A Small Region in the Angiotensin-Converting Enzyme Distal Ectodomain Is Required for Cleavage-Secretion of the Protein at the Plasma Membrane[†]

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ABSTRACT: Both germinal and somatic isoforms of ACE are type I ectoproteins expressed on the cell surface from where the enzymatically active ectodomains are released to circulation by a regulated cleavage-secretion process. Our previous studies have shown that ACE-secretase activity is regulated by the ACE distal ectodomain and not by sequences at or around the cleavage site. In the current study we have identified that the ACE residues encompassing 343 to 655 of the germinal form are needed for its cleavage-secretion. To narrow down this region further, we have examined the cleavage-secretion of ACE-CD4 chimeric proteins in mammalian cells and *Pichia pastoris*. These experiments identified five residues (HGEKL) in the ACE region of the chimeric proteins that were essential for their cleavage-secretion. When the corresponding residues were substituted by alanine in native germinal and somatic ACE, the mutant proteins were not cleaved, although they were displayed on the cell surface and enzymatically active. These results demonstrated that a small region in the ectodomain of ACE is required for its cleavage at the juxtamembrane domain. This conclusion was further supported by our observation that secreted ACE inhibited cell-bound ACE cleavage-secretion, although the secreted form did not contain the cleavage site.

Angiotensin converting enzyme (ACE[1]) is a key player in the renin angiotensin system that controls the blood pressure and fluid homeostasis (*1*). Gene knockout studies have shown that ACE plays pivotal roles in maintaining normal blood pressure, renal functions, male fertility and general growth (*2*). Studies from our laboratory and others have shown that tissue specific expression of ACE in transgenic mouse models can restore specific functions of ACE in the body (*3–8*). Recently ACE has been shown to possess GPIase activity, which is distinct from its peptidase activity and is associated with its role in fertilization (*9*). However, GPIase activity of ACE remains controversial, as reported by Leisle et al. (*10*); moreover, the peptidase function of ACE has been shown to be required for its role in fertilization (*11*). ACE exists as two isoforms, viz., somatic ACE (sACE), the larger isoform which is expressed mainly in the somatic tissues and the smaller isoform, germinal ACE (gACE), which is expressed in sperm cells. Both isoforms are synthesized from a single gene using tissue specific promoters and have distinct and specific functions in the body and they can not substitute for each other (*3, 4, 12*). Both the isoforms of ACE are expressed on the cell surface as type I ectoprotein, with a long ectodomain, short cytoplasmic domain and a transmembrane domain (*13–15*). Enzymatic active site of ACE is located on the extracellular domain, and there are two active sites in the sACE, which has an additional N-domain along with the C-domain, which is identical to the ectodomain of gACE (*16*).

Numerous integral membrane proteins, including ACE, undergo "ectodomain shedding", where the extracellular domain is cleaved off by a membrane bound protease. The ectodomain shedding of transmembrane proteins is a regulated event and is involved in maintaining various cellular functions including growth, development and disease formation (*17, 18*). Among various proteins that undergo ectodomain shedding, amyloid precursor protein has been extensively studied and the protease responsible for its shedding has been identified (*19, 20*). Recently a number of proteases have been identified in human cells and a group of proteases known as ADAMs have been studied extensively for their roles in various cellular functions and disease progression (*21, 22*). ADAM17, also known as TACE, was the first known metalloprotease, which is involved in shedding of TNF_α, and many other cell surface proteins (*23, 24*). Other classes of proteases include matrix metalloproteinases (MMPs) and membrane type metalloproteinases (MT-MMPs), which are also involved in ectodomain shedding of membrane proteins. Ectodomain shedding of ACE has long been known as a regulated event that is stimulated by activation of PKC, inhibitors of tyrosine phosphatases and calmodulin (*25–28*) and is inhibited by a hydroxamate based inhibitor of metalloproteases (*29, 30*). It has been shown earlier that ACE is tyrosine phosphorylated on its extracel-

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[1] Abbreviations: ACE, angiotensin converting enzyme; ADAMs, a disintegrin and matrix metalloproteinases; TACE, TNF_α converting enzyme; PKC, protein kinase C.

