Roles of the juxtamembrane and extracellular domains of angiotensin-converting enzyme in ectodomain shedding

Susan PANG*, Anthony J. CHUBB†, Sylva L. U. SCHWAGER†, Mario R. W. EHLERS‡, Edward D. STURROCK† and Nigel M. HOOPER*[1]

*Proteolysis Research Group, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K., †Department of Medical Biochemistry, University of Cape Town, Rondebosch 7700, South Africa, and ‡BioNebraska, Inc., 3820 NW 46th Street, Lincoln, NE 68524, U.S.A.

Angiotensin-converting enzyme (ACE) is one of a growing number of integral membrane proteins that is shed from the cell surface through proteolytic cleavage by a secretase. To investigate the requirements for ectodomain shedding, we replaced the glycosylphosphatidylinositol addition sequence in membrane dipeptidase (MDP) - a membrane protein that is not shed - with the juxtamembrane stalk, transmembrane (TM) and cytosolic domains of ACE. The resulting construct, MDP–STM_{ACE}, was targeted to the cell surface in a glycosylated and enzymically active form, and was shed into the medium. The site of cleavage in MDP–STM_{ACE} was identified by MS as the Arg^{374}-Ser^{375} bond, corresponding to the Arg^{1203}-Ser^{1204} secretase cleavage site in somatic ACE. The release of MDP–STM_{ACE} and ACE from the cells was inhibited in an identical manner by batimastat and two other hydroxamic acid-based zinc metallosecretase inhibitors. In contrast, a construct lacking the juxtamembrane stalk, MDP–TM_{ACE}, although expressed at the cell surface in an enzymically active form, was not shed, implying that the juxtamembrane stalk is the critical determinant of shedding. However, an additional construct, ACEΔC, in which the N-terminal domain of somatic ACE was fused to the stalk, TM and cytosolic domains, was also not shed, despite the presence of a cleavable stalk, implying that in contrast with the C-terminal domain, the N-terminal domain lacks a signal required for shedding. These data are discussed in the context of two classes of secretases that differ in their requirements for recognition of substrate proteins.

Key words: batimastat, membrane dipeptidase, secretase, zinc metalloprotease.

INTRODUCTION

Numerous integral membrane proteins are shed from the membrane by post-translational proteolysis, including angiotensin-converting enzyme (ACE), the amyloid precursor protein, tumour necrosis factor-α and transforming growth factor-α (TGF-α) (reviewed in [1–3]). The enzymes responsible for cleaving such membrane proteins have been referred to as secretases, sheddases or convertases, the best characterized of which is tumour necrosis factor-α convertase (TACE). TACE is a membrane-bound zinc metalloproteinase [4,5] and is a member of the ADAMs ('a disintegrin and metalloproteinase') family [6], that is involved in the shedding of several proteins [7].

ACE (EC 3.4.15.1) is a type I integral membrane protein that plays a key role in blood pressure homoeostasis, and inhibitors of this zinc metalloproteinase are used routinely in the treatment of hypertension [8,9]. Mammalian ACE is present as two distinct isoforms, somatic and testis ACE, which are transcribed from a single gene at tissue-specific initiation sites [10]. Somatic ACE, which is widely expressed in the lungs, kidney and other tissues, consists of two catalytic domains each bearing a functional, zinc-dependent active site [11–13]. In contrast, testis ACE, which is located exclusively in the testis, consists of only a single domain that corresponds to the C-terminal domain of somatic ACE [14]. A soluble form of ACE is present in blood, cerebrospinal fluid, seminal fluid and other body fluids. This soluble form is derived from the membrane-bound form through the action of ACE secretase [15]. ACE secretase is itself a membrane-bound zinc metalloproteinase, inhibited by hydroxamic acid-based compounds, such as batimastat [16], and has many properties in common with the α-secretase that cleaves the amyloid precursor protein [17]. ACE secretase is distinct from TACE, displaying a different inhibitor profile with a range of hydroxamic acid-based compounds [18], and the release of ACE is not blocked in cells derived from TACE knockout mice [19]. The site of cleavage of somatic ACE by its secretase has been identified as the Arg^{1203}-Ser^{1204} bond (human somatic ACE numbering), 27 residues on the extracellular side of the transmembrane (TM) domain [20].

The requirements for recognition of a membrane protein by its cognate secretase are unclear. For example, juxtamembrane stalk sequences from TGF-α and the amyloid precursor protein endowed betaglycan with the ability to be cleaved, implying that the juxtamembrane stalk domain is the determinant for ectodomain shedding [21]. In contrast, a chimaeric protein containing the ectodomain of ACE and the juxtamembrane stalk, TM and cytosolic domains of CD4, which is not subject to ectodomain shedding, was efficiently cleaved off the cell surface, whereas a chimaera containing the ectodomain of CD4 and the juxtamembrane stalk, TM and cytosolic domains of ACE was not cleaved, implying that the distal ectodomain is the primary determinant for shedding [22], and possibly contains a motif that is recognized by the secretase.

In the present study we have investigated the requirements for recognition of a substrate protein by its cognate secretase. In order to investigate the role of the juxtamembrane stalk region in ectodomain shedding, the C-terminal glycosylphosphatidylinositol (GPI) anchor attachment signal in membrane dipeptidase (MDP; EC 3.4.13.19), which is not subject to proteolytic

Abbreviations used: Abz, *o*-aminobenzoic acid; ACE, angiotensin-converting enzyme; CHO, Chinese hamster ovary; Dnp, 2,4-dinitrophenyl; GPI, glycosylphosphatidylinositol; HER4, human epidermal growth factor receptor 4; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MDP, membrane dipeptidase; TACE, tumour necrosis factor-α convertase; TBS, Tris-buffered saline; TGF-α, transforming growth factor-α; TM, transmembrane; wtMDP, wild-type MDP.

