Establishment of Transfected Cell Lines Producing Testicular Angiotensin-converting Enzyme

STRUCTURAL RELATIONSHIP BETWEEN ITS SECRETED AND CELLULAR FORMS*

Indira Sen‡‡, Himadri Samanta‡‡‡, William Livingston III**, and Ganesh C. Sen**

From the Departments of ‡Heart and Hypertension and **Molecular Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195 and ‡‡‡Eugene Tech, Allendale, New Jersey 07401

Angiotensin-converting enzyme (ACE) is present in endothelial and epithelial cells of various tissues as well as in the circulating plasma. The structural relationship between the cellular and the secreted forms of ACE and the pathways to their biosynthesis have not been determined as yet mainly because of the unavailability of a natural cell line expressing ACE in tissue culture. To circumvent this problem we have permanently transfected a mouse epithelial line with an expression vector containing the recently cloned rabbit testicular ACE cDNA. Clonal derivatives of this line secreted large quantities of enzymatically active ACE.

When these cells were cultured in serum-free medium, the only detectable protein in the culture medium was ACE. It has been suggested that a hydrophobic domain near the carboxyl terminus of the enzyme anchors it to the plasma membrane. To test this hypothesis we established cell lines expressing a truncated form of the active enzyme which is missing the putative anchoring domain. Pulse-chase experiments showed that the truncated ACE was secreted from the cells much faster than the native enzyme. Moreover, the secreted form of the native enzyme had a lower molecular weight than the corresponding cellular form. These results are consistent with the hypothesis that the hydrophobic domain is instrumental in keeping the enzyme cell-bound, and secretion is achieved physiologically by removal of this domain from the enzyme by a specific proteolytic cleavage.

* This work was supported in part by Grants HL-36667 and HD-22526 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Heart and Hypertension Research, FFb-29, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195.

‡‡‡ Current address: Bristol-Myers Squibb, Wallingford, CT 06492.

The abbreviations used are: ACE, angiotensin-converting enzyme; SDS, sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURES

Establishment of Transfected Cell Lines—Mouse C127 cells were transfected with an expression vector containing the rabbit testicular ACE cDNA. For expressing the entire protein, an EcoRI (nucleotide 1) to SacI (nucleotide 2272) fragment of ACEs (7) was used. For expressing a truncated protein an EcoRI to EagI (nucleotide 2022) fragment was cloned. These fragments were ligated to a 238-base pair fragment of SV40 DNA (nucleotides 2533–2770) which provided the polyadenylation signal. The ligated fragments were cloned at the XhoI site of the expression vector pBMT-X. This vector contains bovine papilloma viral sequences, bacterial replication origin, the ampicillin resistance gene, the human metallothionein 1a, gene and the mouse metallothionein 1 gene (15). The Xho cloning site is at the +20 position of the mouse metallothionein transcription.
unit. The cloned ACE transcripts would therefore initiate at −20 and terminate at the SV40 polyadenylation site. Moreover, the cloned genes will be regulated by the mouse MT1 promoter and thus be inducible by heavy metals. The same agents will also induce the synthesis of human metallothionein protein from the other transcription initiation and termination sites as a selection criterion. Translation initiation and termination sites were present within the cloned ACE cDNA, giving rise to a protein of 737 amino acids. From the truncated ACE cDNA, 64 amino acids from the carboxyl terminus of the protein as well as the termination codon had been removed. However, the chimeric gene construction was done in such a way that an in-frame termination codon was provided by the SV40 sequence giving rise to a protein containing 673 amino acids of ACE, plus 7 extra amino acids at the carboxyl terminus.

DNA was transfected into C127 cells by the calcium phosphate precipitation method (16). Transfected cells were selected in the presence of 10 μM CdCl2. Individual colonies were isolated and expanded. The cells were always cultured in the presence of 10 μM CdCl2.

To culture the cells in serum-free media, cells were washed three times with phosphate-buffered saline and incubated at 37 °C in Dulbecco’s modified Eagle’s medium containing 10 μM CdCl2. After 30 min the medium was discarded to remove any remaining serum, and the cells were cultured in fresh media without serum. To purify secreted ACE, the ACE89 cultures were expanded and grown in roller bottles. The culture media from four bottles were collected and each media filtered through a 0.2-μm nonsterile filter. The filtrate was divided into two aliquots; one was treated with 1 unit of neuraminidase (Sigma) for 1 h at 37 °C and the other was mock treated. Both were then acidified to pH 6 with sodium acetate and sequentially digested or mock digested with 1 unit of neuraminidase and 200 units of trypsin (Sigma) for 1 h at 37 °C. The samples were dried, dissolved in sample buffer, and subjected to gel filtration through a column of Sephacryl S-300 (Pharmacia). The ACE activity was analyzed by electrophoresis on 7.5% polyacrylamide gels in the presence of 0.1% SDS and analyzed as described for cell lysates.

