Cell Surface Localization of Proteolysis of Human Endothelial Angiotensin I-converting Enzyme

EFFECT OF THE AMINO-TERMINAL DOMAIN IN THE SOLUBILIZATION PROCESS*

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Angiotensin-converting enzyme (ACE) belongs to the type I class of ectoenzymes and is solubilized by Chinese hamster ovary cells transfected with the full-length human ACE cDNA. ACE release in Chinese hamster ovary cells involves a proteolytic cleavage occurring in the carboxy-terminal region, between Arg-1137 and Leu-1138. The subcellular localization of ACE proteolysis was established by pulse-chase experiments, cell surface immunolabelling, and biotinylation of radiolabeled mature proteins. The proteolysis of ACE takes place primarily at the plasma membrane. The solubilization of ACE is less than 2% within 1 h, is increased 2.4-fold by phorbol esters, but is not influenced by ionophores. An ACE mutant lacking the transmembrane domain and the cytosolic part (ACE<sub>CT</sub><sup>com</sup>) is secreted at a faster rate without a carboxy-terminal cleavage, and phorbol esters or ionophores have no effect on its rate of production in the medium. Therefore, the proteolysis of ACE is dependent on the presence of the membrane anchor and suggests that the secretase(s) involved is also membrane-associated. An ACE mutant lacking the amino-terminal domain (ACE<sub>CF</sub>) is secreted 10-fold faster compared with wild-type ACE. The solubilization of ACE<sub>CF</sub> occurs at the plasma membrane and is stimulated 2.7-fold by phorbol esters, and the cleavage site is localized between Arg-1227 and Val-1228. The amino-terminal domain of ACE slows down the proteolysis and seems to act as a "conformational inhibitor" of the proteolytic process, possibly via interactions with the "stalk" of ACE and the secretase(s) itself.

Proteolytic release of the ectodomain of carboxyl-terminally anchored proteins (type I class ectoenzymes) leads to the production of the protein in the extracellular space and, in some cases, in biological fluids. It is a widespread phenomenon (1), concerning several proteins with different biological functions such as membrane-anchored growth factors like transforming growth factor (TGF-α), c-kit ligand (growth factor receptor like the tumor necrosis factor receptor (4), cell adhesion molecules (1), ectoenzymes such as angiotensin I-converting enzyme (ACE) (1) or lactase-phlorizin hydrolase (5) and the amyloid precursor protein (APP) (6, 7). However, little is known about the molecular basis of this secretion. A common mechanism involving a processing enzyme named "secretase" has been proposed (1, 2, 6). The characteristics of this putative enzyme, which could be itself membrane-associated are broad sequence specificity (2, 6) and cleavage at a certain distance from the membrane (8).

ACE (EC 3.4.15.1) is a zinc-dipeptidyl carboxypeptidase (9) that also displays endopeptidase activity on some peptides (10). ACE is an endoenzyme found in most mammalian tissues bound to the external surface of the plasma membrane of endothelial, epithelial, neural, and neuroepithelial cells.

There are two ACE isoforms derived from a single gene, by the transcription from two alternative promoters (11, 12), which are expressed in a tissue-specific fashion. In somatic tissues, ACE is expressed as a glycoprotein composed of a single polypeptide chain with an apparent molecular mass of 170 kDa, containing two large homologous domains, called the N and C domains, each domain bearing an active catalytic site (13, 14); in male germinal cells, ACE is synthesized as a lower molecular mass form of 110 kDa containing only the active C domain of endothelial ACE (15). Both isoforms belong to the type I class of integral transmembrane ectoenzymes (1, 6) with a large amino-terminal extracellular domain, a 17-amino acid hydrophobic anchor located 30 amino acid residues from the cytosolic carboxyl terminus (13). The role of this α-helix in the membrane anchoring of ACE has been demonstrated by studying a carboxyl-terminal truncated cDNA (ACE<sub>CT</sub><sup>com</sup>), which is secreted faster in the medium of CHO cells (17).