[1] To whom correspondence should be addressed (e-mail n.m.hooper@leeds.ac.uk).

shedding, was replaced with either the juxtamembrane stalk, TM and cytosolic domains of ACE, to generate MDP–STM_{ACE}, or just the TM and cytosolic domains (MDP–TM_{ACE}). Another chimaeric construct, ACEΔC, was produced to determine whether the N-terminal domain of somatic ACE lacks an essential recognition motif for the secretase-mediated cleavage of the ACE stalk. This hypothesis was based on the observation that whereas testis ACE is shed efficiently, somatic ACE, which contains an additional N-terminal domain but is otherwise identical with testis ACE, is shed poorly [20]. All three constructs were expressed at the cell surface in enzymically active forms, but only MDP–STM_{ACE} was shed into the medium. The site of cleavage in the juxtamembrane stalk was identical with that of ACE, and the inhibition profile with a range of hydroxamic-acid based compounds for the release of MDP–STM_{ACE} was essentially identical with that of ACE. These data are discussed in the context of two classes of secretases that differ in their requirements for recognition of substrate proteins.

EXPERIMENTAL

Generation of the cDNA constructs

Construction of MDP–TM_{ACE} and MDP–STM_{ACE} was based on megaprimers synthesized by PCR using the forward primers 5′-CGG ACG AAT TAC GGC TAC TGG CTG CTG CTC TTC CTG-3′ and 5′-CGG ACG AAT TAC GGC TAC TGG ACG CCG AAC TCC GCT-3′ respectively. Human somatic ACE cDNA [12] was used as the template, and the reverse primer 5′-CCA TCG ATT CAG GAG TGT CTC AGC TC-3′ was used in the first PCR for both constructs. Each resulting megaprimer was used as the reverse primer in the subsequent PCR, where 5′-GCG CGC TCT AGA CAG ACG TGA GGA GCG GCT-3′ was used as the forward primer and porcine MDP cDNA [23] was used as the template, to yield the coding sequences of MDP–TM_{ACE} and MDP–STM_{ACE}. The inserts were ligated into the expression vector pBK-CMV and then subcloned into pIRES*hyg*. The ACEΔN coding sequence was subcloned from the pECE vector [12] into pIRES*hyg* by PCR using 5′-ATG GAT CCA TGG GGG CCG CCT CGG GC-3′ as the forward primer and 5′-TAC CAG TGT GCT GGT CAG GAG TGT CTC AGC TC-3′ as the reverse primer.

Construction of pLEN-ACEΔC was achieved with a two-stage PCR strategy similar to that used previously for pLEN-ACE-JMLDL [24], but the unique *Pin*AI site in the N-terminal domain of somatic ACE was used. Suitable PCR primers were used to delete the sequence Pro^{602}–Thr^{1198} in somatic ACE, thereby fusing the somatic ACE N-terminal domain directly on to the stalk region of testis ACE. The primers 5′-CCC GGG AAT TCA TCT ACC GGT CCA CC-3′ and 5′-GCG AGC GGA GTT CGG GTG CCA CTG GTA C-3′ were used to amplify nt 1697–1912 of the full-length somatic ACE cDNA (numbering from GenBank® accession number J04144). Primers 5′-TAC CAG TGG CAC CCG AAC TCC GCT CGC-3′ and 5′-GTC GAC GGT ATC GAT TCA GGA GTG TCT CAG CTC-3′ were used to amplify nt 1982–2221 of full-length testis ACE cDNA containing the testis juxtamembrane stalk sequence, TM domain and cytoplasmic region (numbering from GenBank® accession number M26657). The PCR products were mixed and the flanking primers used to PCR a 'zippered' fusion product that was cloned into pBluescript using *Eco*RI and *Cla*I. After sequencing, the PCR product was cloned into somatic ACE in pBS using *Pin*AI and *Cla*I. The complete ACEΔC construct was then cloned into the mammalian expression vector pLEN-ACEVII [25] using *Bam*HI and *Cla*I.

Cell growth, transfection and lysis

IMR32 cells [26] were cultured in Dulbecco's modified Eagle's medium with glutamax, supplemented with 10% (v/v) foetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin, and maintained in a 5% CO_2-humidified atmosphere at 37 °C. Cells at mid-confluence were harvested with trypsin and resuspended in growth medium, and a 0.7 ml aliquot of cell suspension was placed in a 4 mm electroporation cuvette and incubated for 1 min with 30 μg of linearized DNA prior to the pulse. Cells were pulsed at 1650 μF/250 V using the Easy-Ject electroporator (Flowgen, Ashby-de-la-Zouch, Leics., U.K.) and immediately transferred to fresh medium. Selection for antibiotic resistance was started 24 h after electroporation by incubating the cells with complete medium containing 0.4 mg/ml hygromycin B. Chinese hamster ovary (CHO)-K1 cells were grown and cotransfected with 20 μg of pLEN-ACEΔC and 0.33 μg of pSV2Neo by methods detailed previously [24,25].

Cells were washed with OptiMEM and incubated with either batimastat, SB256636 or SKF109074 (provided by Dr G. Christie, GlaxoSmithKline, Harlow, U.K.) for 7 h. The medium was then harvested and centrifuged at 1000 ***g*** to remove debris. Cell lysates were prepared by treating cells with 1% (v/v) Triton X-100 in 50 mM Hepes/NaOH (pH 7.5), 0.5 M NaCl and 1 mM PMSF as described elsewhere [24]. Membranes were isolated by washing cells twice in PBS, and scraping cells into PBS and centrifuging at 100 ***g*** for 10 min. The cell pellet was resuspended in 50 mM Hepes/NaOH (pH 7.5) and 20 mM $CaCl_2$, and the cells were disrupted by sonicating for two 1 min pulses, with a 30 s cooling period in between. The suspension was centrifuged at 1000 ***g*** for 10 min, and the resulting supernatant was centrifuged at 100000 ***g*** for 90 min. The pellet was resuspended in 10 mM Tris/HCl (pH 7.5) and 0.5 M NaCl, and re-centrifuged at 100000 ***g*** for 90 min. The final membrane pellet was resuspended in 10 mM Hepes/NaOH (pH 7.4), and 2 μl of protease inhibitor cocktail (Sigma, Poole, Dorset, U.K.) was added.

