The ectodomain shedding of angiotensin-converting enzyme is independent of its localisation in lipid rafts

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Summary

Angiotensin-converting enzyme (ACE), a type I integral membrane protein that plays a major role in vasoactive peptide metabolism, is shed from the plasma membrane by proteolytic cleavage within the juxtamembrane stalk. To investigate whether this shedding is regulated by lateral segregation in cholesterol-rich lipid rafts, Chinese hamster ovary cells and human neuroblastoma SH-SY5Y cells were transfected with either wild-type ACE (WT-ACE) or a construct with a glycosylphosphatidylinositol (GPI) anchor attachment signal replacing the transmembrane and cytosolic domains (GPI-ACE). In both cell types, GPI-ACE, but not WT-ACE, was sequestered in caveolin or flotillin-enriched lipid rafts and was released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C. When cells were treated with activators of the protein kinase C signalling cascade (phorbol myristate acetate or carbachol) the shedding of GPI-ACE was stimulated to a similar extent to that of WT-ACE. The release of WT-ACE and GPI-ACE from the cells was inhibited in an identical manner by a range of hydroxamate-based zinc metalloprotease inhibitors. Disruption of lipid rafts by filipin treatment did not alter the shedding of GPI-ACE, and phorbol ester treatment did not alter the distribution of WT-ACE or GPI-ACE between raft and non-raft membrane compartments. These data clearly show that the protein kinase C-stimulated shedding of ACE does not require the transmembrane or cytosolic regions of the protein, and that sequestration in lipid rafts does not regulate the shedding of the protein.

Key words: Angiotensin-converting enzyme, Lipid rafts, Cholesterol, Glycosyl-phosphatidylinositol, Ectodomain shedding

Introduction

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a zinc metallopeptidase that cleaves two vasoactive peptides, angiotensin I and bradykinin (Yang and Erdos, 1967; Yang et al., 1971). Consequently, this enzyme plays an important role in blood-pressure regulation, and in the development of vascular pathology and endothelium remodelling in some disease states (Corvol et al., 1995; Ehlers and Riordan, 1989). There are two distinct isoforms of mammalian ACE, somatic and testis, both of which are transcribed from a single gene by the use of two alternative promoters (Howard et al., 1990). Somatic ACE (180 kDa), which consists of two identical catalytic domains each bearing a functional, zinc-dependent active site, is synthesized by vascular endothelial cells as well as several types of epithelial and neuronal cells (Ehlers and Riordan, 1991; Soubrier et al., 1988; Wei et al., 1991). In contrast, testis ACE (110 kDa), which consists of only a single domain corresponding to the C-terminal domain of somatic ACE, is expressed exclusively in the testis (Ehlers et al., 1989).

Despite the fact that ACE is expressed as a type I integral membrane protein, a soluble form of the enzyme exists in plasma and other body fluids (Hooper, 1991). This soluble form is derived from the membrane-bound form through the action of ACE secretase (Oppong and Hooper, 1993; Parvathy et al., 1997). This process of cleavage and secretion (often referred to as shedding) appears to be an important and widely used post-translational regulatory process (Hooper et al., 1997). A variety of structurally and functionally unrelated cell-surface proteins are proteolytically cleaved from the membrane, including tumour necrosis factor α, transforming growth factor α, and L-selectin (reviewed by Hooper et al., 1997). The secretases that cleave these membrane proteins share several properties including upregulation by phorbol esters and muscarinic agonists, and inhibition by hydroxamate-based zinc metalloprotease inhibitors such as batimastat. The ADAM (a disintegrin and metalloprotease) family of proteins has been implicated in the constitutive and regulated shedding of some of these proteins (Black and White, 1998; Schlondorff and Blobel, 1999; Turner and Hooper, 1999). However, the identity of the enzyme responsible for the cell surface shedding of ACE remains to be determined.

Lipid rafts are regions of the plasma membrane rich in sphingomyelin, glycosphingolipids, cholesterol and acylated proteins (Hooper, 1999; Simons and Toomre, 2000). Rafts are characterised by their relative insolubility at 4°C in certain detergents such as Triton X-100 (Brown and Rose, 1992). Because of their high lipid-to-protein ratio, the detergent-insoluble rafts float to a low density during buoyant sucrose density gradient centrifugation in the presence of Triton X-100. The resulting low-density, detergent-insoluble membrane fraction is enriched not only in cholesterol and glycosphingolipids but also in certain proteins, including multiple glycosylphosphatidylinositol (GPI)-anchored proteins (Hooper and Turner, 1988). Lipid rafts have been implicated

in a range of biological processes, including intracellular trafficking, transmembrane signalling, lipid and protein sorting, and regulated proteolysis (Brown and London, 1998; Wolozin, 2001).

We therefore considered whether lateral segregation in lipid rafts could be a mechanism to regulate the proteolytic shedding of a membrane protein. In order to investigate this we expressed wild-type ACE (WT-ACE) and an ACE construct with a GPI anchor attachment signal sequence replacing the transmembrane and cytosolic domains (GPI-ACE) (Marcic et al., 2000) in two unrelated cell lines (Chinese hamster ovary (CHO) and human neuroblastoma SH-SY5Y). Although only GPI-ACE was sequestered in lipid rafts, the shedding of both constructs was stimulated to a similar extent by PMA or the muscarinic agonist carbachol, and the inhibition profile for a range of hydroxamate-based compounds for the shedding of WT-ACE and GPI-ACE was essentially identical. These data show that the phorbol-ester-stimulated shedding of ACE does not require the transmembrane or cytosolic regions of the protein. Furthermore, the fact that shedding is neither enhanced nor inhibited when ACE is targeted to lipid rafts by the addition of a GPI anchor also shows that lateral segregation in the plane of the membrane is not involved in regulating the shedding of ACE.

Materials and Methods

Generation of ACE plasmids

The expression vector pECE containing WT-ACE cDNA was kindly provided by P. Corvol (Collège de France, Paris, France) and the expression vector pcDNA3 containing the GPI-ACE insert was provided by E. Erdös (University of Illinois-Chicago). For stable transfection in CHO and SH-SY5Y cells the WT-ACE insert was cleaved out of pECE using *Eco*RI and subcloned directly into *Eco*RI digested pIRESneo (Clontech, Oxford, UK). The GPI-ACE insert was cleaved out of pcDNA3 using *Eco*RI and *Bam*HI and blunt-end ligated into *Eco*RI/*Xba*I digested pIRESneo.