ACE is solubilized and circulating in some body fluids such as serum (18). The source of circulating ACE in plasma is thought to be derived mainly from endothelial pulmonary cells. Wei et al. (17) showed that the full-length endothelial recombinant ACE cDNA, expressed in CHO cell lines, is secreted into the culture medium from the membrane form by a post-translational proteolytic cleavage occurring in the carboxy-terminal region. Recently (19), we have established by carboxyl-terminal microsequencing the same carboxyl terminus sequences AGQR for secreted recombinant ACE and for human plasma ACE, which corresponds to a cleavage site between Arg-1137 and Leu-1138 (see Fig. 1).

Sen et al. (20) showed that the rabbit testicular ACE cDNA expressed in a mouse epithelial cell line is secreted in the...
Culture medium by a proteolytic processing of its carboxyl-terminal region. The cleavage of the rabbit testicular ACE also occurs at a monobasic site between Arg-663 and Ser-664 (21). This cleavage site corresponds in endothelial human ACE to positions Arg-1203 and Ser-1204, downstream from the cleavage site we observed for the full somatic ACE (Fig. 1). The mutation of Arg-1137 to a glutamine residue did not prevent the secretion of ACE (19). Altogether, these findings suggest several hypotheses since the enzyme(s) responsible for ACE cleavage is (are) not identified. 1) A single enzyme of broad specificity could be involved, which could accommodate an Arg to Gin substitution in the P1 site of the human ACE. 2) Several related enzymes could be implicated. 3) Species differences related enzymes could be implicated. 3) Species differences could account for the differences in the solubilization of human and rabbit ACE observed in the two cell lines.

The subcellular localization of the proteolytic cleavage of endothelial ACE in CHO cells is unknown and could occur intracellularly or at the plasma membrane. The molecular mechanisms of its regulation are also unknown.

The aim of the present study was to identify the subcellular localization of the proteolytic cleavage of endothelial ACE and its regulation by metabolic labeling in CHO cell lines permanently transfected with the cDNA of endothelial ACE. This study shows that the solubilization takes place at the plasma membrane and that ACE solubilization under basal conditions is low and enhanced by phorbol esters but not by ionophores, implying a calcium-independent protein kinase C. The solubilization rate also depends on the presence of the N domain; an amino-terminal truncated mutant (ACE<sub>CF</sub>) was secreted 10-fold faster compared with the wild-type enzyme comprising both domains, and its cleavage site was different.

**MATERIALS AND METHODS**

**Construction of Expression Plasmids**

The construction of the expression plasmids has been described previously (14, 17). Expression plasmids, pcCHO-ACE containing the full-length ACE cDNA, pcCHO-ACE<sub>ACOOH</sub>, a carboxyl-terminal truncated cDNA in which the last 47 amino acids at the carboxyl terminus and the putative transmembrane domain are not translated (17), and pcCHO-ACE<sub>CF</sub> lacking the entire amino-terminal domain (14), were cotransfected with the neomycin resistance plasmid pSV2neo into CHO cells by calcium phosphate precipitation. Single colonies of primary G418-resistant transformants were assayed for the expression of ACE activity using p-benzoyl-L-glycyl-L-histidyl-L-leucine (Bachem, Switzerland) as substrate (22). The detection and quantification of the liberated hippuric acid was performed by high performance liquid chromatography (23). Cell lines expressing ACEs were selected and purified by subcloning using the limiting dilution technique. Cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

**Metabolic Labeling and Immunoprecipitation**

Wild-type and mutant ACE CHO cell lines with similar cellular contents of ACE activity were seeded in 60-mm dishes at a density of 2 x 10<sup>6</sup> cells/dish and grown to confluence. Cells were then incubated at 37°C for 30 min, in methionine-, cysteine-, and serum-free medium (Ham's F-12). The medium was removed, and the cells were incubated with serum-free UltraCHO medium (Bio-Whitaker), and the cells were washed with ice-cold phosphate-buffered saline (PBS) and were solubilized in 0.5 ml of lysis buffer/dish, 1% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 10 mM EDTA, and 0.15% SDS for 30 min at 4°C. Cell lysates were collected, and insoluble material was removed by centrifugation for 10 min at 10,000 x g at 4°C. Cell lysates supernatants were immediately stored at -70°C until further use.