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lular domain and its cytoplasmic domain is serine phosphorylated (*25, 27*) and the phosphorylation of ACE regulates its cleavage-secretion (*31*). Studies from our laboratory and others have shown that ADAM17 is not involved in the ectodomain shedding of ACE (*32, 33*) and the protease involved in ACE shedding has not yet been identified. ACE secretase activity has been chacarterized and found to be a membrane bound protease (*34*). A structure activity relationship study has been directed toward identification of hydroxamate-based inhibitors of ACE seccrtease (*35*).

A number of studies have been carried out to identify the domains of ACE responsible for recognition of ACE secretase, and the major technical difficulty that we and others have faced during these studies was the incomplete processing of ACE proteins that were generated by deletions of specific domains. Therefore, many of these studies have not resulted in successful conclusions. However, studies from our laboratory indicated that extracellular domain of ACE spanning 1−655 residues have the potential to activate the ACE secretase and the cytoplasmic domain is not only needed but plays negative roles in shedding (*27, 36*). This region, when attached to a nonsecreted protein CD4, made the chimeric protein capable of cleavage-secretion. More importantly, the cleavage was directed to the membrane-proximal CD4 residues, demonstrating that ACE-secretase does not have specificity for the cleavage site.

Studies from others have shown that the juxtamembrane stalk region of ACE also plays a major role in regulating its ectodomain shedding. Stalk glycosylation has been shown to modulate the ectodomain shedding of ACE (*37*). In another study it has been found that a mutation in the juxtamembrane region of ACE (ACE_{NQ} mutant) stimulated the basal ectodomain shedding of ACE by a different secretase (*38*). The juxtamembrane, transmembrane and the cytosolic domains of ACE when attached to the ectodomain of membrane dipeptidase (MDP), a membrane protein that does not shed, were cleaved from the cell surface; however, a chimera containing only the transmembrane and cytosolic domains of ACE and the ectodomain of MDP resulted in a mutant that was displayed on the cell surface but not cleaved (*39*). This showed the involvement of juxtamembrane domain of ACE in the regulation of activity of ACE secretase. This observation was in contrast to our findings that the ACE ectodomain (1−655) was sufficient to activate ACE secretase. In the present investigation we have identified a region within the ectodomain of ACE that is needed for the cleavage-secretion of ACE.

MATERIALS AND METHODS

Cell Culture and Transfection. HeLa cells were maintained in DMEM with 10% fetal bovine serum and transient transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

Generation of ACE/CD4 Chimeric Proteins and ACE Mutants. Rabbit gACE/CD4 chimeric constructs were generated as described before (*36*); gACE(1−655)/CD4 and CD4/ACE(343−737) were used as templates to generate the resultant deletion mutants using PCR in mammalian expression vector pcDNA3.1 or yeast expression vector pHIL-S1. The block alanine substitution mutants of rabbit gACE were generated by site directed mutagenesis using megaprimer PCR method (*27*). The corresponding sACE-5A construct was generated by restriction swapping with the gACE-5A construct.

