thin arrows) with the expected molecular ions above the corresponding peptides. The cleavage site of the ACE secretase (bold arrow) for both the recombinant somatic and seminal ACE as well as previously reported cleavage sites (broken arrows) [23] are shown. Anti-peptide antibodies were raised to sequences (boxed) on either side of the previously reported R^{1137}/L^{1138} cleavage site. The Lys-C peptide L^{1144} to K^{1173} (*) (not found) is probably glycosylated as in the testis isoform. Underlining refers to the TM domain.

observed (Table 1). The calculated mass of the human C-terminal peptide is 1690.8, which was in close agreement with the observed porcine ACE molecular ions. Thus, although the sequence for porcine ACE is unknown, these data together with the HPLC co-elution of the two peptides (results not shown) were highly suggestive that the same C-terminal peptide was generated by both human and porcine ACE secretases, and also by adventitious trypsin-mediated stalk cleavage.

We conclude that membrane-bound human and porcine somatic ACE were cleaved at the Arg^{1203}/Ser^{1204} bond, identical with the Arg^{627}/Ser^{628} bond in testis ACE, at a distance of 24 residues from the membrane (Figure 6). We propose that an identical cleavage generates soluble seminal plasma ACE, but that this protein undergoes secondary carboxypeptidase processing to generate an Ala^{1202} C-terminus.

DISCUSSION

The somatic and testis isoenzymes of ACE are both type I ectoproteins that undergo proteolytic release or shedding when expressed in various eukaryotic cells in culture [8,9,20,23]. An identical process has been observed in ACE-expressing tissues *in situ* [10], and is probably responsible for the generation of soluble somatic ACE that can be detected in most bodily fluids *in vivo* (reviewed in [1,2]). The generation of soluble testis ACE *in vivo* remains to be clearly documented. Moreover, the functions of soluble somatic ACE, or of soluble testis ACE (if it exists), *in vivo* are unknown. This notwithstanding, the release of membrane-bound ACE in transfected cells *in vitro* constitutes a useful model system for the general phenomenon of membrane protein release, which is widespread and involves a specialized class of proteases, variously referred to as sheddases, secretases, or membrane-protein-solubilizing proteases [11,12,37].

The mechanisms that confer specificity and that regulate membrane protein release are unresolved. Considerable data indicate that in diverse membrane proteins a juxtamembrane stalk sequence that is sterically unhindered and of minimum length is required for cleavage (reviewed in [21]). Although similar considerations apply in the case of testis ACE [21], this membrane protein appears to tolerate a remarkable diversity of stalk sequences, including sequences that are disulphide-bridged or glycosylated [24,25] which nevertheless are cleaved with shedding of the protein. A mechanism that could account for the strong propensity for cleavage and release of testis ACE, irrespective of stalk characteristics, is the presence of a secretase-activating recognition motif in the extracellular domain adjacent to the stalk. Evidence for this notion derives from two sources. First, Beldent et al. [23] showed that somatic ACE was released 10-fold slower than a C-domain construct (essentially the testis isoenzyme) when expressed in CHO cells, speculating that the N-terminal domain of somatic ACE acts as a 'conformational inhibitor'.

Second, Sadhukhan et al. [26] showed that when the extracellular domain of testis ACE was fused to the stalk, TM and cytoplasmic domains of CD4 (a membrane protein that is usually resistant to shedding), the construct was efficiently cleaved and released. These data suggest, but do not prove conclusively, that the testis ACE extracellular domain contains a recognition motif that stimulates the ACE secretase. An alternative explanation is that the N-terminal domain of somatic ACE, or the extracellular domain of CD4, sterically hinders access to the stalk, a notion that was apparently supported by different cleavage sites in endothelial somatic ACE versus the C-terminal construct [23].

In the present study we confirm the previous finding [23] that somatic ACE is released less efficiently than testis ACE, indicating that the latter is indeed a poor substrate for the CHO cell secretase. However, this difference could not be ascribed to the use of an alternative stalk cleavage site and hence was not a manifestation of obstruction by the N-terminal domain of a more 'favoured' cleavage site used for the efficient release of testis ACE.

The cleavage site in somatic ACE expressed in CHO cells, as well as in the native human plasma enzyme, was previously reported to be at the Arg^{1137}/Leu^{1138} bond [22]. This was based on C-terminal microsequencing, amino acid analysis of C-terminal peptides, and immunocharacterizations with C-terminal antibodies [22]. eACE and tACE clearly lack the hydrophobic anchoring domain, as shown by their partitioning into the aqueous phase of Triton X-114, their failure to reconstitute into liposomes [29] and their lack of recognition by an antiserum raised against the hydrophobic anchoring domain (Figure 3). These properties were distinctly different from those of the amphipathic mACE, which retains the hydrophobic anchoring domain. However, anti-peptide antibodies raised against sequences on either side of the proposed Arg^{1137}/Leu^{1138} cleavage site (Figure 6) recognized all three solubilized forms of porcine kidney ACE (Figure 4), indicating that neither eACE nor tACE had been cleaved at this site. Moreover, soluble ACE generated *in vivo*, i.e. pACE, probably had a similar or identical cleavage site as eACE and tACE, based on superimposable inhibition curves produced in a competitive ELISA with each of the soluble forms of somatic ACE.