Cell culture

The SH-SY5Y and CHO cells were cultured, respectively, in Dulbecco's modified Eagle's medium (DMEM) or Ham's F12 supplemented with 10% foetal bovine serum, penicillin (50 units/ml), streptomycin (50 mg/ml), and 2 mM glutamate (all Gibco BRL, Paisley, UK). Cells were maintained at 37°C in 5% CO_2 in air. For stable transfections, 30 μg DNA was introduced to cells by electroporation and selection was performed in normal growth medium containing 500 μg/ml of neomycin selection antibiotic. Batimastat and all other hydroxamate-based inhibitor compounds were synthesised at Glaxo SmithKline (Harlow, UK) and were used at 10 μM. Structural details of these compounds have been published previously (Parkin et al., 2002). Carbachol and PMA (Sigma, Poole, UK) were used at 30 μM and 1 μM, respectively. When the cells were confluent the medium was changed to OptiMEM (Gibco BRL) and the cells were incubated for 7 hours at 37°C with the indicated compounds. The medium was then harvested, concentrated and assayed for ACE activity. For analysis of cell-associated ACE, cells were washed with phosphate-buffered saline (PBS; 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4) and scraped from the flasks into PBS. Following centrifugation at 500 ***g*** for 5 minutes, the pelleted cells were lysed in 0.1 M Tris/HCl, 0.15 M NaCl, 1% Triton X-100, 0.1% Nonidet P-40, pH 7.4.

Lipid raft isolation

The following procedures were all performed at 4°C. Harvested cells were resuspended in 2 ml of Mes-buffered saline (MBS; 25 mM Mes/NaOH, 0.15 M NaCl, pH 6.5) containing 0.5% Triton X-100 and homogenised by 15 passages through a Luer 21 gauge needle. Unsolubilized cells were removed by centrifugation at 500 ***g*** for 5 minutes and the supernatant was adjusted to 40% (w/v) sucrose by the addition of an equal volume of 80% (w/v) sucrose in MBS. An aliquot of the sample (1 ml) was then layered beneath a discontinuous sucrose gradient consisting of 2 ml of 30% (w/v) sucrose and 2 ml of 5% (w/v) sucrose both in MBS. The tubes were then centrifuged overnight at 140,000 ***g*** in an SW-55 rotor (Beckman Instruments). Sucrose gradients were harvested from the base of the tubes in 0.5 ml fractions.

SDS-PAGE and immunoelectrophoretic blot analysis

Proteins were separated by SDS-PAGE on 7-17% polyacrylamide gradient gels followed by transfer to Immobilon P poly(vinylidene difluoride) membranes as previously described (Hooper and Turner, 1987). For the detection of ACE the rabbit polyclonal antibody RP183 (Williams et al., 1992) was used at 1:2000 dilution. The monoclonal antibodies against β-actin and flotillin (BD Biosciences, Oxford, UK) were used at 1:5000 and 1:1000, respectively, and the polyclonal antibody against caveolin (Affiniti Research Products, Exeter, UK) was used at 1:4000. Bound antibodies were detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham).

Enzyme and protein assays

ACE was assayed using BzGly-His-Leu as substrate, and the substrate and reaction products were separated and quantified by reverse-phase HPLC as described previously (Hooper and Turner, 1987). Protein was quantified using bicinchoninic acid in a microtitre plate assay with BSA as standard (Smith et al., 1985).

Phosphatidylinositol-specific phospholipase C release of GPI-ACE from cells

Phosphatidylinositol-specific phospholipase C (PI-PLC) purified from *Bacillus thuringiensis* (Low, 1992) was diluted in 10 mM Hepes/NaOH, 150 mM NaCl, pH 7.4. Diluted PI-PLC was filter-sterilized and added to CHO or SH-SY5Y cells at a concentration of 0.17 mg/ml. Following a 7 hour incubation at 37°C the conditioned medium was harvested and assayed for ACE activity as already described.

Immunocytochemistry

CHO cells were cultured on coverslips and then fixed in 3% (w/v) paraformaldehyde prior to blocking in 5% (v/v) goat serum in Tris-buffered saline (TBS; 25 mM Tris, 0.15 M NaCl, pH 7.2). ACE was detected using either polyclonal ACE antibody RP183 or RH179 (both at 1:1000) followed by 1:200 dilutions of goat anti-rabbit secondary antibodies conjugated to either FITC or Texas Red (Stratech Scientific, Soham, UK). The cells were visualised using the Deltavision deconvolution microscope system (Applied Precision, Washington, USA) with an Olympus IX70 inverted microscope base. In the Triton X-100 extraction experiment, cells were incubated for 20 minutes at 4°C in TBS containing 2% (v/v) Triton X-100 prior to paraformaldehyde fixation. In the filipin treatment experiment, cells were incubated with either PMA (1 μM) or PMA (1 μM) and filipin complex (10 nM) (Sigma, Poole, UK) for 1 hour at 37°C prior to paraformaldehyde fixation.

Results

Expression of WT-ACE and GPI-ACE in CHO and SH-SY5Y cells

CHO cells and human neuroblastoma SH-SY5Y cells have both previously been shown to cleave and release ACE in a batimastat-sensitive manner which can be enhanced by

activators of the protein kinase C signalling cascade (Parkin et al., 2002; Woodman et al., 2000). Both cell types were stably transfected with cDNA encoding either WT-ACE or GPI-ACE in which the sequence encoding part of the juxtamembrane stalk, the transmembrane and cytosolic domains was deleted from the cDNA and replaced with the sequence encoding the C-terminal GPI-anchor attachment signal from carboxypeptidase M (Deddish et al., 1990) (Fig. 1). Lysates from mock transfected, WT-ACE or GPI-ACE transfected cells were immunoblotted with the ACE polyclonal antibody RP183 (Fig. 2A) and a monoclonal antibody against β-actin (Fig. 2B). ACE immunoreactivity was not detected in lysates prepared from mock-transfected cells. Both WT-ACE and GPI-ACE migrated with a molecular weight of 180 kDa in CHO and SH-SY5Y cells. In CHO cells the level of GPI-ACE expression was half that of WT-ACE whereas the expression of the two proteins was almost equivalent in the SH-SY5Y cells. A faint, non-specific band at approximately 190 kDa was detected in all cell lysates including from the mock-transfected cells. When the lysates were assayed for ACE activity with BzGly-His-Leu as substrate (Fig. 2C), WT-ACE activity in CHO cells was found to be approximately twice that of GPI-ACE activity (2.08±0.04 versus 1.08±0.12 mmol/minute). However, no significant difference was detected between the activities of WT-ACE (5.01±0.71 mmol/minute) and GPI-ACE (3.99±0.41 mmol/minute) in the SH-SY5Y cell lysates. Negligible ACE activity was detected in the mock-transfected cells.