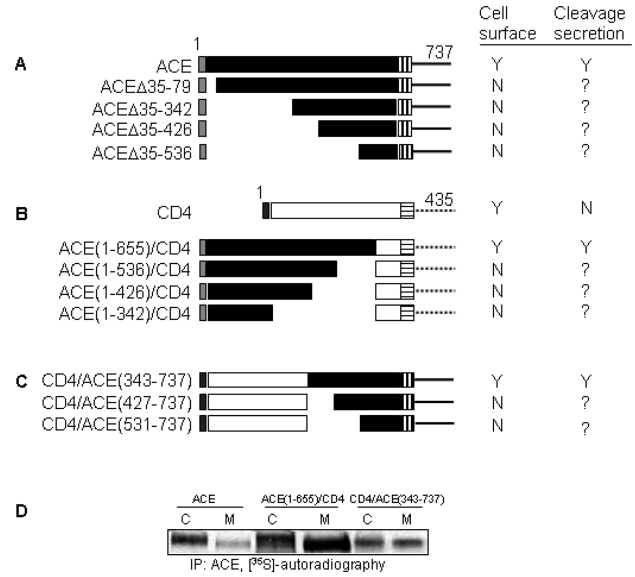

FIGURE 1: ACE domain containing 343−655 residues is needed for cleavage-secretion of ACE. (A) ACE and its N-terminal deleted mutants were transiently expressed in HeLa cells, labeled with ^{35}S-methionine, and the label was chased for 4 h; cleavage-secretion was analyzed as described before (*27*). (B) ACE domain containing its 1−655 residues was fused with C-terminal domain of CD4 (312−435 residues), and this chimeric protein was expressed transiently in HeLa cells and its cleavage was tested similarly as in (A). Several other chimeric proteins were generated using ACE residues as shown and were expressed similarly to test their cleavage activity. (C) CD4 N-terminal domain (1−352 residues) was fused with C-terminal domain of ACE (343−737), and its cleavage was tested in HeLa cells by similar assay as indicated in (A). Other chimeric proteins were generated by deletion of ACE domain and the CD4 domain as shown, and their cleavage was tested in HeLa cells. (D) ACE, and ACE/CD4 chimeric proteins (see B and C for residues of ACE and CD4 in the chimera) were transiently expressed in HeLa cells, labeled with ^{35}S-methionine for 30 min, and the label was chased for 4 h. The cell extracts (C) and the culture media (M) were immunoprecipitated with anti-ACE and analyzed by SDS−PAGE and autoradiography.

^{35}S-Methionine Labeling of Cells, Immunoprecipitation. Transiently transfected HeLa cells were first incubated in methionine/cysteine-free medium for 1 h, and then labeled with ^{35}S-methionine/cysteine (Perkin-Elmer Life Sciences) for 0.5 h, and the label was chased for the time periods as indicated in the figures. At the end of the chase period cells were washed in cold phosphate buffered saline and total proteins were extracted in RIPA buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X100, 1% SDS, and 1 mM EDTA. ACE was immunoprecipitated from the cell extracts and culture medium with anti-ACE. Immunoprecipitated ACE was then analyzed by 8% SDS−PAGE, followed by autoradiography (*27*).

Expression of ACE and ACE/CD4 Chimera in Pichia pastoris. Expression vector pHIL-S1 (Invitrogen) containing methanol inducible alcohol oxidase (AOX1) promoter and PHO1 signal sequence was used to express ACE and ACE/CD4 chimeric proteins. Briefly, the recombinant plasmid containing ACE or ACE/CD4 proteins was linearized with *Bgl*II and transformed into *P. pastoris* by *Pichia* expression kit following manufacturer's instructions (Invitrogen). After

transformation, several transformants were screened for ACE expression. Cells were grown in BMGY medium (yeast extract, peptone, glycerol, biotin, yeast nitrogen base and potassium phosphate buffer, pH 6.0) at 30 °C. After 2 days, cells were harvested, resuspended in BMMY medium (same as BMGY with 0.5% methanol in place of glycerol) and incubated at 30 °C for 1−5 days to induce ACE expression. Expression of ACE or ACE/CD4 proteins was assessed by measuring ACE enzymatic activity in the culture medium. For this purpose, the cultures were centrifuged and the supernatant media were dialyzed extensively against 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl, prior to enzyme activity measurements (*40*).

Cell Surface Biotinylation of P. pastoris Expressing ACE/CD4 Proteins. P. pastoris cells expressing ACE/CD4 were harvested, washed with phosphate-buffered saline (pH 8.0) and biotinylated with 0.5 mg/ml sulfo-succinimydyl-6-(biotin-amido) hexanoate (Pierce) in phosphate buffered saline for 1 h at 4 °C. After biotinylation, cells were washed three times with phosphate buffered saline to remove unreacted biotin. Biotinylated cells were extracted and were immunoprecipitated with anti-ACE and immunoblotted with anti-ACE or streptavidin as indicated.

Western Blotting and Enzyme Activity Measurement. Membrane bound and secreted ACE was immunoblotted using antirabbit lung ACE (anti-ACE) antibody (*41*). ACE enzyme activity was assayed using hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as a substrate and measuring fluorimetrically the His-Leu liberated at 5 mM Hip-His-Leu (*41*).

