Mouse Angiotensin-converting Enzyme Is a Protein Composed of Two Homologous Domains*

(Received for publication, December 22, 1988)

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Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase that converts angiotensin I into the potent vasoconstrictor angiotensin II. We have used cDNA and genomic sequences to assemble a composite cDNA, ACE.315, encoding the entire amino acid sequence of mouse converting enzyme. ACE.315 contains 4838 base pairs and encodes a protein of 1278 amino acids (147.4 kDa) after removal of a 34-amino acid signal peptide. Within the protein, there are two large areas of homologous sequence, each containing a potential Zn-binding region and catalytic site. These homologous regions are approximately half the size of the whole ACE protein and suggest that the main ACE gene is the duplicated product of a precursor gene. Mouse ACE is 83% homologous to human ACE in both nucleic acid and amino acid sequence, and like human ACE, contains a hydrophobic region in the carboxyl terminus that probably anchors the enzyme to the cell membrane (Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., and Corvol, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9386-9390). Northern analysis of mouse kidney, lung, and testis mRNA demonstrates that the testicular isozyme of ACE is encoded by a single, smaller RNA (250 bases) rather than the two message sizes found in kidney or lung (4800 and 4150 bases), and that this testicular RNA hybridizes to the 3' portion of ACE.315.

Angiotensin-converting enzyme (ACE) is a Zn (II) containing dipeptidyl carboxypeptidase that converts angiotensin I into the potent vasoconstrictor angiotensin II (1-3). ACE is also capable of cleaving many other small peptides, an example being the vasodilator bradykinin which is converted into an inactive fragment (4). While renin and other components of the renin-angiotensin system have been the subject of numerous studies, the biochemistry of ACE is less understood, and such basic questions as its amino acid structure and 18 bp, base pairs.

* This work was supported in part by National Institutes of Health Grants DK39777, DK38834, and BRSO S07 RR05364, Biomedical Research Support Grant Program. 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.

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§ The abbreviations used are: ACE, angiotensin-converting enzyme; bp, base pairs.

‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04946 and J04947.

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sequence of human ACE and while the sequences of mouse and human ACE are highly homologous, the two regions in each molecule containing putative catalytic sites are the most highly conserved.

MATERIALS AND METHODS

mRNA was prepared from the kidneys of male NIH Swiss mice and selected by oligo(dT) chromotography (17). Mouse CD1 lung, testis, and kidney mRNA was also purchased from Clontech Laboratories, Inc., Palo Alto, CA. The construction of a mouse kidney cDNA library, the screening of this library with ACE-specific oligonucleotide probes, the subcloning of double-stranded cDNA into the EcoRI site of Bluescript (+) (Stratagene, San Diego, CA), and the rescue and sequencing of single-stranded DNA have been described (15). During the construction of cDNA, internal EcoRI sites were not protected by methylation resulting in the separate cloning of sequences on either side of internal EcoRI sites (GAATTTC). ACE.11 was completely sequenced on both strands using a combination of three approaches, subcloning EcoRI restriction fragments of ACE.11 into the Smal site of mp10 M13, priming single-stranded DNA with complementary ACE oligonucleotides and unidirectional progressive deletions using ExoIII nuclease (Erase-a-Base, Promega (18). The unidirectional progressive deletions were prepared by cutting both orientations of ACE.11 in Bluescript with XhoI followed by end repair with α-phosphorothioate deoxyribonucleotides. The DNA was digested with BamHI, phenol extracted, and then digested with EcoIII and recloned as described in the protocols of the Erase-a-Base kit. The sequence of ACE.5 was determined by priming single-stranded DNA with specific oligonucleotides. Where sequence was identical to that of ACE.5 with the exception of one nucleotide at position 706, the 3' Utr of ACE.11 is identical to that of ACE.5 for 187 bases (indicated by *). The 3' Utr of ACE.5 and ACE.11 is indicated. G4.3200 is a cloned portion of mouse genomic DNA. The position of introns is indicated by the open circle (C) while exons are indicated by the bold line. Analysis of this sequence indicates that ACE.31 and ACE.5 encode contiguous RNA sequence. This is indicated as ACE.31. The position of exons encoded by G4.3200 is indicated beneath ACE.31. No sequence discrepancies were noted between ACE.315 and the genomic sequence.

