A novel human zinc metalloprotease that has considerable homology to human angiotensin-converting enzyme (ACE) (40% identity and 61% similarity) has been identified. This metalloprotease (angiotensin-converting enzyme homolog (ACEH)) contains a single HEXXII zinc-binding domain and conserves other critical residues typical of the ACE family. The predicted protein sequence consists of 805 amino acids, including a potential 17-amino acid N-terminal signal peptide sequence and a putative C-terminal membrane anchor. Expression in Chinese hamster ovary cells of a soluble, truncated form of ACEH, lacking the transmembrane and cytosolic domains, produces a glycoprotein of 120 kDa, which is able to cleave angiotensin I and angiotensin II but not bradykinin or Hip-His-Leu. In the hydrolysis of the angiotensins, ACEH functions exclusively as a carboxypeptidase. ACEH activity is inhibited by EDTA but not by classical ACE inhibitors such as captopril, lisinopril, or enalaprilat. Identification of the genomic sequence of ACEH has shown that the ACEH gene contains 18 exons, of which several have considerable size similarity with the first 17 exons of human ACE. The gene maps to chromosomal location Xp22. Northern blotting analysis has shown that the ACEH mRNA transcript is ~3.4 kilobase pairs and is most highly expressed in testis, kidney, and heart. This is the first report of a mammalian homolog of ACE and has implications for our understanding of cardiovascular and renal function.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF241254. § To whom correspondence should be addressed: School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK. Tel.: 44 113 233 3160; Fax: 44 113 242 3187; E-mail: s.r.tipnis@leeds.ac.uk.

Angiotensin-converting enzyme (ACE), 1 peptidyl-dipeptidase A, EC 3.4.15.1) is a well characterized zinc metalloprotease of the M2 family (1). The predominant physiological function of ACE is in cardiovascular homeostasis through cleavage of the C-terminal dipeptide from angiotensin I to produce the potent vasoconstrictor, angiotensin II (2). ACE also inactivates the vasodilator, bradykinin, by the sequential cleavage of two C-terminal dipeptides (3). ACE can also hydrolyze a wide range of other endogenous bioactive peptides (4).

Two forms of mammalian ACE have been identified to date: the two-domain somatic ACE, containing two catalytic sites and a single domain germline ACE (5–7). Both enzymes are derived from the same gene through the use of alternative promoters, and it has been suggested that the ACE gene arose from the duplication of an ancestral gene coding for a single domain enzyme (8). Somatic ACE exists as a type I integral membrane protein anchored to the plasma membrane through a transmembrane domain near the C terminus (9). However, it can also be found in plasma and other body fluids as a soluble enzyme lacking the transmembrane and cytosolic domains (10–12). This form is thought to arise predominantly by post-translational proteolytic cleavage at the cell surface through the action of ACE secretase (9, 13, 14).

ACE-like enzymes have also been found in other non-mammalian species. In particular Musca domestica and Drosophila melanogaster have both been shown to contain single domain, ACE-like proteins (AnCE) (15, 16), and more recently, a second ACE-like protein, termed AceR, has been identified in D. melanogaster (17). AceR and AnCE appear to be alternatively expressed during D. melanogaster pupal development (18), suggesting different roles for the two enzymes.

In this study we have identified a novel, single domain, human zinc metalloprotease cDNA (ACEH) whose predicted amino acid sequence has significant similarity with mammalian ACE. Furthermore, the genomic structure of ACEH indicates a remarkable exon size similarity with the first 17 exons of ACE. Expression of a soluble, truncated form of ACEH, lacking the transmembrane and cytosolic domains, produced a 120-kDa glycosylated protein that hydrolyzed the C-terminal residue from angiotensins I and II. The transcript for this cDNA is highly expressed in heart, kidney, and testis, implying that the translated protein may play a role in the regulation of cardiovascular and renal function, as well as fertility.

