Unaltered Cleavage and Secretion of Angiotensin-converting Enzyme in Tumor Necrosis Factor-α-converting Enzyme-deficient Mice*

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Mammalian angiotensin-converting enzyme (ACE) is one of several biologically important ectoproteins that exist in both membrane-bound and soluble forms as a result of a post-translational proteolytic cleavage. It has been suggested that a common proteolytic system is responsible for the cleavage of a diverse group of membrane ectoproteins, and tumor necrosis factor-α-converting enzyme (TACE), a recently purified disintegrin-metalloprotease, has been implicated in the proteolytic cleavage of several cell surface proteins. Mice devoid of TACE have been developed by gene targeting. Such mice did not show reduced ACE activity or ACE protein levels, indicating that TACE is responsible for the cleavage of other ectoproteins.

Cultured fibroblasts without TACE activity, when transfected with cDNA encoding for the testicular isozyme of ACE (ACET), synthesized and secreted ACE normally after a proteolytic cleavage near the C terminus. In addition, similar quantities of the soluble, C-terminally truncated somatic isozyme of ACE (ACEp) were present in the serum of wild-type and TACE-deficient mice. These results demonstrate that TACE is not essential in the generation of soluble ACE under physiological conditions. Finally, we also report solubilization of ACE-secretase, the enzyme that cleaves ACE, from mouse ACE89 cells and from rabbit lung. We demonstrate that soluble ACE-secretase from both sources failed to cleave its substrate in solution, suggesting a requirement for anchoring to the membrane.

Many proteins exist in both membrane-bound and soluble forms as a result of post-translational proteolytic processing. The responsible proteases, variously called secretases, sheddases, or convertases, cleave the membrane-bound form at sites near the plasma membrane on the extracellular side of the single membrane-spanning domain. The entire extracellular domain, often physiologically active, is released into the medium or the circulation. This process of cleavage and secretion appears to be an important and widely used cellular post-translational regulatory process because a structure of functionally unrelated cell-surface proteins undergoes this process. They include tumor necrosis factor α (TNF-α), involved in inflammatory responses, several cytokine receptors, β-amyloid precursor protein (β-APP) implicated in Alzheimer’s disease, a number of growth factors and their receptors, adhesion molecules, and the vasoregulatory enzyme angiotensin-converting enzyme (ACE) (1, 2). Since a number of these proteins are involved in disease processes such as inflammation, neurodegeneration, hypertension, apoptosis, and oncogenesis, the secretases could also provide novel therapeutic targets.

Despite the biological importance of this process, little is known about the identity of the responsible proteases. We have been studying the characteristics of one such protease, ACE-secretase, the enzyme involved in cleavage and secretion of ACE (3–7). Although ACE is primarily a cell-associated protein, under normal physiological conditions soluble ACE is found in serum and other body fluids (8). The cleavage and secretion of ACE is particularly significant because tissue-bound ACE and soluble ACE in circulation may have different physiological roles. ACE has two structurally related isozymes: testicular ACE (ACEp) and pulmonary ACE (ACEp), involved in male fertility and blood pressure regulation, respectively (9, 10). We and others have shown that both ectoproteins, ACEp and ACEp, undergo specific cleavage and secretion (3, 11–13). The nature of the ACE-secretase activity has been studied in vitro using cell lines that do not express ACE, as well as primary cultures of cells that generate ACE in vivo. The results obtained from these studies are corroborated by observations made with human and animal tissue expressing natural ACE (6, 13). ACE89, a mouse epithelial cell line permanently transfected with a rabbit ACEp expression vector, synthesizes, glycosylates, and secretes enzymatically active soluble ACEp (3). The secretion process is slow, and ACEp accumulates on the cell surface. However, secretion can be enhanced by treatment of cells with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, indicating the existence of cellular mechanisms that regulate the conversion of cell-bound ACEp to its soluble form. The secreted protein has a molecular mass lower by 8 kDa than the corresponding cellular form, suggesting that a proteolytic cleavage is involved. The exact site of cleavage in this system has been determined. The cleavage occurs in the ectodomain near the transmembrane domain between Arg663 and Ser664, generating the short form, soluble ACEp.