RESULTS

In a screening of 70,000 primary plaques of a mouse kidney cDNA library, three oligonucleotide probes, 37.21, 43.9, and 50.53, identified 26 plaques referred to as ACE group 1 cDNA (15). Nine cloned cDNA ranged in size from 2900 to 3800 bp. One additional cDNA, ACE.11, was 4700 nucleotides. cDNA ACE.5 is 3820 nucleotides. It contains one large open reading frame beginning at the 5' EcoRI site and encoding 980 amino acids. ACE.31 is not described (Figs. 1 and 2). The DNA sequence of ACE.11 has also been determined and with the exception of one nucleotide at position 706 (GCC-Ala in ACE.11, ACC-Thr in ACE.5), is identical to that of ACE.5 for the 5' first 3130 nucleotides. This encompasses 2943 nucleotides of coding sequence and 187 nucleotides of 3' untranslated region (Figs. 1 and 2). Further, 3' these two cDNA are completely divergent with ACE.5 encoding a total of 573 nucleotides of 3' Utr and ACE.11 encoding 1781 nucleotides of 3' Utr (Fig. 2).

The cDNA library used in these studies was such that internal EcoRI sites were cleaved during library construction. Thus, we asked if ACE.31 and ACE.5 were contiguous within genomic DNA. A genomic library was probed with ACE.31 and an oligonucleotide from the 5' end of ACE.5. One clone called G4 was purified, and a 3200-bp PstI fragment was subcloned and sequenced (Fig. 3). This demonstrates that the EcoRI sites at the 3' end of ACE.31 and the 5' end of ACE.5 are in fact a single restriction site within mouse genomic DNA (Fig. 3). Further evidence that ACE.31 and ACE.5 encode contiguous mRNA sequence is found in the amino acid sequence of the ACE tryptic peptide 40.1 (15). The amino terminus of this peptide, VSEEFFTSLGLSP,% is exactly the sequence found by abutting and translating ACE.31 and ACE.5 with the EcoRI site encoding Glu and Phe. We refer to the combined sequences of ACE.31 and ACE.5 as ACE.315.

This sequence is 4836 bp and encodes a protein of 150.9 kDa composed of 1512 amino acids (Fig. 4). The mature protein without a signal peptide is predicted to contain 1278 amino acids with a molecular mass of 147.4 kDa. ACE.315 encodes

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The table and diagram are not transcribed due to the nature of the content. For the table and diagram, please refer to the original document. 

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% In an earlier publication (Bernstein, K. E., Martin, B. M., Bernstein, E. A., Linton, J., Striker, L., and Striker, G. (1988) J. Biol. Chem. 263, 11021–11024) this sequence was incorrectly published as VSEEFTSLGLPS.
Finally, ACE-specific oligonucleotides were cated by exception of position are indicated by an into the EcoRI site of the vector Bluescript (KS) in both orientations. Deletional clones were prepared as described under "Materials and Methods." Potential sites of N-glycosylation are indicated in (*).

C

\[ \text{DNA Sequence of Angiotensin-converting Enzyme} \]

A

\[ \text{CAGACGCCCAAGGGCTACACCATGTGTGACATCTACCAGTCGCCCAGGCGGGGGCC} \]

B

\[ \text{AACCGGAAGGACTTCCGGATTAAGCAATGCACACACGGGTACGATGGAACAGCTGGCCACA} \]

\[ \text{CTGTTCTCAGCCATCCTGACCTCTTTGCTGTCCCCATCCACGCCCTGCTCGTGGCCACCGTGGGTCTCGCCCATCGGCTCTACMCATCCGTMCCATCAC} \]

\[ \text{GCCCTGCTCGTGGCCACCGTGGGTCTCGCCCATCGGCTCTACMCATCCGTMCCATCAC} \]

\[ \text{GCCCTGCTCGTGGCCACCGTGGGTCTCGCCCATCGGCTCTACMCATCCGTMCCATCAC} \]

\[ \text{TGTGACCTCTCTGTGGCTGGGTGTCGGAGGMGGCTCTGGATGCCCTTCCCTGCTACA} \]

\[ \text{GTGTCCCTTGTGGGMGCCAGGGACAGGACCGDCTDCTTOC} \]

\[ \text{CAGGAGTGGTGGAGCCTCAGGCTGMGTA~CAGGG~CTG~CCCCCCAGTGCCMGATC~} \]