Immunodetection of ACE by Immunofluorescence. HeLa cells grown on glass coverslips were transfected with appropriate expression vector as mentioned in the figure legends, fixed and treated with anti-ACE antibody and fluorescein conjugated goat IgG as described (*42*).

RESULTS

Requirement of Residues 343−655 of ACE for its Cleavage-Secretion. One major property of ACE-secretase, that distinguishes it, is that ACE cleavage is regulated by distal residues of the ACE extracellular domain and not by the residues around the actual cleavage sites in the membrane proximal region (*36, 39, 43–45*). In the next series of experiments we attempted to identify the minimum region of ACE that is needed for the cleavage-secretion of ACE. For this purpose, we generated a nested set of N-terminal deletion mutants that expressed proteins combining the signal sequence (residues 1−34) and increasing deletions of the residues from residue 35 onward (Figure 1A). All proteins were expressed in HeLa cells. but only the Wt protein was transported to the cell surface, as determined by immunofluorescence, and cleaved. Because the deletion mutant proteins were not transported to the plasma membrane, we could not determine whether they could be cleaved.

To circumvent this problem, we expressed various chimeric proteins containing N-terminal regions of gACE and C-terminal regions of CD4 (ACE/CD4) or vice-versa (CD4/ACE). As expected, CD4, by itself, was not cleaved at all (Figure 1B) and among the ACE/CD4 mutant proteins that were expressed, only ACE(1−655)/CD4 was transported to the cell surface and cleavage-secreted (Figures 1B and 1D). Among the tested CD4/ACE mutant proteins, CD4/ACE-(343−737) was transported to the cell surface and cleaved (Figures 1C and 1D). From these experiments we concluded that the region between the N-terminal residue 343 and the C-terminal residue 655 is needed for the cleavage-secretion of ACE and the presence of this region enabled the ACE secretase to cleave the membrane-proximal peptide bonds in the CD4 region of ACE/CD4 chimera and in the ACE region of CD4/ACE chimera.

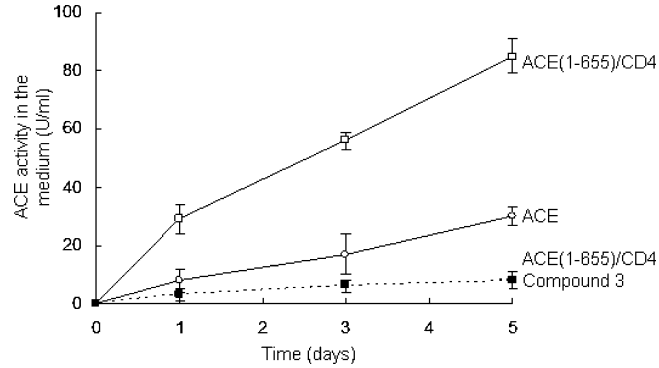

FIGURE 2: Stable expression and cleavage of ACE and ACE/CD4 proteins in *P. pastoris*. Recombinant *P. pastoris* expressing ACE or ACE/CD4 proteins was induced by methanol for the indicated period either in the absence or in the presence of Compound 3 (100 μM). At the indicated time, culture media were collected, dialyzed against 50 mM potassium phosphate buffer, pH 7.0, containing 150 mM NaCl, and ACE activity was measured by its enzymatic activity to liberate His-Leu from the substrate Hip-His-Leu (*41*).