Enzyme assays

MDP enzymic activity was assayed using the dipeptide Gly-D-Phe (1 mM) as the substrate in 0.1 M Tris/HCl (pH 8.0). ACE enzymic activity was determined using 5 mM benzoyl-Gly-His-Leu as the substrate in 0.1 M Tris/HCl (pH 8.3), 0.3 M NaCl and 10 μM $ZnCl_2$. The reaction mixtures were incubated at 37 °C and terminated by heating at 100 °C for 4 min. Substrate and reaction products were separated and quantified by reverse-phase HPLC as described previously [27,28]. ACEΔC enzymic activity was assayed at 37 °C using the internally quenched fluorogenic peptide Abz-SDK(Dnp)P-OH (8 μM) (which is specific for the ACE N-terminal domain [29]; Abz corresponds to *o*-aminobenzoic acid and Dnp corresponds to 2,4-dinitrophenyl) as the substrate in 0.1 M Tris/HCl (pH 7.0), 50 mM NaCl and 10 μM $ZnCl_2$. Substrate hydrolysis was monitored continuously by measuring the fluorescence at an excitation wavelength of 320 nm and an emission wavelength of 420 nm for 5 min after 50 μl of enzyme solution was added to 2.5 ml of temperature-equilibrated substrate solution. The initial slope was converted into mol of substrate hydrolysed/min using fluorescence curves for standard peptide solutions after total hydrolysis [30].

SDS/PAGE and Western-blot analysis

Samples were mixed with an equal volume of either reducing or non-reducing electrophoresis sample buffer and boiled for 5 min.

Proteins were resolved by SDS/PAGE using a 7–17% (w/v) acrylamide gradient gel and transferred to PVDF membranes (Immobilon P), as described previously [28]. The membranes were blocked by incubation in PBS containing 0.1% (v/v) Tween 20, 5% (w/v) dried milk powder and 2% (w/v) BSA for 1 h at 24 °C. The polyclonal antibody (RP209) raised against purified porcine kidney MDP was prepared as described previously [27]. The polyclonal antibody (RP147) raised against purified human kidney ACE was prepared as described elsewhere [13]. All primary and secondary antibody incubations were performed in PBS containing 2% (w/v) BSA and 0.1% Tween 20. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham Life Sciences, Little Chalfont, Bucks., U.K.). Protein was quantified using bicinchoninic acid [31] in a microtitre plate assay with BSA as a standard.

Immunofluorescence confocal microscopy

Cells were seeded on sterile acid-washed coverslips in 6 well plates, cultured to 50% confluency, and then incubated in OptiMEM overnight (with or without 20 μM batimastat). The cells were washed in Tris-buffered saline [TBS; 25 mM Tris/HCl (pH 7.5), 137 mM NaCl and 2.68 mM KCl], and then either permeabilized, fixed using methanol/acetone (1:1, v/v) for 10 min and then washed in TBS, or fixed using 3% (w/v) paraformaldehyde in PBS and washed with PBS. The cells were then incubated with blocking buffer [TBS containing 5% (v/v) goat serum] for 30 min and rewashed in TBS. The coverslips were incubated in 0.1 ml of the primary antibody (1:100 dilution) in TBS containing 1% (v/v) goat serum for 2 h at 24 °C. Cells were washed in TBS and incubated with the appropriate biotin anti-primary antibody (1:100 dilution) in TBS containing 1% (v/v) goat serum for 1 h. Following another wash in TBS, the cells were incubated with the FITC-conjugated anti-biotin antibody (1:400 dilution) in TBS containing 1% (v/v) goat serum for 30 min at 24 °C. The coverslips were finally washed in TBS, mounted on to glass microscope slides using Vectashield, and viewed under a scanning confocal microscope (Leitz diaplan model).

Purification and MS analysis of soluble MDP–STM$_{ACE}$

Cilastatin–Sepharose affinity resin was prepared as described previously [27], and was pre-equilibrated in 50 mM Tris/HCl (pH 7.5) and 0.1 M NaCl. The cell medium sample containing the soluble form of MDP–STM$_{ACE}$ was applied to the cilastatin–Sepharose column, and unbound material was removed with 50 mM Tris/HCl (pH 7.5) and 0.5 M NaCl. MDP–STM$_{ACE}$ was eluted from the column with cilastatin (10 mg) in 10 ml of the same buffer. The cilastatin was removed from the enzyme by extensive dialysis against 10 mM Tris/HCl (pH 7.6). MDP–STM$_{ACE}$ was then concentrated using Vivaspin 15 centrifugal concentrators (10000 molecular mass cut off; Vivascience, Sartorious Group, Stonehouse, Gloucestershire, U.K.). Purified soluble MDP–STM$_{ACE}$ was reduced and protected with vinylpyridine, followed by hydrolysis with endoproteinase Lys-C. The total digest was analysed directly by matrix-assisted laser-desorption ionization–time-of-flight (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 mass spectral analysis [32,33].

RESULTS

Construction and expression of MDP and ACE constructs

In order to investigate the role of the juxtamembrane stalk region of ACE in ectodomain shedding, two chimaeras of MDP and ACE were made (Figure 1). In MDP–STM$_{ACE}$ the GPI anchor attachment signal of MDP was replaced with the C-terminal 81 amino acid residues of ACE including the juxtamembrane stalk, the TM domain and the cytosolic domain. In MDP–TM$_{ACE}$ the GPI anchor attachment signal was replaced with the C-terminal 47 amino acid residues of ACE encompassing only the TM and cytosolic domains. Each of the constructs, along with wild-type