Enzyme Activity Measurements—ACE enzyme activity was determined using hippuryl-l-histidyl-l-leucine (Hip-His-Leu) as substrate and measuring fluorometrically the His-Leu liberated under standard conditions of 5 mM Hip-His-Leu in 0.4 M sodium borate buffer, pH 8.3, and 0.3 M NaCl for 15 min at 37 °C (18). For the determination of Km and Vmax, ACE purified from the culture media was incubated under similar conditions but with varying concentration of Hip-His-Leu (100 μM to 250 mM). The results were plotted according to the method of Lineweaver and Burke. Protein was measured according to the method of Lowry et al. (19).

RESULTS

Establishment of ACE-producing Cell Lines—Cloned rabbit testicular ACE cDNA was inserted into the expression vector shown in Fig. 1. This vector contained a bovine papilloma virus autosomal replication origin which enabled it to replicate to high copy numbers in transfected cells. The ACE cDNA was inserted into a transcription unit containing mouse metallothionein transcription initiation and termination signals as well as an intron. The vector also carried a human metallothionein gene whose product helped the transfectant grow in the presence of Cd2+. Mouse C127 cells were transfected with this expression vector, and permanent transfec-
tants were selected in the presence of CdCl2. Seven independent clonal derivatives of the transfec-
tants were isolated and tested for ACE mRNA expression (Fig. 2A). All clones expressed variable but high quantities of ACE mRNA. As expected, ACE mRNA was larger than the in vitro ACE88 transcript (2,534 base pairs) which lacked the poly(A) tail. The inclusion of known quantities of the in vitro transcripts in the Northern blot provided us with a rough quantitative estimate of the level of ACE mRNA in different transfec-
tants. Clones ACE88 and ACE89 samples contained about 10 ng of
Angiotensin-converting Enzyme Secretion

Fig. 1. Expression vector for rabbit testicular ACE. SV40 polyadenylation signal (hatched bars) was provided at the 3' end of the ACE cDNA, and the chimeric DNA was inserted at the XhoI site of the expression plasmid pBMT3X. mMT, mouse metallothionein I gene; hMT, human metallothionein Ia gene; BPV, complete bovine papilloma virus genome; Amp', ampicillin resistance gene.

Fig. 2. Expression of ACE mRNA and enzymatically active protein in transfected cells. Seven transfected clones were examined for ACE mRNA expression (panel A) by Northern analysis, and three of them were examined for ACE protein expression (panel B) by Western analysis. Panel A: lane 1, 1 ng of in vitro ACE mRNA transcript; lane 2; 10 ng of in vitro transcript; lanes 3–9, 20 μg of total cytoplasmic RNA from clones ACE81, 82, 85, 86, 88, 89, and 810 transfected with the complete ACE construct. The blots were hybridized with an antisense ACE riboprobe. Panel B, Western analysis of cell culture medium. 10 μl of culture medium was analyzed in each lane. Lane 1; C127 cells; lane 2, ACE89; lane 5, ACE82; lane 4, ACE810; lanes 5 and 6, 50 and 10 ng of pure rabbit pulmonary ACE (140 kDa). Positions of molecular weight markers are shown on the left. Panel C, culture media of C127, ACE89, ACE82, and ACE810 cells were tested for ACE enzyme activity. These media were collected after overnight incubation of 10 ml of medium/100-mm confluent plates. The relative levels of enzyme activities are presented in the bar graph. C127 medium had less than 0.2% of enzyme activity present in the medium of ACE89 cells. Several dilutions of media were assayed to ensure a linear range of measurement. The enzyme activity present in 1 ml of culture medium of ACE89 cells could cleave 1,800 nmol of substrate/min.

ACE mRNAs in 20 μg of total cytoplasmic RNA isolated from three million cells, which amounts to about 5% of total poly(A)+ RNA being ACE mRNA assuming that 1% of total RNA is poly(A)+. Several of these clones were tested for ACE production in the medium by Western blotting, three of which are represented in Fig. 2B. All of them secreted large quantities of ACE protein in the medium. The protein bands were diffused, suggesting that the protein could be glycosylated. Large amounts of enzymatically active ACE were secreted by these clones (Fig. 2C) whereas untransfected C127 cells did not produce any detectable ACE activity. CdCl₃ present in the culture medium did not have any effect on ACE enzyme activity measurements as judged by adding 10 μM CdCl₃ to an assay mixture containing pure natural ACE (data not shown).