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1 The abbreviations used are: ACE, angiotensin-converting enzyme; ACEp, testicular ACE; ACEp, pulmonary ACE; PAGE, polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-α; TGF-α, transforming growth factor-α; TACE, tumor necrosis factor-α-converting enzyme; β-APP, β-amyloid precursor protein; PMA, phorbol 12-myristate 13-acetate; ConA, concanavalin A.
ACEp, which lacks the membrane-anchoring C-terminal tail of the cell-associated ACEp (4).

Recently we have developed a cell-free in vitro assay system to quantitate and characterize ACE-secretase, utilizing membranes prepared from ACE89 cells and rabbit lung. The in vitro secretase activity was resistant to high salt extraction and to inhibitors of serine, chymotrypsin, trypsin, cysteine, aspartate, and elastase type proteases, but it is susceptible to Compound 3, a hydroxamic acid-based inhibitor of certain metalloproteases (5). These results indicate that ACE-secretase is an integral membrane metalloprotease. We extended the observation made with ACEp in a transfected system to explain the mechanism of ACEp secretion in vitro. A similar pattern of inhibition of ACEp production was observed in natural and transfected vascular endothelial cells and freshly isolated kidney epithelial cells, the two major sources of ACEp in the body. These observations, together with analysis of ACEp proteins present in rabbit serum, lung, and kidney, established that ACEp secreted in vivo is also caused by the cleavage removal of the C-terminal region of the cell-associated protein (6). In this article, we report the use of the same in vitro assay to characterize, for the first time, detergent-soluble ACE-secretase from both ACE89 cells and rabbit lung membranes.

The secretases studied so far share certain general properties, such as stimulation by PMA and sensitivity to hydroxamic acid-based metalloprotease inhibitors. These common properties suggest that the shedding phenomena may be mediated by the same cellular components since mutant cell lines have been isolated that are defective in hydroxamic acid inhibitor-susceptible, PMA-stimulated cleavage and secretion of many proteins, including TGF-α, TNF-α, L-selectin, interleukin-6 receptor α-subunit, and β-APP (14). These observations led to the suggestion that a single secretase or a small number of similar secretases rather than multiple independent secretases are involved in the shedding process. The most well characterized secretase is TNF-α-converting enzyme (TACE), the one that cleaves proTNF-α. TACE has recently been purified and cloned (15, 16), and mice lacking this protease were generated (17).

TACE-deficient cell populations derived from these mutant mice are grossly deficient in shedding of several other ectoproteins in addition to TNF-α. These include TGF-α, L-selectin, p75 TNF receptor, and β-APP (17–19). These results reinforce the suggestion that the shedding process, which involves various structurally and functionally diverse proteins, is probably mediated by the same or a similar type of proteases.

In this report, we show that, unlike the release of several other ectoproteins mentioned above, the cleavage and secretion of either isozyme of ACE does not require TACE. Also, the process of cleavage and secretion of ACE in vivo in mice is not affected by the absence of TACE. These results indicate that ACE-secretase is a unique enzyme, different from TACE. Here, for the first time, we also report solubilization of ACE-secretase and characterization of some of the unusual properties of the solubilized enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**Lisinopril (N-(1S)-carboxy 3-phenylpropyl-1-lysyl-L-proline) was provided by Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Compound 3 (N-{dl-[2-(hydroxaminocarbonyl)]-methyl}[4-methylpentanoyl]-L-3-(tert-butyl)-alanyl-L-alanine, 2-aminoethyl amide) was provided by Immunex Research and Development (Seattle, WA).

**Cells, Transient Transfections, Pulse-Chase Analysis, Immunoprecipitation, and Quantitation of Secretion—**TACE-deficient ear fibroblasts were isolated from TACE-deficient mouse, as described (19). Wild-type or TACE-deficient fibroblasts were transiently transfected with ACEcDNA using the vaccinia virus-T7 RNA polymerase system, as described previously for HeLa and Chinese hamster ovary cells (20, 21). Labeling with [35S]methionine, pulse-chase analysis, immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography have been described previously (21). For the co-transfection experiments (Fig. 2B and Table III), ACEp and TACE were subcloned into pcDNA3zeo expression vector from Invitrogen and transiently transfected using DEAE-dextran (22). For quantitatively accurateavage and secretion of prohormone, ACE was subjected to phosphorImager analysis by using ImageQuant software (both from Molecular Dynamics, Sunnyvale, CA). The amount of mature ACE or chimeric proteins (measured in arbitrary PhosphorImager units) present in each experiment was then determined. Results are expressed as the percentage of activity at each stage relative to the total activity. Western Analysis and Enzyme Activity Measurement—Two different goat polyclonal antibodies were used for Western analysis. The first antibody was generated against purified rabbit lung ACE (anti-ACE antibody). The second antibody was generated against a synthetic peptide corresponding to the 14 amino acids of the C-terminal sequence of rabbit ACE (anti-C-terminal peptide antibody). Both of these antibodies have been used previously (4, 6). ACE enzyme activity was assayed by using hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as substrate (21).