**EXPERIMENTAL PROCEDURES**

**Materials—**Captopril was a gift from Bristol-Myers Squibb Co. Enalaprilat (MK422) and lisinopril were gifts from Merck. Other chemicals were obtained from Sigma.

cDNA Cloning—A partial cDNA of 2885 nucleotides encoding ACEH was obtained following identification of a zinc metalloprotease with homology to ACE from a proprietary EST database and subsequent screening of a human lymphoma cDNA library. To establish whether the clone was a full-length cDNA, 5'- and 3'-RACE were carried out on a SUPERSCRIPT™II human kidney cDNA library (Life Technologies, Inc.). Gene-specific primers deduced from the partial cDNA library

33238 This paper is available online at http://www.jbc.org
clone GSPl (5‘CCCATATGCTCATATAGATTGGG3‘), GSPl2 (5‘CC-
AGTACTGATTAGGTTGC3‘), S3RACE1 (5‘GGACTCTTAAACAGG-
CCTCTTTTTPCC3‘), S3RACE2 (5‘GGATCTCGGAGCCGTATCA-
TGTG3‘), and primers to the pmCSVsport Library Vector, cSpor-
t (5‘GGCTAATCTATTTCTCTACC3‘), cSpor2 (5‘GGGAGAGA-
GCTTATACGACTCACTATTAGGC3‘), cSpor3 (5‘GGTATTTGAGCTAC-
TAGAAGG3‘), and cSpor4 (5‘CTTATAGGTCGTACCGTGAGTAC-
3‘) were designed. 5‘-RACE was carried out with an initial com-
bination of primers cSpor3 and GSPl, followed by a nested PCR 
using primers GSPl and GSPl2. 3‘-RACE was carried out with an initial com-
bination of primer pairs cSpor1 and S3RACE2, followed by nested PCR 
using primers cSpor2 and S3RACE2. The PCR products were digested 
with XhoI and NotI and sequence obtained from 5mU units/ml penicillin, and 100 
mU/g ml streptomycin, at 37 °C, with 5% CO2. A 943-nucleotide cDNA fragment of ACE was obtained by 
XmnI restriction endonuclease digestion. A 614-nucleotide cDNA fragment of ACE 
was obtained using a Scal digest. The cDNA fragments were labeled with 
[32P] using a random primed DNA labeling kit (Roche Molecular 
Biochemicals).

**Construction of Expression Plasmid pSTMyc-TM7**—The truncated cDNA encoding ACE was isolated from the human lymphoma cDNA library in plasmid pmCSVsport2 which contains cDNA inserts between 
NcoI and SalI restriction sites. By using these sites the partial ACE cDNA 
was excised from pmCSVsport2 and cloned into pBluescript SK(+) 
plasmid. This plasmid was designated pBSKACE10. Additional 5‘ 
sequence obtained from 5‘-RACE was added to pBSKACE10, using 
SalI and HindIII to remove the extra sequence from the RACE product in 
PBSKACE10 construct using the same sites, generating the plasmid pBSKACE10+S. The cDNA 
with the additional 5‘ sequence was then excised from pBSKACE10+S using 
NotI and SalI restriction sites and cloned into vector pc1-neo 
(Promega, Southampton, UK) using the same sites. PCR was then 
carried out using antisense primer SMYCPR (5‘CGAGGGCCCGAAA-
CAGGGGCGTTGTTAGCAGG3‘) (nucleotides 2300–2323 which also 
oxonates site) and sense primer NeoT7 (5‘GGACCTCCTAACCAG-
GCTTATAATACGACTCACTATTAGGC3‘). This gave a truncated cDNA encoding ACE, which lacks 
the transmembrane and cytosolic domains. The PCR product was digested 
with Apoi and Xhol and ligated, in frame, into expression vector 
pcDNA3.1Mye-His A which was also digested with Xhol and Apoi. This 
construct, designated pSTMyc-TM7, gave a truncated ACE cDNA 
coiled with an in frame fusion tag encoding the e-Myc epitope and a 
hexahistidine tag.

**Expression of ACE in CHO Cells**—CHO cells were obtained from 
the European Collection of Cell Cultures (Salisbury, Wiltshire, UK) and 
were cultured in Ham’s F-12 nutrient mix (Life Technologies, Inc.) supplemented with 2 mM (i-glutamine, 10% (v/v) fetal bovine serum, 100 
units/ml penicillin, and 100 µg/ml streptomycin, at 37 °C, with 5% CO2 
for 24 h prior to transfection cells were seeded at a density of 1 × 106 cells 
per 75-cm2 flask. For transient transfection, the monolayer was washed 
twice with OPTI-MEM (Life Technologies, Inc.) before transfection with 
3 µg of pSTMyc-TM7 plasmid DNA per flask. LipofectAMINE (Life 
Technologies, Inc.) was used as cationic lipid at a ratio of DNA/lipid, 1:10 (w/w), this was added to the flasks in 2.5 ml of OPTI-MEM and incubated for 4 h before the addition of Ham’s F-12 nutrient 
containing 10% (v/v) fetal bovine serum. The medium was removed after 24 h 
and the addition of OPTI-MEM, and then 5 ml of OPTI-MEM was added to each flask. This 
was incubated for a further 16 h before harvesting of the medium, contain-
ing soluble secreted ACE protein. The media samples containing 
protein were concentrated using 4-m1 Vivaspin columns (Vivaspace, 
Birnbrook, Lincoln, UK).