Three different rabbit antisera were used for immunoprecipitation (see Fig. 1). Antiserum Y1 was obtained from rabbits immunized against pure human kidney ACE (13). Antiserum 28A and Clo were raised in rabbits against synthetic peptides corresponding to amino acids 1258-1277 at the carboxyl terminus and 1-18 at the amino terminus of the human endothelial ACE sequence, respectively (14, 19). Cell lysates or culture media were incubated overnight at 4°C with undiluted antisera and protein A-Sepharose (Pharmacia Biotech Inc.) (50 μl of a 50% suspension in lysis buffer). The immune complex protein A-Sepharose was collected by centrifugation and washed 4 times with 0.1%
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Triton X-100 in 50 mM Tris-HCl containing 1 mM EDTA, 0.15% SDS and once with 20 mM Tris-HCl, pH 6.8, and then dried. Samples were heated for 5 min at 100 °C in 25 µl of SDS-PAGE electrophoresis sample buffer. The Sepharose was removed by centrifugation, and proteins were resolved by 6 or 10% SDS-polyacrylamide gel electrophoresis (24) and revealed by autoradiography. Proteins were quantified from autoradiograms using a video densitometer (MC View Color, Agfa).

In some experiments a two-step immunoprecipitation method was used. Cells were pulse-labeled as described above and then chased in some cases in a serum-free medium for 4 or 16 h. Cell lysates (20 µl) were immunoprecipitated overnight with the antibody 28A, at the concentrations indicated in the figure legends. The immune complex protein A-Sepharose was removed, and the supernatant was heated at 100 °C for 5 min and immunoprecipitated for 4 h with antibody Y1 (3 µl).

Cell Surface Localization

Cells were then washed 4 times with ice-cold PBS. After washing, 10 µl of antibody Y1 or 28A in 2 ml of ice-cold PBS were added to the cell cultures. After 1 h of incubation with gentle agitation at 4 °C, the cells were washed 4 times with PBS. Cells were lysed in lysis buffer as described previously, and the lysates containing antigen-antibody complexes were immediately adsorbed on protein A-Sepharose (50 µl) at 4 °C for 4 h and analyzed by SDS-PAGE. To test the presence of the carboxyl-terminal part of the cell surface ACE immunolabeled with antibody Y1, antigen-antibody complexes from direct immunoprecipitation were heat-denatured at 100 °C for 5 min and then immunoprecipitated by protein A-Sepharose coupled to antisera 28A (3 µl).

Biotinylation of Cell Surface ACEs—For cell surface biotinylination experiments, the three CHO cell lines were metabolically labeled as described above and chased for different periods in UltraCHO medium. Cells were then washed 4 times with PBS. Cells were lysed in lysis buffer as described previously, and the lysates containing antigen-antibody complexes were immediately adsorbed on protein A-Sepharose (50 µl) at 4 °C for 4 h and analyzed by SDS-PAGE. To test the presence of the carboxyl-terminal part of the cell surface ACE immunolabeled with antibody Y1, antigen-antibody complexes from direct immunoprecipitation were heat-denatured at 100 °C for 5 min and then immunoprecipitated by protein A-Sepharose coupled to antisera 28A (3 µl).

Biotinylation of Cell Surface ACEs—For cell surface biotinylation experiments, the three CHO cell lines were metabolically labeled as described above and chased for 24 h (wild-type ACE) or 4 h (ACE_{ECHO} and ACE_{CF}). At the end of the chase period, subsequent steps were performed at 4 °C. Cell were washed 4 times with PBS containing 1 mM CaCl_2 and 1 mM MgCl_2 (PBS-CM) for 10 min. Cell surface proteins were biotinylated (1 mg/ml NHS-LC-Biotin: Pierce) for 30 min and washed 3 times with PBS-CM containing 10 mM glycine to quench biotinylation. One group of cells was solubilized, and the other group was incubated at 37 °C for 24 h in UltraCHO medium. Finally, solubilized cells and media were immunoprecipitated as described above. ACEs were eluted from washed antibody-protein-A Sepharose with 2% SDS in 50 mM Tris-HCl, pH 6.8. Biotinylated proteins were isolated by incubation with streptavidin-Sepharose beads (Sigma) for 30 min at 4 °C. The streptavidin-Sepharose beads were washed 3 times with 1% Triton X-100, 5 mM EDTA, 250 mM NaCl in Tris-HCl 25 mM, pH 7.8. Pellets were boiled in electrophoresis sample buffer for 10 min, and proteins were resolved by SDS-PAGE.

