Cleavage Processing of Angiotensin-Converting Enzyme by a Membrane-Associated Metalloprotease†

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ABSTRACT: Angiotensin-converting enzyme (ACE) is synthesized as a type 1 ectoprotein. It is released from the cell surface by a proteolytic cleavage−secretion process which is enhanced by treatment of the cells with phorbol esters. Here, we report the development of an *in vitro* cell-free assay system for the cleavage−secretion, its characterization, and the identification of a potent inhibitor of this process. Membranes prepared from ACE89 cells secreted the testicular isozyme of ACE (ACE_T) in a temperature- and time-dependent fashion. As expected, the *in vitro* secreted ACE_T lacked the membrane-anchoring carboxy-terminal tail of the cell-associated ACE_T. The *in vitro* secretase activity was resistant to high salt extraction and to inhibitors of serine, chymotrypsin, trypsin, cysteine, aspartate, and elastase type proteases. However, the activity was sensitive to metal ion chelators and to a synthetic hydroxamic acid derivative, compound 3, a known inhibitor of certain metalloproteases. Compound 3 very efficiently blocked both basal and phorbol ester-stimulated ACE_T secretion by ACE89 cells. The inhibition was rapid, dose-dependent, and reversible, and ACE_T synthesis, glycosylation, and transport were not affected. Cleavage−secretion of ACE_T in transiently transfected HeLa cells was also inhibited by compound 3. Finally, *in vitro* cleavage−secretion of the other isozyme of ACE, ACE_P, by membranes isolated from rabbit lungs was strongly inhibited by compound 3. These results indicate that the cleavage−secretion of both isozymes of ACE is carried out by an integral membrane metalloprotease which is specifically inhibited by compound 3.

Angiotensin-converting enzyme (ACE,[1] EC 3.4.15.1) is a Zn-containing peptidyl dipeptidase that participates in the regulation of blood pressure by converting the inactive decapeptide angiotensin I into the potent vasopressor octapeptide angiotensin II. It also inactivates bradykinin, a potent vasodilator peptide (Erdös & Yang, 1967; Cushman & Ondetti, 1980; Skeggs et al., 1956). There are two isozymes of ACE derived from a single gene, by tissue-specific choice of the alternative transcription initiation sites (Kumar et al., 1991; Howard et al., 1990; Hubert et al., 1991). The larger isozyme, ACE_P, is a 140−170 kDa glycoprotein composed of a single polypeptide chain containing two large homologous domains (Wei et al., 1991a; Thekkumkara et al., 1992). Each domain bears an active catalytic site. The smaller isozyme ACE_T, on the other hand, is a 100−110 kDa glycoprotein containing only the active C-terminal domain of ACE_P (Thekkumkara et al., 1992; El-Dorry et al., 1982). Thus, rabbit ACE_P has 1309 amino acid residues, and rabbit ACE_T has 737. The C-terminal 665 residues of the two rabbit isozymes are identical, whereas the N-terminals are unique. In all species, both isozymes contain a hydrophobic transmembrane domain of 17 amino acids near their C-termini. There is ample experimental evidence to indicate that, like a typical type 1 ectoprotein, ACE remains anchored in the cellular plasma membrane through this hydrophobic domain, the putative cytoplasmic tail has 30 residues, and the rest of the protein is extracellular (Sen et al., 1991; Wei et al., 1991b; Ehlers et al., 1991a). Since the C-terminal residues of ACE_T and ACE_P are identical, the proteins are expected to be similarly oriented on the cell surface and be released from it by the same mechanism.

Although ACE is primarily a cell-associated protein, soluble form of ACE_P exists in plasma and other body fluids (Hooper, 1991). The source of circulating soluble ACE_P in plasma has not been established unequivocally, but it is thought to be derived from vascular endothelial cell-associated ACE_P. The mechanism controlling the conversion of the membrane-anchored ACE to soluble form are unknown, and the processing enzyme(s) responsible for this conversion has not been identified. The results of the current study provide new information about this aspect of ACE processing.

To facilitate studies on ACE biosynthesis, we and others have established permanently transfected cell lines which can produce large quantities of ACE in culture (Sen et al., 1991; Wei et al., 1991b; Ehlers et al., 1991a). ACE89, a mouse epithelial cell line permanently transfected with a rabbit ACE_T expression vector, synthesizes, glycosylates and secretes enzymatically active ACE_T (Sen et al., 1991). A large portion of the membrane-anchored ACE, as predicted from its structure, is extracellular, and it can be cleaved off these cells by treatment with exogenous proteolytic enzyme (Ramchandran et al., 1994). Since the transfected cell system produces both cell-bound and secreted forms of ACE_T, it has been used to understand the process of secretion. By

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[1] Abbreviations: ACE, angiotensin-converting enzyme; ACE_T, testicular angiotensin-converting enzyme; ACE_P, pulmonary angiotensin-converting enzyme; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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doing pulse−chase experiments, we have shown that secretion of natural soluble ACE_T is a slow process. However, when the putative anchoring domain is deleted, the modified ACE_T is secreted much faster confirming the function of the anchoring domain. The secreted form of native ACE_T is slightly smaller than the cell-bound form suggesting that the process of secretion may involve proteolytic removal of the C-terminus, including the anchoring domain (Sen et al., 1991). Indeed, we have shown that an antibody raised against a C-terminal peptide of ACE_T reacts with the cell-bound form but not with the secreted form of ACE_T (Sen et al., 1993). Wei et al. (1991b) have shown that a similar antibody did not react with cell-bound ACE unless the cells were permeabilized, whereas a polyclonal antibody against ACE_P reacted with the ACE on the cell surface, thus confirming the topology of cellular ACE.