\[ \text{CTCATCGACCAGTGGCGCTGGGG~C~TGAT~MGCATCAC~GGAGMCTATMC} \]

\[ \text{CCGACTTCCCTGCCCAGTCTC~~MTACMTT~~TCCTCCCCCACACCTGAGTCT} \]

\[ \text{CTGTACCTGGAGCCACAGCAGGCCCGCGTGGGCCAGTGGGTGCTGCTCTTCCTG~CGTC} \]

\[ \text{E~CANPCPHEAIGDIIALSVS} \]

\[ \text{LIDQWRRVFDFGITEKKNR} \]

\[ \text{LLEPQDARYKLNPE} \]

\[ \text{N*E T} \]

Fig. 2—continued

amino acid sequence identical or highly homologous to each of nine ACE tryptic peptides previously reported (Fig. 4) (15). The method of Kyte and Doolittle (23) was used to analyze hydrophobic-hydrophilic regions of the protein encoded by ACE.315. Both the signal peptide region and the carboxyl terminus encode highly hydrophobic regions (Fig. 5). The hydrophobic carboxyl domain is followed by a highly hydrophilic region; it is probably here that ACE, a known membrane protein, is anchored to the cell membrane. Similar results were found in the study of human ACE (16).

Fig. 6 A and B are dot matrix plots of the nucleic acid and the predicted amino acid sequence of ACE.315 compared against itself. These show that ACE.315 encodes a protein with two large regions of internal homology, each approximately half the size of the mature ACE protein (the signal sequence is not homologous to other sequences within ACE.315). When a Needleman and Wunsch algorithm (24) (Genepro) is used to align ACE.315 amino acids 47–610 with amino acids 650–1208, 300 amino acids are identical and an additional 47 are conservative substitutions (leucine-isoleucine, glycine-alanine etc.). Thus, mouse ACE is composed of two homologous domains.
Fig. 3. Sequence of mouse genomic DNA. A genomic library prepared from adult BALB/c mouse liver DNA was purchased from Clontech Laboratories. Plaques were screened with ACE.31. Positive plaques were re-screened with a 28-base oligonucleotide from the portion of ACE3 (ACCAACGGATGGACGGGAGGTGGTGTGC, bp in human ACE catalytic activity. Similar amino acids are encoded proteins demonstrate that these are also highly conserved between human and mouse. X-ray analysis of thermolysin has implicated His-142, His-146, and Glu-166 in Zn binding. Within both mouse and human ACE, these may correspond to His-361, His-365, Glu-389, and His-959, His-963, Glu-987, Glu-143 and His-211 of thermolysin, thought involved in the active site of the enzyme, may correspond to Glu-362, Glu-960, and His-404, His-1002 within both mouse and human ACE. Thus, both human and mouse ACE are composed of two homologous domains each containing a potential catalytic site.

Northern analysis of kidney and lung RNA with ACE.11 demonstrates a pattern similar to that observed with ACE.31 (Fig. 8A). Both probes identify two bands, one of 4900 and one of 4150 bases. In contrast when hybridized with mouse genomic DNA, ACE.11 identifies a strong band of 2500 bases and a weaker band of 1350 bases. These bands are not identified by ACE.31. Liver mRNA shows virtually no hybridization to ACE.11 (data not shown). Restriction fragments comprising ACE.11 from bp 672 to 1186 and the partial sequence of nine ACE tryptic peptides (sequence in lowercase) and exons (sequence in uppercase). The EcoRI site present at the 3′ end of ACE.31 and the 5′ end of ACE.5 is underlined and in bold type. Regions of amino acid sequence similar to the Zn binding and catalytic domains of thermolysin are in bold type and underlined (24, 25). Potential sites of N-glycosylation are at amino acids 9, 25, 45, 82, 117, 131, 288, 480, 494, 648, 666, 685, 731, 913, and 1162.

**DISCUSSION**

We have previously described the purification of mouse kidney ACE and the partial sequence of nine ACE tryptic

**FIG. 5. Hydrophobic analysis of mouse ACE.** The protein encoded by ACE.315 was analyzed using the method of Kyte and Doolittle (22) with a window size of 12. Both the signal peptide and the carboxyl terminus are highly hydrophobic. The hydrophobic region at the carboxyl terminus is followed by a hydrophilic region, and it is probably here that ACE is anchored to a cell membrane.

highly conserved between human and mouse. X-ray analysis of thermolysin has implicated His-142, His-146, and Glu-166 in Zn binding. Within both mouse and human ACE, these may correspond to His-361, His-365, Glu-389, and His-959, His-963, Glu-987, Glu-143 and His-211 of thermolysin, thought involved in the active site of the enzyme, may correspond to Glu-362, Glu-960, and His-1002 within both mouse and human ACE. Thus, both human and mouse ACE are composed of two homologous domains each containing a potential catalytic site.