Deglycosylation

For the deglycosylation of ACEs, cell lysates and media from the three cell lines were immunoprecipitated as described above. The immune complex with protein A-Sepharose was suspended in 20 µl of 1% SDS and heated at 100 °C for 3 min. Water was added (150 µl), and the sample was heated at 100 °C for 1 min. The supernatant was adjusted to 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 100 mM EDTA, 0.1% SDS and acidified to pH 6 with sodium acetate (1 M). The samples were divided into two aliquots; one was treated with 1 milliunit of endoglycosidase H (endo-H) (Boehringer Mannheim) overnight at 37 °C, and the other was mock treated. Both samples were dried and then dissolved in sample buffer and analyzed by SDS-PAGE and autoradiography.

Western Blot Analysis

Western blot analysis was performed using a milliblot SDE (semidry transfer cell, Millipore) to transfer ACE to a polyvinylidene difluoride membrane (Immobilon P, Millipore). Immunoelectrophoretic blot analysis was carried out as described by Wei et al. (17). Three different rabbit antisera were used to characterize the ACE isoenzymes. Antisera Y1 and 28A were described above. Rabbit anti-sera 5 and 3 were directed against synthetic peptides containing the sequence 1126–1137 and 1214–1227, respectively, corresponding to the two putative cleavage sites of human endothelial ACE, as described previously (19), see Fig. 1.
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Fig. 3. Two-step immunoprecipitation of endo-H sensitive forms of ACE. Cells were pulse-labeled 30 min without chase to obtain endo-H sensitive forms. The same volume of cell lysates (20 μl) were first immunoprecipitated overnight without or with increased concentrations of 28A, from 0.01 to 5 μl. The supernatant of the first immunoprecipitation was heat-denatured and then immunoprecipitated with Y1 (3 μl) for the tsIP procedure. The 28A and Y1 immune complexes were resolved by SDS-PAGE. Results are expressed as the percentage (% ± S.D., n = 3) of cell lysates immunoprecipitated with 28A. The inset, autoradiograms of two tsIP are shown. One tsIP experiment is represented with bracket; the first lane is the result of the first immunoprecipitation with the 28A antibody, the second lane is that of the supernatant immunoprecipitation with Y1 antibody (3 μl) and are indicated by 28A/Y1. Cell lysates were first immunoprecipitated without (0) or with 5 μl of 28A (5).

Fig. 4. Subcellular localization of ACE proteolysis: endoplasmic reticulum and Golgi compartments. A, cells were labeled and chased for 4 h in the absence (−) and presence (+) of BFA (5 μg/ml), and then 20 μl of cell lysates were immunoprecipitated with Y1 (3 μl). Moreover, a tsIP with 20 μl of BFA-treated cell lysates was performed as described in the legend to Fig. 3 using 28A (5 μl) and Y1 (3 μl). Cells were labeled and chased for 4 h. Cell cultures were shifted from 37 to 20 °C at the beginning of the chase period (20 °C) for 4 h. Cells were then harvested and immunoprecipitated with 3 μl of Y1. A tsIP was performed as described in the legend to Fig. 3 using 20 μl of 20 °C cell lysates. ⋆, cell lysates.

Cell Surface Immunolabeling—Cell surface immunolabeling (4 °C during 1 h) was performed after a pulse-chase of 16 h. It was verified that the cell surface immunolabeling conditions used did not detect intracellular proteins. The 160-kDa EHS form of ACE (Fig. 5A, lane 2) is not immunolabeled by the Y1 antibody (Fig. 5A, lane 1). Less than 50% of the mature forms of ACE (170 kDa) were immunolabeled by the Y1 antibody (Fig. 5A, compare lane 1 with lane 2). Y1 immunolabeled ACE cross-reacted with the 28A antibody (Fig. 5A, lane 3). Moreover, the 28A antibody added in the medium in place of the Y1 antibody under the same conditions failed to detect any form of ACE (data not shown).