The cleavage−secretion of ACE_T in ACE89 cells is slow, but the process can be enhanced by treatment of cells with tumor-promoting phorbol esters. The cleavage-processing occurs only after the protein has reached the cell surface and is not affected by disruption of the Golgi apparatus or the lysosomal compartments. The exact peptide bond cleaved has been identified by sequencing the amino-terminal residues of the purified C-terminal tail left in the cells after ACE is secreted, and the C-terminal residues of secreted ACE. The cleavage occurs at a monobasic site between Arg-663 and Ser-664 generating the soluble enzyme and leaving a cell-bound protein of 74 residues. These results demonstrate the existence of cellular mechanisms that regulate the conversion of cell-bound ACE to a soluble enzyme (Ramchandran et al., 1994).

In this paper we report development of a cell-free assay system for the conversion of cell-bound ACE to the secreted form. We have also determined that this "ACE-secretase" activity has the characteristics of a metalloprotease and identified a potent inhibitor of this putative proteolytic activity. In addition to inhibiting ACE_T secretion from ACE89 cells, the inhibitor also effectively stopped secretion of naturally occurring ACE_P from rabbit lung membranes.

EXPERIMENTAL PROCEDURES

Materials. Lisinopril [N^{α}-[1-(*S*)-Carboxy-3-phenylpropyl]-L-lysyl-L-proline] was provided by Merck Sharp and Dohme Research Laboratories. Compound 3 [*N*-[D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl]-L-3-(*tert*-butyl)alanyl-L-alanine, 2-aminoethyl amide) was provided by Roy A. Black, Immunex Research and Development Corp. This is identical to compound 2 (Mohler et al., 1994) with the exception that napthylalanine side chain is replaced by a *tert* butyl group.

Preparation of Membrane Fractions. ACE89 cells (Sen et al., 1991) were scraped and suspended in 20 mM Hepes buffer, pH 7.0, containing 0.2 M sucrose (1 mL/100 mm plate). The cell suspension was homogenized in a polytron for 30 s with intermittent cooling and centrifuged at 700*g* for 10 min. The pellet was discarded, and the supernatant was centrifuged again at 36000*g* for 40 min. The sedimented membranes were washed twice with the same buffer by centrifugation and suspended in 0.1 M borate buffer, pH 8.3 (0.4 mL/plate), using a glass-glass homogenizer and used as the membrane preparation. For the preparation of labeled membranes, a similar procedure was followed, except that the cells were pulse-labeled with [^{35}S]methionine for 0.5 h (Sen et al., 1991) and the label was chased in unlabeled medium for 3 h before cells were harvested.

For the preparation of lung membranes freshly isolated rabbit lung was homogenized as described above in 50 mM Hepes buffer, pH 7.0, containing 0.33 M sucrose (8 mL/g). The homogenate was centrifuged at 8000*g* for 15 min, and the resulting supernatant was centrifuged again at 36000*g* for 1.5 h. The pellet was washed twice and suspended in 0.1 M borate buffer, pH 8.3 (0.5 mL/g), and used as lung membrane preparation. ACE enzyme activity was measured as described before (Sen et al., 1993).

Purification of [^{35}S]Methionine-Labeled Uncleaved and Cleaved ACE_T. For the purification of labeled uncleaved ACE_T, confluent plates of ACE89 cells were labeled overnight with [^{35}S]methionine, extracted with 0.1% Triton X-100 in 50 mM Tris-HCl, pH 7.5, and centrifuged at 50000*g* for 1 h. The supernatant containing the solubilized uncleaved ACE_T was purified using a lisinopril affinity column (Ehlers et al., 1991b). The ACE_T was subjected to phase separation with Triton X-114 (described below), and the detergent phase containing only the uncleaved ACE_T was repurified by lisinopril column chromatography. The purified ACE_T was exclusively of the uncleaved variety as determined by its molecular weight. As we have shown previously (Sen et al., 1991), the culture medium of ACE89 cells contains virtually pure secreted ACE_T; such medium from labeled ACE89 cells was used without further purification as the source of labeled secreted ACE_T.

Assays for ACE-Secretase Activity. For the detergent-extraction assay, 80−100 μL of a membrane fraction was incubated for an indicated time at 37 or 4 °C. The incubation mixture was transferred to ice, and an equal volume of cold 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2% (v/v) Triton X-114 was added to it and incubated on ice for an additional 5 min. The samples were then transferred to 30 °C for 3 min, when the mixture separates into aqueous and detergent phase. The samples were centrifuged at 3000*g* for 5 min, and the resulting aqueous and the detergent phases were separated and diluted appropriately before ACE enzyme activity assay.

For the deglycosylation-PAGE assay, labeled membranes (80−100 μL) were preincubated without or with various inhibitors or agents, for 15 min on ice in a final volume of 100 μL and transferred to 37 °C. After 0.5 h the reaction was stopped by the addition of ice-cold RIPA buffer (Sen et al., 1991), and ACE-related proteins were immunoprecipitated, boiled with SDS, and deglycosylated by sequential treatment with 400 milliunits of N-glycosidase F, 10 milliunits of neuraminidase, and 1 milliunit of O-glycosidase (Kasturi et al., 1994), before being analyzed by PAGE.

Cell Labeling and Immunoprecipitation. ACE89 cells were pulse-labeled with [^{35}S]methionine for 0.5 h, and the label was chased for the indicated periods. ACE-related proteins were immunoprecipitated from the media and cell extracts and analyzed by PAGE as described earlier (Sen et al., 1991, 1993). Transient expression of ACE_T protein in HeLa cells and pulse−chase analysis in this system have also been described (Sen et al., 1993; Kasturi et al., 1994).