Expression of ACE in P. pastoris for Further Analysis. Previously we showed that ACE can be efficiently expressed in *P. pastoris*; moreover, it is cleavage-secreted from the cell surface by a yeast secretase with properties similar to the mammalian ACE secretase (*40*). To take advantage of this knowledge, we expressed Wt ACE and ACE(1−655)/CD4 in yeast and tested their cleavage-secretion. Both proteins were expressed and cleavage-secreted. Surprisingly, the chimeric protein was secreted more efficiently than the Wt protein; and its secretion was completely blocked by Compound 3, an inhibitor of ACE-secretease in the mammalian cells (Figure 2). The elevated secretion of the chimera might be due to the involvement of multiple secretases that could recognize it and were inhibited by Compound 3. As expected, CD4 was displayed on the cell surface but as in the mammalian system, it was not cleaved in yeast (Figure 3A). However, unlike the situation in human cells, all chimeric ACE/CD4 proteins expressed in yeast were transported to the cell surface (Figures 3A and 3B). But among them only ACE(1−655)/CD4 was secreted to the medium (Figure 3B). To rigorously compare the properties of ACE(1−655)/CD4 and the closest mutant, ACE(1−640)/CD4, we examined their levels of expression in the cells (Figure 3C, top panel), their levels of expression on the cell surface (Figure 3C, middle panel) and their levels of expression in the media (Figure 3C, lower panel). Both proteins were expressed in the cell at similar levels and transported to the cell surface equally well, but only one of them was cleavage-secreted. These results strongly indicated that deletion of the residues 640−655 in the gACE/CD4 chimera resulted in a mutant protein that was a poor substrate for the ACE secretase and, therefore, was not cleaved.

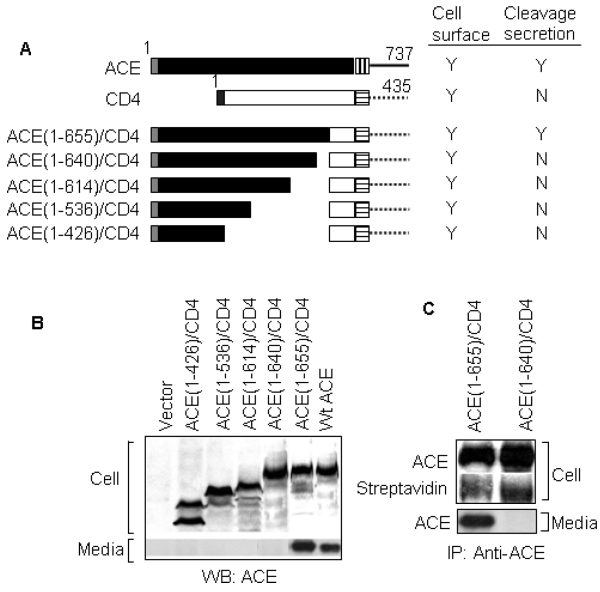

FIGURE 3: ACE domain containing residues 640−655 was needed for cleavage-secretion of ACE in *P. pastoris*. (A) Schematic representation of ACE/CD4 chimeric proteins, the numbers of ACE residues present in the chimera are indicated on left. These chimeric proteins were expressed in *P. pastoris* and assayed for ACE activity in the culture medium after induction. (B) The ACE/CD4 chimeric proteins (as shown in A) were expressed in *P. pastoris*, after induction the culture medium, and the cell extracts were analyzed by SDS−PAGE and Western blotted with anti-ACE. (C) *P. pastoris* expressing ACE(1−655)/CD4 and ACE(1−640)/CD4 chimeric proteins was collected after 5 days, and cells were biotinylated using sulfosuccinimidyl-6-(biotin-amido) hexanoate (Pierce) using the manufacturer's instructions. The biotinylated proteins were analyzed by SDS−PAGE and immunoblotted with ACE or streptavidin as indicated.

