Specific Cellular Proteins Associate with Angiotensin-converting Enzyme and Regulate Its Intracellular Transport and Cleavage-Secretion*

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Angiotensin-converting enzyme (ACE) is an extensively glycosylated type I ectoprotein anchored in the plasma membrane by a hydrophobic transmembrane domain. In tissue culture as well as in vivo, the extracellular domain of ACE is released into the culture medium by a regulated proteolytic cleavage. To identify the cellular proteins that regulate ACE processing and cleavage-secretion, ACE-bound proteins were purified by affinity chromatography and characterized by microsequencing and Western blotting. One protein was identified as ribophorin and another as immunoglobulin-binding protein (BiP), a chaperone. Metabolic labeling and immunoprecipitation of ACE confirmed its interaction with BiP. Overexpression of BiP inhibited ACE secretion, an effect accentuated by the expression of an enzymatically inactive mutant BiP. This inhibition was caused by the retention of ACE precursors by BiP in the endoplasmic reticulum, as revealed by immunoprecipitation and immunofluorescence experiments. However, treatment with a phorbol ester, phorbol 12-myristate 13-acetate, enhanced ACE secretion even from cells overexpressing BiP. Western blot analysis of ACE-associated proteins with antibodies to protein kinase C (PKC) revealed the presence of its specific isozymes. Treatment with phorbol 12-myristate 13-acetate caused marked reduction in ACE association of selective PKC species. Thus, our studies have identified PKC and BiP as two proteins that directly interact with ACE and modulate its cell-surface expression and cleavage-secretion.

Angiotensin-converting enzyme (ACE) is a member of a group of transmembrane ectoproteins that is proteolytically processed near the plasma membrane on the extracellular side of a single membrane-spanning domain. The entire extracellular domain is released into the culture medium, or circulation, converting a membrane-anchored enzyme, growth factor, or receptor into a diffusible protein (1, 2). In several physiological and pathological situations such as normal mouse development (3), Alzheimer’s disease (4), response to inflammation (5–7) and wound healing (8), the cleavage event has been demonstrated to be biologically significant. Despite emerging evidence that this process of ectodomain shedding is an important and widely used cellular post-translational regulatory process, little is known about the responsible proteases, their mode of activation, or probable regulation by other cellular proteins.

It is important that the factors regulating the secretory processing of ACE be fully understood, as tissue-bound ACE and soluble ACE in the circulatory system may have different physiological roles. ACE has two structurally related isozymes: testicular ACE (ACE_T) and somatic, or pulmonary, ACE (ACE_P) (9–12). The former is involved in male fertility, the latter in blood pressure regulation (13). Studies by us and others (14–20) have shown that both ACE_P and ACE_T undergo specific cleavage-secretion. We have used extensively human, bovine, and mouse cells transfected with ACE_T cDNA to study this process for ACE_T. In one such system of a mouse epithelial cell line transfected with a rabbit ACE_T expression vector, the ACE 89 cells (14) synthesize, glycosylate, and express ACE_T on the cell surface, where it is cleaved off in a regulated fashion (15, 21). The responsible enzyme, ACE secretae, is a membrane-associated metalloprotease (16, 22) which we recently obtained in an enzymatically active, detergent-solubilized form (23). The ACE secretae activity was enhanced by treatment of cells with phorbol 12-myristate 13-acetate (PMA) (15), an activator of protein kinase C (PKC). PMA has been shown to stimulate most of the known shedding processes (2), but the mode of activation of PMA or any other regulator of shedding, such as serum (24) or pervanadate (25–27), has not yet been determined. It has been reported that PKCδ directly binds to a metalloprotease disintegrin, MDC9, leading to the suggestion that these two proteins might be involved in PMA-induced shedding of membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) (28). On the other hand, Raf/MAP kinase cascade has been implicated in PMA-activated shedding of HB-EGF (29). Moreover, activation of receptor tyrosine kinase and MAP kinase signaling cascades has also been suggested as a general mechanism to induce shedding of tumor growth factor-α, tumor necrosis factor-α, and l-selectin (30). Thus, it is not yet clear which cellular component(s) regulate the constitutive or induced cleavage-secretion of this group of transmembrane ectoproteins.