These results indicate that only mature cellular ACE proteins reached the plasma membrane and that the intracellular carboxyl terminus of the protein is not exposed to the extracellular space.

Cell Surface Biotinylation—Biotinylation of cell surface proteins (4 °C during 1 h) was performed after a pulse-chase of 16 h. Biotinylated ACE was localized at the plasma membrane and cross-reacted with the two antibodies Y1 and 28A (Fig. 5B, lanes 1 and 2). It was verified that the method was selective for membrane proteins and not for cellular proteins since intracellular EHS forms or BFA-treated ACE were not biotinylated (data not shown). After biotinylation, 15% of the initially biotinylated proteins appeared in the medium after 16 h (Fig. 5B, lane 3). The soluble biotinylated proteins did not cross-react with the 28A antibody (Fig. 5B, lane 4), indicating that a proteolytic cleavage occurred at the plasma membrane.

Using this method, the biotinylated ACE solubilization at 1 and 16 h was estimated at 1.9 ± 0.3% and 13.8 ± 3.1%, respectively. Furthermore, ACE solubilization quantified by the ratio of total soluble ACE in the medium after 1 and 16 h of chase over the total initially labeled cellular ACE at the chase time 0 was superimposable, estimated at 1.5 ± 0.2 and 18 ± 3%, respectively. Thus, the secretion of ACE in the medium closely corresponds to that of proteolytic cleavage occurring at the plasma membrane.

Regulation of the Solubilization by Phorbol Esters

The time-course of ACE secretion in the medium was studied by following ACE enzymatic activity under the influence of different pharmacological reagents after verification that these products did not modify the enzymatic activity. In absence of phorbol 12-myristate 13-acetate (PMA), ACE solubilization expressed as the ratio of enzymatic activity in the medium after

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This was studied by cell surface immunolabeling and biotinylation of cell surface proteins.
60 min over the cellular activity is measured as 2.25 ± 0.05% (n = 6) (Fig. 6). PMA at 10−6 M increased ACE solubilization 2.4 ± 0.3-fold (n = 6). This stimulation is specific since it is not observed with the inactive analogue of PMA at 10−6 M (data not shown). Furthermore, this stimulation is inhibited (95%) by staurosporin (10−6 M). In contrast, the ionophore A23187 at 10−6 M (data not shown) had no effect on solubilization (Fig. 6).

To test whether PMA treatment altered the proteolytic process of ACE, a pulse-chase of 16 h was used. After this period, cells were washed and then incubated for 1 h in the absence or presence of PMA (10−6 M). The secretion of ACE was increased with PMA in the same range, 2.2 ± 0.3 (n = 3), and soluble proteins did not cross-react with the 28A antibody (data not shown). These results suggest that the proteolysis of ACE in CHO cells is controlled by a calcium-independent protein kinase C.

Effects of Membrane Anchoring and of the Amino-terminal Domain on the Solubilization Process

To test whether the membrane anchoring and the amino-terminal domain of the protein influence solubilization, two truncated mutants stably expressed in CHO cell lines were used. ACECOOH is a carboxyl-terminal truncated mutant in which the transmembrane domain and the cytosolic part of ACE are not translated; ACECHO is a amino-terminal truncated mutant lacking the entire amino-terminal domain (Fig. 1).

Role of Membrane Anchoring: Biosynthesis and Secretion of Metabolically Labeled ACE and ACECOOH

It was established previously that enzymatically active ACECOOH was secreted in large amounts from transfected cells (17). In order to determine whether or not the plasma membrane insertion influences the intracellular traffic and the secretion, the biosynthesis of ACECOOH was compared with that of the wild-type.

The nonglycosylated form of ACECOOH migrated as a 135-kDa protein after endo-H treatment (Fig. 2B). Intracellular traffic of ACECOOH is similar to that of the wild-type ACE. The EHS form of ACECOOH (155 kDa) is entirely converted in the EHR form (165 kDa) after 16 h of chase (Fig. 2B). 8 h were required for formation of 30-min pulse-labeled ACE and ACECOOH molecules to the EHR forms.