**Deglycosylation of ACE**—30 µg of total protein containing soluble 
secreted ACE from CHO cell media was incubated overnight at 37 °C 
with 1 µl of PNGase F (Oxford Glycosystems, Abingdon, Oxford, UK).

**Protein and Enzymic Assays**—Protein concentrations were deter-
mined using the biochromic acid assay (21) with bovine serum albu-
mmin as standard. Assays for ACE activity were carried out in a total 
volume of 1 mL, containing 100 nM Tris-HCl, pH 7.4, 20 µg of protein 
and either 100 µM angiotensin I or II or 500 µM bradykinin, Hip-Phe, 
or Hip-His-Leu as substrates. Where appropriate, inhibitors were added 
to give final concentrations of 10 µM lisinopril, 10 µM captopril, 10 µM 
enalaprilat, 100 µM benzyl succinate, or 10 mM EDTA. Reactions were 
carried out at 37 °C, for 2 h and stopped by heating to 100 °C for 5 min 
followed by centrifugation at 11,600 × g for 10 min. Carboxypeptidase A 
assays were carried out at room temperature for 30 min, using 
0.1 units of enzyme per assay. 

**HPLC Analysis of Cleavage Products**—Peptide hydrolysis products 
were separated using reverse-phase HPLC (MBondapak C-18 reverse 
phase column, Waters) with an UV detector set at 214 nm. All separa-
tions were carried out at room temperature, with a flow rate of 1.5 
ml/min. Mobile Phase A consisted of 0.08% (v/v) phosphoric acid and 
mobile phase B consisted of 40% (v/v) acetonitrile in 0.08% (v/v) phosphoric 
acid. A linear solvent gradient of 11% B to 100% B over 15 min 
with 5 min at final conditions, and 8 min re-equilibration was used. The 
products of angiotensin I cleavage was collected and analyzed by 
matrix-assisted laser desorption/ionization/time of flight mass 
spectrometry.

**SDS-PAGE and Western Blotting**—Proteins were separated by 
SDS-PAGE, using a 10% resolving gel and a 5% stacking gel according to 
the method of Laemmli (22). Blotting was carried out as described (23) 
using an anti-N-acetylglucosaminidase antibody (Invitrogen, Leek, 
The Netherlands). Bound antibody was detected using the enhanced 
chemiluminescence system (Amersham Pharmacia Biotech).

**RESULTS**

**cDNA Sequence Analysis of ACE**—A partial cDNA encoding 
ACE was originally identified as a zinc metalloprotease with homology to ACE, in an EST data base. Following isolation of 
this partial clone from a human lymphoma cDNA library, the full cDNA encoding ACE (Fig. 1) was deduced in 
combination with 3‘- and 5‘-RACE. Sequence analysis of 5‘- 
RACE DNA products revealed an appropriate product that 
consisted of 100 nucleotides of extra 5‘ sequence. This gave rise 
to a possible new initiating methionine codon 61 amino acids 
upstream of the original putative ATG. Two products containing 
extra 3‘ sequence were identified from sequence analysis of 
3‘-RACE DNA products. The sequence differed at the termina-
codon of the original clone and downstream from there on. 
Product 132 contained an extra 207 nucleotides of sequence, 
including 36 extra codons. Product UB4 contained this 
sequence plus an additional 774 nucleotides of downstream 3‘ 
sequence, a total of 981 nucleotides of additional sequence. 
Hence, the cDNA encoding ACE consists of 3405 nucleotides 
with 103 nucleotides of 5‘-untranslated sequence, 2418 nucleo-
tides of open reading frame, and 884 nucleotides of 3‘-un-
translated region (Fig. 1). The ATG codon at nucleotide position 
104 has been assigned as the initiating methionine as it is 
preceded by two in-frame termination codons, TAA at nucleo-
tide 26 and TAG at nucleotide 59. A polyadenylation signal is 
located 24 nucleotides upstream of the poly(A) tract.