**Preparation of the Membrane Fractions and Solubilization of ACE-secretase Activity—**Confluent ACE89 cells (3), labeled overnight with [35S]methionine (100 μCi/150-mm plate) were scraped and suspended in 0.02 m HEPES buffer, pH 7.0, containing 0.2 m sucrose, protease inhibitor mixture (Boehringer Mannheim, prepared according to manufacturer’s instructions), phenylmethylsulfonfluoride (1 mM), leupeptin (1 μg/ml), and phosphoramidon (50 μM). The cell suspension was homogenized in a Polytron and centrifuged at 700 × g for 10 min. The pellet was discarded, and the supernatant was centrifuged again at 36,000 × g for 40 min. The sedimented membranes were washed and used as the membrane preparation, as described previously (5). For solubilization, the membrane pellet was suspended in 0.05 m Tris- HCl, pH 8.0, containing 0.3 m NaCl, the protease inhibitors and 0.1% Triton X-100 (0.25 ml/150-mm plate). The suspension was stirred at 4 °C for 1 h and centrifuged at 36,000 × g for 45 min. The supernatant was used as the source of solubilized ACE-secretase.

**Rabbit lung membranes were prepared as described previously (5), and the secretase activity was solubilized from these membranes by detergent extraction, as described above for ACE89 cell membranes.**

**Assay for ACE-secretase Activity—**In the deglycosylation-PAGE assay (Fig. 3A), detergent extracts of ACE89 cell membranes (0.25 ml) were incubated with or without 300 μl of lisinopril-Sepharose (6) or concanavalin A (ConA)-agarose (Sigma, 200 μl) in a final volume of 1.5 ml for 1 h at 37 °C. For incubations using lisinopril-Sepharose, 0.02 m HEPES, 100 μM ZnCl2, 0.5 m NaCl, and 0.3 m NaCl were included as areas for incubations using ConA-agarose, 0.05 m Tris- HCl, pH 7.5, and 0.2 m NaCl were included. Protease inhibitors were present in all incubations. The reaction was stopped by boiling with SDS (1%) for 3 min; the incubation mixture was then centrifuged, and ACE-related proteins were immunoprecipitated from the supernatant and deglycosylated using N-glycosidase F, neuraminidase, and O-glycosidase before being analyzed by PAGE (5). ACE-secretase activity in the ACE89 cell membranes was assayed, as described previously (5).

For the detergent-extraction assay (Table IV), membranes or Triton X-100 extract of the membranes prepared from rabbit lung were incubated with or without lisinopril-Sepharose, as described above, at 4 °C or 37 °C for 1 h. Uncleaved and cleaved ACEp proteins were physically separated by the temperature-induced phase-separation method utilizing Triton X-114, as described previously (5), and ACEp enzyme activity was measured in both the detergent and the aqueous phase. In the incubations containing lisinopril-Sepharose, phase separation was performed after ACE proteins were eluted from the lisinopril-Sepharose by borate buffer (6). As determined previously (5), cleaved ACE partitions to the aqueous phase almost exclusively (more than 98%), whereas the majority (more than 66%) of the uncleaved ACE is present in the detergent phase. A considerable amount (34%) of uncleaved ACE also partitions to the aqueous phase. Thus, the corrected value for cleaved ACE was calculated by subtracting this latter value from the total ACE activity determined in the aqueous phase.