All newly synthesized proteins that are destined for the cell surface and secretion are folded and assembled in the endoplasmic reticulum (ER). This process is facilitated by interaction of the nascent proteins with various chaperones, of which immunoglobulin-binding protein (BiP) (31, 32) is the most...
abundant. BiP binds instantly to nascent proteins and releases them upon folding and assembly. Such interactions often alter the processing and secretion of several biologically important proteins (33, 34), including amyloid precursor protein (35), a transmembrane protein that is also cleavage-secreted. But not all proteins are affected by such interactions (34). A mutant BiP, in which ATP hydrolysis is impaired, retains the ability to bind nascent peptides but almost completely loses the capacity to release the bound protein, thus impeding its processing and cell surface expression. Such a recently developed mutant (GRP78 T37G, MBiP) (36) has been very helpful in analyzing the processing of certain membrane associated secretory proteins.

In this study, our aim was to identify cellular proteins that regulate ACE processing and cleavage-secretion. We show here that the ER resident protein BiP and specific PKC isoforms directly interact with ACE and modulate its cell-surface expression and shedding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hamster BiP (GRP78) and mutant BiP (GRP78 T37G), cDNAs encoding rabbit testicular ACE (14) cloned into pcDNA3 (Invitrogen), hamster BiP, and MBiP (GRP78 T37G, MBiP) (36) were obtained from Affinity BioReagents (Golden, CO) and Transduction Laboratories (Lexington, KY), respectively.

**Purification of ACE-associated Proteins from ACE 89 Cells**—Detergent extract of ACE 89 cell membranes (mouse epithelial cells transfected with the testicular isozyme of ACE (14)) were prepared essentially as described earlier (23). ACE and the associated proteins (from 15 × 10⁶ cells) were purified by lisinopril affinity chromatography (37), and the purified eluate was analyzed by 10% SDS-PAGE, electroblotted onto Problot membranes (Applied Biosystems), and either stained with Coomassie Brilliant Blue or analyzed by Western blotting. The stained protein bands were cut out and used for N-terminal sequence analysis after Edman degradation using a Procise model 492 protein sequencer (Applied Biosystems) attached to a 140C microgradient system and a 610A, version 2.1, data analysis system in the Molecular Biotechnology Core of The Lerner Research Institute. Western blot analysis was performed according to the manufacturer’s instructions, and signals were detected by chemiluminescence using the ECL system (Amersham Pharmacia Biotech). The effect of PMA on the binding of PKC isoforms to ACE was quantified by densitometric scanning of films obtained after ECL using a Microtect ScanMaker scanner and the ImageQuant program.

To purify ACE-associated proteins by coinmunoprecipitation, 12 μl of anti-ACE antiserum and 200 μl of protein A-Sepharose (1:1, v/v) were added to the detergent extract (prepared from 3 × 10⁶ cells). The slurry was gently mixed for 12 h at 4°C, and the protein A-Sepharose was collected by centrifugation. The precipitate was washed extensively, and the bound proteins were eluted by boiling with SDS-sample buffer and analyzed by SDS-PAGE. Similar lisinopril affinity chromatography and coinmunoprecipitation experiments were also performed using detergent extracts of C127 cells.

**Transfection, Metabolic Labeling, and Immunoprecipitation**—Human embryonic kidney (HEK) 293 cells were cultured as described by Yang et al. (35). cDNA encoding rabbit testicular ACE (14) cloned into the EcoRI restriction site of pcDNA3 (Invitrogen), hamster BiP, and mutant (MBiP) (35) were used for transfection. HEK 293 cells (0.9 × 10⁶ cells/60-mm dish coated with poly-L-lysine hydrobromide, Sigma), split 1 day prior to transfection, were grown to 70% confluency. For transfection, 15 μg of DNA (3 μg of ACE cDNA and 10 μg of vector, BiP, or MBiP (DNA) and 30 μl of Lipofectin (Life Technologies, Inc.) were added to 0.2 ml of Opti-MEM in separate tubes and incubated at 22°C. After 30 min, the mixture was diluted with an additional 0.8 ml of Opti-MEM. Cells (washed with Opti-MEM) were overlaid with the diluted DNA-Lipofectin mixture and incubated at 37°C for 5 h. At the end of the transfection period, an equal volume of Dulbecco’s modified Eagle’s medium containing 20% serum was added to the cells. After 1 and 18 h, the medium was replaced with Dulbecco’s modified Eagle’s medium containing 10% serum. 36 h after transfection, HEK 293 cells were labeled with [35S]methionine/cysteine (NEN Life Science Products), 150 μCi/60-mm dish for 8 h. The culture medium was recovered, and cell lysates were prepared using RIPA buffer (14). After centrifugation, the clear lysates were equally distributed into two tubes and immunoprecipitated using anti-ACE antibody (2.5 μl) or anti-BiP antibody (5 μl) and 50 μl of 1:1 protein A-Sepharose. The anti-rodent BiP antibody does not cross-react with endogenous human BiP. Immunoprecipitation and analysis by SDS-PAGE (mini-gels) were done as described earlier (38), and the radiolabeled proteins were detected by fluorography.