We have reported a general methodology for determining the C-terminus of shed, soluble wild-type and stalk-mutated testis ACE, thereby establishing the juxtamembrane cleavage sites [21,24,25]. The method depends on chemical or enzymic fragmentation of the soluble protein, reverse-phase HPLC fractionation and identification of the C-terminal peptide, followed by MALDI–TOF MS, which enables the precise assignment of the C-terminal amino acid residue. Using this procedure we have been able to assign the C-terminus of soluble somatic ACE to Arg^{1203} with very high precision (Figure 6). It is possible, but unlikely, that we have failed to detect peptides C-terminal to Arg^{1203}. It is also possible that we have failed to detect peptides ending in Arg^{1137}, representing the C-terminus of a fraction of released ACE. However, if present, these are likely to be quantitatively minor, because the C-terminal peptide and the adjacent peptide were observed as strong signals on all mass spectra performed on several independent digests. Furthermore, cleavage at Arg^{1137} would generate a protein 7.6 kDa smaller than the protein cleaved at Arg^{1203}, and no such second protein was observed on regular SDS/PAGE gels or on autoradiographs of affinity precipitates of conditioned media (Figures 2 and 5). Previously we have shown that the hydrophobic anchoring domain released from mACE by trypsin has a size of 6.9 kDa [15], which is inconsistent with a cleavage at Arg^{1137}. Lastly, cleavage at Arg^{1137} is 43 residues N-terminal to the distal limit of a 47 residue stalk deletion mutant of testis ACE, which was catalytically inactive [21]. Since the C-terminal (i.e. testis) domain contributes 80–90 % of the catalytic activity of somatic ACE, a cleavage at Arg^{1137} would not be consistent with the known catalytic properties of soluble somatic ACE.

It is likely that the Arg^{1203}/Ser^{1204} site is also the cleavage site used *in vivo* to generate soluble somatic ACE by shedding from epithelial cell surfaces. Porcine eACE, shown here to be cleaved at Arg^{1203}/Ser^{1204}, is generated by the action of an endogenous secretase located on renal brush border epithelial surfaces [10]. Similarly, seminal plasma ACE, which is derived from epididymal and prostatic epithelia (reviewed in [1]), was shown here to end in Ala^{1202}. Although this may indicate a cleavage at the

Ala^{1202}/Arg^{1203} bond, we favour the view that the primary cleavage is at Arg^{1203}/Ser^{1204}, with secondary carboxypeptidase processing, as was found for the amyloid precursor protein [38]. Soluble plasma ACE, derived from shedding of endothelial ACE, is probably also cleaved at the Arg^{1203}/Ser^{1204} bond. Although we did not determine the C-terminus of plasma ACE by MS, the inhibition curve for pACE by competitive ELISA was superimposable on the curves for eACE and tACE, indicating similar or identical sequences.

The finding that tACE has a C-terminus that is identical with that of eACE and soluble hsACE is interesting. Trypsin has long been used as a specific and convenient means to solubilize tissue-bound ACE, especially from kidney and lung homogenates [36,39]. As the endogenous secretase cleaves C-terminal to an arginine, it is fortuitous but not surprising that trypsin cleaves at the same site. Nevertheless, the proposed ACE stalk contains three arginine residues (Arg^{627}, Arg^{637} and Arg^{651}) and one lysine residue (Lys^{613}) (testis ACE numbering, see Ehlers et al. [21]). Exclusive use of the Arg^{1203}/Ser^{1204} bond by a highly active protease such as trypsin implies that this bond offers a unique degree of accessibility in the ACE stalk. A similar conclusion can be drawn from results with diverse stalk-deletion mutants of ACE expressed in CHO cells. Whenever the native Arg/Ser cleavage site is preserved, irrespective of adjacent deletions, the CHO cell secretase cleaves at that site Ehlers et al. [21] (and A. J. Chubb, S. L. U. Schwager, M. R. W. Ehlers and E. D. Sturrock, unpublished work). We propose that the Arg^{1203}/Ser^{1204} site in somatic ACE, and the homologous Arg^{627}/Ser^{628} site in testis ACE, provides a unique point of access for the secretase. Control of shedding is thus not the result of constraints on access to the stalk, but due to factors that lie beyond the boundaries of the stalk itself.

Identical stalk cleavage sites for somatic and testis ACE do not rule out the possibility that the slow release rate of the former results from steric inhibition of the secretase by the N-terminal domain. However, our results lend weight to the alternative view that the C-terminal domain contains a recognition motif that is obscured by the N-terminal domain of somatic ACE. The identity of such a motif is unknown. An obvious candidate would be the Ser/Thr-rich heavily O-glycosylated N-terminal sequence unique to testis ACE [40]. However, this possibility was ruled out by the C-terminal domain mutant of Beldent et al. [23], which did not contain this sequence, and by a testis ACE construct lacking the sequence [25]. Neither differed from wild-type testis ACE in terms of rapid release rates. This question is of considerable interest, in light of increasing evidence that the ACE secretase differs from the TNF-α convertase [16,17] and this may also extend to its recognition and activation characteristics.

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