The secreted form of ACE appeared in the medium after a 4-h chase, whereas the secreted form of ACECOOH appeared after a 1-h chase, in the same experimental conditions (Fig. 7, A and B). Quantification of secretion using the ratio of total soluble ACEs after a 16-h chase, over initially labeled cellular ACEs at chase time 0, revealed that 97 ± 17% of the labeled glycosylated ACECOOH was secreted compared with 18.5 ± 1.8% of ACE after a chase of 16 h when 90% of cellular proteins are mature.

ACECOOH is not found at the plasma membrane by cell surface immunolabeling and biotinylation (data not shown). Western blot analysis showed that both antisera 5 and 3 cross-react with soluble forms of ACECOOH (Fig. 8), suggesting that ACECOOH is secreted into the medium without modification of its carboxyl-terminal end.

In addition, the percentage of ACECOOH secretion after 1 h, in absence of PMA, determined by enzymatic activity, was 20.6 ± 1.01% (n = 6). No differences were observed under PMA (10−6 M) or A23187 (10−6 M) reagents: the secretion was estimated, respectively, to 23.2 ± 1.4% and 19.2 ± 0.78%.

Thus, the proteolysis of wild-type ACE depends on the presence of its membrane-anchor and possibly on a plasma membrane-associated secretase.

Role of the N-Terminal Domain: Biosynthesis and Secretion of Metabolically Labeled ACECF

The possible effect of the amino-terminal domain on ACE solubilization was first investigated in different clones expressing ACECF, where a 10-fold increase in the solubilization process was observed.

Since the rate of biosynthesis of ACECF was faster than that of ACE, the conditions of the metabolic labeling were adapted. Cells were labeled for 15 min with [35S]methionine-cysteine and chased in serum-free medium for 0.5, 1, 2, and 4 h (Fig. 7C). After 4 h of chase, 90% of the initially labeled ACECF was converted to the EHR form of the protein. Under these conditions, the biosynthesis and secretion of ACECF could be compared with that of a 16-h chase of ACE, where 90% of the initially labeled ACE was converted to the EHR form of ACE.

The nonglycosylated form of the ACECF migrated as a 75-kDa protein after endo-H treatment (Fig. 2C). ACECF was initially synthesized as an EHS protein (95 kDa), which was
ACE proteins were mature, 35% of ACE CF was secreted as a carboxy-terminal truncated protein with a decrease of 5 kDa in molecular mass (105 kDa). Western blot analysis of ACE CF is localized between Arg-1227 and Val-1228 (see Fig. 1). This cleavage did not occur intracellularly, as shown by the two-step immunoprecipitation (Fig. 9A).

Like ACE, ACE CF is expressed at the cell plasma membrane (Fig. 9B, lane 1) and was studied by biotinylation (Fig. 9B, lanes 2–6). After 4 h of chase and 1 h of incubation at 37°C in a serum-free medium, 17.7 ± 9.2% of initially labeled proteins appeared in the medium (Fig. 9B, lanes 3 and 4). This protein did not cross-react with the 28A antibody.

Solubilization of biotinylated ACE CF in the medium and 4 h after the biotinylation was 17.7 ± 9.2 and 35.1 ± 2%, respectively. The solubilization, quantified by the ratio of soluble ACE CF in the medium after 1 and 4 h of chase over the initially labeled cellular ACE CF at the chase time 0, was similar, estimated at 19.4 ± 7% and 33.0 ± 2.7%, respectively. Thus, as for wild-type ACE, the proteolytic cleavage of ACE CF takes place at the plasma membrane and occurs at the carboxy-terminal end.

ACE CF solubilization is also stimulated by phorbol esters like PMA (10−6 M) increases the solubilization 2.7 ± 0.8-fold, which is inhibited by staurosporin (10−6 M). The ionophore A23187 has no effect on ACE CF solubilization (data not shown).

In order to verify that PMA stimulates the proteolysis of membrane-bound ACE CF, cells were pulse-labeled, chased 4 h and biotinylated as described above. After 1 h in the presence of PMA (10−6 M), the solubilization of biotinylated ACE CF was increased (Fig. 9B, lane 5) compared with control (Fig. 9, lane 3). Moreover, soluble biotinylated ACE CF, after PMA treatment, did not cross-react with the 28A antibody (Fig. 9, lane 6).