Characteristics of ACE Produced by a Transfected Line—The ACE89 cell line was selected for further characterization of transfected ACE. For this purpose, cells were labeled with [³⁵S]methionine, both the cell extracts and culture medium were immunoprecipitated with ACE antibody, and the immunoprecipitates were analyzed by gel electrophoresis. The secreted form of ACE had a lower molecular mass (approximately 106 kDa) than the cellular mature ACE (approximately 116 kDa) (Fig. 3A). Both cellular and secreted forms of ACE were heavily glycosylated as evidenced by the considerable change in their apparent molecular weights upon deglycosylation (Fig. 3C). The deglycosylation was achieved by sequential treatment of the immunoprecipitated proteins with N-glycosidase F, neuraminidase, and O-glycosidase. Such a treatment removes both N-linked and O-linked sugars from a glycoprotein. The deglycosylated cellular form of ACE had the same electrophoretic mobility (lane 3, Fig. 3C) as newly synthesized ACE (lane 1, Fig. 3C), which is the precursor of mature cellular ACE (see Fig. 8).

The observed difference in the apparent molecular weights of the secreted and the cellular forms of ACE could not be attributed to a difference in their degrees of glycosylation. Upon complete deglycosylation, the secreted form still had a slightly lower molecular weight than the corresponding deglycosylated cellular form (compare lanes 3 and 5, Fig. 3C). This observation suggested that the polypeptide chain of secreted ACE might be shorter than that of cellular ACE and that the secretion process may involve a proteolytic processing step. This putative proteolytic processing could be mediated by either a cellular protease or an activity present in the serum.
added to the culture medium. To address this issue, ACE89 cells were cultured in a medium free of serum, and ACE production by cells cultured in the presence or absence of serum was monitored. As shown in Fig. 3B, cellular ACE was processed to the secreted form efficiently even in the absence of serum in the culture medium. This observation suggests that the processing activity was provided by the cells rather than the medium. Since ACE89 cells could be cultured for at least 36 h without serum in the medium and they secreted active ACE, we wondered whether the only protein present in its culture medium was ACE. For this purpose, cells grown in serum-free medium were labeled with [35S]methionine, and a portion of the culture medium was analyzed by gel electrophoresis without immunoprecipitation. As shown in Fig. 4, lane 1, the only radiolabeled protein present in the medium had the same electrophoretic mobility as ACE. Medium from a parallel culture, which had not been labeled with [35S]methionine, was analyzed by staining with Coomassie Brilliant Blue (Fig. 4, lane 2). ACE was the predominant, if not the exclusive, protein present in the culture medium. These results suggested that almost pure ACE can be harvested from this medium assuming that a secreted contaminant did not comigrate with ACE in this electrophoretic system. The enzymatic properties of secreted ACE were determined by its ability to hydrolyze Hip-His-Leu. As earlier kinetic studies have shown that chloride ions are activators of ACE activity (3), the hydrolysis of Hip-His-Leu by the purified enzyme was analyzed in presence of 0–1 M NaCl. The enzyme activity is undetectable in the absence of added NaCl, reaches a maximum at 150 mM, and decreases by 20% at 0.9 M NaCl (data not shown). Hence 300 mM NaCl was used for all subsequent experiments. Hydrolysis of Hip-His-Leu by ACE at varying concentrations of the substrate is shown in Fig. 5, plotted according to the method of Lineweaver and Burke. The enzyme exhibited a $K_m$ of 1.74 ± 0.1 × 10^−3 M and a $V_{max}$ of 85.5 ± 8.7 μmol of His-Leu liberated/min/mg of protein at 37 °C. The apparent turnover number, $K_{cat}$, based on a molecular mass of 106 kDa, is 9,096 mol/min/mol of enzyme. The requirement of chloride ions as well as the $K_m$ of purified secreted ACE is in reasonable accord with the value reported for purified testicular enzyme ($K_m$, 2.6 mM (3)). The $K_{cat}$, on the other hand, is only 50% of the reported value (18,500 mol/min/mol (3)) even though the enzyme was electrophoretically homogeneous. This could reflect a homogeneous population of molecules with impaired catalytic activity or a heterogeneous preparation containing a comigrating contaminant, or a mixture of both active and inactive species of ACE. The latter interpretation is favored because the culture medium for the purification of ACE was collected over a period of 20 h, and it is possible that this long exposure of the secreted

**Fig. 4.** Secreted ACE from ACE89 cells grown in serum-free medium. Lane 1, 2 ml of the culture media from ACE89 cells grown under serum-free conditions and labeled with [35S]methionine as described in the legend of Fig. 3B, was lyophylized and analyzed directly by SDS-PAGE and autoradiography without immunoprecipitation. Lane 2, medium from a parallel culture, which was not labeled, was analyzed by staining with Coomassie Brilliant Blue.