GPI-ACE is released from cells by exogenous PI-PLC

To confirm the GPI-anchored nature of GPI-ACE, cells were incubated with bacterial PI-PLC and the conditioned medium was harvested and assayed for ACE activity (Table 1). The release of WT-ACE from both CHO and SH-SY5Y cells in the presence of PI-PLC was indistinguishable from that which occurred in controls when PI-PLC was omitted from the incubations. In contrast, the amount of GPI-ACE released by PI-PLC from CHO cells was increased 6.9-fold over control cells incubated in the absence of PI-PLC and the release from SH-SY5Y cells was increased 14.6-fold.

GPI-ACE is sequestered in lipid rafts

In order to determine whether GPI-ACE was localised in lipid rafts, cells were solubilized in Triton X-100 at 4°C and rafts isolated by buoyant sucrose density-gradient centrifugation as described in Materials and Methods. In both CHO and SH-SY5Y cells most of the total cellular protein was effectively solubilized and subsequently located in fractions 1-3 of the sucrose gradients (Fig. 3A). A lesser amount of protein (between 21 and 28% of the total) was located in a high-density detergent-insoluble pellet at the base of the centrifuge tube (fraction 0). The assay technique employed was not sensitive enough to detect protein in the low-density raft region of the gradients (fractions 4-6). The position of lipid rafts in the sucrose gradients prepared from CHO cells was determined by immunoblotting with an antibody against caveolin (Lisanti et al., 1993). The majority of caveolin (WT-ACE cells, 87.5%; GPI-ACE cells, 66.5%) was located in fractions 4-6 of the sucrose gradients (Fig. 3B). Neuronal cells do not express caveolin (Gorodinsky and Harris, 1995; Parkin et al., 1997), therefore, an antibody against flotillin (Bickel et al., 1997) was used to determine the position of lipid rafts in sucrose gradients prepared from SH-SY5Y cells. Like caveolin in CHO cells, the majority of flotillin (WT-ACE cells, 75.0%; GPI-ACE cells, 65.5%) in SH-SY5Y cells was located in fractions 4-6 (Fig. 3B). The sucrose gradient fractions were also assayed for ACE (Fig. 3C). In CHO cells the majority (94.3%) of WT-ACE was excluded from lipid rafts whilst the same construct was completely excluded from rafts in SH-SY5Y cells. In contrast, 52.8% and 55.0% of GPI-ACE activity was located in lipid rafts isolated from CHO and SH-SY5Y cells, respectively.

In order to confirm that GPI-ACE was localised in lipid rafts, CHO cells expressing either WT-ACE or GPI-ACE were subjected to immunocytochemistry as described in the Materials and Methods using an anti-ACE polyclonal primary antibody (Fig. 4). WT-ACE (Fig. 4A) exhibited a relatively diffuse staining pattern at the cell surface whilst GPI-ACE (Fig. 4B) had a much more punctate staining pattern consistent with a raft localisation. When the cells were incubated with 2% (v/v) Triton X-100 prior to fixation, the level of fluorescence in WT-ACE expressing cells was dramatically reduced (Fig. 4C)

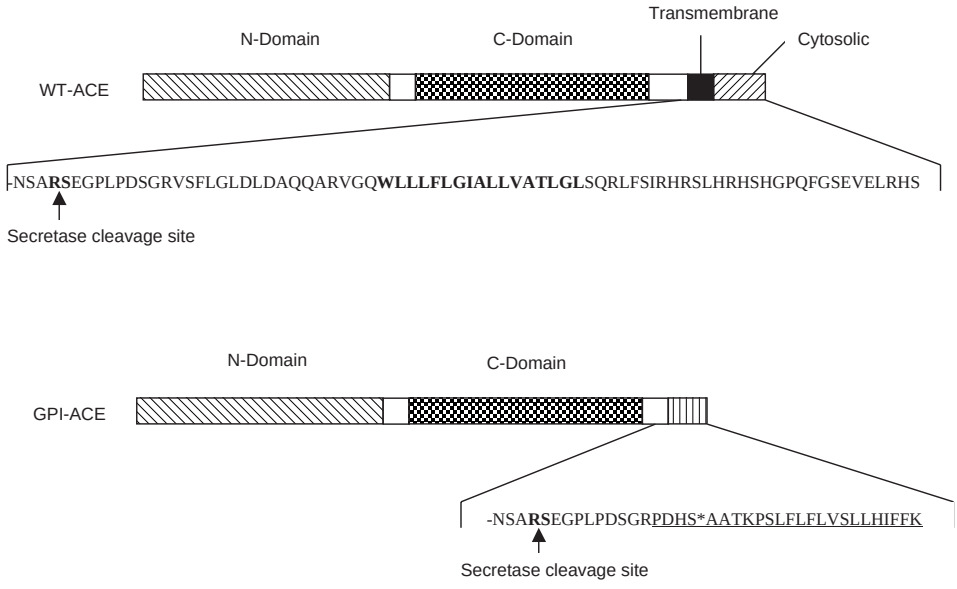

Fig. 1. Schematic diagram of WT-ACE and GPI-ACE. The domain structures of human somatic ACE (WT-ACE) and the GPI-anchored ACE (GPI-ACE) construct are shown. WT-ACE is a type I transmembrane protein with a C-terminal transmembrane domain (bold). The site of cleavage by the secretase is indicated. In GPI-ACE, the C-terminal 64 residues, including the transmembrane and cytosolic domains, were replaced with the 24 residue GPI anchor signal sequence (underlined) of human carboxypeptidase M (CPM). The site of GPI anchor attachment is indicated (*) (Tan et al., 2003).

consistent with this construct residing in detergent-soluble regions of the plasma membrane. In contrast, GPI-ACE staining remained punctate even when cells were pre-treated with Triton X-100 (Fig. 4D) consistent with its localisation in detergent-resistant rafts.

