Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1009–1013, February 1991
Biochemistry

Spontaneous solubilization of membrane-bound human testis angiotensin-converting enzyme expressed in Chinese hamster ovary cells

(hydrophobic anchor/proteolysis/transmembrane proteins/ectoenzymes)

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Communicated by Bert L. Vallee, November 9, 1990

ABSTRACT **The testis isozyme of angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a membrane-bound protein that, apart from the first 35 N-terminal residues, is identical to the C-terminal half of somatic ACE and contains the same putative C-terminal membrane anchor. Stable transfection of Chinese hamster ovary (CHO) cells with an expression vector containing the full-length human testis ACE cDNA results in the expression of two forms of recombinant human testis ACE (hTACE): membrane-bound ACE and, surprisingly, large quantities (up to 3 mg/liter) of soluble hTACE in the conditioned medium. Both forms are fully active and are physicochemically similar. However, by phase separation in Triton X-114, the soluble enzyme is hydrophilic, as is an anchor-minus mutant hTACE recovered from the medium of CHO cells transfected with a vector that contains a 3′-truncated testis ACE cDNA lacking the sequence encoding the membrane anchor. In contrast, the membrane-bound hTACE is amphipathic but is converted to a hydrophilic form on treatment with trypsin. The data establish that in ACE the hydrophobic sequence near the C terminus is necessary for membrane anchoring. Moreover, in CHO cells, membrane-bound hTACE is apparently solubilized by proteolytic cleavage of this anchor. A similar mechanism may account for the release of endothelial ACE *in vivo* to generate serum ACE and more generally for the constitutive processing and solubilization of analogously anchored proteins such as the amyloid precursor protein, among others. The release of membrane-bound ACE in CHO cells may, therefore, provide a useful system for the study of membrane-protein-solubilizing proteases.**

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a zinc-metallopeptidase that plays a critical role in blood pressure regulation by converting the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II. There are two isoforms: Somatic or "lung" ACE is a ubiquitous ectoenzyme bound to the plasma membranes of predominantly fluid-bathed cells, such as in endothelium and the intestinal and renal brush borders. Testis ACE is uniquely present in developing spermatozoa as a membrane-bound intravesicular enzyme (1, 2).

Molecular cloning revealed the somatic isozyme to be a single polypeptide consisting of two homologous domains, each containing a putative metal-binding site (3, 4). In contrast, the smaller testis isozyme is identical, except for a unique 35-residue sequence making up its N terminus, to the C-terminal half of somatic ACE. It therefore contains the second of the two putative metal-binding sites of somatic ACE and the same C-terminal hydrophobic sequence thought to constitute the membrane anchor (5–7).

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There appears to be an *in vivo* mechanism that solubilizes the ACE bound to various luminal surfaces and, consequently, most body fluids contain the enzyme (8), sometimes abundantly, as in human seminal plasma (9) and guinea pig serum (10). Such a mechanism may involve proteolytic cleavage and appears to be responsible for the solubilization of other similarly anchored transmembrane proteins, including the Tac protein (11), leukemogenic glycoprotein (12), and the amyloid β protein precursor (13). Thus, the membrane attachment of ACE and its release are of considerable interest.

To investigate the membrane anchoring of ACE, we expressed recombinant human testis ACE (hTACE) in stably transfected Chinese hamster ovary (CHO) cells. A significant proportion of the hTACE appears as a soluble form in conditioned medium. This soluble enzyme is hydrophilic on phase separation in Triton X-114, as is an anchor-minus mutant of hTACE. The present data suggest that the soluble ACE is likely derived from the membrane-bound enzyme by proteolytic cleavage of its C-terminal hydrophobic sequence.