**Immunodetection of ACE in Transfected HEK 293 Cells by Indirect Immunofluorescence**—HEK 293 cells (0.1 × 10⁶) grown on glass coverslips were either transfected with ACE or co-transfected with ACE and MBiP constructs. After 36 h, cells were fixed with formaldehyde (3.6% for 10 min) and permeabilized with Nonidet P-40 (1% for 5 min) for internal staining where indicated. They were then treated with anti-ACE antibody (1:4000) and fluorescein-conjugated secondary antibody (1:200 Vectastain) before being visualized with a TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany).

**RESULTS**

**Identification of ACE-binding Proteins**—We have used ACE 89 cells in the past to study the regulation of ACE biosynthesis (14, 15, 21). These cells synthesize large quantities of ACE that are transported to the plasma membrane and cleavage-secreted into the culture medium. The rate of secretion can be enhanced by treating the cells with PMA (15), an activator of PKC, or pervanadate, an inhibitor of tyrosine phosphatases. As a source of ACE and its associated proteins, we used the membrane fraction of the ACE 89 cell extract. A detergent extract of these membranes was used for either immunoprecipitation with anti-ACE antibody or binding to an affinity resin consisting of lisinopril, a competitive inhibitor of ACE, covalently bound to Sepharose. After the binding of ACE and its associated proteins, the affinity resin was extensively washed, and the bound proteins were then eluted and separated by SDS-PAGE, which was followed by Coomassie blue staining (Fig. 1). At least seven distinct proteins were eluted from the lisinopril column (lane 4). The same proteins were eluted from the ACE antibody resin, although their proportions were different (lane 2). For example, protein 1 is much more prominent in lane 4 than protein 2, whereas protein 7 is prom-

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*2 I. Sen, unpublished observation.*
mature and immature ACE (Fig. 2). Along with BiP, a doublet of apparent molecular mass of 78 kDa was also present in this precipitate (Fig. 2, lane 3). Its apparent molecular mass of 78 kDa was also that of BiP. The N-terminal sequence of protein 7 perfectly matched that of ribophorin 1 (Table I). Ribophorin 1, or dolichyl-diphosphooligosaccharide-protein glycosyltransferase, is involved in sugar modification of proteins, and thus its association with ACE probably occurs during ACE protein modification.

BiP Associates with Underglycosylated ACE and Affects Its Further Transport—The functional consequences of the association of BiP with ACE were examined in a series of experiments. In these experiments, ACE and BiP were expressed in the HEK 293 cell line by transfection; the proteins were metabolically labeled and analyzed after immunoprecipitation. When an antibody to the transfected BiP was used for immunoprecipitation, a prominent BiP band was present in the precipitate (Fig. 2, lane 3). Along with BiP, a doublet of apparent molecular mass of about 90 kDa was also present in this precipitate. This doublet probably represents the endoglycosidase H-sensitive immature form of ACE, as reported by us previously (21). Neither BiP nor ACE was immunoprecipitated from empty vector-transfected cells (Fig. 2, lane 1) or ACE only-transfected cells (Fig. 2, lane 2), thus confirming the specificity of the ACE-BiP interaction. The same extracts were analyzed reciprocally in the experiments shown in Fig. 3. When ACE was immunoprecipitated from the transfected and labeled cell extracts, two species of ACE, the mature 115-kDa species and its 90-kDa precursor, were detected in ACE-transfected cells (Fig. 3A, lane 2). When BiP or its mutant, MBiP, which has no ATPase activity (36), was expressed along with ACE, a protein of apparent molecular mass of 78 kDa co-precipitated with ACE (Fig. 3A, lanes 3 and 4). Western blotting confirmed that this protein was indeed BiP (Fig. 3C).