**RESULTS AND DISCUSSION**

ACEp Is Cleaved and Secreted from TACE-deficient Cells—To determine if TACE is required for the cleavage and secretion of ACE, we investigated the ability of TACE-deficient fibroblasts to secrete ACE. These mutant cells were generated by immortalizing fibroblasts derived from TACE-deficient mice and comparing them with similarly immortalized cells from...
Cleavage and Secretion of ACE in TACE-deficient Mice

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Table I

| Transfection            | Addition         | Secretion<sup>a</sup> |
|-------------------------|------------------|-----------------------|
|                         | ($)              | (%)                   |
| ACET                   | None             | 16/18                 |
| ACE<sub>T</sub>         | PMA              | 20/28                 |
| ACE<sub>T</sub>/CD4–5F  | Compound 3       | 7/4.4                 |
| ACE<sub>T</sub>/CD4–5F  | None             | 55/45                 |
| ACE<sub>T</sub>/CD4–5F  | PMA              | 69/66                 |
| ACE<sub>T</sub>/CD4–5F  | Compound 3       | 12/10                 |

<sup>a</sup> Values presented are mean of two separate experiments.

Fig. 1. Cleavage and secretion of ACET from wild-type and TACE-deficient cells. Wild-type (+/+) and TACE-deficient (−/−) fibroblasts were transiently transfected with ACET<sub>T</sub> cDNA using the vaccinia virus T7 RNA polymerase system. A, 4 h after transfection, cell extracts (C) and culture media (M) were analyzed by SDS-PAGE followed by Western blot analysis using anti-ACE (lanes 1 and 2) or anti-C-terminal peptide (lanes 3 and 4) antibodies. B, following transfection, cells were pulse-labeled with [35S]methionine for 30 min, and the label was chased for 4 h in the absence (lanes 3 and 4) or presence of Compound 3 (lanes 5 and 6) or PMA (lanes 7 and 8). Cell extracts (C) and culture media (M) were immunoprecipitated with anti-ACE antibody and analyzed by SDS-PAGE. Lanes 1 and 2, without cDNA. The molecular mass of the mature ACET is indicated in kilodaltons. Secretion was quantitated by PhosphorImager analysis (see Table I).

We have shown previously that ACE-secretase activity present in ACE98 cells, as well as in the primary cultures of rabbit renal proximal tubular epithelial cells, is resistant to inhibitors of various known classes of proteases. None of the inhibitors of trypsin, chymotrypsin, elastase, cysteine, serine, aspartase, and certain metalloprotease inhibitors had any effect on cleavage and secretion of ACET<sub>P</sub> by ACE98 cells (4) or ACET<sub>P</sub> by renal epithelial cells (6). In contrast, a hydroxamic acid-based metalloprotease inhibitor, Compound 3, significantly inhibited secretion of both ACET<sub>T</sub> and ACET<sub>P</sub>. In addition, cleavage and secretion of both isozymes of ACE was enhanced by treatment of cells with PMA. Thus, to further characterize the properties of ACE-secretase present in TACE-deficient cells, the effects of Compound 3 and PMA on ACE-secretase activity in TACE-deficient cells were studied. The wild-type and the mutant cells were transfected with ACET<sub>T</sub> cDNA, pulse-labeled with [35S]methionine, and chased in the absence (Fig. 1B, lanes 3 and 4) or in the presence of Compound 3 (lanes 5 and 6) or PMA (lanes 7 and 8), added to the culture media. PMA increased secretion moderately, but significantly (p < 0.05), whereas Compound 3 inhibited secretion almost completely (Fig. 1B and Table I). Thus, the ACE-secretase present in TACE-deficient cells exhibited biochemical characteristics similar to those of wild-type cells. It should be noted that, unlike in ACE98 cells (4), the enhancement of secretion by PMA in fibroblasts is less pronounced.