MATERIALS AND METHODS

Construction of Expression Vectors. Details of the construction of plasmids pΩ-ACE, pLEN-ACEVII, and pLEN-ACE6/5, and all relevant sources, will be given elsewhere (unpublished results). Briefly, a full-length human testis ACE cDNA was constructed from overlapping clones 10A and RI.2 (5) and subcloned into the expression vector pJ4Ω to give pΩ-ACE (Fig. 1*A*). A second expression cassette, pLEN-ACEVII, was constructed in the vector pLEN (based on pMThGHSV40 2; ref. 14) (Fig. 1*B*). Lastly, the deletion mutant pLEN-ACE6/5 was generated by replacing the 3′ end of the ACE cDNA in pLEN-ACEVII with an oligoduplex containing a stop codon at nucleotide (nt) 2105 (see Fig. 1 *B* and *C*).

Cell Culture and Gene Transfer. CHO-K1 cells (American Type Culture Collection, CCL 61 CHO-K1) were maintained in Ham's F12/Dulbecco's modified Eagle's medium supplemented with 15 mM Hepes (pH 7.0) and 10% (vol/vol) fetal calf serum. Calcium phosphate precipitates (15) of each expression plasmid (10 μg) plus pSV2NEO (2 μg) (16) were used to cotransfect cells grown to 60–70% confluence. Cells were split 1:20 after 24–36 hr and allowed to grow for 24 hr prior to addition of the neomycin analogue G418 at 0.4 mg/ml. Cells stably transfected with either pLEN-ACEVII or pLEN-ACE6/5 were induced by the addition of 80 μM $ZnCl_2$ to the medium with or without 2% fetal calf serum.

Abbreviations: ACE, angiotensin-converting enzyme; hTACE, recombinant human testis ACE; Fa-FGG, 2-furanacryloyl-L-phenylalanylglycylglycine; nt, nucleotide(s); SV40, simian virus 40; Hip, hippuryl.

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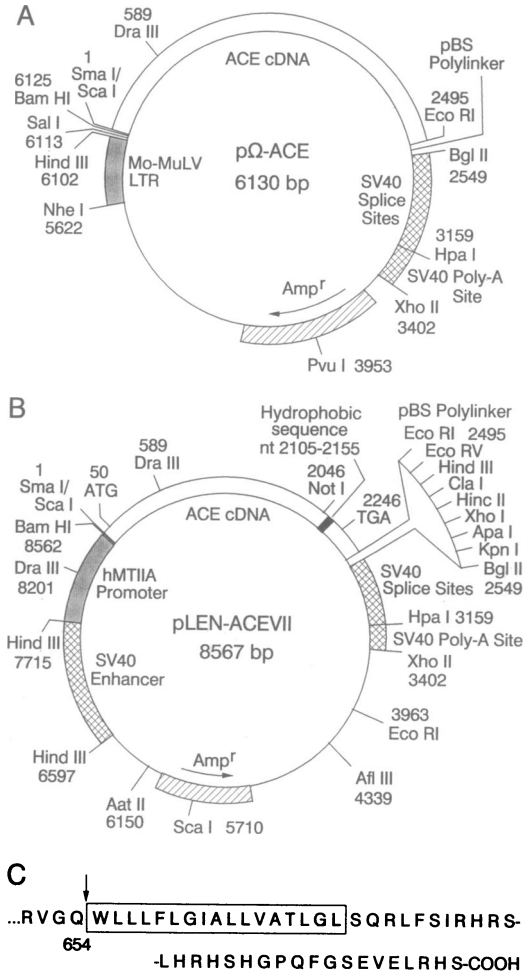