**Fig. 5.** Catalytic activity of secreted ACE. Purified secreted ACE (15 ng) was assayed as described under “Experimental Procedures” with varying levels of Hip-His-Leu. $K_m$ and $V_{max}$, as determined from a double-reciprocal Lineweaver-Burke plot, was 1.8 mM and 85.5 μmol/min/mg.

**Fig. 6.** Expression of a truncated ACE mRNA (EACE) and protein in transfected cells. Panel A, the arrow indicates the position of truncation on the sequence of ACE. Eight transfected clones were examined for EACE mRNA expression (B) by Northern analysis, and three of them were examined for EACE protein expression (C) by Western analysis. Panel B: lane 1, 10 ng of in vitro transcript; lanes 2–9, 20 μg of total cytoplasmic RNA from clones EACE81, 82, 84, 85, 86, 88, 89, and 810. The blots were hybridized with an antisense ACE riboprobe. Panel C, Western analysis of cell culture medium 10 μl of culture medium was analyzed in each lane. Lane 1, C127 cells; lane 2, ACE89; lane 3, EACE81; lane 4, EACE810.

ACE to 37 °C causes partial inactivation.

**Role of the Putative Membrane Anchoring Domain**—A strongly hydrophobic region present near the carboxyl terminus of ACE has been suggested to be responsible for anchoring the protein in the plasma membrane (21). For the purpose of testing this hypothesis, we constructed an ACE cDNA clone (EACE) which would encode a truncated ACE missing the carboxyl-terminal 63 residues including the hydrophobic region (Fig. 6A). C127 cells were transfected with an expression vector containing EACE. Several clones of these transfectants were analyzed. Eight such clones produced high levels of EACE mRNA (Fig. 6B), and all three tested clones secreted ACE protein in the culture medium (Fig. 6C). The secreted EACE had enzyme activity similar to secreted ACE (data not shown).

Once we established that native and truncated forms of enzymatically active ACE are secreted by the transfected cells, we studied the routes of their biosynthesis. For this purpose, cells were labeled with [35S]methionine; both cell extracts and culture media were immunoprecipitated with ACE antibody, and the immunoprecipitates were analyzed by gel electrophoresis. In ACE89 cells, 30-min labeling produced almost exclu-
sively a broad band of approximately 86 kDa (Fig. 7A, lane 1). Longer labeling periods resulted in appearance of another broad band of approximately 116 kDa. The upper band represented the glycosylated molecules whereas the lower band was of the unglycosylated form. This conclusion is supported by the fact that complete deglycosylation of the upper band changed its electrophoretic mobility to that of the lower band (see Fig. 3C, lanes 1–3). It is not clear why the lower band was heterogeneous. A shorter exposure of the autoradiogram revealed at least three distinct bands in this region. They may represent various intermediates in the maturation of the protein which, in addition to glycosylation, involves removal of the signal peptide, and may also involve other as yet unidentified post-transcriptional modifications such as phosphorylation. In the medium, only the glycosylated form was secreted (Fig. 7A, lanes 7 and 8). Moreover, long labeling periods (2–3 h) were needed before the secreted form appeared in the medium. In contrast, the truncated EACE was secreted very efficiently from the cell (Fig. 7B). Unglycosylated EACE had an approximate molecular weight of 76,000. The majority of glycosylated EACE (approximately 110 kDa) was secreted in the medium whereas most of glycosylated ACE stayed in the cell (Fig. 7, A and B). As shown above in Fig. 3A, secreted ACE had a lower molecular weight than cellular ACE whereas secreted and cellular glycosylated EACE had similar electrophoretic mobilities (Fig. 7 and data not shown).

The experiment shown in Fig. 8 clearly demonstrated that EACE is secreted from the cells much faster than ACE. Cells were pulse labeled for 30 min and then chased with unlabeled medium for increasing lengths of time. This allowed us to monitor the fate of the newly synthesized ACE and EACE. Newly synthesized unglycosylated molecules were slowly glycosylated, and this process was virtually complete by 4 h (Fig. 8, A and B, lane 5). Glycosylated ACE was partially released in the medium. Even 8 h after labeling, glycosylated ACE was equally distributed between the cell and the medium (Fig. 8A, lanes 6, 12). In contrast, hardly any glycosylated EACE remained in the cell. EACE appeared in the medium 30 min after labeling (Fig. 8B, lane 8) and by 4 h all EACE was secreted out.