These data show that basal ACE solubilization is clearly impaired by the presence of the amino-terminal domain since deletion of this domain increases the rate of solubilization of ACE CF without altering the PMA-induced solubilization process.
DISCUSSION

The general phenomenon of the solubilization of carboxyl-terminal membrane-anchored proteins involves a carboxyl-terminal proteolytic cleavage that takes place near or at the plasma membrane. This is the case for the secretion of the carboxyl-terminally truncated secreted APP (8, 29), or TGF-α (2). The mechanism of ACE secretion in CHO cells involves a proteolytic cleavage of the carboxyl-terminal part of the protein between Arg-1137 and Leu-1138 (19). The present study shows that the processing of membrane-anchored ACE operates primarily at the cell surface. Indeed, all intracellular ACE retained in the ER or in Golgi compartment before the membrane, cross-reacts with the carboxyl-terminal antibody, and the two-step immunoprecipitation method failed to detect any soluble intracellular forms of ACE even when intracellular blockers (brefeldin A or lowering temperature to 20 °C treatments) were used. After 16 h of chase, 90% of initially radiolabeled ACE was present as endo-H resistant form, N-glycosylated, and present at the plasma membrane. Immunolabelling of mature radiolabeled ACE shows that the amino-terminal part of ACE is exposed in the extracellular space, whereas the carboxyl-terminal end remained intracellular, excluding the possibility that the carboxyl terminus forms a hairpin loop and is exposed in the extracellular space. Biotinylation of mature radiolabeled ACE present at the cell surface clearly shows that the proteolytic cleavage of ACE takes place at the plasma membrane since the biotinylated ACE secreted in the medium is carboxyl-terminally truncated. The solubilization rate estimated for biotinylated proteins was the same as that determined for total cellular ACE. Therefore, the cleavage at the plasma membrane appears to be the predominant pathway for the generation of soluble ACE. However, we cannot exclude the possibility that some cleavage takes place in a specialized vesicular compartment near the plasma membrane via a degradation/recycling pathway, as described for APP (30, 31). If this processing occurs, it would involve another sequence than the consensus endocytic sequence SXQYRL, which is not present in the cytosolic part of ACE.

Experiments using an ACE mutant with its transmembrane α-helix and cytosolic parts deleted (ACE1-647) show a faster rate of secretion than ACE, without a difference in glycosylation or pattern of cellular traffic. This mutant is not expressed at the plasma membrane and is not cleaved intracellularly between Arg-1137 and Leu-1138, showing that membrane-anchoring of the protein and its cytosolic region are necessary for the proteolytic cleavage to take place at the plasma membrane, as for other proteins of this class.

Very little is known about the molecular mechanisms that may influence the secretion rate of C domain anchored proteins. One general finding, however, seems to be the increase in the solubilization rate induced by phorbol esters. The solubilization of the membrane-bound form of APP, pro-TGF-α, and ACE in CHo cells, transfected with the cDNA of APP, pro-TGF-α (32), or ACE (present study) is low, being 5%, 1%, and 2%, respectively, after 1 h of chase. The proteolytic cleavage of pro-TGF-α (33–35) and APP (36–38) is enhanced in both cases by phorbol esters or ionophores. A series of experiments were designed to evaluate this observation with respect to the ACE mutants. Proteolytic cleavage of recombinant human ACE in CHO cells is stimulated 2.4-fold by phorbol esters but is not influenced by ionophores. These results are in agreement with those published by Sen and co-workers (21) on the phorbol ester-induced stimulation of rabbit testicular ACE secretion. In the present study, the degree of this stimulation does not depend on the amino-terminal part of ACE, since phorbol ester stimulation is quantitatively similar to that observed for ACECF. In addition, the secretion of ACE1-647 is not regulated by phorbol esters and ionophores, suggesting that the PMA effect depends on the membrane-anchoring of the protein. Experiments using biotinylated mature radiolabeled ACECF show that phorbol esters stimulate the proteolysis of the membrane-bound protein, suggesting that the implicated secretase(s) is probably also membrane-associated, a hypothesis consistent with the membrane-associated secretase(s) proposed for ACE (39), APP (8, 40), or pro-TGF-α (41).