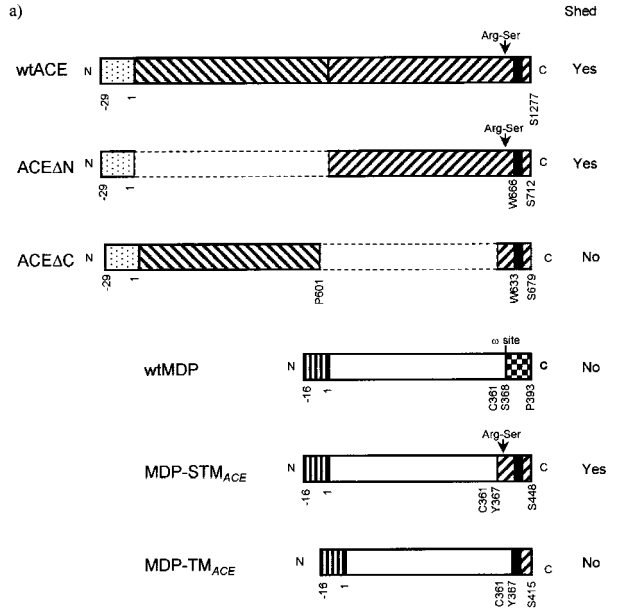

Figure 1 Constructs used in the present study

(**a**) Somatic ACE (wtACE) has an N-terminal signal peptide (dotted box), two catalytic domains (diagonally hatched boxes) and a C-terminal transmembrane domain (black box). ACEΔN has an N-terminal signal peptide (diagonally hatched box), the C-terminal domain of somatic ACE and the C-terminal TM domain (black box) [12], i.e. it lacks the N-terminal catalytic domain of somatic ACE. ACEΔC comprises the N-terminal domain of somatic ACE (Leu^{1} to Pro^{601}) fused to the juxtamembrane stalk, TM and cytosolic domains; i.e. it lacks the C-terminal catalytic domain of somatic ACE. wtMDP has an N-terminal signal peptide (vertically hatched box) and a C-terminal GPI anchor addition sequence (chequered box) [23]. MDP–STM$_{ACE}$ and MDP–TM$_{ACE}$ possess the N-terminal portion of porcine MDP up to and including the $\omega-1$ site for GPI anchor addition (Tyr^{367}). MDP–STM$_{ACE}$ contains the C-terminal 81 amino acid residues of human ACE, including the juxtamembrane stalk region, the TM domain and the cytosolic domain. MDP–TM$_{ACE}$ contains the C-terminal 47 amino acid residues of human ACE from the TM domain onwards. The secretase cleavage site in wtACE, ACEΔN and MDP–STM$_{ACE}$ is indicated between the arginine and serine residues by an arrow. Cys^{361} is the sole residue involved in the interchain disulphide linkage of the MDP homodimer. (**b**) The C-terminal sequence of wtMDP from Cys^{361} to the C-terminus is shown. The GPI anchor addition sequence, including the ω residue (Ser^{368}) is underlined. The juxtamembrane stalk region, including the secretase cleavage site (arrow), is shown for ACEΔN, MDP–STM$_{ACE}$, MDP–TM$_{ACE}$ and ACEΔC. The transmembrane region is in italics, although there is uncertainty about its precise start, and VGQ is an alternative [20,24].

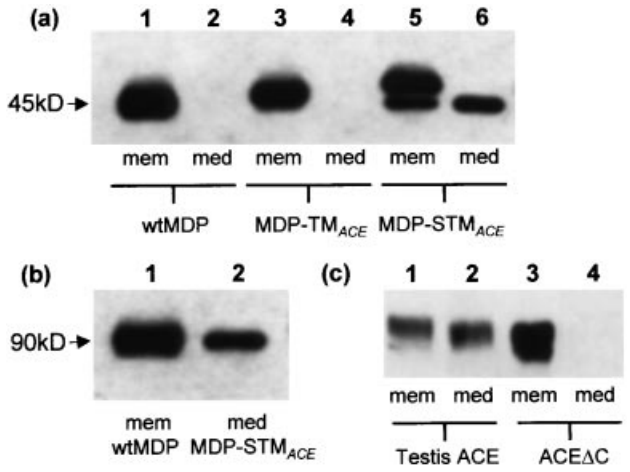

Figure 2 MDP–STM$_{ACE}$, but not MDP–TM$_{ACE}$ or ACEΔC, is shed from transfected cells

IMR32 cells expressing either wtMDP, MDP–STM$_{ACE}$, or MDP–TM$_{ACE}$ were incubated in OptiMEM for 7 h. The medium (med) was harvested and the membranes (mem) prepared from the cells as described in the Experimental section. Samples (8 μg) were resolved by SDS/PAGE under (**a**) reducing or (**b**) non-reducing conditions and immunoblotted with an anti-MDP antibody. (**c**) CHO cells expressing either testis ACE or ACEΔC were induced with 1 μM phorbol ester for 4 h. The medium was harvested and the cell extract was prepared as described in the Experimental section. Samples were subjected to SDS/PAGE and immunoblotted with an anti-ACE polyclonal antibody. kD, kDa.

MDP (wtMDP), were stably expressed in the IMR32 cell line that we have used previously for the characterization of the ACE secretase [17,34]. The ACE N-terminal domain mutant was designed to test whether there may be a secretase recognition motif in the C-terminal domain of ACE that is absent in the N-terminal domain. To achieve this, ACEΔC was constructed by splicing the N-terminal domain of somatic ACE (Leu^{1}–Pro^{601}) on to the juxtamembrane stalk, TM and cytosolic domains of testis ACE (Asn^{624}–Ser^{701}). This is identical with the corresponding region in somatic ACE (Asn^{1200}–Ser^{1277}). This construct was expressed in CHO cells as described elsewhere [24].

MDP–STM$_{ACE}$, but not MDP–TM$_{ACE}$ or ACEΔC, is shed from cells

Confirmation of the expression of the ACEΔC mutant and the MDP–ACE chimaeras was obtained by Western-blot analysis. An anti-MDP antibody [27] was used to detect MDP in membranes and medium derived from the transfected cells (Figure 2). A polypeptide of 45 kDa was detected in the membrane fraction (Figure 2a, lane 1), but not in the medium (Figure 2a, lane 2), from cells expressing wtMDP, indicating that this GPI-anchored form of the protein is not constitutively shed from the cells. Similarly a polypeptide of approx. 46 kDa was detected in the membranes (Figure 2a, lane 3) but not the medium (Figure 2a, lane 4) from cells expressing MDP–TM$_{ACE}$. The slight increase in molecular mass between MDP–TM$_{ACE}$ and wtMDP is due to the presence of the TM and cytosolic domains of ACE in the construct. Similarly, MDP–STM$_{ACE}$ appeared as a slightly larger polypeptide in the membrane fraction (Figure 2a, lane 5), reflecting the presence of the additional juxtamembrane stalk region in this construct. In addition, a polypeptide of approx. 45 kDa was detected in the medium from the cells expressing MDP–STM$_{ACE}$ (Figure 2a, lane 6), indicating that MDP–STM$_{ACE}$ was shed from the cells. The observed reduction in size between the polypeptide in the membranes compared with that in the medium is consistent with removal of the TM and cytosolic domains upon shedding of MDP–STM$_{ACE}$.