Fully mature ACE was cleaved from the cell surface and secreted into the culture medium (Fig. 3B). Less ACE was secreted from cells expressing BiP (Fig. 3B, lane 3), and an even lesser amount was secreted from the cells expressing the mutant BiP (Fig. 3B, lane 4). We reasoned that this could be the result of defective transport of ACE to the cell surface because of its enhanced retention in the internal membranes through its interaction with BiP, which is known to reside in the ER. Immunofluorescence analysis confirmed this scenario (Fig. 4). In cells expressing ACE only, most of the ACE protein was on the cell surface, although some was detected in the internal organelles of permeabilized cells as well (Fig. 4, top panel). In contrast, in cells expressing MBiP along with ACE, massive amounts of ACE were trapped intracellularly, and less

![Fig. 2. BiP binds to ACE precursor protein.](image)

**TABLE I**

| Band | Sequence obtained | Matches with |
|------|-------------------|-------------|
| 2    | RRVTNQGTTSSQA     | N-terminal sequence of ACE |
| 3    | DDEAESFPVEYY      | ACE sequences: 76–87 |
| 4    | EEEKDKEQTVVGG     | N-terminal sequence of BiP |
| 7    | SSEAPPLVNEKDVKRT  | N-terminal sequence of dolichyl-diphosphooligosaccharide-protein glycosyltransferase (ribophorin 1) |

![Fig. 3. Overexpression of BiP and its inactive mutant species impairs ACE secretion.](image)
ACE was present at the cell surface (Fig. 4, bottom panel).

**Regulation of ACE Secretion by PMA**—We have previously shown that ACE cleavage-secretion from the cell surface is accelerated by treating cells with PMA. In the experiment shown in Fig. 3E, we asked whether this process was affected by overexpression of BiP or MBiP. As expected, PMA treatment enhanced ACE secretion (compare Fig. 3, E and B, lane 2). ACE secretion was similarly enhanced by PMA in cells expressing BiP and MBiP as well (Fig. 3, B and E, lanes 3 and 4). As shown previously, as a consequence of enhanced secretion, the amount of cell-associated ACE decreased slightly in PMA-treated cells (compare Fig. 3, D and A). These results showed that BiP does not affect the PMA-mediated up-regulation of the ACE secretion process. The same conclusion was reached from the results presented in Fig. 5. In this experiment, secreted ACE was quantitated by measuring its enzyme activity. Although less ACE was secreted from cells expressing BiP or MBiP, PMA enhanced this secretion from all cells.

To explore the mechanism of PMA-mediated regulation of ACE secretion, we considered the possibility that PKC, the target of PMA activation, may directly bind to cellular ACE. To test this hypothesis, ACE-associated proteins in ACE 89 cells were analyzed by Western blotting with PKC antibodies using the procedure described for Fig. 1. Five antibodies that recognized six specific isozymes of PKC were used for this experiment, and purified PKC isoforms were used as positive controls. As shown in Fig. 6, PKCα and PKCβ, which were recognized by the same antibody, did not co-purify with ACE. In contrast, PKCγ, PKCδ, and PKCε were all present in the anti-ACE immunoprecipitate, suggesting their specific association with ACE. When a similar analysis was carried out with cells that had been treated with PMA, much less PKCγ and PKCδ co-precipitated with ACE (3- and 9-fold less, respectively), and no PKCγ was detectable. The amount of ACE-associated PKCα, however, did not change significantly. These results clearly demonstrate that PMA-mediated up-regulation of ACE secretion is accompanied by selected dissociation of specific PKC isozyme from the ACE proteins. When a similar analysis was carried out with membrane extracts of the parental C127 cells, which do not produce ACE, none of the PKC isoforms were detected (data not shown).