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peptides. Based on this information, five oligonucleotides were used to screen a mouse kidney cDNA library. The positive cDNA clones fell into two groups based on the patterns of hybridization to the oligonucleotides. The analysis of mouse genomic DNA presented here now establishes that this was artificial, due to cleavage of an internal EcoRI site during cDNA construction. We refer to the 4838-bp fusion of ACE.31 and ACE.5 as ACE.315 and ask whether ACE.315 encodes the protein sequence of mouse angiotensin-converting enzyme. ACE.315 encodes protein sequence identical or very similar to that found in nine separate tryptic peptides from purified ACE protein. These nine sequences are found throughout the encoded protein sequence of ACE.315. Review of the amino acid chromatographic patterns for those regions where there are discrepancies between the protein microsequencing results and the predicted sequence of ACE.315 shows that these differences are due to difficulties or errors in protein sequence determination; no definitive differences are noted between the microsequencing results and the sequence predicted by cDNA analysis. In addition Northern analyses using ACE.31, ACE.11, and smaller restriction fragments from these cDNA detects hybridizing mRNA in kidney and lung (organs rich in ACE) and very little hybridization to liver RNA (an organ poor in ACE). Perhaps the only difficulty in unconditionally accepting ACE.315 as encoding mouse converting enzyme is the unexpectedly large molecular mass of the encoded mature protein (147.4 kDa). Generally the molecular mass of ACE has been reported as being between 140-150 kDa (6). Nonglycosylated ACE has been studied by El-Dorry et al. (26) who translated rabbit lung mRNA in vitro and determined that the converting enzyme produced had a molecular mass of 129 kDa. Despite this result, there are several good reasons to believe that ACE.315 does encode ACE protein, the most convincing being that the amino acid sequences found in ACE peptides are scattered throughout the protein predicted by ACE.315. ACE.5 and ACE.11, two independently isolated ACE cDNA, predict a virtually identical protein when coordinated with the DNA sequence of ACE.31. Studies in the rabbit using cDNA selected with anti-ACE antisera have identified ACE mRNA of 5000 and 2600 bp in lung and testis mRNA, respectively (27). These data are very similar to those reported in this paper and corroborate ACE.315 as encoding mouse angiotensin-converting enzyme. Finally, the recently reported sequence of human ACE is highly homologous to that of ACE.315 (16).

One difference between the results found in the rabbit and those reported here is the presence in mouse lung and kidney of two ACE mRNA bands, one of 4900 and one of 4150 bases. We do not know yet the difference in structure between these two message sizes but the results of Northern analyses with restriction fragments of ACE.31 and ACE.11 (ACE.31/557 and ACE.11/514) demonstrates that both bands contain sequences homologous to ACE.315 positions 1-557 and 1699-2213. As reported by Soubrier et al. (16), Northern analysis of RNA from cultured human umbilical vein endothelium shows only a single ACE band of 4.3 kilobases. It is also not known at this time why ACE.11 contains such
When probed with ACE.11. These bands are not identified by ACE.31. Northern analysis of NIH Swiss mouse kidney washing are described in the text. The BRL RNA ladder was used as published in Ref. 15. Conditions of probe labeling, hybridization and ACE mRNA. Different set of 3' Ut exons than that of the typical forms of lane fragments ACE.31/557 and ACE.11/514. Aliquots of 5 pg of NIH Lanes probed with ACE.11 and ACE.31. Testis mRNA contains a strongly hybridizing band at 2500 bases and a much weaker band at 1350 bases with both ACE.11 and ACE.31. Rabbit lung ACE has enzyme activity. In contrast Northern analysis of testis RNA (Fig. 8A) suggests that the testis-specific form of ACE may only contain a single catalytic site homologous to that of the carboxyl portion of kidney ACE. These data coupled with the analysis of protein structure presented here suggest that only one of the large homologous regions is sufficient for enzyme activity. A reasonable speculation is that at some point during the molecular evolution of converting enzyme, the ancestral gene encoding this protein underwent a genetic duplication. It is also possible that during evolution (or in some species even now) converting enzyme may have had two substrate-binding sites. And perhaps modern ACE can use either of its catalytic sites though not both concurrently in a single molecule.