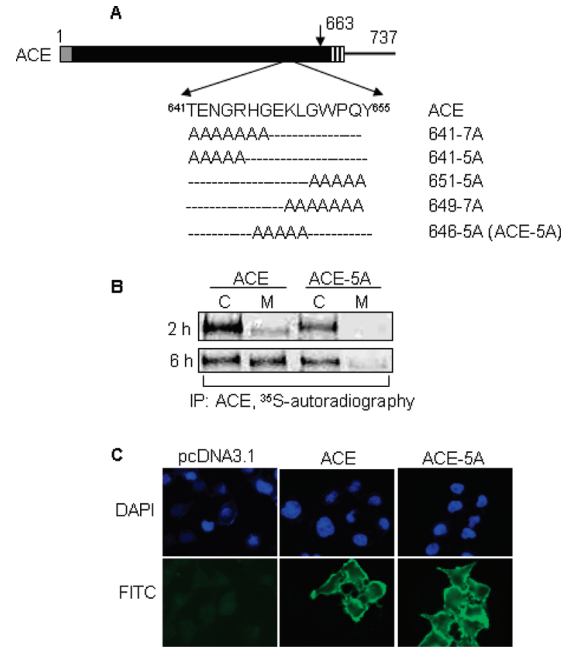

FIGURE 4: Substitutions of ACE 646−650 residues by alanines inhibited ACE secretase activity. (A) Schematic diagram of ACE and the residues in ACE that were mutated to alanines to test their cleavage-secretion. (B) ACE and ACE-5A were transiently expressed in HeLa cells, after transfection cells were labeled with ^{35}S-methionine for 30 min and the label was chased for 2 and 6 h. Cell extracts (C) and culture media (M) were immunoprecipitated with anti-ACE and analyzed by SDS−PAGE and autoradiography. The figure is a representative of three independent experiments. (C) HeLa cells were transiently transfected with empty vector (pcDNA3.1), ACE and ACE-5A expression vectors on coverslips, after transfection cells were fixed with paraformaldehyde and stained for cell surface ACE using anti-ACE antibody.

Requirement of Residues 646−650 for Cleavage-Secretion of ACE in Mammalian Cells. We used the information gathered from the yeast expression system to design appropriate mutants to be tested in human cells. Because residues 640−655 were identified as critical, we deleted these residues from ACE and expressed the internally deleted protein in HeLa cells. Unfortunately, the protein failed to reach the cell surface as well (data not shown). In our further attempts to study this region, we introduced several block alanine substitution mutations in ACE (Figure 4A) and tested their cleavage-secretion in HeLa cells. The 641-7A and 641-5A mutant proteins were not transported to the cell surface, whereas the other three mutant proteins, 651-5A, 651-7A and 646-5A (ACE-5A) were transported. However, cleavage-secretion of ACE-5A but not the other two mutant proteins on the cell surface was severely inhibited (Figure 4B), although it was displayed on the cell surface as prominently as the Wt protein (Figure 4C). These results clearly showed that the residues HGEKL are critical for the cleavage-secretion of gACE.

Up to this point all our analyses have been carried out with germinal isoform of ACE, the smaller of the two, which is physiologically not expressed in somatic cells, although when artificially expressed, it is cleavage-secreted with the same characteristics as somatic ACE. Because the C-terminal half of sACE is identical to gACE, we wanted to examine whether mutation of the residues in sACE that correspond to gACE residues 646 to 650 can affect its cleavage-secretion. For this purpose, these residues of sACE (HGEKL) were substituted by Ala residues. Similar to Wt sACE, the sACE-5A mutant was expressed well in HeLa cells; but, unlike Wt protein, it was not cleavage-secreted (Figure 5A). As before, we verified that both proteins were expressed on the cell surface (Figure 5B). These results demonstrated that substitution in these regions of both gACE and sACE produced cell surface proteins that were poor substrates of ACE secretase. To provide further evidence for our model, we took advantage of the molecular weight difference between gACE and sACE. We argued that the secreted Wt gACE, which contains the region 646−650, should be recognized by the secretase, thus serve as a decoy substrate and prevent cleavage-secretion of sACE. Indeed, this was the result (Figure 5C). Added secreted gACE strongly inhibited sACE secretion to the medium (top panel, Figure 5C). This result indicates that secreted ACE has a negative feedback effect on further cleavage-secretion of cell-bound ACE.

DISCUSSION

ACE plays an important role in maintaining normal blood pressure, kidney functions and male fertility. Transgenic studies have shown that expression of ACE in vascular endothelial cells is absolutely necessary for maintaining normal blood pressure (*3, 4*). ACE is a type-I transmembrane protein that undergoes ectodomain shedding to generate an enzymatically active soluble form, which is found in the body fluids including serum (*29, 32, 46–48*). A suitable balance