MDP is a homodimer with Cys^{361} the sole cysteine residue involved in the interchain disulphide link (Figure 1b) [35]. Thus under non-reducing conditions wtMDP migrates with an apparent molecular mass of 90 kDa (Figure 2b, lane 1). Analysis of the medium from the cells expressing MDP–STM$_{ACE}$ revealed that the shed form of the protein also existed as a disulphide-linked dimer (Figure 2b, lane 2), indicating that the protein had been cleaved C-terminal to Cys^{361}. The lower polypeptide band in the membrane fraction from the cells expressing MDP–STM$_{ACE}$ (Figure 2a, lane 5) may be due to generation of the cleaved form of the protein during the isolation of the membranes, or to an incompletely cleaved MDP–STM$_{ACE}$ homodimer that has a cleaved monomer tethered to the membrane-anchored monomer by the interchain disulphide. MDP has two N-glycosylation sites, both of which are modified with carbohydrate [36]. Deglycosylation with peptide *N*-glycosidase F revealed that, like wtMDP, both MDP–STM$_{ACE}$ and MDP–TM$_{ACE}$ were glycosylated to a similar extent (results not shown).

An anti-ACE polyclonal antibody was used to detect the membrane-bound and soluble forms of ACE (Figure 2c). Testis ACE was detected in the cell extract, as well as the culture medium, after 4 h of induction with phorbol ester (Figure 2c, lanes 1 and 2). The cellular form of the ACEΔC mutant was also detected after 4 h of induction, but as a much stronger band than the testis ACE (Figure 2c, lane 3). In marked contrast with the soluble testis ACE results, the ACEΔC mutant was not detected in the medium after 4 h (Figure 2c, lane 4). The immunoblotting data suggested that the ACEΔC mutant was expressed in the CHO cells. To investigate whether it was enzymically active, the cell lysate and medium were assayed using Hip-His-Leu and the N-terminal domain-specific substrate, Abz-SDK(Dnp)P-OH [29]. Continuous fluorometric assays with the substrate Abz-SDK(Dnp)P-OH could not detect ACE N-terminal domain activity in the medium of phorbol ester-stimulated CHO cells, whereas the cell lysates contained significant activity (8.99 ± 0.69 nmol/min per ml; almost 16-fold greater than that observed in cells expressing testis ACE, 0.58 ± 0.33 nmol/min per ml). Similar data were obtained using Hip-His-Leu as the substrate (results not shown), although the activity was much lower than that of the C-terminal domain, owing to the lower k_{cat} of the N-terminal domain for the substrate Hip-His-Leu [12]. Thus ACEΔC, like MDP and MDP–TM$_{ACE}$, is not shed, in spite of the presence of the ACE juxtamembrane stalk region. Collectively, these data indicate that the juxtamembrane stalk of ACE, but not its TM and cytosolic domains, can confer secretase cleavage on an otherwise non-shed protein. However, interactions between the secretase and regions outside the ACE juxtamembrane stalk region, in the extracellular domain, also appear to be important for ectodomain shedding.

MDP–STM$_{ACE}$ is cleaved at the same Arg-Ser site as ACE

Soluble MDP–STM$_{ACE}$ was purified from the conditioned medium of transfected cells by chromatography on cilastatin–Sepharose [27]. The protein was digested with endoproteinase Lys-C, fractionated by HPLC and analysed by MALDI–TOF MS (Table 1). The use of endoproteinase Lys-C, as opposed to trypsin, is preferable because the natural secretase cleavage site in ACE follows an arginine residue (Figure 1b) [20]. The spectra revealed a $[M+H]^+$ ion at m/z 7978.2, which corresponds to the calculated mass of the peptide Leu^{305}–Arg^{374} (m/z 7974.9). Furthermore, the identity of this peptide was confirmed by partial N-terminal sequence analysis (32 cycles). Hence, the

Table 1 Observed $[M+H]^+$ ions of peptides generated by endoproteinase Lys-C digestion of MDP–STM_{ACE}

Bold characters indicate the C-terminal peptide.

Peptide	Calculated *m/z*	Observed *m/z*
57–80	2723.0	2722.3
81–117	4446.1*	4447.2
118–170	5866.6*	5863.1
171–185	1573.7	1572.5
186–209	2628.2	2627.5
210–241	3684.2	3685.3
242–261	2333.6*	2333.1
277–304	2681.9	2681.4
305–374	**7974.9***	**7978.2†**

* Cysteines modified with vinylpyridine.
† Confirmed by N-terminal sequencing.