Acknowledgments—We would like to thank Dr. Juha P. Kokko, Emory University for support and encouragement of this research effort. Sarah Lehner aided in the compilation of DNA sequence data.

REFERENCES
1. Erdos, E. G. (1975) Circ. Res. 36, 247–255
2. Skeggs, L. T., Dorer, F. E., Kahn, J. R., Lentz, K. E., and Levine, M. (1976) Am. J. Med. 60, 737–747
3. Patchett, A. A., and Cordes, E. H. (1985) in Advances in Enzymology (Meister, A., ed) Vol. 57, pp. 1–84, John Wiley & Sons, New York
4. Yang, H. Y. T., Erdos, E. G., and Levine, Y. (1970) Biochim. Biophys. Acta 214, 374–376
5. Case, D. B., Wallace, J. M., Keim, H. J., Weber, M. A., Drayer, J. I. M., White, R. P., Sealey, J. E., and Laragh, J. H. (1975) Am. J. Med. 61, 790–796
6. Soffer, R. L. (1981) in Biochemical Regulation of Blood Pressure (Soffer, R. L., ed) pp. 123–164, John Wiley & Sons, New York
7. Ryan, J. W., Ryan, W. S., Schultz, D. R., Whitaker, C., Chung, A., and Dorer, F. E. (1975) Biochem. J. 146, 497–499
8. Aiken, J. W., and Vane, J. R. (1970) Nature 228, 30–34
9. Johnson, A. R., and Erdos, E. G. (1977) J. Clin. Invest. 59, 684–695
10. Vane, J. R. (1969) Br. J. Pharmacol. 35, 209–242
11. Del Vecchio, P. J., and Smith, J. R. (1981) J. Cell. Phys. 108, 337–345
12. Mendelsohn, F. A. O., Lloyd, C. J., Kachel, C., and Funder, J. W. (1982) J. Clin. Invest. 70, 684–692
13. Forslund, T., Fyhquist, F., Gronhage-Riska, C., and Tikkanen, L. (1983) Eur. J. Pharmacol. 80, 121–125
14. Bernstein, K. E., Martin, B. M., Striker, L., and Striker, G. (1988) Kidney Int. 33, 652–655
15. Bernstein, K. E., Martin, B. M., Bernstein, E. A., Linton, J., Striker, L., and Striker, G. (1988) J. Biol. Chem. 263, 11021–11024
16. Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allesgrini, J., John, M., Tregnear, G., and Corvol, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9386–9390
17. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408–1412
18. Henikoff, S. (1984) Gene (Amst.) 28, 351–359
19. Bankier, A. T., and Barrell, B. G. (1983) in Techniques in the Life Sciences (Flavell, R. A., ed) Vol. B5, pp. B505/1-B508/34, Elsevier Scientific Publishing Co., Ireland
20. Vogelstein, B., and Kalasipie, D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 615–619
| Reference                                                                 |
|--------------------------------------------------------------------------|
| 21. Hamaguchi, K., and Geiduschek, E. P. (1962) *J. Am. Chem. Soc.* 84, 1329-1338 |
| 22. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* 132, 6-13   |
| 23. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* 157, 105-132       |
| 24. Needleman, S. B., and Wunsch, C. D. (1970) *J. Mol. Biol.* 48, 443-453   |
| 25. Kester, W. R., and Mathews, B. W. (1977) *Biochemistry* 16, 2506-2516   |
| 26. El-Dorry, H. A., Pickett, C. B., MacGregor, J. S., and Soffer, R. L. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 4295-4297 |
| 27. Roy, S. N., Kusari, J., Soffer, R. L., Lai, C. Y., and Sen, G. C. (1988) *Biochem. Biophys. Res. Commun.* 155, 678-684 |
| 28. Das, M., and Soffer, R. L. (1975) *J. Biol. Chem.* 250, 6762-6768        |
| 29. Iwata, K., Blancher, R., Soffer, R. L., and Lai, C-Y. (1985) *Arch. Biochem. Biophys.* 227, 188-201 |