Western Analysis. For the immunological characterization of deglycosylated uncleaved and cleaved ACE_T, two different antipeptide antibodies were used. One was generated against 14 C-terminal amino acid residues of uncleaved ACE_T (Sen

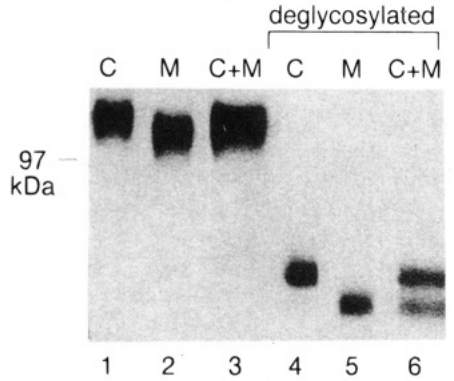

FIGURE 1: Deglycosylation of ^{35}S-labeled cell extract and media from ACE89 cells. Labeled cell extract (lane 1) or medium (lane 2) or a mixture of both (lane 3) were immunoprecipitated and analyzed by PAGE. For deglycosylation, immunoprecipitates were boiled with SDS and sequentially treated with N-glycosidase F, neuraminidase, and O-glycosidase, dried, and analyzed (lanes 4–6). C, cell extract; and M, media. The position of the molecular weight marker is shown on the left.

et al., 1993) and the other generated against 16 residues toward the N-terminus of rabbit ACE_T (TTHQTTAHQTTQSPNG). Immunostaining of the blots were performed by a peroxidase procedure (Vectastain ABC Kit, Vector Laboratories) as described earlier (Sen et al., 1991) using a 1:500 dilution of the two antipeptide antibodies.

RESULTS

Development of Assays for ACE-Secretase Activity. In ACE89 cells the enzyme ACE-secretase cleaves cell-bound ACE_T near its C-terminal (between Arg-663 and Ser-664) to generate the soluble C-terminally truncated ACE. To characterize the ACE-secretase activity, it was necessary to develop a rapid, quantitative, and reproducible assay system. Such a system has to distinguish between the uncleaved cell-bound ACE_T and the cleaved secreted ACE_T, the substrate and the product, respectively, of the putative enzyme. Cell-bound uncleaved ACE_T and secreted cleaved ACE_T differ in molecular mass by 8 kDa. Being glycosylated, both forms appeared as broad diffuse bands (Figure 1, lanes 1 and 2). Mixed together, they appeared as an even broader band and could not be resolved into separate bands (lane 3). Thus, straight PAGE analysis of the proteins was not suitable for quantitating the activity of our interest. On the contrary, when these proteins were completely deglycosylated by treatment with glycosidases after immunoprecipitation but before PAGE analysis, the cleaved and the uncleaved forms were resolved clearly into two separate bands (lane 6). It was possible to quantitate these bands by phosphorimager analysis and thus to measure the rate of cleavage–secretion.

Although the above procedure can measure the substrate and the product ACE proteins quantitatively, it is cumbersome and expensive due to the need of the deglycosylation step. An alternative quicker assay was developed following the procedure of Bordier (1981), modified by Hooper (Hooper et al.,1987; Oppong & Hooper, 1993). This method takes advantage of the fact that uncleaved ACE is hydrophobic in nature, whereas cleaved ACE, having lost its transmembrane domain, is hydrophilic. Thus, the ACE-related proteins, in this procedure, are physically separated by partitioning them between an aqueous phase and a detergent-rich phase using Triton X-114. Since both forms

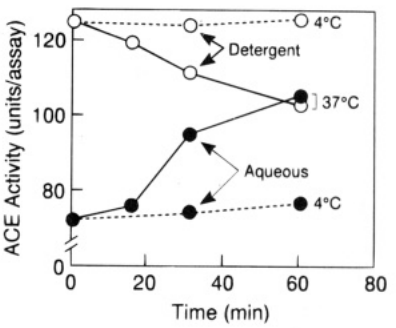

FIGURE 2: ACE-secretase activity in ACE89 cell membranes measured by the detergent extraction procedure. Aliquots (100 μL) of membrane preparations (see Experimental Procedures) were incubated at 37 or 4 °C for the indicated time and subjected to phase separation, and ACE_T proteins in aqueous and the detergent phase were quantitated by enzyme activity measurements. (—●— and - - -●- - -) Aqueous phase at 37 and 4 °C, respectively. (—○— and - - -○- - -) Detergent phase at 37 and 4 °C, respectively.

of ACE have enzyme activity, subsequently the amount of ACE protein present in each phase could be quantified by ACE activity measurements. For standardizing this procedure, radiolabeled ACE_T proteins were produced by metabolically labeling ACE89 cells with [^{35}S]methionine, and ACE_T present in the medium was used as pure labeled secreted ACE_T (Sen et al., 1991). Uncleaved cell-bound ACE_T was purified from the cells by detergent extraction followed by lisinopril affinity chromatography (see Experimental Procedures). The two labeled ACE_T proteins, the substrate and the product, were then separately subjected to the detergent extraction assay. The hydrophilic secreted ACE_T partitioned to the aqueous phase almost exclusively (more than 98%), whereas the majority (more than 66%) of the uncleaved hydrophobic ACE was present in the detergent phase. A considerable (about 34%) amount of uncleaved ACE_T, however, partitioned to the aqueous phase. This was considered to be the background value and subtracted from the observed experimental readings of aqueous phase ACE activities.

Thus, two different methods have been developed by which uncleaved and cleaved ACE_T can be distinguished and quantitated. In the first method, the two ACE_T 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.