**DISCUSSION**

Many membrane-anchored ectoproteases are proteolytically converted to soluble factors on the cell surface by a regulated process of cleavage and secretion; these include ACE, β-amyloid precursor protein, pro-transforming growth factor-α, colony stimulating factor, pro-tumor necrosis factor-α, KL-1, KL-2, cell adhesion receptor Mel-14, and several others. Although the solubilization processes of these proteins share common characteristics, such as being sensitive to inhibitors of a specific class of metalloproteinase and being stimulated by PMA treatment of cells, the identities of most of the secretases and their modes of regulation remain unknown. The only well characterized cell-surface secretase is the one responsible for
the processing of tumor necrosis factor-α.

This enzyme, however, is not required for cleavage-secretion of ACE, because ACE is normally processed in cells missing the tumor necrosis factor-α secretase (23). Thus, it appears that related but distinct secretases may be responsible for the cleavage of different ectoproteins, and each member of this family may have a distinct mode of regulation. We have been studying the mode of ACE cleavage-secretion and, in the current study, have sought to identify some of the cellular proteins that regulate the intracellular transport and secretion of ACE.

The putative ACE-regulating proteins were identified by their physical association with ACE. We chose to use ACE 89 cells, which produce a large quantity of ACE proteins, to purify ACE. As ACE is a glycoprotein, it is always associated with various cell membranes during biosynthesis. Maturation of the protein, from the precursor to the final product present on the various cell membranes during biosynthesis. Maturation of the cells, which produce a large quantity of ACE proteins, to purify the mode of ACE cleavage-secretion and, in the current study, related but distinct secretases may be responsible for the cleavage-secretion of ACE. The results presented in this paper have shed some light on this process. Because PMA treatment of cells is known to activate PKC and cause its translocation from the plasma membrane to the cytosol, we wondered whether this process was linked to the observed enhanced ACE cleavage-secretion in PMA-treated cells. We hypothesized that PKC may be specifically bound to mature ACE or the ACE secretase, both of which are known to be plasma membrane-anchored proteins, and that PMA treatment may decrease this association, causing an enhanced action of the secretase on the ACE protein. The results shown in Fig. 6 validated some features of this hypothesis. Co-immunoprecipitation experiments showed that PKC was bound to ACE. The fact that this binding was highly specific was indicated by the observed association of specific isoforms of PKC, but not others, with ACE. PKCɛ was the most prominent ACE-associated PKC isoform, and its migration in gel electrophoresis was in the range of Band 5 and 6 of Fig. 1 (data not shown). As postulated, the ACE association of PKCɛ was greatly reduced upon PMA treatment. The same was true for PKCγ and PKCδ, but not for PKCα, again demonstrating the specificity of the PMA effect. Thus, it appears that ACE associates with specific isoforms of PKC and that PMA treatment causes dissociation of only a subset of these PKC isoforms. How this dissociation translates into enhanced cleavage of ACE remains an open question. Whether the cleavage process is affected by a regulation of the phosphorylation status of the ACE cytoplasmic domain, as a consequence of PKC dissociation, is a relevant and important question to be addressed in the future.

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22. Band 4. Western blotting (Fig. 1) and co-immunoprecipitation (Fig. 2) further confirmed that it was the protein chaperone BiP. As expected from the location of BiP in the ER, it was associated primarily with the underglycosylated ER form of ACE. Overexpression of BiP caused enhanced retention of ACE in the ER. Consequently, less protein was secreted. These observations suggest that optimal intracellular transport of ACE requires only a transient interaction with BiP and that their continued association, because of a higher abundance of BiP, impairs the proper transit of ACE to the next stage of processing. Results obtained with the enzymatically inactive BiP confirmed this reasoning. Because the ATPase activity is required for BiP functioning in protein folding, and not for binding to nascent proteins in the ER, the mutant BiP blocked the intracellular transit of ACE even more effectively. The observed effect of BiP on ACE transport and secretion is distinct from that observed for the amyloid precursor protein, in which secretion was also decreased upon BiP overexpression (35). This decrease is because the amyloid precursor protein cleavage occurs in the ER membrane, whereas ACE cleavage occurs on the plasma membrane. Thus, the effect of BiP on ACE biosynthesis seems to be exclusively at the transport level. The mature ACE that reached the cell surface in BiP-expressing cells was efficiently cleaved and, as shown in Fig. 5, this process was normally regulated by PMA.

Although the up-regulation of cleavage-secretion of ectoproteins by PMA is well documented, the underlying mechanism is obscure. The results presented in this paper have shed some
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