We have generated (7) a chimeric type 1 ectoprotein (ACE<sub>T</sub>/CD4–5F) containing the distal extracellular domain of ACET<sub>T</sub> and the membrane-proximal, transmembrane, and cytoplasmic domains of CD4, another type 1 ectoprotein that is not cleavage-secreted. This chimera was cleaved extremely efficiently, with a rate of secretion much higher than the natural substrate, ACET<sub>T</sub>. The cleavage occurred in the membrane-proximal CD4 sequences. We transfected TACE-deficient and wild-type cells with ACE<sub>T</sub>/CD4–5F construct to study its secretion in the absence of TACE. As shown in Fig. 2A, ACE<sub>T</sub>/CD4–5F was also cleavage-secreted in a similar fashion (lanes 3 and 4) from wild-type and TACE-deficient cells. We have shown previously that transfected HeLa cells secreted ACE<sub>T</sub>/CD4–5F more efficiently than ACET<sub>P</sub>. ACE-secretase present in either TACE-deficient or wild-type fibroblasts exhibited similar characteristics, as 45–55% ACET/CD4–5F as compared with 18–21% of ACET<sub>P</sub> was secreted by these cells (Table I). Cleavage and secretion was stimulated by PMA (Fig. 2A, lanes 7 and 8) and inhibited by Compound 3 (lanes 5 and 6). Thus, the TACE-deficient cells can cleave and secrete two different substrates of ACE-secretase, ACET<sub>T</sub> and the chimeric protein ACET<sub>T</sub>/CD4–5F. In addition, the cleavage and secretion of both substrates was stimulated by PMA and inhibited by Compound 3 to the same extent in both wild-type and TACE-deficient cells (Table I).

Soluble ACE<sub>p</sub> Is Generated in Vivo in TACE-deficient Mice—ACE<sub>p</sub> is present in the body both as a cell-associated protein in endothelial, epithelial, and monocyte cells and as a soluble protein in various body fluids, including serum. To delineate the mechanism by which soluble ACE<sub>p</sub> is produced in vivo, we have previously analyzed soluble ACE<sub>p</sub> present in rabbit serum and cell-associated ACE<sub>p</sub> in tissues such as lung and kidney. From these, we have established that ACE<sub>p</sub> secretion in vivo is caused by the cleavage removal of the C-terminal region of the cell-associated protein (6). To characterize the proteolytic enzyme responsible for ACE<sub>p</sub> secretion, we employed rabbit renal proximal tubular epithelial cells and demonstrated significant inhibition of secretion by Compound 3.
The experiments described in Figs. 1 and 2 fibroblasts were transfected with ACE T/CD4–5F cDNA using vaccinia virus T7 RNA polymerase system. Pulse-chase analysis was performed in the absence (lanes 3 and 4) or presence of Compound 3 (lanes 5 and 6) or PMA (lanes 7 and 8), followed by immunoprecipitation, as described in the legend for Fig. 1B. The molecular mass of the chimeric protein is indicated in kilodaltons. B, cleavage and secretion of ACE T from TACE-deficient cells co-expressing ACE and TACE. TACE-deficient fibroblasts (−/−) were transiently co-transfected with either ACE p and an irrelevant cDNA (lanes 3 and 4) or co-transfected with ACE p and TACE cDNA (lanes 5 and 6). Lanes 1 and 2, no cDNA. Unlike the experiments described in Figs. 1 and 2A, a cytomegalovirus promoter-driven system was used in these experiments (22). Cells were pulse labeled with [35S]methionine and chased in the presence of PMA. Cell extracts (C) and culture media (M) were immunoprecipitated and analyzed, as described for Fig. 1B. Quantitation of secretion by PhosphorImager analysis is presented in Table III (left panel).

These and other (13) results suggest that soluble serum ACE is generated by an ACE-secretase-like enzyme. Thus, if TACE is the major protease in the body that generates soluble ACEp, then serum obtained from a TACE-deficient mouse should have little or no soluble ACEp. But if soluble ACEp levels are comparable in wild-type and mutant mice, it would indicate that ACE cleavage and secretion was unaffected by the absence of TACE and was carried out instead by a different enzyme, ACE-secretase.

To test this hypothesis, we quantitated, by enzyme activity assay, the soluble ACEp present in serum samples from TACE-deficient mice and their normal littermates. Although most TACE-deficient mice die at birth, we were able to collect serum from three such mutant mice, and as shown in Table II, comparable amounts of soluble ACEp were present in the serum of normal and TACE-deficient mice (390 and 302 units/ml of serum, respectively). These results indicate that TACE is not the major enzyme responsible for solublization of ACEp, in vivo. The slightly lower level of soluble ACEp consistently observed in mutant mice might indicate that in vivo TACE could play a minor role in ACEp production. This possibility was further explored in the experiments described below.