ACE-Secretase Activity in ACE89 Cell Membranes. We applied these techniques of separating the substrate and the product for developing cell-free *in vitro* assay systems to measure ACE-secretase activity. We have shown previously that ACE89 cells express ACE_T on the cell surface and secrete the active enzyme very efficiently into the culture medium after cleavage processing of its C-terminal tail (Ramchandran et al., 1994). Hence, for the purpose of assaying ACE-secretase activity, membranes were prepared from ACE89 cells and incubated at 37 °C for various lengths of time. Cleaved and uncleaved ACE_T molecules were partitioned into aqueous and detergent phase, and each form was quantitated by measuring enzyme activity. Before incubation, 66% of the activity partitioned to the detergent phase, whereas less than 34% partitioned to the aqueous phase (Figure 2, 0 h). Given the limitations of the assay, this probably indicates that all ACE proteins present in the

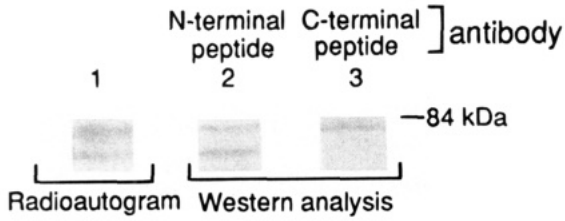

FIGURE 3: Immunological characterization of the product generated by *in vitro* ACE-secretase activity. [^{35}S]Methionine-labeled ACE89 cell membranes were incubated at 37 °C for 0.5 h, immunoprecipitated, deglycosylated, analyzed by PAGE, and transferred to nitrocellulose membranes. Lane 1 shows a radioautogram of the blot. Lanes 2 and 3 are Western analysis performed by using an anti N-terminal peptide antibody (lane 2) or an anti C-terminal peptide antibody (lane 3).

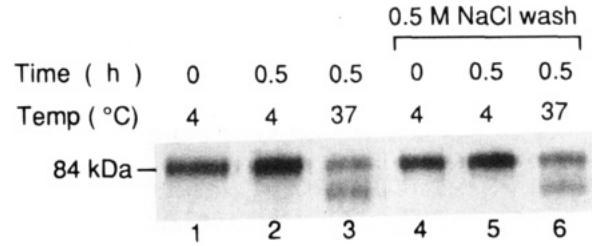

FIGURE 4: ACE-secretase is an integral membrane protein. [^{35}S]-Methionine labeled ACE89 cell membranes were isolated and washed twice with the homogenization buffer (lanes 1−3) or the same buffer containing 0.5 M NaCl (lanes 4−6). Aliquots (100 μL) of both membrane preparations were incubated for 0.5 h (lanes 2, 3, 5, and 6) or 0 h (lanes 1 and 4) at 37 °C (lanes 3 and 6) or at 4 °C (lanes 1, 2, 4, and 5). ACE-related proteins were immunoprecipitated, deglycosylated, and analyzed on PAGE. The molecular weight of the deglycosylated uncleaved ACE_T is shown on the left.

preparation were of the uncleaved variety. With increasing lengths of incubation at 37 °C, but not at 4 °C, increasing amounts of ACE_T activity partitioned to the aqueous phase with a concomitant loss from the detergent phase. After incubation for 60 min, almost equal amounts of activity were recovered from both the phases. These experiments illustrate that the ACE-secretase activity can function *in vitro* in a membrane preparation and that the extent of ACE cleavage can be measured rapidly and quantitatively by our methods.

Characterization of the in Vitro Activity. To characterize the product generated in the cell-free assay system, two different antibodies, one generated against a peptide representing 16 amino acid residues near the N-terminal of ACE_T and the other generated against a peptide representing the 14 C-terminal amino acid of the uncleaved ACE_T, were used. The anti N-terminal peptide antibody recognizes both the uncleaved and the cleaved ACE_T (Sen et al., unpublished observation), whereas the anti C-terminal peptide antibody recognizes only the uncleaved, but not the cleaved, form of ACE_T (Sen et al., 1993). Membranes prepared from [^{35}S]-methionine-labeled ACE89 cells were incubated at 37 °C for 0.5 h, and the ACE proteins were immunoprecipitated, deglycosylated, and analyzed by PAGE. The resolved proteins were transferred to a nitrocellulose membrane and radioautographed to detect the labeled uncleaved and cleaved ACE_T proteins. The same blot was subjected to Western analysis using the two peptide antibodies described above. The radioautogram (Figure 3, lane 1) showed that approximately 50% of the uncleaved ACE_T had been converted to the cleaved form, both of which reacted with the anti N-terminal peptide antibody in the Western analysis (lane 2). On the contrary, the anti C-terminal peptide antibody recognized only the upper band, i.e., the uncleaved ACE_T, but not the lower band which represents the cleaved ACE_T (lane 3) indicating that the C-terminal tail of the uncleaved ACE_T is missing from the cleaved form. Thus, similar to the ACE_T secreted by ACE89 cells in culture, the cleaved ACE_T generated in the *in vitro* assay system is also C-terminally truncated.

In the next experiment we used the *in vitro* assay system to determine if the ACE-secretase enzyme is an integral membrane protein. Uncleaved ACE_T is an integral membrane protein and thus is not released from the membranes by washing with buffers containing a high concentration of NaCl. If ACE-secretase is also an integral membrane protein, it should also be retained in the membrane while peripheral membrane proteins will be removed by such washes. ^{35}S-Labeled ACE89 cell membranes were washed extensively with 0.5 M NaCl, and ACE-secretase activity in the washed membrane was compared with that in the unwashed membrane using the deglycosylation−PAGE analysis method (Figure 4). With unwashed membranes, no cleaved ACE_T was generated upon incubation at 4 °C for 0.5 h (lane 2). Much of the membrane-bound ACE_T was, however, cleaved when the membranes were incubated at 37 °C for the same length of time (lane 3). No such cleavage was observed if the membranes were heated to 100 °C for 1 min before the incubation (data not shown), thus suggesting that the cleavage process is enzymatic. The characteristics of the 0.5 M NaCl-washed membranes, were the same as the unwashed membranes (lane 4−6). The secretase activity was still retained in the membranes, thus suggesting that the responsible enzyme, like its substrate, is an integral membrane protein.