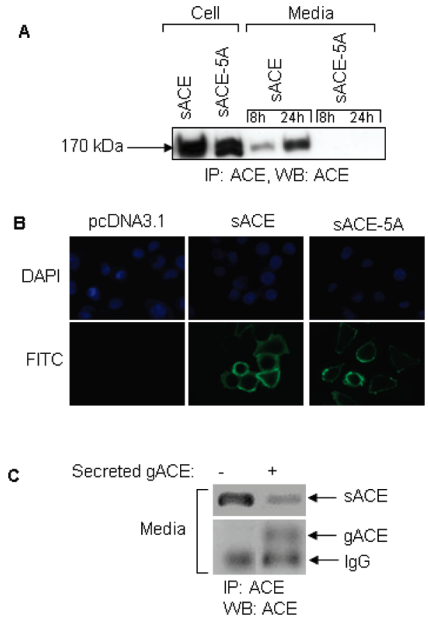

FIGURE 5: Alanine substitutions in sACE inhibited ACE secretase. (A) HeLa cells were transiently transfected with sACE or sACE-5A. Cells (C) and culture media (M) (after 8 and 24 h) were immunoprecipitated with anti-ACE and immunoblotted with anti-ACE. (B) HeLa cells were transfected with empty vector (pcDNA3.1) or sACE, sACE-5A on coverslips, after transfection cells were fixed with paraformaldehyde and stained with anti-ACE antibody. (C) ACE/CD4 chimera (ACE1−655/CD4) was transiently expressed in HeLa cells, after transfection, culture medium containing the secreted form of gACE/CD4 (+) or from an untransfected HeLa cells (−) were collected and used to treat HeLa cells expressing sACE for 48 h. Media were collected and immunoprecipitated with anti-ACE and immunoblotted with anti-ACE.

between the cell bound and secreted ACE is critical for maintaining normal functions of ACE. Our previous studies have demonstrated that ACE shedding is a regulated cellular event; this can be activated by activators of protein kinase C (*30*), inhibitors of tyrosine phosphatases (*25*) and inhibitors of calmodulin (*27*) and inhibited by metalloprotease inhibitors (*29*). Several cellular proteins including calmodulin, specific PKC isoforms, BiP, are associated with ACE molecule and these interactions regulate the ACE shedding process (*26*).

The secretase that is responsible for cleaving ACE has not yet been identified. Previous studies have established that ACE secretase is a membrane-bound metalloprotease (*34*), and its activity was not dependent on the membrane localization of ACE protein in the lipid rafts (*47*). ACE secretase activity can be inhibited by hydroxamate-based compounds (e.g., Compound 3, in this study) which can inhibit a variety of other metalloproteases (*35*). Unless a specific ACE secretase is identified, it is difficult to rule out the possibility that multiple secretases could be involved in ACE cleavage. Moreover, unlike several other secretases, the ACE-secretase does not require specific amino acid residues at or around the cleavage site (*36, 37, 39*). Instead, the site is at a fixed distance from the transmembrane domain and the cleavage is dictated by the distal ectodomain of the protein. These principles were established by expressing a chimeric protein containing the ACE distal ectodomain attached to CD4, an uncleaved ectoprotein; the chimeric protein was cleaved in the CD4 membrane-proximal region by the ACE-secretase.

As mentioned above, the action of the ACE-secretase on its ectoprotein substrate is determined by the presence of a specific sequence of amino acids at the cleavage site. In our attempts to narrow down the region of ACE that is responsible for triggering the secretase, we have used as substrates fusion proteins of ACE and CD4 containing either N-terminal or C-terminal region of ACE. Systematic analysis of such chimeric proteins as potential substrates of ACE-secretase was hampered by their faulty transport to the plasma membrane. In mammalian cells, after synthesis many of these artificial chimeric proteins were arrested at internal membranes of the endoplasmic reticulum or golgi and did not reach the cell surface; consequently, their suitability as substrates for ACE-secretase could not be evaluated. This was possibly because the deletion mutants were not properly folded and degraded before reaching the cell surface. To circumvent this problem, we tested some of these proteins in *P. pastoris*, which expresses a secretase with characteristics similar to ACE-secretase. Because yeast and mammalian cells can transport membrane proteins differently, we were more successful in expressing several chimeric proteins on the surface of yeast and determine their potential as substrates of the secretase. This series of experiments led to the identification of a region containing 15 residues that was essential for the recognition of the protein by the secretase. This mutant and its derivatives were subsequently tested in