Co-expression in the TACE-deficient Cells—Experiments described above using TACE-deficient cells, and serum from mice devoid of TACE suggest that TACE is not required, in vitro or in vivo, for cleavage and secretion of either isozyme of ACE. The next experiment was designed to determine if a TACE-like protein might participate in solubilization of ACE. For this purpose, the TACE-deficient cells were transfected with ACEp cDNA and cleavage and secretion of ACEp by these cells was compared with those that were co-transfected with TACE cDNA as well. The cell extracts and the media were immunoprecipitated and analyzed by SDS-PAGE (Fig. 2B). In these experiments, secretion was quantitated in two different ways. In addition to the PhosphorImager analysis of immunoprecipitated ACE, the cell extracts and the culture media were also assayed for ACE enzyme activity. Both methods of estimating secretion indicated (Table III) that co-expression of TACE with ACE in these cells increased ACE secretion to some extent (from 49–58% to 71%). Thus, although TACE is not required for ACE secretion, under certain circumstances ACE could be a substrate for TACE. Indeed, TACE was able to cleave a synthetic peptide spanning the cleavage site of ACEp when used in large excess (1:100 relative to ACE; data not shown). It should be noted that the rate of PMA-stimulated ACEp secretion in the experiments described in Table III was significantly higher than rates observed in the experiments shown in Fig. 1B and Table I (28% versus 49–58%). The reason for this discrepancy is not yet clear. One possible explanation is that a cytomegalovirus promoter-driven system (Fig. 2B and Table III) instead of a vaccinia virus T7 polymerase-based system (Fig. 1, A and B, and Table I) was used for the cotransfection experiment, leading to different levels of protein expression, which in turn might affect secretion.

Solubilization of ACE-secretase Activity.—The next series of experiments were designed to solubilize ACE-secretase activity from membranes, the first step toward its purification. Previously, we have developed an in vitro cell-free assay system for the measurement of ACE-secretase activity in membranes. Two different methods were used by which uncleaved and cleaved ACEp (the substrate and the product, respectively, of the putative enzyme) were distinguished and quantitated. In the first method, the two [35S]methionine-labeled ACE proteins were quantitated by PhosphorImager analysis after separation by PAGE. In the second method, the two forms were physically separated and quantitated by ACE enzyme activity measurements. Here we utilized both these methods to assay ACE-secretase activity solubilized from membranes isolated from either ACE89 cells or rabbit lung by detergent extraction.

In ACE89 cells, the enzyme ACE-secretase cleaves cell-bound ACE T near its C terminus (between Arg663 and Ser664 to

| Source of ACEp | ACE activity |
|---------------|-------------|
|              | units/ml    |
| (+/+)        | 390 ± 51    |
| (−/−)        | 302 ± 16    |

TABLE III
Co-transfection of ACEp and TACE in TACE-deficient fibroblasts

TACE-deficient fibroblasts were co-transfected with ACEp cDNA and an irrelevant cDNA or co-transfected with ACEp and TACE cDNA. Cleavage-secretion was estimated by PhosphorImager analysis, as described in the legend of Fig. 1B. In parallel experiments where pulse-labeling was omitted, the cell extract and the culture media were assayed for ACE enzyme activity and cleavage-secretion was assessed (right panel), as described previously (7).