FIG. 1. Expression vectors for hTACE. (*A*) A full-length testis ACE cDNA was derived from overlapping clones 10A and RI.2 (5) and ligated with part of the pBluescript (pBS) polylinker into the vector pJ4Ω to give pΩ-ACE. This vector contains the Moloney murine leukemia virus (Mo-MuLV) promoter [long terminal repeats (LTRs)] and two fragments of simian virus 40 (SV40) DNA: SV40 positions 4038–4648 containing splice donor and acceptor sites and SV40 positions 2461–2704 containing the SV40 tumor antigen polyadenylylation signal. The remaining sequence corresponds to nt 2066–4286 from pBR322. (*B*) pLEN-ACEVII was constructed by excising the testis ACE cDNA with the SV40 splice and termination signals from pΩ-ACE and ligating them into the vector pLEN. This vector contains, in addition to the described elements, the *Hin*dIII C fragment from SV40 containing the enhancer element, the human metallothionein IIA (hMTIIA) promoter, and the complete pUC8 sequence. pLEN-ACE6/5 (not shown) is identical to pLEN-ACEVII, except that the fragment between *Not* I (nt 2046) and *Hin*dIII (nt 2507) containing the sequence encoding the putative membrane anchor (solid area) is replaced by a synthetic oligoduplex that introduces a stop codon at nt 2105. Open bars, full-length testis ACE cDNA; stippled bars, promoter sequences; cross-hatched bars, SV40-derived sequences; hatched bars, ampicillin-resistance gene (Amp^r). (*C*) C-terminal sequence of human testis ACE, from Arg-651 to the C terminus, illustrating the putative transmembrane sequence (boxed) and short cytoplasmic tail. In pLEN-ACE6/5 a stop codon was introduced (arrow) after the codon encoding Gln-654.

Membrane fractions, prepared by extracting cells with 1% Triton X-100/50 mM Hepes, pH 7.5/1 mM phenylmethylsulfonyl fluoride at 4°C for 2 hr, and conditioned media were assayed for activity.

ACE Purification and Enzyme Assays. ACE from all sources was purified with a lisinopril-Sepharose affinity resin (5). ACE activities were generally measured with the substrate 2-furanacryloyl-L-phenylalanylglycylglycine (Fa-FGG) 17) under standard conditions: 0.1 mM Fa-FGG/50 mM Hepes, pH 7.5/0.3 M NaCl at 25°C; average specific activity of hTACE was 80 units/mg, where 1 unit of activity is defined as 1 ΔA_{334} unit/min (18). The presence of ACE activity on the surfaces of transfected cells was determined by incubating 5 mM Hip-His-Leu (where Hip is hippuryl)/0.1 M potassium phosphate, pH 8.3/0.3 M NaCl with a washed monolayer of $\approx 3 \times 10^5$ cells for 10 min at 37°C, and estimating the extent of hydrolysis by a fluorometric assay of the product His-Leu (19).

ACE Deglycosylation. ACE (4 μg) was denatured by boiling for 4 min in 0.5% SDS/1% 2-mercaptoethanol, then incubated with 0.4 unit N-Glycanase (Genzyme)/0.25 M sodium phosphate, pH 8.6/10 mM 1,10-phenanthroline/1.25% Nonidet P-40 for 24 hr at 37°C, acidified with sodium acetate to pH 6.0, and sequentially digested with 1 unit of neuraminidase (Sigma) for 1 hr at 37°C and 80 milliunits *O*-Glycanase (Genzyme) for 18 hr at 37°C. Reactions were terminated by addition of SDS electrophoresis buffer, boiled for 5 min, and the products were analyzed by SDS/PAGE.

Phase Separation. This was carried out by the procedure of Bordier (20). Typically, 2–3 μg of ACE was made up to 200 μl with 10 mM Tris·HCl, pH 7.4/0.15 M NaCl/1% Triton X-114 (Sigma) and incubated at 30°C for 3 min. The detergent-rich and detergent-poor phases were separated through a 6% (wt/vol) sucrose cushion by centrifugation at 3000 × *g* for 3 min and assayed in duplicate for enzyme activities by hydrolysis of Hip-His-Leu.

RESULTS

Expression of hTACE in CHO Cells. In general, pools of G418-resistant CHO cells expressed comparable amounts of hTACE, irrespective of the introduced vector. However, for the pLEN vectors, individual high-producing clones could be identified that, compared to the pools, expressed up to 6-fold higher levels of hTACE after induction of the metallothionein promoter with 80 μM $ZnCl_2$ (Table 1). Surprisingly, CHO cells transfected with either pΩ-ACE or pLEN-ACEVII, both of which code for recombinant ACE complete with its putative membrane anchor, secreted significant amounts of soluble ACE in addition to expressing the expected membrane-bound ACE (Table 1). The soluble hTACE appeared in the medium regardless of the presence or absence of fetal calf