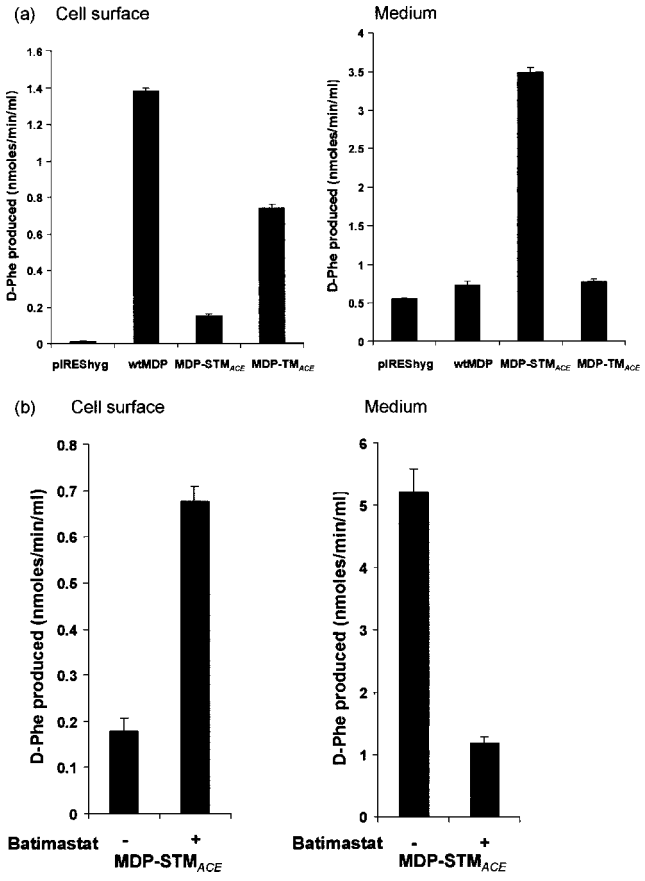

Figure 3 Release of MDP–STM_{ACE} from the cell surface is blocked by batimastat

(**a**) IMR32 cells transfected with either empty vector (pIRES*hyg*) or vector containing wtMDP, MDP–STM_{ACE} or MDP–TM_{ACE} were incubated in OptiMEM for 7 h. The medium was then harvested and assayed for MDP activity with the substrate Gly-D-Phe, and the cells were washed twice with PBS, prior to the measurement of cell surface MDP activity by the addition of 1.5 ml of 3 mM Gly-D-Phe in 0.1 M Tris/HCl (pH 8.0) for 45 min. The data shown are the means ± S.E.M. for three determinations, and are representative of three separate experiments. (**b**) MDP–STM_{ACE}-transfected IMR32 cells were incubated in the presence (+) or absence (−) of batimastat (20 μM) for 7 h. The medium was harvested and assayed for MDP activity. The cells were washed twice with PBS prior to measurement of cell surface MDP activity. The data shown are the means ± S.E.M. for three determinations, and are representative of three separate experiments.

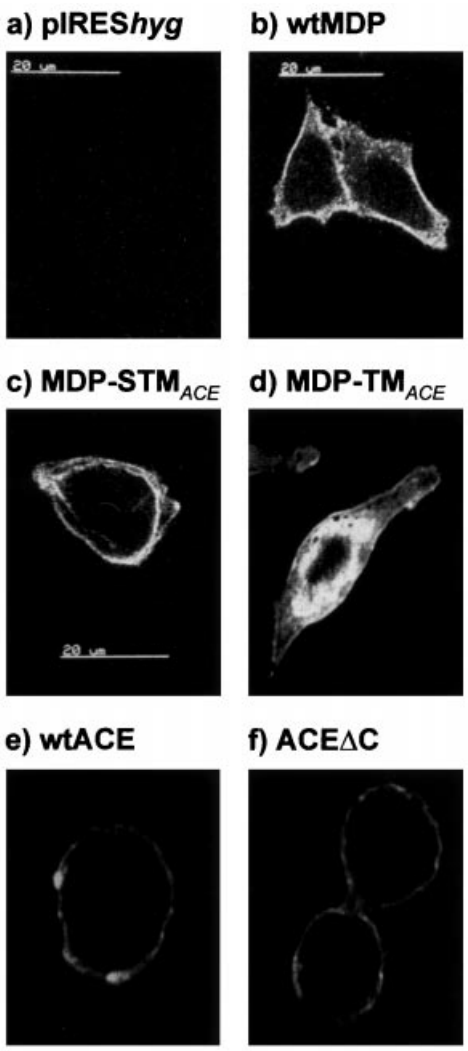

Figure 4 Confocal immunofluorescence microscopy of transfected cells

IMR32 cells transfected with either (**a**) empty vector (pIRES*hyg*) or vector containing (**b**) wtMDP, (**c**) MDP–STM_{ACE} or (**d**) MDP–TM_{ACE} were cultured on coverslips, and incubated with batimastat (20 μM) for 24 h prior to immunocytochemistry. Following incubation with the anti-MDP antibody, the cells were stained with fluorescein and analysed by confocal immunofluorescence microscopy. CHO cells transformed with either (**e**) human testis ACE (wtACE) or (**f**) ACEΔC were grown on coverslips and fixed with paraformaldehyde. Following incubation with the anti-ACE antibody, the cells were stained with fluorescein and analysed by confocal immunofluorescence microscopy.

major site of cleavage of MDP–STM_{ACE} was at the Arg^{374}-Ser^{375} bond (see Figure 1b), corresponding to the secretase cleavage site in somatic ACE [20]. Thus even though the ectodomain of ACE had been replaced with that from the unrelated protein MDP, the juxtamembrane stalk region was still subject to cleavage at the identical Arg-Ser site found in testis and somatic ACE.

MDP–STM_{ACE}, MDP–TM_{ACE} and ACEΔC are localized at the cell surface

The secretase that cleaves ACE is localized to the plasma membrane [15], and therefore any potential substrate protein must also be localized there. Following transfection of the cells with either wtMDP, MDP–STM_{ACE} or MDP–TM_{ACE}, the distribution of enzymically active MDP at the cell surface and in the cell medium was analysed using the selective substrate Gly-D-Phe

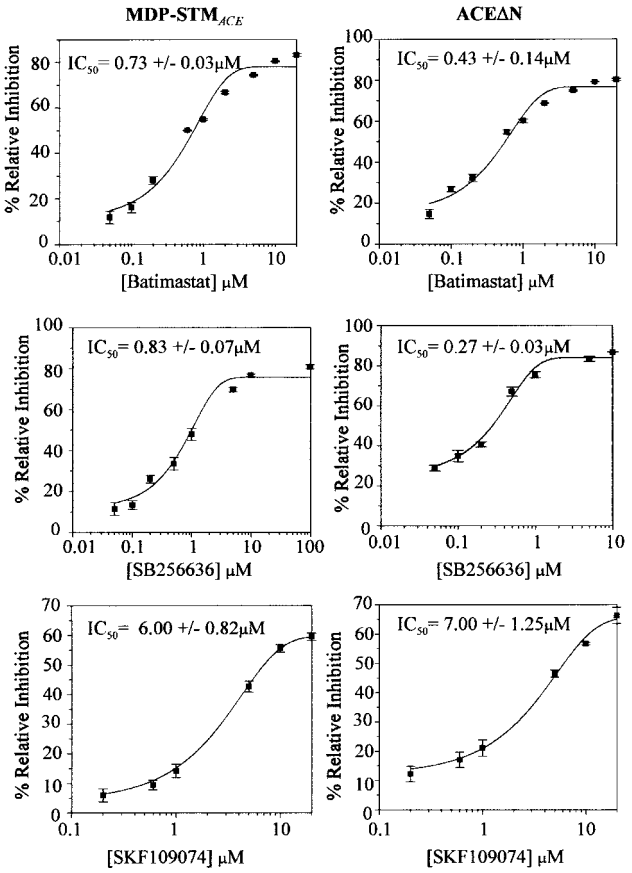

Figure 5 Effect of inhibitors on the cleavage and release of MDP–STM$_{ACE}$ and ACE