The increased solubilization of ACE (endothelial or testicular) by phorbol esters (2-fold over basal conditions) is 2.5 times less than the solubilization observed for pro-TGF-α or APP in CHO cells (32). Another difference is that the secretion of pro-TGF-α and APP in CHO cells is stimulated by ionophores involving a calcium-dependent protein kinase C, whereas a calcium-independent protein kinase C appears to be implicated in the proteolytic processing of rabbit testicular ACE (21) and endothelial ACE (present study).

Each class I ectoprotein could possess its own specific determinants to control the plasma membrane proteolysis (2, 6). One mechanism involved is the primary structure of the cytoplasmic tail, since a specific role for the carboxyl-terminal valine has been clearly demonstrated for pro-TGF-α (42) but is not involved in the solubilization of the tumor necrosis factor receptor (43). The role of the cytoplasmic tail of ACE has not yet been described. A second mechanism involved is the extracellular ectodomain of the protein. Deletion of 285 amino acids from the amino-terminal part of APP increases the solubilization of secreted ACE (8). Construction of a chimeric protein of APP, where amino acids 1–647 of APP ectodomain were replaced by a human secreted alkaline phosphatase derivative, (44) shows that the amino-terminal part of APP could inhibit the solubilization of secreted APP.

The role of the amino-terminal part of APP (8) in its solubilization as well as the increased rate of solubilization of testis ACE (21) compared with endothelial ACE (present study) prompted us to study the specific effect of the ACE amino-terminal domain in the solubilizing process. The proteolytic release of ACE is increased 10-fold by the deletion of the amino-terminal domain of ACE, reaching 35% (Fig. 10) after 4 h of chase. Several hypotheses could be envisaged to explain this increase of solubilization. 1) The intracellular traffic of ACECF could be faster than that of ACE, resulting in an artificial increase in solubilization rate expressed as the ratio of soluble ACECF in the medium over cellular ACECF. However, ACECF is expressed at the plasma membrane, and biotinylated mature ACECF is secreted in the medium after 1 h of chase 10-fold faster than ACE, under the same experimental conditions, i.e. when 90% of all mature proteins have reached the plasma membrane. Moreover, ACECF, like ACE, is primarily cleaved at the cell surface and not intracellularly. Thus, the increased solubilization observed with ACECF is not a consequence of accelerated biosynthesis or another cellular proteolytic process. 2) Through structural interactions with ACE itself or the secretase(s), the amino-terminal domain of ACE could act as a "conformational inhibitor" of the proteolytic process of membrane-anchored ACE. The amino-terminal domain could act on extracellular ACE conformation, near the anchoring domain; when the amino-terminal domain is present, the region near the membrane anchor may have a conformation distinct from that when the N domain is absent. Indeed, the amino-terminal amino acids of the native protein cannot be reached by immunolabeling with the C6 antibody, directed against the first 18 amino acids of the human endothelial sequence (data not shown). Accordingly, the amino-terminal part of the protein is more or less masked by the rest of the protein and/or in close-
and ACECF are cleaved by one or two secretase(s) (Fig. 10), and Val-1228. Therefore, a model can be proposed where ACE is cleaved between Arg-1137 and Leu-1138, whereas ACECF is probably cleaved between Arg-1227 and Val-1228. It is possible to envisage a general model for the processing of ACE. ACE could be cleaved at the plasma membrane by a secretase(s), localized like ACE to the plasma membrane. The amino-terminal domain would act as a conformational inhibitor of the proteolytic solubilization via structural interactions between the ACE stalk and the secretase(s) itself. Under these conditions, endothelial ACE would be slowly solubilized in plasma leading to the presence of most ACE activity at the endothelial cell level. Since protein-protein interactions vary in the plasma membrane and in the extracellular space, it is possible to conceive a "conformational" change in the cleavage site of ACE, leading to an increased ACE solubilization, under certain biological circumstances.

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