Table 1. Expression of hTACE in CHO cells

ACE-encoding plasmid	ACE			
	G418^r pool		G418^r high-producing clones	
	Uninduced	Induced	Uninduced	Induced
	Soluble			
pΩ-ACE	0.5			
pLEN-ACEVII	0.2	0.57	0.86	2.5
pLEN-ACE6/5	0.15	0.5	0.75	3.3
None (control cells)	0			
	Membrane bound			
pΩ-ACE	6.6			
pLEN-ACEVII		6.5		32
pLEN-ACE6/5		0		0.5
None (control cells)	0			

CHO cells were cotransfected with pSV2NEO and one of the following: pΩ-ACE, pLEN-ACEVII, or pLEN-ACE6/5. Control cells were transfected with pSV2NEO alone. G418-resistant (G418^r) pools and clones were analyzed for ACE activity in the conditioned medium (soluble ACE expressed as μg/ml) and Triton X-100-extracted cell pellets (membrane-bound ACE expressed as μg per 3×10^7 cells). Protein quantities were deduced from specific activities. Cells were induced with 80 μM $ZnCl_2$.

serum. Much of the membrane-bound ACE-VII (expressed from the plasmid pLEN-ACEVII) was expressed as an ectoenzyme, as shown by the hydrolysis of Hip-His-Leu added to a washed monolayer of CHO cells transfected with pLEN-ACEVII; these cells generated >20 nmol of His-Leu per min per 3×10^5 cells. Based on a specific activity of 90 nmol per min per μg for this substrate (21), these cells contained >22 μg of cell membrane-bound ACE-VII per 3×10^7 cells, which was >68% of the total membrane-bound ACE-VII recovered from detergent-extracted cell pellets (≈32 μg, Table 1). Control cells transfected with pSV2NEO alone did not hydrolyze Hip-His-Leu. pLEN-ACE6/5 was constructed to direct the expression of an hTACE deficient in its C-terminal hydrophobic anchor (termed ACE-6/5) (Fig. 1 *B* and *C*) and, as expected, virtually all the ACE-6/5 was found as a soluble form in the conditioned medium; the detergent-solubilized membrane fraction from these cells contained <2% of the activity recovered from cells transfected with pLEN-ACEVII (Table 1).

Physicochemical Properties of hTACE. Recombinant ACE purified from conditioned medium (soluble form) and from Triton X-100 extracts of CHO cell pellets (membrane-bound form) migrated with the same mobility as the native human testis ACE isozyme on SDS/PAGE (≈100 kDa, Fig. 2*A*). However, unlike the native isozyme, all forms of hTACE ran as broad, diffuse bands, and may consist of overlapping, differently glycosylated species. Complete deglycosylation reduced all forms of hTACE and native human testis ACE to an ≈70-kDa polypeptide (Fig. 2*B*, native ACE data not shown), consistent with a polypeptide size inferred to be 75 kDa (5) and with a reported carbohydrate content of 20–30% by weight (2).

The amino acid composition of hTACE is within experimental limits of the predicted integer values (Table 2). The compositions of soluble ACE-VII and ACE-6/5 are virtually identical and differ from that of membrane-bound ACE-VII. Automated Edman degradation of hTACE failed to yield an N-terminal sequence, suggesting that the N terminus is blocked, as is that of native human testis ACE (5). The catalytic activities of soluble ACE-VII and ACE-6/5 are similar or identical to those of native human kidney and rabbit testis ACE in terms of substrate hydrolysis (Fa-FGG and angiotensin I), chloride activation, and lisinopril inhibition (unpublished results).