WT-ACE and GPI-ACE shedding is stimulated by activators of protein kinase C

The ability of activators of the protein kinase C signalling cascade to stimulate WT-ACE and GPI-ACE shedding from CHO and SH-SY5Y cells was assessed (Fig. 5). When CHO cells expressing WT-ACE were incubated with the protein kinase C agonist PMA the release of ACE into the medium was stimulated 2.0-fold (Fig. 5A). The hydroxamate-based zinc metalloprotease inhibitor batimastat prevented the PMA-induced release of WT-ACE into the medium. Similarly, the release of GPI-ACE from CHO cells was stimulated 2.8-fold upon PMA treatment and this PMA-induced release was completely inhibited by batimastat.

SH-SY5Y cells express muscarinic receptors at the cell surface, which can be activated by agonists such as carbachol. The resultant signalling cascade leads to the activation of protein kinase C (Canet-Aviles et al., 2002). The ability of carbachol to stimulate WT-ACE and GPI-ACE release from SH-SY5Y cells was assessed (Fig. 5B). When SH-SY5Y cells expressing WT-ACE were incubated with carbachol the release of ACE into the medium was stimulated 2.3-fold (Fig. 5B). Batimastat prevented the carbachol-induced release of WT-ACE into the medium. Similarly, the release of GPI-ACE from SH-SY5Y cells was stimulated 2.0-fold upon carbachol treatment and this carbachol-induced release was inhibited by batimastat.

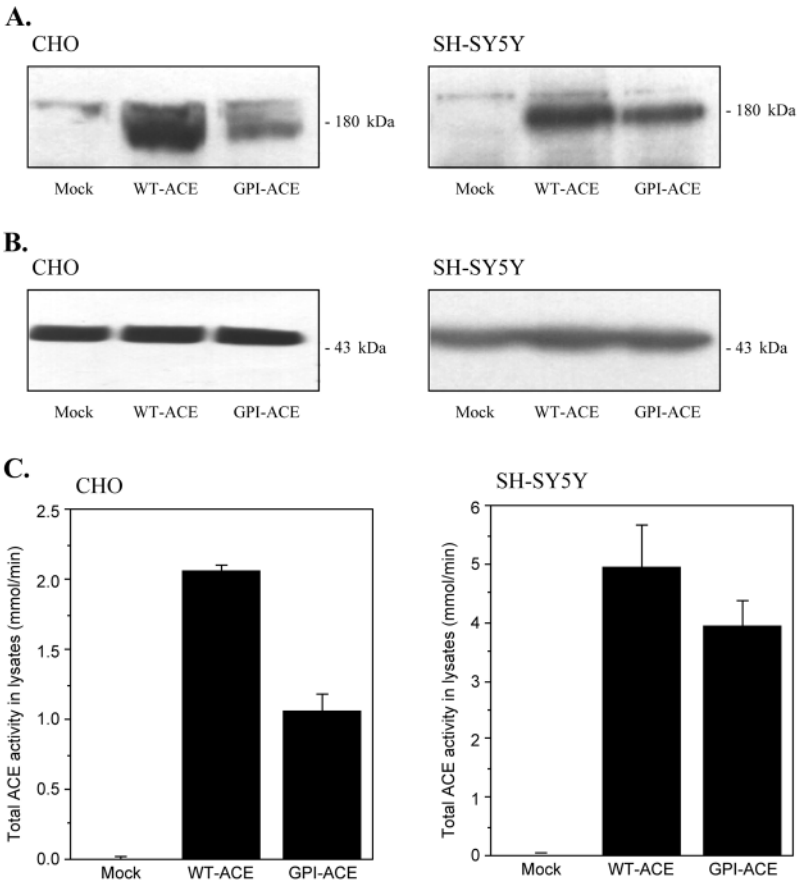

Fig. 2. Characterisation of WT-ACE and GPI-ACE expression in CHO and SH-SY5Y cells. CHO and SH-SY5Y cells were transfected with either empty pIRESneo vector (mock), WT-ACE cDNA or GPI-ACE cDNA. Cell lysates were prepared as described in Materials and Methods. (A) Lysates were immunoblotted using the ACE antibody, RP183. (B) Lysates were immunoblotted using a monoclonal β-actin antibody. (C) Lysates were assayed for ACE activity with BzGly-His-Leu as substrate. Results are the mean ± s.d. of three experiments.

Regulated shedding of WT-ACE and GPI-ACE is inhibited in an identical manner by a range of hydroxamate-based metalloprotease inhibitors

CHO cells expressing either WT-ACE or GPI-ACE were co-incubated with PMA and a range of hydroxamate-based zinc metalloprotease inhibitors that we have characterised previously against the ACE secretase (Parkin et al., 2002). The medium was then harvested and samples assayed for ACE activity. The results (Fig. 6) show that the inhibition profile of these seven compounds for the regulated shedding of WT-ACE and GPI-ACE was essentially identical.

Filipin treatment does not alter the shedding of GPI-ACE

Although the majority of GPI-ACE was located in the lipid raft fractions of the sucrose density gradients, approximately 40% of the enzyme activity was recovered in the detergent-soluble fractions (Fig. 3C). We considered the possibility that this detergent-soluble pool of GPI-ACE may be shed more effectively than the lipid raft pool such that, although a substantial proportion of GPI-ACE was sequestered in lipid rafts, the overall shedding of GPI-ACE in comparison to WT-ACE shedding was not altered. In order to assess this, CHO cells transfected with either GPI-ACE or WT-ACE were incubated with PMA in the absence or presence of the lipid

Table 1. Effect of PI-PLC on the release of ACE constructs from the surface of CHO and SH-SY5Y cells

		Activity released into medium (nmol/min/ml)	
Cell line	Construct	– PI-PLC	+ PI-PLC
CHO	WT-ACE	85.1±5.4	80.3±5.4
	GPI-ACE	93.4±6.3	642.4±242.4
SH-SY5Y	WT-ACE	145.2±33.3	157.0±14.2
	GPI-ACE	178.9±18.2	2606.3±460.9

CHO and SH-SY5Y cells transfected with either WT-ACE or GPI-ACE were incubated for 7 hours in the absence or presence of bacterial PI-PLC. The medium was then harvested and the samples assayed for ACE activity with BzGly-His-Leu as substrate. Results are the mean ± s.d. of three experiments