Effect of Protease Inhibitors on ACE-Secretase Activity. Since the nature of ACE-secretase is unknown, we studied the effects on ACE-secretase activity of inhibitors of various known classes of proteases. ^{35}S-Labeled membranes isolated from ACE89 cells were preincubated for 15 min on ice, with various protease inhibitors at the concentration they are known to inhibit the cognate cell-bound protease activities. The treated membranes were then incubated at 37 °C for 0.5 h. Figure 5a shows that inhibitors of serine (PMSF, 3:4 DCI), trypsin (STI, TLCK, leupeptin), chymotrypsin (TPCK), cysteine (E-64), aspartate (pepstatin), or elastase (elastatinal, $\beta_1\beta Me_2$-6-Br) type proteases did not block the cleavage of ACE_T. The only effective inhibitors were EDTA, EGTA, and 1,10-phenanthroline, agents which chelate cations associated with metalloenzymes (Figure 5b). 1,7-Phenanthroline, an inactive analog, did not affect cleavage of ACE_T indicating that the effect is specific. A panel of more specific metalloprotease inhibitors were tested next. Several compounds such as phosphoramidon and captopril or lisinopril (data not shown), which inhibit other well characterized metalloproteases, did not inhibit cleavage of ACE_T. But a hydroxamic acid-based inhibitor of certain selective metalloproteases, compound 3, very effectively blocked the cleavage-processing activity (Figure 5b). Another commercially available hydroxamate, ZINCOV (Calbiochem), inhibited cleavage−secretion significantly but to a lesser extent than compound 3 (data not shown). α_2-Macroglobulin, a general inhibitor of several endoproteases (Barrett & Starkey, 1973), also inhibited the process, as did the reducing agents DTT and 2-mercaptoethanol (Figure 5c).

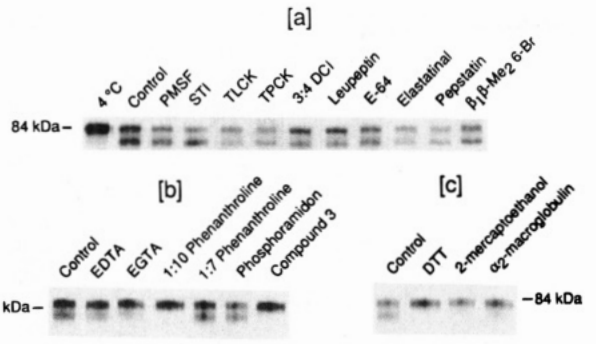

FIGURE 5: Effect of protease inhibitors on ACE-secretase activity. [^{35}S]Methionine-labeled ACE89 membranes (80 μL aliquots) were preincubated on ice for 15 min without (4 °C and control) or with various inhibitors in a final volume of 100 μL and kept at 4 °C (a, first lane) or transferred to 37 °C. After 0.5 h ACE-related proteins were analyzed as described in legend of Figure 4. Final concentrations of the agents added were as follows: (a) phenylmethanesulfonyl fluoride (PMSF), 0.5 mM; soybean trypsin inhibitor (STI), 100 μg/mL; 1-chloro-3-tosylamide-7-amino-2-heptanone (TLCK), 100 μM; L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), 50 μM; 3,4-dichloroisocoumarin (3,4-DCI), 40 μg/ml; leupeptin, 1 μM; L-*trans*epoxysuccinyl-leucylamide(4-guanidino)butane (E-64), 1 mg/mL; elastatinal, 100 μM; pepstatin, 10 μM; and $\beta_1\beta$-$Me_2$6-Br, 500 μM. (b) EDTA, 10 mM; EGTA, 10 mM; 1,10-phenanthroline, 5 mM; 1,7-phenanthroline, 5 mM; phosphoramidon, 50 μM; compound 3, 50 μM. (c) DTT, 1 mM; 2-mercaptoethanol, 1 mM; α_2-macroglobulin 1 unit.

Effects of Compound 3 on ACE$_T$ Secretion in ACE89 Cells. Among the several inhibitors identified by the *in vitro* assay, we chose to test the effects of compound 3 *in vivo* on ACE$_T$ secretion by ACE89 cells. In the first experiment, ACE89 cells were pulse-labeled with [^{35}S]methionine for 0.5 h followed by 1.5 h of chase with unlabeled medium. We have previously shown that this protocol allows the newly synthesized labeled ACE$_T$ protein to be fully glycosylated and transported to the plasma membrane, thus creating a labeled substrate analogous to the *in vitro* membrane preparations we used before. These cells were further incubated in the absence (Figure 6a, lanes 1 and 2) and the presence (lanes 3 and 4) of 50 μM compound 3. ACE$_T$ cleavage−secretion was completely blocked by compound 3. When compound 3 was added during the entire chase period, it blocked the cleavage−secretion but not the glycosylation and transport of the ACE$_T$ protein (lanes 5−8). Synthesis of the ACE polypeptide backbone and its glycosylation were also not affected by compound 3 when it was added with the label (lanes 9−12). These results demonstrated that compound 3 does not affect the synthesis, glycosylation, and intracellular transport of ACE$_T$, but it specifically inhibits the terminal step of cleavage−secretion. As a result, more labeled ACE$_T$ remained cell-associated in compound 3-treated cells (lanes 3 and 7). Compound 3 was effective in blocking ACE$_T$ release from ACE89 cells at a concentration as low as 100 nM (Figure 6b). Complete inhibition was observed between 20 and 50 μM concentration (data not shown).