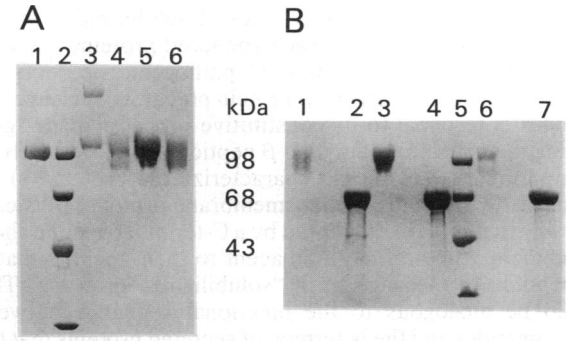

FIG. 2. SDS/PAGE of testis ACE on a 4–20% gradient gel stained with Coomassie brilliant blue. (*A*) Native and recombinant enzymes. Lanes: 1 and 3, native ACE from rabbit and human testes, respectively (note that human testes contain equimolar quantities of the somatic and testis isozymes); 4–6, membrane-bound ACE-VII, soluble ACE-VII, and ACE-6/5, respectively; 2, molecular mass markers. (*B*) hTACE sequentially deglycosylated with N-Glycanase, neuraminidase, and *O*-Glycanase. Lanes: 1, 3, and 6, untreated enzymes (ACE-6/5, soluble ACE-VII, and membrane-bound ACE-VII, respectively); 2, 4, and 7, deglycosylated enzymes (ACE-6/5, soluble ACE-VII, and membrane-bound ACE-VII, respectively); 5, molecular mass markers.

Table 2. Amino acid composition of hTACE

Amino acid	Residues per mol		
	Membrane-bound ACE-VII	Soluble ACE-VII	ACE-6/5
Asx	63.1 (64)	62.8 (64)	64.4 (64)
Glx	72.2 (87)	77.3 (83)	75.8 (83)
Ser	49.3 (49)	46.2 (43)	46.5 (43)
Gly	45.9 (39)	35.9 (35)	35.1 (35)
His	23.0 (28)	23.3 (23)	23.3 (23)
Arg	34.7 (34)	29.7 (29)	29.7 (29)
Thr	42.2 (43)	42.3 (42)	41.6 (42)
Ala	57.9 (53)	54.3 (51)	54.4 (51)
Pro	37.6 (37)	35.8 (36)	36.8 (36)
Tyr	25.5 (26)	25.3 (26)	25.1 (26)
Val	34.4 (34)	32.2 (32)	33.6 (32)
Met	17.9 (17)	17.2 (17)	17.4 (17)
Ile	27.4 (27)	24.5 (25)	25.1 (25)
Leu	76.4 (77)	66.8 (66)	67.3 (66)
Phe	27.6 (29)	26.4 (26)	26.0 (26)
Lys	36.8 (30)	30.9 (30)	30.5 (30)

Samples were hydrolyzed for 18–24 hr at 110°C in 6 M HCl and analyzed by reverse-phase high performance liquid chromatography after derivatization with phenylisothiocyanate. The results are the average of two, five, and four analyses for membrane-bound ACE-VII, soluble ACE-VII, and ACE-6/5, respectively. The values are presented as residues per mol and integer (shown in parentheses), the latter based on the inferred polypeptide sequence of the mature hTACE (5) for membrane-bound ACE-VII and from the sequence with the membrane anchor and cytoplasmic tail deleted (Fig. 1*C*) for soluble ACE-VII and ACE-6/5.

Phase Separation of ACE in Triton X-114. Soluble ACE-VII, ACE-6/5, and guinea pig serum ACE partitioned predominantly (>97%) into the detergent-poor phase on treatment with Triton X-114, whereas the membrane-bound ACE-VII partitioned favorably (>48%) into the detergent-rich phase (Table 3). Under identical conditions, detergent-solubilized native human kidney and testis ACE, which can be expected to retain their membrane anchors, partitioned 20–30% into the detergent-rich phase. Treatment of membrane-bound ACE-VII with trypsin converted it from an amphipathic to a hydrophilic form that partitioned into the detergent-poor phase (Table 3).