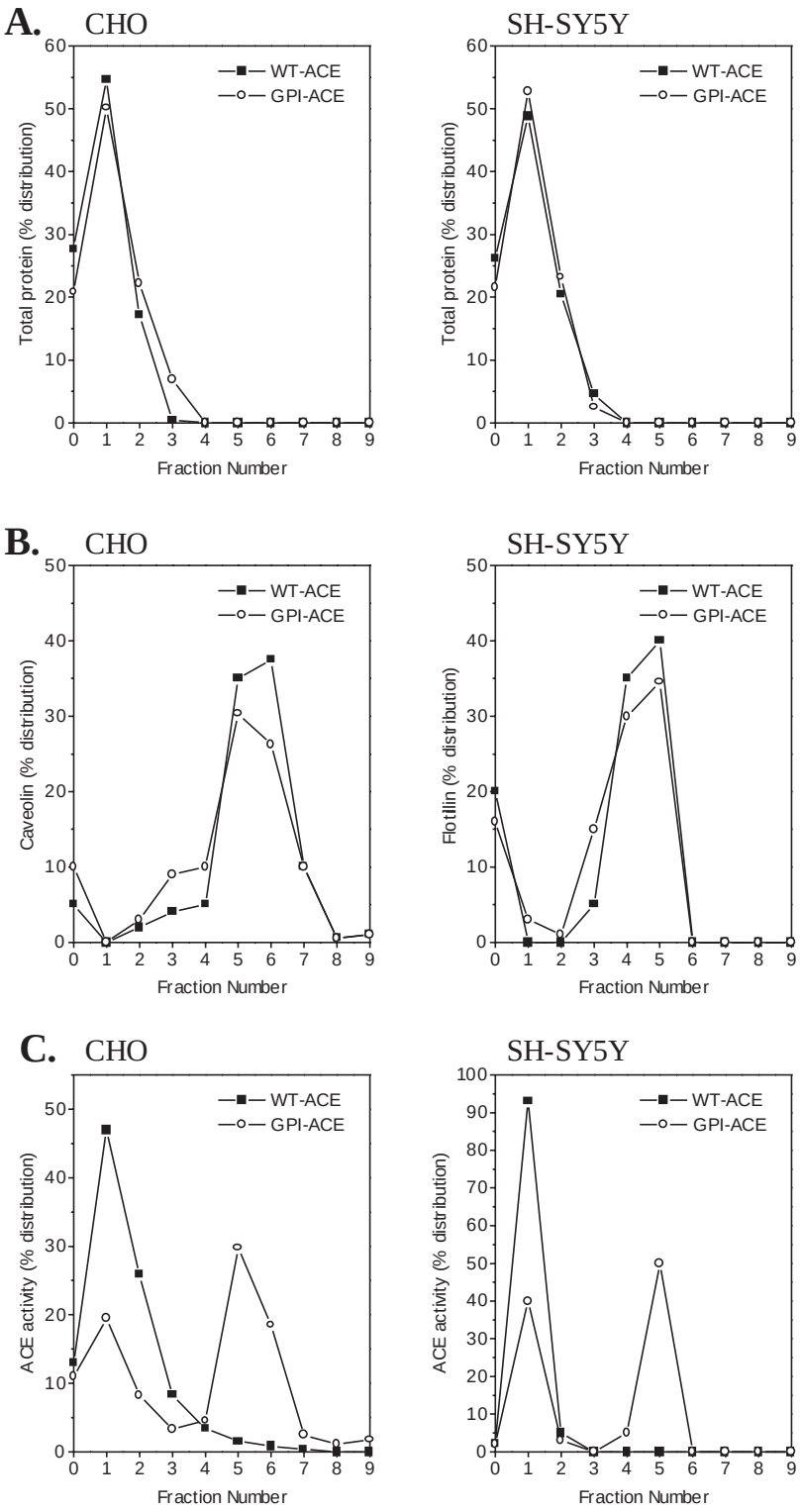

Fig. 3. Distribution of WT-ACE and GPI-ACE in lipid rafts. CHO and SH-SY5Y cells expressing either WT-ACE or GPI-ACE were used to prepare lipid rafts by buoyant sucrose density-gradient centrifugation in the presence of Triton X-100 as described in Materials and Methods. The sucrose gradients were fractionated in 0.5 ml aliquots (0, insoluble pellet; 1, bottom of tube; 9, top of tube). (A) Distribution of total protein in sucrose gradient fractions. (B) Distribution of caveolin (CHO cells) and flotillin (SH-SY5Y cells) in sucrose gradient fractions. (C) Distribution of ACE activity in sucrose gradient fractions. The results are representative of triplicate experiments.

raft-disrupting compound, filipin. When filipin was used to disrupt rafts the diffuse staining pattern of WT-ACE at the cell surface did not change (Fig. 7A,C). In contrast, the punctate distribution of GPI-ACE (Fig. 7B) changed to a more diffuse staining pattern (Fig. 7D) consistent with filipin disrupting the lipid rafts. However, the PMA-regulated shedding of GPI-ACE from CHO cells remained unchanged whether or not the cells were co-incubated with filipin (Fig. 7E).

PMA does not alter the membrane compartmentalisation of WT-ACE and GPI-ACE

Although GPI-ACE is present in rafts (Fig. 3C and Fig. 4B), we considered the possibility that upon PMA stimulation of the cells, the protein might move laterally into non-raft regions of the membrane where it is subsequently cleaved. In order to address this, CHO cells transfected with either WT-ACE or GPI-ACE were incubated with or without PMA. In order to retain ACE in the cell membrane the incubations were all performed in the presence of batimastat. Cells were then harvested and lipid rafts were prepared in the presence of batimastat as described in Materials and Methods. The resultant sucrose gradient fractions were assayed for ACE activity and the results show that there was no change in the distribution in rafts of either WT-ACE (Fig. 8A) or GPI-ACE (Fig. 8B) when cells were incubated in the presence of PMA.