We have shown previously (Ramchandran et al., 1994) that ACE$_T$ cleavage−secretion can be enhanced by a treatment of ACE89 cells with phorbol-12-myristate 13-acetate (PMA). In the experiment shown in Figure 6c, we examined the effects of compound 3 on PMA-induced ACE$_T$ secretion. ACE89 cells were pulse-labeled, and the label was chased for 3 h in the presence or the absence of compound 3. The media was changed, and the chase was continued for another 0.5 h in the presence or the absence of compound 3 and PMA. Analysis of the cells and the media revealed normal ACE$_T$ cleavage−secretion in untreated cells which was greatly enhanced by PMA treatment (Figure 6c, lanes 1−4). Compound 3 blocked both basal and PMA-induced cleavage−secretion, when present during the entire chase (lanes 5−8). However, when compound 3 was removed from the last 0.5 h of chase, PMA-induced cleavage-secretion was entirely unaffected (lanes 9 and 10). These results showed that the effect of compound 3 is quickly reversed once it is removed from the culture medium.

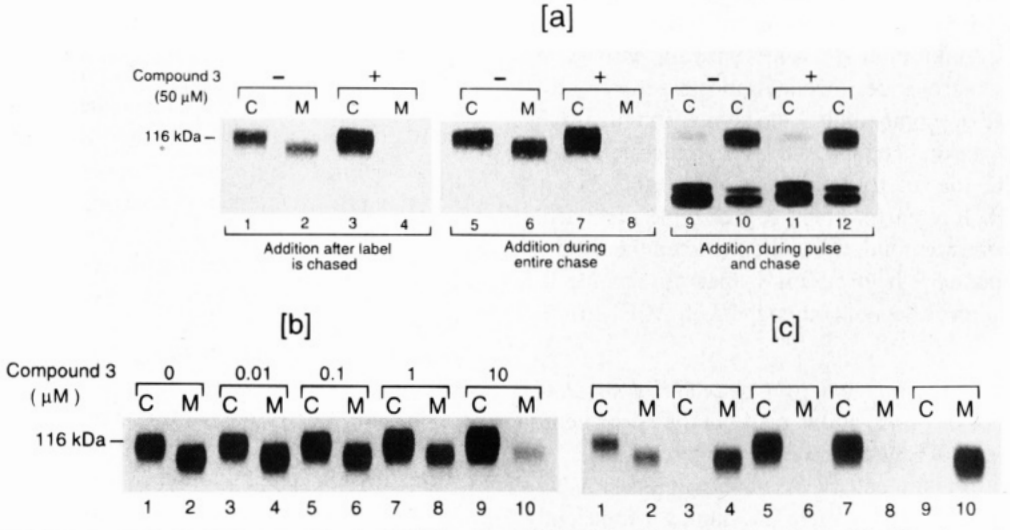

FIGURE 6: Effects of compound 3 on ACE$_T$ secretion in ACE89 cells. (a) ACE89 cells were pulse-labeled for 30 min and chased as follows with (+) or without (−) the addition of 50 μM compound 3: Lanes 1−4, chased for 1.5 h, in regular medium, medium was then changed, and fresh medium without (lanes 1 and 2) or with (lanes 3 and 4) compound 3 was added and the chase continued for an additional 1.5 h. Lanes 5−8, chased for 3 h without (lanes 5 and 6) or with (lanes 7 and 8) compound 3 for the entire chase period. Lanes 9−12, chased for 0 h (lanes 9 and 11) or 0.5 h (lanes 10 and 12); compound 3 was absent (lanes 9 and 10) or present (lanes 11 and 12) during the entire period of pulse and chase. (b) Cells were chased for 3 h in the absence (lanes 1 and 2) or the presence (lanes 3−10) of different concentrations of compound 3. (c) Cells were pulse-labeled and chased for 3 h in the absence (lanes 1−4) or the presence (lanes 5−10) of 50 μM compound 3. The media was changed, and chase was continued for another 0.5 h in the absence (lanes 1−4, 9, and 10) or presence (lanes 5−8) of compound 3 with added 500 μM PMA in lanes 3, 4, and 7−10.

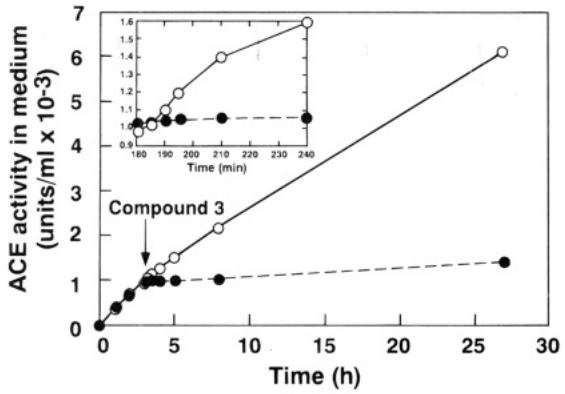

FIGURE 7: Kinetics of the onset of inhibition of secretion of ACE_T from ACE89 cells by compound 3. Two 100 mm plates of ACE89 cells (80% confluent, with 2 mL of culture media) were incubated at 37 °C. After 3 h, 50 μM compound 3 (−●−) or DMSO (−○−) was added to the plates and the incubation continued for another 23 h. At the indicated time, 3 μL aliquots were withdrawn from the medium, and ACE-enzyme activity was measured after diluting the samples 1000−5000 fold. The inset in an expanded scale shows the secretion of ACE_T between 3 and 4 h.