Table 3. Triton X-114 phase separation of recombinant and native ACE

Form of ACE	Enzyme activity in detergent phase, % of total activity
ACE-6/5	1.2 ± 1.3
Soluble ACE-VII	1.5 ± 1.3
Guinea pig serum ACE	3.0 ± 1.1
Membrane-bound ACE-VII treated with trypsin*	3.0 ± 1.2
Membrane-bound ACE-VII	48.5 ± 5.2
Native human testis ACE	30.0 ± 5.4
Native human kidney ACE	23.4 ± 3.2

ACE was subjected to phase separation in Triton X-114 (20) and the detergent-rich and detergent-poor phases were assayed in duplicate for ACE activities. Activity recovered in the detergent-rich phase is expressed as a percentage of the total starting activity and given as the mean ± SEM for four phase separations. Form of ACE: hTACE, purified from conditioned medium (ACE-6/5 and soluble ACE-VII) or from detergent-extracted CHO cell pellets (membrane-bound ACE-VII); native ACE, purified from detergent extracts of human kidney and testis homogenates; and soluble ACE, from guinea pig serum.

*ACE (1 μg) was incubated with 0.03 μg of trypsin in 10 mM Hepes (pH 7.0) for 10 min at 37°C and then immediately subjected to phase separation.

DISCUSSION

Somatic ACE is generally thought to be an ectoenzyme resident on the surfaces of fluid-bathed cells in numerous mammalian tissues, most notably endothelium and the renal, intestinal, and choroid plexus brush borders (1, 2). The microvillus enzyme is a member of a dense population of brush border hydrolases that are variously anchored by N- and C-terminal transmembrane peptides or glycolipid moieties (22). The unique testis ACE isozyme, limited to developing spermatozoa, is also membrane-bound but is intravesicular (5). Membrane anchoring is thought to involve a hydrophobic transmembrane sequence located near the C terminus of both isozymes (Fig. 1*C*). However, ACE is also present in most body fluids (8), although the mechanism of production and purpose of this soluble form remains obscure.

We have expressed human testis ACE in CHO cells to address a number of structural problems, including the cellular targeting and anchoring of this protein. Although the testis isozyme is identical to the C-terminal domain of somatic ACE, it contains a unique 35-residue serine- and threonine-rich N-terminal sequence that may target it to its unusual intravesicular position in spermatozoa (5). This signal is apparently inoperative in CHO cells, since most or all of the hTACE appeared to be transported to the cell surface, as shown by the high rate of hydrolysis of Hip-His-Leu added to a washed monolayer of transfected cells. Moreover, a significant proportion of the membrane-bound hTACE is secreted into the medium as a soluble form (Table 1).

To examine this phenomenon, the vector pLEN-ACE6/5 was constructed; it codes for hTACE lacking the C-terminal hydrophobic putative membrane-spanning sequence and cytoplasmic tail. As expected, ACE-6/5 appears only in the medium as a soluble protein and virtually no activity is recovered from the membrane fraction (Table 1). This confirms that the C-terminal hydrophobic sequence anchors the enzyme in the membrane. These data are consistent with the structure of this sequence (Fig. 1*C*), which, typical of membrane anchors, consists of a 17-residue hydrophobic stretch followed by a highly charged intracytoplasmic tail (3). The amphiphilicity of soluble ACE-VII and ACE-6/5, estimated by phase separation in Triton X-114, was compared to that determined for membrane-bound ACE-VII harvested from the membrane fraction by Triton X-100 solubilization as well as to detergent-solubilized human testis and kidney ACE and guinea pig serum ACE (a convenient source of abundant, naturally occurring soluble ACE). Soluble ACE-VII appears to have lost its membrane anchor since it is equivalent in terms of hydrophilicity to the anchor-minus mutant ACE-6/5 and to the naturally soluble guinea pig serum ACE (Table 3). On the other hand, membrane-bound ACE-VII is strongly amphipathic, similar to the detergent-solubilized tissue enzymes from human testis and kidney, indicating that it has retained its hydrophobic membrane anchor. Treatment of membrane-bound ACE-VII with trypsin converts it from an amphipathic to a hydrophilic protein on phase separation in Triton X-114 (Table 3); trypsin is commonly used to solubilize tissue-bound ACE from various sources and apparently removes the membrane anchor without affecting activity (9).