Discussion

The shedding of transmembrane-anchored proteins involving a proteolytic cleavage within the juxtamembrane stalk is seen with a range of proteins including tumour necrosis factor-α, transforming growth factor-α, amyloid precursor protein and L-selectin (reviewed by Hooper et al., 1997). Although ectodomain shedding is a widespread phenomenon, the mechanism(s) by which it is regulated are not clear. One possible mechanism to regulate the shedding process would be for the secretase and its substrate to be anchored in distinct domains within the plasma membrane. Upon cell activation, the secretase and/or its substrate would move laterally into the same domain where shedding can then take place. Cholesterol-rich lipid rafts are distinct domains of the plasma membrane that are involved in a range of biological processes including regulated proteolysis (Brown and London, 1998; Wolozin, 2001). As we (Parkin et al., 1996) and others (Schnitzer et al., 1995) have previously shown that the transmembrane anchored WT-ACE is excluded from lipid rafts, we considered whether targeting ACE to rafts by replacing the transmembrane polypeptide with a GPI anchor would regulate its

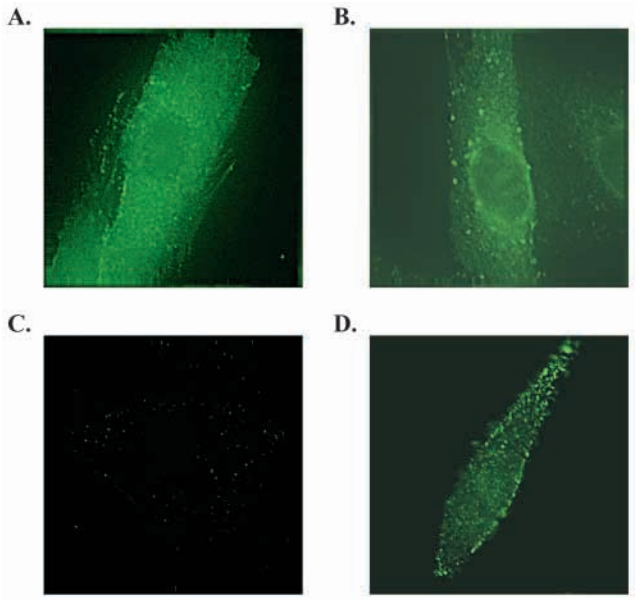

Fig. 4. Immunocytochemistry of CHO cells expressing WT-ACE or GPI-ACE. (A) and (B) CHO cells transfected with WT-ACE and GPI-ACE, respectively were subjected to immunocytochemistry using the polyclonal anti-ACE antibody RP183 as described in Materials and Methods. (C and D) CHO cells transfected with WT-ACE and GPI-ACE, respectively, were incubated for 20 minutes at 4°C with 2% (v/v) Triton X-100 prior to paraformaldehyde fixation and subsequent immunocytochemistry using the polyclonal anti-ACE antibody RP183, as described in Materials and Methods.

ectodomain shedding. Replacing the transmembrane and cytosolic domains of ACE with a GPI anchor did not alter either the specific activity of the enzyme, as the signals detected by immunoelectrophoretic blotting correlated directly with the ACE activity assay results (Fig. 2), or the expression of the protein at the cell surface (Table 1 and Fig. 4). Using the well-established criterion of detergent-insolubility, as assessed by buoyant sucrose density gradient centrifugation (Fig. 3) and immunocytochemistry (Fig. 4), GPI-ACE was predominantly localised in lipid rafts in both the CHO and human neuroblastoma SH-SY5Y cells.

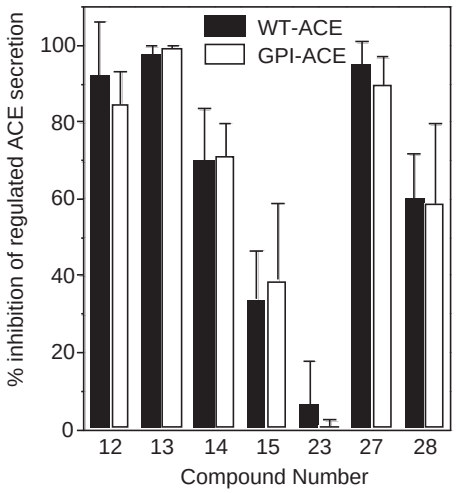

Fig. 6. Inhibition of the regulated shedding of WT-ACE and GPI-ACE by a range of hydroxamate-based inhibitors. CHO cells transfected with either WT-ACE or GPI-ACE were incubated for 7 hours in the presence of 1 μM PMA and 10 μM concentrations of a range of structurally variant hydroxamate-based inhibitors [numbers as in Parkin et al. (Parkin et al., 2002)]. The medium was then harvested and concentrated and samples were assayed for ACE activity with BzGly-His-Leu as substrate. Results are the mean ± s.d. of three experiments.

The basal shedding of GPI-ACE from SH-SY5Y cells was indistinguishable from that of WT-ACE. However, whereas the total GPI-ACE activity in CHO cell lysates was approximately half that of the WT-ACE activity (Fig. 2C) the basal shedding of the former protein (Table 1) was not significantly different from that of WT-ACE. At first sight these data would seem to imply that the basal shedding of GPI-ACE from CHO cells was more efficient than that of WT-ACE. However, as the amount of the proteins at the cell surface was not determined we cannot rule out that the apparent increase in the basal shedding of GPI-ACE is due to enhanced trafficking of the protein to the surface of the CHO cells where the secretase acts (Parvathy et al., 1999).

Upon incubation of the CHO or SH-SY5Y cells with PMA

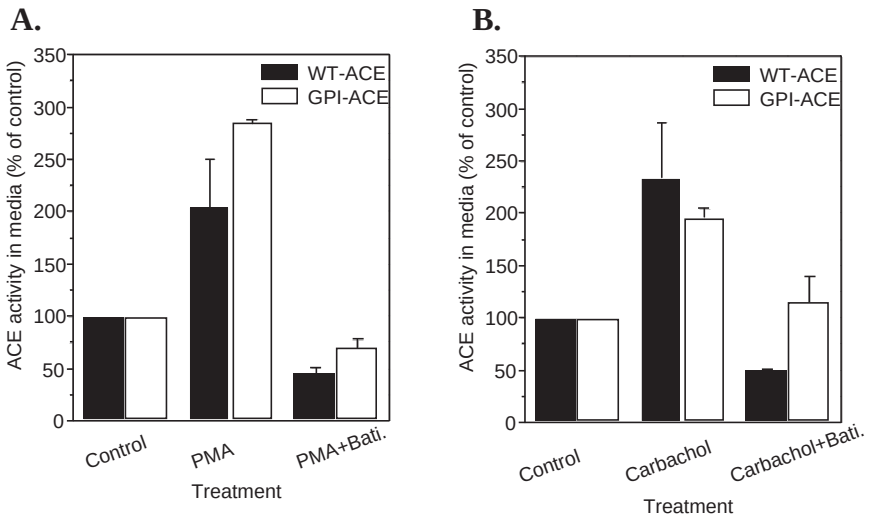

Fig. 5. Phorbol ester stimulates the shedding of WT-ACE and GPI-ACE. (A) CHO and (B) SH-SY5Y cells transfected with either WT-ACE or GPI-ACE were incubated for 7 hours in the absence (control) or the presence of either PMA (1 μM), PMA (1 μM) and batimastat (10 μM), carbachol (30 μM), or carbachol (30 μM) and batimastat (10 μM) as indicated. The medium was then harvested and concentrated as described in Materials and Methods. The samples were assayed for ACE activity with BzGly-His-Leu as substrate. Results are the mean ± s.d. of three experiments.