| Transfection | Addition | PhosphorImager analysis | Activity assay |
|--------------|----------|-------------------------|---------------|
| ACEp         | PMA      | 49 ± 4                  | 58 ± 3        |
| ACEp + TACE  | PMA      | 71 ± 4                  | 71 ± 8        |
generate the soluble, C-terminally truncated form of ACE. Cell-bound uncleaved ACE<sub>P</sub> and secreted, cleaved ACE<sub>T</sub> differ in molecular mass by 8 kDa. Being glycosylated, both forms appeared as broad diffuse bands and could not be resolved into separate bands by PAGE analysis after immunoprecipitation. However, when these proteins were completely deglycosylated by treatment with glycosidases after immunoprecipitation but before PAGE analysis, the cleaved and the uncleaved forms resolved clearly into two separate bands (5). We quantitated these bands by PhosphorImager analysis and thus measured the rate of cleavage and secretion. Membranes isolated from [35S]methionine-labeled ACE<sub>89</sub> cells, when analyzed by the deglycosylation–PAGE analysis method, showed that almost all the ACE proteins present in the membrane preparation were of the uncleaved, 84-kDa variety (5) (Fig. 3A, lane 1). Much of the membrane-bound ACE<sub>T</sub> was cleaved when the membranes were incubated at 37 °C (lane 2), indicating the presence of ACE-secretase activity. No such cleavage was observed if Compound 3 was present during incubation, confirming its authenticity (lane 3). To solubilize ACE-secretase, these labeled membranes were extracted with the nonionic detergent Triton X-100, and the detergent extract was assayed for ACE-secretase activity in a similar manner. Unlike incubation containing membranes (lane 2), generation of cleaved ACE was not observed when detergent extracts of membranes were incubated at 37 ºC (lane 4). This indicated that ACE-secretase either was not extracted from the membrane or was rendered inactive during the extraction procedure. A third possibility is that the interaction of ACE-secretase and ACE is hampered in the detergent-solubilized state. Indeed, it has been shown that, although solubilized TACE cleaves various peptide substrates in solution, it failed to cleave any of its putative full-length protein substrates (other than TNF-α) when both the substrate and TACE were in solution (17). This is consistent with the evidence that shedding generally requires the anchoring of the secretase and its target substrate (or both) in the plasma membrane. Thus, we immobilized soluble ACE on agarose beads by the lectin ConA or lisinopril, the competitive inhibitor of ACE, by including ConA-agarose or lisinopril-Sepharose in the incubation. Under both conditions, much of the uncleaved ACE present in the detergent extract was converted to the cleaved form (lanes 5 and 6). Again, the presence of Compound 3 completely inhibited this conversion (lane 7, data for lisinopril-Sepharose and Compound 3 not included). These results indicate that ACE-secretase, similar to TACE, is unable to cleave its substrate in solution. However, if either the substrate or the secretase (or both) was anchored to a solid surface, such as agarose beads, efficient cleavage was observed. This cleavage was blocked in the presence of Compound 3, confirming its authenticity. Incidentally, much more cleaved ACE was generated when lisinopril-Sepharose rather than ConA-Sepharose was used (compare lanes 6 and 5). In both situations, however, as determined in separate experiments, all of the ACE present in the detergent extract was bound to agarose beads (data not shown). The reason for this difference is not known. In fact, different domains of the ACE molecule are involved in binding to lisinopril or to ConA, which in turn might alter the accessibility of ACE-secretase, explaining the difference in efficiency of cleavage.

To examine the cleavage and secretion of ACE<sub>P</sub> by solubilized ACE-secretase, membranes were prepared from rabbit lung, an organ rich in vascular tissue and thus in ACE<sub>P</sub>. Detergent extracts of rabbit lung membranes were prepared and incubated, as described above, to measure the ACE-secretase activity. Cleaved and uncleaved ACE<sub>P</sub> proteins were physically separated by temperature-induced phase separation using Triton X-114. This method takes advantage of the fact that uncleaved ACE is hydrophobic in nature, whereas cleaved ACE, having lost its transmembrane domain, is hydrophilic. The separated ACE<sub>P</sub> proteins were quantitated by ACE activity measurements. Assayed by this method (Table IV), 55% of ACE<sub>P</sub> in rabbit lung membrane was converted to the cleaved

![Fig. 3. Solubilization of ACE-secretase activity from ACE<sub>89</sub> cell membranes. A, [35S]methionine-labeled ACE<sub>89</sub> cell membranes (lanes 1–3) or Triton X-100 extract of the labeled membranes (lanes 4–7) were incubated at 37 °C with added ConA-Sepharose (lanes 5 and 7), lisinopril-Sepharose (lane 6), and/or Compound 3 (lanes 3 and 7, 50 μM). ACE-related proteins were immunoprecipitated, deglycosylated, and analyzed on SDS-PAGE. The molecular mass of the deglycosylated uncleaved ACE<sub>T</sub> is shown on the left. B, immunological characterization of the product generated by solubilized ACE-secretase. Detergent extracts of [35S]methionine-labeled ACE<sub>89</sub> cell membranes were incubated at 37 °C in the presence of lisinopril-Sepharose, followed by immunoprecipitation, deglycosylation, SDS-PAGE, and transfer to nitrocellulose membranes. Lane 1 shows a autoradiogram of the blot. Lanes 2 and 3 show results of Western analysis performed by using an anti-ACE antibody (lane 2) or an anti-C-terminal peptide antibody (lane 3).