The similarity between soluble ACE-VII and anchor-minus ACE-6/5 is further demonstrated by their amino acid compositions, which are in good agreement and differ from the composition of membrane-bound ACE-VII (Table 2). Indeed, the compositions of soluble ACE-VII and ACE-6/5 are so similar that their C termini must be within a few residues of each other (see Fig. 1*C*). Thus these data suggest that the membrane anchor is removed by proteolysis to generate soluble ACE-VII. However, these results do not distinguish between proteolysis occurring at the cell surface and intracellular processing of the membrane-bound hTACE.

The apparent proteolytic cleavage of the membrane anchor and release of soluble hTACE has parallels *in vivo*. ACE belongs to a growing number of proteins that exist naturally as both soluble and membrane-bound forms. This is particularly evident with the glycosyl-phosphatidylinositol-anchored proteins. Indeed, one function of this unusual glycolipid anchor might be to allow the rapid release of membrane-bound proteins by the action of a specific anchor-degrading phospholipase D (23).

Proteins anchored by a transmembrane peptide are also known to have soluble counterparts. In some case, as in dopamine β-monooxygenase (24) and neutral endopeptidase 24.11 (25), the membrane anchor consists of an uncleaved N-terminal signal peptide, and release of such proteins may be mediated by a signal peptidase-like protease. In other cases, such as ACE, the proteins are anchored by a hydrophobic C-terminal sequence. Soluble ACE *in vivo* likely derives from membrane-bound ACE by loss of the C-terminal anchor, as shown by its hydrophilic properties on phase separation (Table 3) and gel filtration (9). It is unlikely that the *in vivo*-soluble enzyme arises from a distinct biosynthetic pathway, as no "anchor-minus" ACE cDNAs have been isolated from the various libraries screened (3–7). Furthermore, Hooper *et al.* (26) found that an endogenous "ACE-solubilizing" activity, inhibitable by EDTA but not by ACE inhibitors, removes the C-terminal membrane anchor during purification of porcine kidney ACE, as demonstrated by conversion of the tissue enzyme from an amphipathic to a hydrophilic form.

Other examples of proteins anchored by C-terminal peptides that are proteolytically cleaved with release of their extracellular domains include: the Tac protein, which is a glycoprotein that binds interleukin 2 (11); the leukemogenic glycoprotein encoded by Friend spleen focus-forming virus (12); and the amyloid β protein precursor, a large glycoprotein that is constitutively secreted by a specific cleavage near its membrane anchor (13). It may well be that a general mechanism is responsible for the release of extracellular glycoproteins that like ACE are anchored in cell membranes by a C-terminal hydrophobic peptide. Whether or not the solubilized proteins perform functions that are distinct from those of their membrane-bound counterparts remains to be established.

Whatever the physiological roles of solubilized proteins, aberrant cleavage of membrane-anchored proteins could be responsible for the formation of pathogenic products. In Alzheimer disease, amyloid β protein precursor is cleaved 16 residues N-terminal to its constitutive processing site, generating the neurotoxic amyloid β peptide (13, 27). Clearly, it is important to isolate and characterize the protease(s) responsible for solubilizing transmembrane proteins. It is conceivable that proteins anchored by a C-terminal peptide share a common structural motif adjacent to their anchors that is susceptible to cleavage by a "solubilizing" protease. This would be analogous to the junctional sequence between signal peptides and the N termini of secreted proteins that the signal peptidase recognizes. The release of membrane-bound ACE from CHO cells may provide a convenient system for investigating this protease(s) and the structural basis for its limited proteolytic action.

We thank Drs. Frank S. Lee, Daniel J. Strydom, and Edward A. Fox for advice, Dr. Stana Weremowicz for assistance with the cell cultures and transfections, Dr. Werner Dafeldecker for the DNA syntheses, and Wynford Brome for technical help. We are indebted to Drs. C. L. Cepko and P. J. Kushner for providing the vectors pJ4Ω and pLEN, respectively. This work was supported in part by Grant HL 34704 from the National Institutes of Health.

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