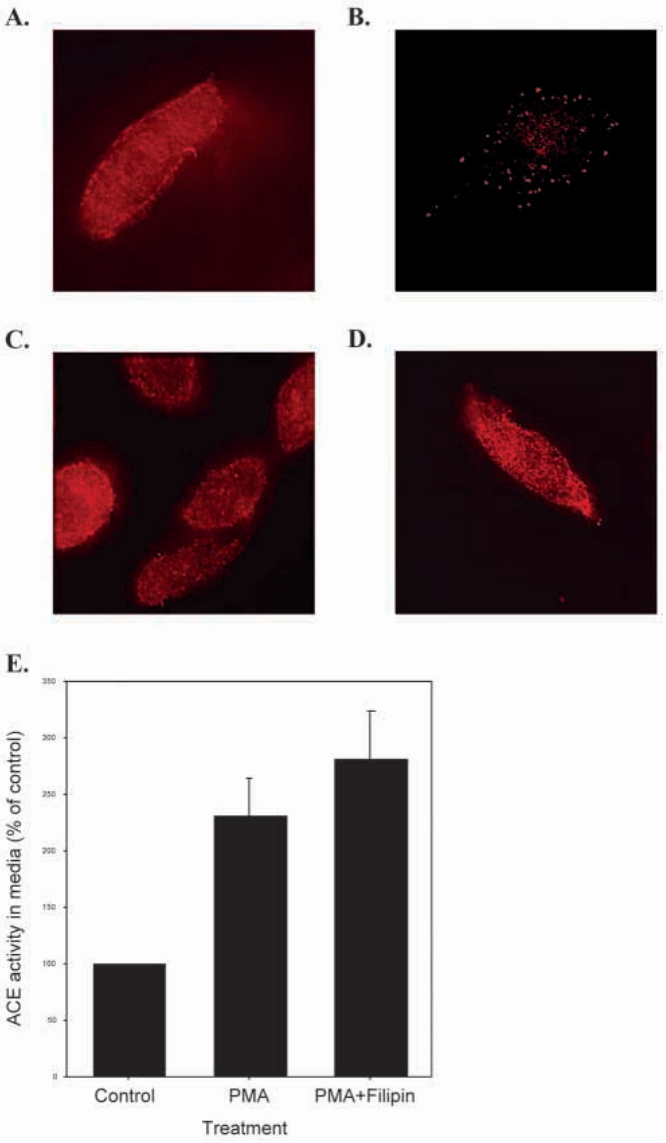

Fig. 7. Lack of effect of filipin on the shedding of GPI-ACE. CHO cells transfected with either WT-ACE or GPI-ACE were incubated for 1 hour in the presence of PMA (1 μM) with or without filipin (10 nM). The conditioned medium was harvested and concentrated and the cells then fixed in paraformaldehyde and subjected to immunocytochemistry using the polyclonal anti-ACE antibody RP179 as described in Materials and Methods. (A) WT-ACE-transfected CHO cells incubated in the presence of PMA alone. (B) GPI-ACE-transfected CHO cells incubated in the presence of PMA alone. (C) WT-ACE-transfected CHO cells incubated in the presence of PMA and filipin. (D) GPI-ACE-transfected CHO cells incubated in the presence of PMA and filipin. (E) Conditioned media samples were assayed for ACE activity with BzGly-His-Leu as substrate. Results are the mean ± s.d. of three experiments.

or carbachol the shedding of WT-ACE was enhanced 2.0- and 2.3-fold, respectively (Fig. 5) in agreement with previously published data relating to the shedding of human somatic ACE from CHO cells (Beldent et al., 1993) and rabbit testicular ACE from mouse epithelial cells (Ramchandran et al., 1994). Surprisingly, the shedding of GPI-ACE from CHO and SH-SY5Y cells was also stimulated 2.0- and 2.8-fold by PMA and carbachol, respectively (Fig. 5). One possibility is that this enhanced secretion of GPI-ACE upon PMA or carbachol treatment was due to the release of a pre-existing intracellular pool of cleaved ACE. However, multiple lines of evidence argue against this possibility. First, an ACE mutant lacking any form of membrane anchorage does not exhibit enhanced secretion from CHO cells upon phorbol ester treatment (Beldent et al., 1993). Second, the entire ACE content of GPI-ACE expressing cells in the present study was shown, by temperature-induced phase separation in Triton X-114, to be hydrophobic, possessing the membrane-anchoring domain (data not shown). Third, the PMA/carbachol-induced shedding of GPI-ACE from CHO and SH-SY5Y cells was prevented by multiple hydroxamate-based compounds which have previously been identified as ACE secretase inhibitors (Parkin et al., 2002). Therefore, it is clear that treatment of cells expressing GPI-ACE with PMA or carbachol results in enhanced de novo secretase-mediated ACE release.