**DISCUSSION**

Establishment of permanent cell lines, which continuously synthesized ACE, was needed for studying its biosynthesis. Natural vascular endothelial cells that produce ACE in cultures lose this capacity with cell passages (13, 14). Moreover, even at an early passage, the quantity of ACE production in culture is very low. Our transfected cells are of mammary epithelial lineage. However, like vascular endothelial and renal epithelial cells, they were able to synthesize, process, and secrete enzymatically active ACE. We used the testicular ACE cDNA for establishing these lines, but it is safe to conclude that they will also produce and process the pulmonary form upon transfection of the requisite cDNA. The carboxyl-terminal halves of both forms are identical, and the putative membrane anchoring domain resides in this half of the molecule.

The experiments reported here provided strong support for the hypothesis that the hydrophobic domain near the carboxyl terminus of ACE is indeed a membrane anchoring domain which keeps the enzyme cell-bound (7, 8). As shown here, its artificial removal resulted in total and fast secretion of ACE into the medium. Such truncated ACE also had enzymatic activity, indicating that the carboxyl-terminal region of the native enzyme is not needed for enzyme activity. The exact length of the anchoring domain remains to be defined. The point of truncation in the EACE construct used in the studies reported here was chosen for experimental convenience. Since it encoded a nonanchored protein with enzyme activity, we have in hand the starting point for designing the next generation of deletion and substitution mutations for narrowing down the anchoring domain.

It was interesting that secreted ACE had a molecular weight lower than that of cellular ACE. This difference in molecular weight was not eliminated by deglycosylation of the proteins (Fig. 3C), suggesting a difference in the lengths of their polypeptide chains. It is conceivable that secretion was achieved by proteolytic removal of the anchoring domain of the native enzyme. Indeed release of cellular ACE by an endogenous “ACE-solubilizing” activity has been reported in the literature (21). Involvement of a proteolytic process in ACE secretion is also supported by the evidence presented in two recent papers (22, 23) that were published after completion of the work being presented here. Wei et al. (22) reported that an antisera raised against a synthetic peptide corresponding to a sequence present at the carboxyl terminus of human pulmonary ACE recognizes cellular ACE in a transfected cell line, but it does not recognize secreted ACE, thereby suggesting an absence of the corresponding region in the latter form. This antibody also failed to recognize ACE present in human plasma, thus providing physiological relevance to the cleavage secretion process. The exact point of cleavage, however, remains to be determined. The specific orientation of ACE on the cell surface was also revealed by...
their antibody experiments. An antibody to the whole enzyme recognized cellular ACE present on the surface of intact cells whereas the carboxyl-terminal-specific antibody could do so only after cell disruption. These results strongly support the view that the region between the anchoring domain and the carboxyl terminus of ACE is cytoplasmic whereas the bulk of the molecule is on the outside cell surface.

The fact that cleavage processing in our system was not affected by culturing the cells in a serum-free medium (Fig. 3B) suggests that the responsible protease is a cellular one, possibly a resident of the plasma membrane. Such specific proteolytic processing of membrane-bound glycoproteins is well documented for many viral envelope proteins. The regulated release of ACE by a plasma membrane-bound protease may have profound physiological significance since ACE activity is present in circulation as well as in tissues. It is known that endothelial and epithelial cells of different tissues can synthesize ACE (1). In principle, the relative abundance of the putative protease in these tissues may determine the extracellular fate of locally synthesized ACE. One can imagine that cells of a particular organ, such as the blood vessels, may produce mostly secreted ACE for the functioning of the circulatory renin-angiotensin system whereas cells in the kidney may produce mostly cellular ACE, thus keeping the system extremely localized. We anticipate that we will now be able to analyze these processes further using the transfected cells that appear to synthesize and process this protein in a physiological fashion. Using our in vitro system, it will be possible to study the responsible protease. Such a protease may have a broad range of substrates whose functions it may regulate by controlling the degree of their secretion.

Our transfected cell lines synthesized and secreted large quantities of ACE. We could harvest 0.5 mg of pure ACE in a day from the culture medium of a confluent roller bottle. Since the cells could be temporarily maintained in a serum-free medium, it was relatively easy to obtain pure ACE. Enough pure ACE, especially the secreted form, can probably be harvested to attempt its crystallization. The transfected cells will also provide a convenient system for radiolabeling ACE as well as for producing the protein without secondary modification such as glycosylation.

Acknowledgements—We are grateful to Richard L. Soffer for providing the ACE antiserum and to Donald W. Jacobsen for advice on ACE enzyme assays. We thank Margaret Leet and Patricia Martin for secretarial assistance.

REFERENCES