### Table IV

ACE-secretase activity in detergent extract of rabbit lung membranes

| Membrane        | Detergent Extract | Lisinopril-Sepharose | Cleaved ACE generated (%) |
|-----------------|-------------------|----------------------|---------------------------|
| 4 °C            | 37 °C             | 4 °C                 | 37 °C                     |
| 8 ± 0.3         | 55 ± 1            | 9 ± 1                | 17 ± 4                    |

| Membrane        | Detergent Extract | Lisinopril-Sepharose | Cleaved ACE generated (%) |
|-----------------|-------------------|----------------------|---------------------------|
| 4 °C            | 37 °C             | 4 °C                 | 37 °C                     |
| 8 ± 3           | 80 ± 3            |                      |                           |
form when incubated at 37 °C. Very little cleaved ACE was generated (8%) at 4 °C. When the detergent extracts of these membranes were incubated in a similar fashion at 37 °C, only 17% of \( \text{ACE}_p \) was cleaved, but inclusion of lisinopril-Sepharose in the incubation increased conversion to the cleaved form to 80%. Incidentally, significant conversion was observed even at 4 °C in the presence of lisinopril.

**Immunological Characterization of the Product Generated by Solubilized ACE-secretase**—To characterize the product generated by the solubilized ACE-secretase, we took advantage of the two different antibodies we used in experiments described in Fig. 1A. Detergent extract of membranes prepared from metabolically labeled ACE89 cells were incubated at 37 °C for 1 h in the presence of lisinopril-Sepharose. The ACE proteins were immunoprecipitated, deglycosylated, and analyzed by SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane and autoradiographed to detect the labeled uncleaved and cleaved \( \text{ACE}_p \) proteins. The same blot was subject to Western analysis using the two antibodies described above. The autoradiogram (Fig. 3B, lane 1) showed that approximately 40% of the initial uncleaved \( \text{ACE}_p \) had been converted to the cleaved form, both of which reacted with the anti-ACE antibody in the Western analysis (lane 2). In contrast, the anti-C-terminal peptide antibody recognized only the upper band, i.e. the uncleaved \( \text{ACE}_p \), but not the lower band, the cleaved \( \text{ACE}_p \) (lane 3), indicating that the C-terminal tail is missing from the cleaved form. Thus, similar to the \( \text{ACE}_T \) secreted by ACE89 cells in culture or the cleaved \( \text{ACE}_T \) generated in the \textit{in vitro} assay system using ACE89 membranes (5), the \( \text{ACE}_p \) generated by the solubilized ACE-secretase is also C-terminally truncated.

The results presented above, demonstrating the solubilization of ACE-secretase, constitute a major technical advancement toward identification of this enzyme. The observed need for anchoring of the substrate protein on a solid support for the cleavage to occur displays a unique property of the catalysis process. The property of the solubilized system mimics the physiological reaction between the enzyme and the substrate, both of which are presumably anchored in the plasma membrane. This matrix-reconstituted soluble system will be used in the future to purify the ACE-secretase.

From our previous studies and the information in the literature, there were strong indications that there are important differences in the properties of different secretases. Cleavage sites are often different. Moreover, for ACE-secretase, the distal extracellular domain of ACE was shown to be the important determinant that governs cleavage and secretion; a protein containing the distal extracellular domain of ACE and the juxtamembrane, transmembrane, and cytoplasmic domains of CD4 (another type 1 ectoprotein that does not undergo cleavage and secretion) was efficiently cleaved from the cell surface. The reciprocal protein, which contained the extracellular domain of CD4 attached to a membrane-anchored ACE protein containing the authentic cleavage site, was not at all cleaved (7). In contrast to these results, for TGF-\( \alpha \) precursor and \( \beta \)-APP, the determinants are the juxtamembrane domains. The structurally unrelated juxtamembrane domains of the two proteins can mediate cleavage and secretion of an uncleaved protein when substituted for the corresponding domain of that protein (23). These important differences between ACE-secretase and the secretase(s) that cleave TGF-\( \alpha \) precursor or \( \beta \)-APP, on the other hand, implicate involvement of multiple proteases. The current study establishes in a definitive way that ACE-secretase is distinct from TACE. Future isolation and cloning of ACE-secretase will reveal whether the two enzymes bear a structural resemblance. Similarly, further studies will be required to identify the secretases that catalyze the cleavage and secretion of other membrane-bound proteins. One can speculate that many related but distinct proteins belong to this family of enzymes that carry out similar functions in cells.

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