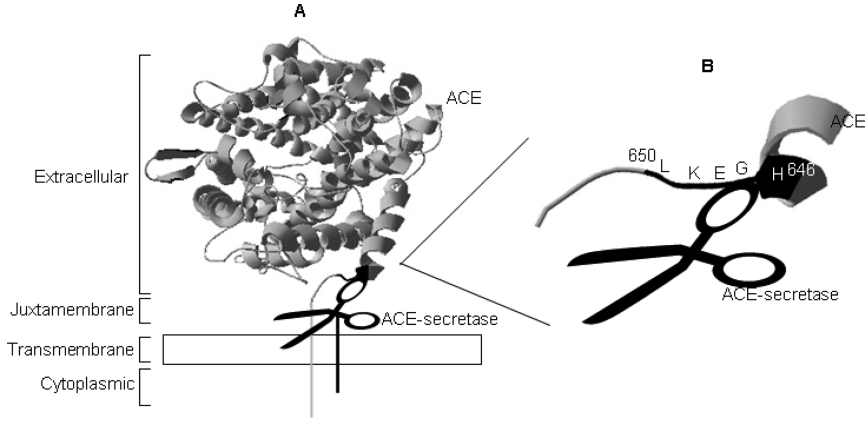

FIGURE 6: ACE-secretase activation model. (A) Crystal structure of gACE and its various domains are shown with the ACE-secretase activation domain indicated in black. Putative ACE-secretase is shown as a transmembrane protein. (B) A closer view of the ACE-secretase activation domain containing the critical amino acid residues.

mammalian cells, and the essential region was narrowed down to only five residues, whose substitution by Ala made the protein uncleavable by the secretase. This region is located 17 residues away on the N-terminal side of the major cleavage site on ACE extracellular domain. The residues in this region are conserved in rabbit and human ACE and have substantial sequence homology in mouse and rat ACE. Four of the five amino acid residues are present in an unstructured region of gACE ectodomain whose structure is known (Figure 6 and ref *49*). The fifth residue is at the end of an α-helix; the alanine substitutions in the mutant protein should extend this α-helix further by including the four residues that are in the unstructured region of the wild type protein. The mutant protein had no gross structural abnormality as indicated by the fact that it was transported to the cell surface and enzymatically active. Hence, it is likely that the local change in structure induced by the alanine substitutions was strong enough to alter the recognition of ACE-5A by the secretase, suggesting that this region of ACE comes in direct contact with the secretase (Figure 6). Structure of the full-length ACE is not yet known, and therefore, it is possible that the structure in this region may play a substantial role in activating the ACE secretase.

Most of our experiments used gACE as the substrate which contained the secretase-activation domain as well as the cleavage site. We chose to use gACE for the biochemical studies because this is a relatively smaller protein and, therefore, it is easier to perform biochemical manipulations, and also the smaller isoform has been found to be cleaved more efficiently by the ACE secretase as compared to the larger isoform (sACE). However, we confirmed that the basic conclusion was also true for sACE, a much larger protein. Our experiments showed that the same residues present in the region shared between sACE and gACE were responsible for sACE cleavage as well. This observation is consistent with those made by others; sACE missing the C-terminal half, that is equivalent to gACE, was displayed on the cell surface, but not cleaved (*39*). Moreover, the N-terminal sACE specific domain was shown to negatively regulate ACE cleavage-secretion (*44*). Substitution of specific residues in the juxtamembrane domain of ACE has been shown to enhance its cleavage, possibly by additional secretase (*38*). In contrast, our 5A substitution mutant was not cleaved by ACE secretase or other secretases. Thus, it is safe to conclude that these residues are required for ACE cleavage-secretion.

For other secretases, the substrate specificity is determined by the amino acid sequences at and around the cleavage site. For p75 neurotrophin receptor, a domain containing 15 residues has been identified as the target for α- and γ-secretases (*50*). Similarly, for TGFα and β-APP, a domain containing 14 residues in membrane proximal region determined recognition by the secretase (*51*). Our studies, presented here, demonstrate that the ACE protein contains a region containing five residues that dictates recognition by the ACE-secretase; however, unlike other secretases the ACE-secretase does not cleave a peptide bond in this region.

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