Once we established that the effects of compound 3 can be rapidly reversed upon its removal (Figure 6c), we wanted to measure the kinetics of onset of its effects upon addition to the culture medium. ACE89 cells continuously secrete ACE_T into the culture medium, and the amount of secreted ACE can be conveniently measured by assaying ACE enzyme activity present in a small aliquot of the culture medium. The kinetics of ACE_T secretion was almost linear over 26 h (Figure 7). When compound 3 was added at 3 h, the secretion of ACE_T was immediately inhibited. This inhibition was noticeable within 10 min of compound 3 addition (inset, Figure 7). The activity of the already secreted ACE_T remained constant over the time period of the experiment, thus demonstrating that the inhibitor does not affect the activity of the secreted protein. Since ACE is also a metalloprotease, we had to ensure that the observed inhibition of ACE enzyme activity in the medium was not due to a direct inhibition of ACE enzyme activity by compound 3. Control experiments established that, up to 1 μM concentration, compound 3 does not inhibit ACE_T enzyme activity in the medium, although higher concentrations (100 μM) do partly inhibit it (data not shown). Consequently, before performing ACE enzyme assays, the cell culture media was routinely diluted to keep the concentration of compound 3 below 50 nM, thus eliminating the possibility of a direct effect of compound 3 on ACE_T enzyme activity.

Inhibition of ACE Secretion by Compound 3 in Other Systems. The above observations made in the permanently transfected mouse ACE89 cells were extended to transiently transfected human HeLa cells. We have previously used a vaccinia virus−T7 RNA polymerase system for transiently expressing ACE_T in many cell lines, including HeLa cells (Sen et al., 1993). ACE_T is synthesized, glycosylated, and cleavage-secreted efficiently in these cells as demonstrated by a pulse−chase experiment (Figure 8, lanes 1 and 2). Inclusion of compound 3 during the chase period strongly inhibited the cleavage−secretion process (lanes 3 and 4). Consequently, more ACE_T remained cell-associated in the

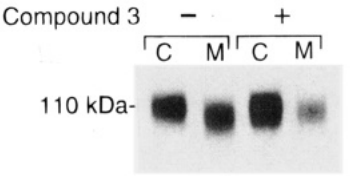

FIGURE 8: Inhibition of ACE secretion by compound 3 in HeLa cells. HeLa cells were transiently transfected with ACE_T cDNA by the vaccinia virus T7-RNA polymerase system. The cells were pulse-labeled and chased for 15 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 100 μM compound 3.

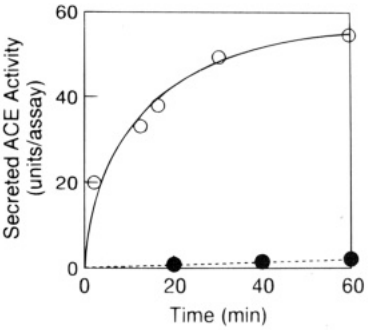

FIGURE 9: Effect of compound 3 on ACE_P secretion from rabbit lung membranes. Membranes prepared from rabbit lung (80 μL aliquots) were incubated at 37 °C in the absence (−○−) or the presence (−●−) of 100 μM compound 3 in a final volume of 100 μL. Uncleaved and cleaved ACE_P proteins were separated by detergent extraction method, and ACE_P enzyme activity was measured. The corrected value for secreted ACE_P was calculated by assuming that, in addition to the secreted ACE_P, 34% of cell-associated ACE_P also partitions into the aqueous phase. The latter value was subtracted from the total ACE_P activity determined in the aqueous phase.

treated cells (lanes 1 and 3). These results suggest that the effect of compound 3 is not cell specific. The inhibition of secretion was, however, not complete in HeLa cells. This could be due to several reasons. These cells might be less permeable to compound 3, or the human secretase may be less sensitive to compound 3 than the mouse secretase present in ACE89 cells. Moreover, the HeLa cells were infected with vaccinia virus, which might have an effect on the secretion process. Finally, it is possible that the secretion process in the HeLa cells is mediated by a combination of compound 3-sensitive and -insensitive enzymes.

In the next experiment, we examined the effects of compound 3 on the secretion of ACE_P, the other isozyme of ACE synthesized in vascular endothelial cells. For this purpose, rabbit lung membranes were prepared and assayed for ACE_P-secretase activity *in vitro*. Cleaved and uncleaved ACE_P proteins were separated by the detergent extraction procedure, and the kinetics of ACE_P secretion were measured (Figure 9). The secretion process was highly efficient at 37 °C but not at 4 °C (data not shown). Addition of compound 3 completely inhibited the secretion process. The experiments demonstrated that the enzyme machinery responsible for ACE_P cleavage−secretion from endothelial cells is also highly sensitive to the inhibitory effects of compound 3.

DISCUSSION

Many membrane-anchored ectoproteins are proteolytically converted to soluble factors on the cell surface, by a regulated process (Ehlers & Riordan, 1991). Previous studies revealed that proteolytic cleavage of ACE_T on the surface of ACE89

cells is stimulated by pharmacologic agents such as PMA. Such stimulation has also been reported for protransforming growth factor α (Pandiella & Massague, 1991), colony-stimulating factor (Stein & Rettenmier, 1991), KL-1, KL-2 (Huang et al., 1992), P75 transforming growth factor α receptor (Porteu & Nathan, 1990), β-amyloid precursor protein (Buxbaum et al., 1990), and cell adhesion receptor Mel-14 (Kishimoto et al., 1989). However, the mechanism of the regulated cleavage of ACE and other membrane-anchored proteins to generate soluble forms is not well understood, and the responsible protease(s) and other cellular components involved in the regulated cleavage system have not been identified. In the present work, we have characterized a membrane-associated protease activity which converts membrane-anchored ACE to the soluble form.