IMR32 cells expressing either MDP–STM$_{ACE}$ or ACEΔN were incubated in OptiMEM in the absence or presence of the indicated compound for 7 h. The medium was then harvested and assayed for either MDP or ACE as described in the Experimental section. Results are the means ± S.E.M. for three determinations at each concentration of inhibitor. The curves are the best fits to the experimental points using the program Origin with Boltzmann Best Fit. The IC$_{50}$ values were determined from three separate inhibition curves and are the means ± S.E.M.

[37,38]. All three cell lines had MDP activity at the cell surface that was significantly above that of the vector-only transfected cells (Figure 3a), indicating that there was no gross defect in either the folding or trafficking of the proteins. However, only in the medium from the cells expressing MDP–STM$_{ACE}$ was the activity of MDP above that of the vector-only transfected cells (Figure 3a). The lower level of MDP–STM$_{ACE}$ at the cell surface compared with MDP–TM$_{ACE}$ was probably due to the shedding of MDP–STM$_{ACE}$ into the medium. Consistent with the data from the Western-blot analysis (Figure 2a), the more sensitive enzymic activity assay revealed no evidence for the shedding of either wtMDP or MDP–TM$_{ACE}$ from the cells (Figure 3a).

Confocal immunofluorescence microscopy was used to confirm the cell surface localization of the MDP–ACE and ACEΔC chimeras. Both wtMDP and MDP–STM$_{ACE}$ were localized predominantly at the surface of the transfected cells (Figures 4b and 4c). Although the cells expressing MDP–TM$_{ACE}$ clearly had some of the protein present at the cell surface, there was a significant intracellular pool of the enzyme (Figure 4d). This result agreed with the lower level of cell surface MDP enzymic activity in the cells expressing MDP–TM$_{ACE}$ compared with those expressing wtMDP (Figure 3a), suggesting that the lack of the juxtamembrane stalk region impairs, but does not completely prevent, the trafficking of MDP–TM$_{ACE}$ to the cell surface. However, the lack of shedding of MDP–TM$_{ACE}$ was clearly not due to its inability to reach the cell surface where the secretase is located. Similarly, cell surface localization of both testis ACE and ACEΔC was visible as a ring of immunofluorescence in paraformaldehyde-fixed but unpermeabilized cells (Figures 4e and 4f). Thus the ACEΔC mutant was also processed efficiently to the cell surface, where it appeared to accumulate.

Shedding of ACE and MDP–STM$_{ACE}$ is inhibited similarly by secretase inhibitors

The secretase that cleaves and releases ACE from the cell surface is inhibited by hydroxamic acid-based compounds, such as batimastat [16]. Incubation of the cells with 20 μM batimastat significantly inhibited the release of MDP–STM$_{ACE}$ into the cell medium (Figure 3b), with a corresponding increase in cell surface MDP activity. In order to determine whether the secretase that was cleaving and releasing MDP–STM$_{ACE}$ from the cells had the same inhibitor profile as the secretase that sheds ACE, the effect of three hydroxamic acid-based zinc metalloproteinase inhibitors on the release of both proteins was examined in more detail. For this purpose, cells were stably transfected with the cDNA encoding the single C-terminal domain of human ACE, ACEΔN (which is essentially equivalent to testis ACE, see Figure 1) [12,17]. The effect of a range of concentrations of batimastat and two other hydroxamic acid-based compounds, SB256636 and SKF109074, on the release of MDP–STM$_{ACE}$ and ACEΔN was compared. All three compounds inhibited the release of MDP–STM$_{ACE}$ and ACEΔN in a dose-dependent manner (Figure 5) with remarkably similar IC$_{50}$ values, implying that the same, or a very closely related activity, was cleaving both ACE and MDP–STM$_{ACE}$.

DISCUSSION

In the present study we investigated the requirements for recognition and cleavage of a membrane protein by its secretase by investigating the cleavage characteristics of wild-type and chimaeric constructs of MDP and ACE. wtMDP is known to be a membrane protein that is not shed; ACE is shed, but there are significant differences in rates of shedding of the somatic and testis ACE isoenzymes, despite identical juxtamembrane stalk, TM and cytosolic domains.

To test the role of the juxtamembrane stalk in ectodomain shedding, we fused the C-terminal regions of ACE to the GPI-anchored protein, MDP [23,36]. Although having the same membrane topology as ACE, the GPI-anchored MDP is not subject to proteolytic cleavage from the membrane [35,37]. Replacing the GPI anchor addition signal in MDP with the juxtamembrane stalk, TM and cytosolic domains of ACE resulted in a construct, MDP–STM$_{ACE}$, that was targeted to the cell surface in an enzymically active, N-glycosylated form. This construct was efficiently shed from the cell surface by proteolytic cleavage at the same Arg-Ser site in the juxtamembrane stalk as utilized in ACE [20]. Furthermore, the secretase involved in the cleavage and secretion of MDP–STM$_{ACE}$ had a remarkably similar sensitivity to three hydroxamic acid-based inhibitors as the secretase that releases ACE. Previously we have reported that the secretase(s) that cleaves ACE and the amyloid precursor protein has a different sensitivity to certain hydroxamic acid-based compounds, including batimastat and SKF109074, compared with TACE [18]. Thus our data are strongly suggestive that it is the same, or a very closely related, zinc metalloproteinase

that cleaves both ACE and MDP–STM_{ACE}. This is in contrast with the results with a mutant of ACE containing a single amino acid change in the juxtamembrane stalk, which is cleaved by a mechanistically and spatially distinct secretase [34].