ACE secretase requires an accessible stalk region in ACE of at least 11 residues in length and a minimum distance of 3

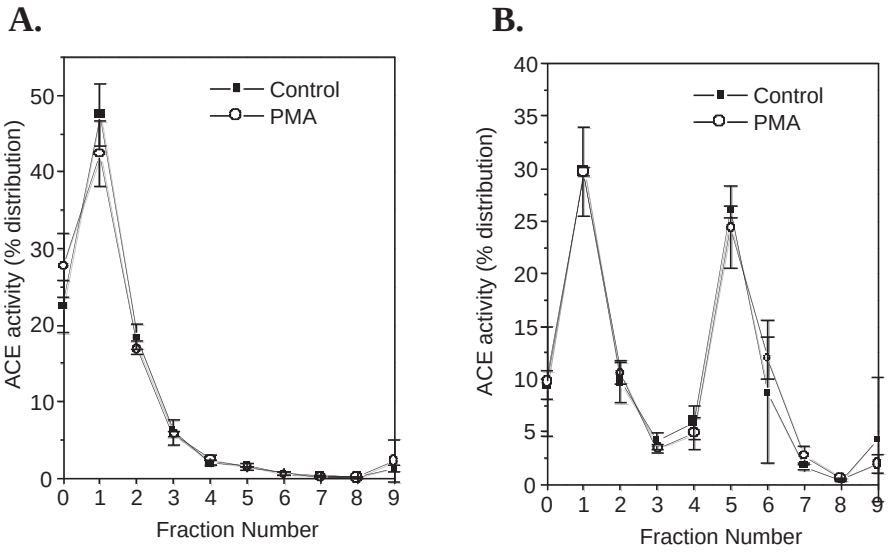

Fig. 8. Effect of PMA on the membrane compartmentalisation of WT-ACE and GPI-ACE. CHO cells transfected with either WT-ACE or GPI-ACE were incubated for 7 hours in the presence of batimastat (10 μM) with or without PMA (1 μM). Lipid rafts were prepared by buoyant sucrose density-gradient centrifugation in the presence of batimastat (10 μM) as described in Materials and Methods. The sucrose gradients were fractionated in 0.5 ml aliquots (0, insoluble pellet; 1, bottom of tube; 9, top of tube) and each fraction was assayed for ACE activity with BzGly-His-Leu as substrate. (A) Distribution of WT-ACE activity in sucrose gradient fractions. (B) Distribution of GPI-ACE activity in sucrose gradient fractions. Results are the mean ± s.d. of three experiments.

residues from the proximal extracellular domain and 8 residues from the transmembrane domain (Ehlers et al., 1996). These distances are essentially preserved in GPI-ACE, as there are 14 residues between the Arg-Ser bond cleaved by the secretase (Woodman et al., 2000) and the site of GPI attachment on the Ser residue in the Ser-Ala-Ala (ω, ω + 1, ω + 2) motif of carboxypeptidase M (Tan et al., 2003) (Fig. 1). Also, deletion of 17 residues from the end of the transmembrane domain and into the juxtamembrane stalk, up to the same Arg residue to which the carboxypeptidase M GPI signal was attached, did not alter the site of cleavage by the secretase in CHO cells (Ehlers et al., 1996). Thus there is no obvious alteration to either the sequence or structure of the juxtamembrane stalk that should prevent the normal secretase from cleaving GPI-ACE. This was essentially confirmed by examining the effect of a range of hydroxamate-based zinc metalloprotease inhibitors on the shedding of GPI-ACE and WT-ACE (Fig. 6). Using these compounds we were unable to distinguish between the shedding of the two constructs indicating that both WT-ACE and GPI-ACE are probably released from the cell surface by the same secretase.

As a proportion of GPI-ACE was not localised in the detergent-insoluble rafts, we considered the possibility that this non-raft pool of GPI-ACE may be preferentially shed over that localised in the rafts. However, disruption of the rafts with the cholesterol-binding agent filipin did not lead to an increase in the shedding of GPI-ACE (Fig. 7), and we could obtain no evidence for the lateral movement of GPI-ACE from the detergent-insoluble rafts into the detergent-soluble regions of the membrane upon PMA stimulation of the cells (Fig. 8). Collectively these data show that sequestration of ACE in lipid rafts does not regulate its ectodomain shedding. Following on from this, our data also imply that the secretase is present in both raft and non-raft domains of the plasma membrane where it can act on available substrate, although the lack of any effect on the shedding process may indicate that the levels of available secretase are not the rate-limiting factor in this process. Although the identity of the secretase that sheds ACE remains unknown, it has the properties of a member of the ADAM family of membrane-bound zinc metalloproteases (Hooper et al., 1999), and in this context it is interesting to note that ADAM10 has been localised to both raft and non-raft domains following buoyant sucrose density gradient centrifugation in the presence of Triton X-100 (Kojro et al., 2001).

It has been suggested that upon cell activation attachments between the cytoskeleton and the cytoplasmic domains of the transmembrane substrates (and their cognate secretases) change, co-clustering the transmembrane protein and its secretase and allowing them to interact (Werb and Yan, 1998). In addition, it has been proposed that activation of the protein kinase C signalling cascade could modify the cytoplasmic domain of the substrate protein causing a conformational change in it which makes the secretase cleavage site more accessible (Werb and Yan, 1998). Our results argue against both of these as possible mechanisms whereby the shedding of ACE is increased upon activation of protein kinase C. GPI-ACE, which lacks a cytoplasmic domain, is clearly not capable of direct interaction with the cytoskeleton as the GPI anchor only interacts with the outer leaflet of the bilayer and yet ACE shedding is still enhanced upon incubation of cells with PMA or carbachol (Fig. 5). Thus the cytosolic domain of ACE is not required for the regulated shedding of its ectodomain or involved in regulating this process. It has recently been demonstrated (Kohlstedt et al., 2002) that protein kinase CK2 phosphorylates the cytoplasmic tail of ACE and retains it in the plasma membrane, thus reducing the basal shedding of the protein. The authors also demonstrated that PMA was still able to stimulate the shedding of a form of ACE in which the phosphorylatable serine residue at position 1270 had been mutated to alanine. In support of the present study, the authors concluded that regulated ACE shedding could be attributed to the activation of a secretase whereas the basal shedding of the enzyme was regulated by the CK2-mediated phosphorylation of the cytoplasmic tail.

The role of the transmembrane domain of a substrate protein in regulating shedding has been investigated by deleting the whole of this region (Cheng and Flanagan, 1994; Deng et al., 1996). However, the resultant constructs were not membrane-anchored and/or trafficked to the cell-surface where shedding occurs (Parvathy et al., 1999). In the present study we overcame this problem by replacing the transmembrane domain of ACE with an alternative membrane-anchoring domain, a GPI anchor. The resultant construct was membrane-anchored and located at the cell surface similarly to WT-ACE (Table 1) (Marcic et al., 2000), and was similarly subject to ectodomain shedding, indicating unequivocally that the transmembrane domain is not involved in the shedding process.

In conclusion, we have shown using a GPI-anchored form of ACE that sequestration of the protein in lipid rafts does not regulate its shedding, and that neither the cytoplasmic nor the transmembrane domains are required for the shedding of ACE via activation of the protein kinase C signalling cascade.

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