We have developed two analytical methods for separating the substrate and the product of the secretase activity. If the proteins are metabolically labeled, cleaved and uncleaved ACE_T can be distinguished by regular SDS−PAGE analysis when they are already separated (Figure 1, lanes 1 and 2). However, because of the close similarity in their polypeptide lengths and the heavy glycosylation, this method is not suitable for clearly separating the substrate and the product when they were present in the same mixture (Figure 1, lane 3), a prerequisite for studying the enzyme reaction of our interest. When they were completely deglycosylated, the two proteins were resolved completely, thus providing us with a quantitative method for measuring the secretase activity. As documented in many experiments described here, this method was very useful for studying the characteristics of the secretase reaction. It was especially useful for kinetic studies which required pulse-labeling of the substrate (Figure 6) as well as for screening the effects of many potential inhibitors. It is, however, a laborious and expensive process due to the high cost of the glycosidases. Moreover, a labeled substrate is needed for the assay, since after deglycosylation, detection of the proteins by Western blotting is neither sensitive nor quantitative. Thus, this method may not be ideal for routine assays of the secretase for its future purification. In contrast, the detergent extraction method was rapid, inexpensive, and quantitative. It does not require a labeled substrate, because the ACE enzyme activity is retained in the physically separated substrate and product fractions. Thus, natural tissues and cells which are difficult to metabolically label can be easily analyzed by this method. Moreover, since immunoprecipitation is not involved, samples from different species of animals can be analyzed by this method without the need for the cognate ACE antibody. Thus, the detergent extraction method is very suitable for enzyme purification purposes. The only drawback of this method is that the uncleaved form of ACE does not completely segregate to the detergent phase thus creating a background noise in the aqueous phase. Introduction of appropriate correction factors in the calculations, however, can easily rectify this problem (Figure 9). A similar detergent extraction procedure has been used earlier to identify a metal chelator sensitive secretase activity, which releases ACE from membrane preparation (Oppong & Hooper, 1993). We have modified the detergent extraction method and complemented it with an *in vitro* assay utilizing radiolabeled substrate to convincingly identify and characterize the ACE-secretase activity as well as identify a potent inhibitor which blocks this activity.

The cell-free membrane system for ACE-secretase activity offers several advantages over a whole cell system. It allows us to study the secretase activity in isolation from ACE synthesis and processing. Thus, an observed inhibition by an added agent in the *in vitro* system can safely be interpreted as a direct inhibition of the secretase activity. Moreover, the enzyme system is more readily accessible to putative inhibitors in the *in vitro* system. The purified membrane fraction will also be the starting material for purification of the secretase. Our experiments with membranes isolated from rabbit lungs (Figure 9) demonstrated that natural tissues which are inexpensive and available in large quantities can potentially be used for purification of this enzyme. The specificity of the cleavage−secretion *in vitro* was indistinguishable from the *in vivo* cleavage as judged by the characteristics of the product and the inhibitor profiles of the two processes. Our *in vitro* system also demonstrated that both ACE_T and ACE_P on one hand, and the secretase activity on the other, are co-localized on the plasma membrane. This explains our earlier observation that in brefeldin-treated cells, ACE_T arrested in intracellular compartments is not cleavage-secreted (Ramchandran et al., 1994). The observation that high salt wash does not remove the secretase activity from the membranes confirms our earlier conclusion that this activity is cell-derived and not provided by the culture medium (Sen et al., 1991). Since the substrate and the enzyme are both integral membrane proteins, one can speculate that PMA may promote the cleavage reaction by promoting their encounters in the plasma membrane. Our assay used the natural substrate, i.e., the full-length glycosylated membrane-associated ACE proteins. At the initial stages, it is a better substrate than synthetic peptides containing the cleavage site which may serve as surrogate substrates. Use of the natural substrate ensures maintenance of the specificity; as a result, this approach will not lead to the purification of an irrelevant peptidase. However, the potential use of this system for purifying the secretase will require separation of the substrate from the enzyme which coexist in the membrane fraction used here. Furthermore, we will need a reconstituted assay system containing the substrate, the enzyme source, and possibly specific lipid materials to mimic the natural environment of the enzyme reaction. Such reconstituted assay systems have been used for purifying other membrane-anchored proteases (Jackson, 1983).

The inhibitor study identified several potential inhibitors of ACE-secretase. Among them, compound 3 proved to be very efficient in blocking the action of the secretase rapidly and at micromolar concentrations. More importantly, it did not have any toxic effects on the cells, and ACE synthesis was unaffected by its presence. The effect of the drug was also completely reversible in a very short time. Thus, it will be a very useful drug for studying the effects of inhibiting ACE secretion at cellular and whole animal levels. Compound 3 may also be a useful affinity ligand for ACE-secretase and thus be a valuable reagent for purification of this enzyme.

With the limited information available, it appears that ACE-secretase is a plasma membrane-bound metalloprotease. Its properties only partially overlap with other known secretases. TNF-α secretase is also inhibited by hydroxamates (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994) such as compound 3 (Mohler et al., 1994) and

stimulated by PMA. It, however, cleaves TNF-α between Ala and Val residues, whereas the ACE-secretase cleaves between Arg and Ser residues. The other partially characterized secretases, which act upon TGF-α, KL-1, KL-2, and β-APP, have cleavage specificities and inhibitor profiles different from those of ACE-secretase. Since none of these enzymes have been purified, their substrate profiles are unknown. It is probably safe to speculate that ACE-secretase belongs to a family of cell-surface metalloproteases which share some characteristics but have different substrate specificities. The sequence around the ACE cleavage site displays some of the sequence characteristics that govern monoarginyl cleavage in several other proteins. Among the four rules suggested for cleavage at monoarginyl site (Devi , 1991), ACE_T and ACE_P display three: no cysteine is present in the vicinity of the cleavage site, aromatic residues are not present at the position N-terminal to the monobasic amino acid, and hydrophobic aliphatic amino acid is not present in the position C-terminal to the monobasic amino acid. However, unlike the other substrates, a basic residue is not present in ACE at 3, 5, or 7 amino acids N-terminal to the cleavage site. Further purification of the activity will be required for understanding its mechanism of action at the biochemical level and its physiological function in different ACE-expressing tissues.

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