MDP is a disulphide-linked homodimer, with Cys^{361} the only residue involved in the interchain link [35], whereas ACE resides in the plasma membrane as a monomer [39]. Clearly the presence of the interchain disulphide bond, only 14 residues N-terminal to the secretase cleavage site, did not impede cleavage at the Arg-Ser bond in MDP–STM_{ACE} by the secretase. Moreover, the observation that MDP–STM_{ACE} was relatively efficiently cleaved from the cell surface (e.g. addition of batimastat to the culture medium was required prior to immunofluorescent staining of the cells in Figure 4 to observe significant cell surface staining) implies that the covalent dimerization of the substrate protein does not significantly perturb the shedding process, and may even enhance substrate recognition or cleavage by the secretase.

Efficient cleavage of MDP–STM_{ACE} appears to contrast with a previous study using a chimaera containing the ectodomain of CD4, which is also not subject to ectodomain shedding, and the juxtamembrane stalk, TM and cytosolic domains of ACE [22]. Although the CD4–ACE chimaera was transported to the cell surface, it was not cleaved. The MDP–STM_{ACE} chimaera contained only 34 amino acid residues of the juxtamembrane stalk region of ACE, whereas the CD4–ACE chimera contained 77 amino acid residues N-terminal to the TM domain. On the basis of experiments with truncation mutants, the juxtamembrane stalk of ACE has been estimated to be greater than 24 residues but less than 47 residues [24]. The lack of cleavage of the CD4–ACE chimaera may have been due to aberrant folding of the relatively large portion of ACE that includes part of the globular ectodomain, thus preventing access or cleavage by the secretase.

The 14-amino-acid residue juxtamembrane stalk sequences of TGF-α and the amyloid precursor protein have been shown to endow betaglycan with the ability to be cleaved by the regulated shedding system. As both juxtamembrane stalk regions were equally effective at supporting betaglycan shedding, whereas the TM and cytosolic domains of TGF-α alone were not sufficient, it was concluded that the short juxtamembrane stalks were the major determinants of ectodomain shedding [21]. Our results with the MDP–ACE constructs appear to support this conclusion, in that MDP–STM_{ACE} containing the juxtamembrane stalk, TM and cytosolic regions of ACE was cleaved, whereas MDP–TM_{ACE} containing only the TM and cytosolic domains, although transported to the cell surface in an enzymically active, N-glycosylated form, was not cleaved. Recently it was also reported that the human epidermal growth factor receptor 4 (HER4) exists in two isoforms, HER4-JM-a and HER4-JM-b, that differ solely in their juxtamembrane stalk regions: HER4-JM-a contains a 23-amino-acid residue stalk and is cleaved by TACE [40], whereas HER4-JM-b contains a 13-amino-acid residue stalk and is not cleaved [41]. Moreover, the HER4-JM-a stalk sequence also conferred cleavage susceptibility on HER2, which contains a 14-amino-acid residue, uncleaved stalk [40].

However, the juxtamembrane stalk does not appear to constitute the only determinant of ectodomain shedding. It was shown, using interleukin-6 receptor chimaeras containing the pro-tumour necrosis factor-α cleavage site, that the amino acid sequence at the juxtamembrane cleavage site contributes to the cleavage characteristics of a protein, but, significantly, that regulation of cleavage could not be ascribed solely to characteristics of the stalk [42,43]. This is supported by the ACEΔC construct in which the N-terminal domain of somatic ACE, including Pro^{601}, the last common amino acid of the two domains, was fused directly to the juxtamembrane stalk, TM and cytosolic domains. Although ACEΔC accumulated on the cell surface in an enzymically active form, ectodomain shedding was abolished. One explanation for this observation is that there is a recognition motif in the C-terminal domain, that is absent from the N-terminal domain, which is essential for cleavage of ACE by the secretase. This requirement for a recognition motif in the extracellular domain that the secretase binds to, in addition to the substrate cleavage site in the juxtamembrane stalk, is in agreement with the work of Sadhukhan et al. [22]. These authors showed that a chimaeric protein containing the C-terminal domain of ACE and the juxtamembrane stalk, TM and cytosolic domains of CD4 was efficiently cleaved at the cell surface.

Another possibility is that in CD4, and the CD4–ACE and ACEΔC chimaeras, the overall structures of the distal extracellular domains prevent the ectodomain cleavage in the juxtamembrane stalk region by steric hindrance of protease accessibility as suggested by Deng et al. [44]. In contrast, in chimaeras such as MDP–STM_{ACE} and ACE–CD4 the extracellular domains allow for accessibility of the protease to the juxtamembrane region of otherwise uncleaved proteins, thereby permitting efficient cleavage. However, although this explanation may account for the different cleavage rates of somatic and testis ACE [20], we believe that this explanation is unlikely in the case of the ACEΔC chimaera, as the testicular 'C-terminal domain' was replaced with the 55% identical N-terminal domain of somatic ACE in an identical position. This ACEΔC mutant shows enzymic activity characteristic of the N-terminal domain and is cell-surface localized, and is thus likely to be correctly folded into a structure similar to that of the C-terminal domain. Therefore we favour the view that the ACE N-terminal domain lacks an essential, as yet unidentified, recognition motif.

These apparently disparate data can be rationalized by postulating two distinct classes of secretases: type A secretases, that cleave any stalk comprising the requisite unhindered juxtamembrane stalk sequence between the TM and the proximal region of the ectodomain [24]; and type B secretases, that require some interaction with the ectodomain prior to proteolysis, as is the case in the cleavage of ephrin A2 by the metalloprotease Kuzbanian [45]. Based on all the available data, the type A and B secretases have broadly similar stalk requirements for cleavage, but given an adequate substrate recognition motif, type B secretases can clearly cleave certain stalks that are refractory to cleavage by type A secretases. The type A secretases, exemplified by TACE, will cleave a wide variety of stalks that comply with minimum distance constraints from both the TM and proximal extracellular domains [24]. In contrast, type B secretases, exemplified by ACE secretase and Kuzbanian [19,45], require a recognition motif in the proximal extracellular domain to activate stalk cleavage. In the presence of such a motif, the type B secretases will cleave a wide range of stalks and pseudo-stalks, including the CD4 stalk and disulphide-bridged and glycosylated sequences [32,33].

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