The open reading frame encodes 805 amino acids, including 
a potential 17-amino acid N-terminal signal sequence, and has 
a predicted size of 92.4 kDa. There are 7 potential N-glycosyla-
tion sites within the protein sequence. Hydropathy analysis has 
also revealed a hydrophobic region toward the C terminus of 
ACE, indicating that the latter is likely to be membrane 
bound. The predicted amino acid sequence exhibits significant 
homology to existing members of the ACE family with ~60% 
similarity and 40% identity to the N- and C-terminal domains 
of human ACE and ~56% similarity and 36% identity to AnCE 
and ACEr. In addition to the conserved zinc metalloprotease 
consensus sequence, HEXXH, at amino acid positions 374–378,
there is also a conserved glutamate residue (Glu-402) predicted to serve as the third zinc ligand (corresponding to Glu-389 and Glu-987 of the N and C domains of ACE, respectively) 24 amino acids downstream from histidine residue 378. Asp-393 and Asp-991 of the N and C domains of ACE, respectively, which have been proposed to function in the positioning of the first histidine ligand (24), are replaced by a glutamate in ACEH, which may fulfill a similar role. Mutagenesis of the Asp-991 to glutamate in ACE reduces, but does not eliminate, activity (24). There are potential casein kinase II and tyrosine kinase phosphorylation sites in residues 787–790 and 775–781, respectively.

Expression of ACE mRNA and ACEH mRNA in Human Tissues—

The expression of mRNA encoding ACEH and ACE was examined in human tissues. Multiple tissue Northern blots were probed with a 32P-labeled fragment of the cDNA encoding either ACEH or ACE. Autoradiography revealed that expression of ACEH was greatest in kidney, testis, and heart, and moderate levels were also detected in colon, small intestine, and ovary (Fig. 2A). A single mRNA species of 3.4 kb was detected in these tissues, and an additional, less abundant, 5.9-kb species was also detected in kidney and testis. Two mRNA species were detected in tissues probed with endothelial ACE, of 4.3 and 3.5 kb, corresponding to the previously described alternatively spliced variants of endothelial ACE (25). Expression of ACE mRNA appeared to be more widespread than ACEH, being found in colon, small intestine, ovary, testis, prostate, heart, placenta, liver, skeletal muscle, and pancreas, with the highest levels of expression in lung and kidney (Fig. 2B). The Northern blots were also probed with β-actin as a control (Fig. 2C).

Expression and Enzymic Activity of an ACEH Construct—To examine the function of ACEH, we expressed a truncated, secreted form of the enzyme in CHO cells. Expression of the

![Fig. 1. Nucleotide sequence and deduced amino acid sequence of the cDNA encoding ACEH.](image)

![Fig. 2. Northern blot analysis of ACEH mRNA expression in various human tissues.](image)
protein without its putative C-terminal membrane binding domain and in conjunction with a C-terminal Myc-His fusion protein allowed detection of the protein by Western blotting (Fig. 3). When subjected to SDS-PAGE, the expressed secreted Myc-His tagged enzyme migrated with a molecular mass of ~120 kDa (Fig. 3, lane 2), indicating that the protein was glycosylated. Deglycosylation of ACEH with PNGase F resulted in the migration of the protein at the predicted molecular mass of ~85 kDa (Fig. 3, lane 1). No protein expression was detected in media taken from untransfected CHO cells. Secreted ACEH protein obtained from the medium was also used to identify potential substrates for the enzyme. High performance liquid chromatography (HPLC) was used to analyze the cleavage products. A parallel preparation taken from the medium of untransfected CHO cells was unable to hydrolyze angiotensin I (Fig. 4A). However, in the presence of ACEH, angiotensin I (retention time 9.4 min) was hydrolyzed to give a single product with a retention time of 6.7 min (Fig. 4B). Mass spectrometric analysis of the peptide recovered from the product peak gave an observed $M_r$ of 1183.9 which indicated that ACEH was acting as a carboxypeptidase to cleave the C-terminal leucyl residue from angiotensin I, producing angiotensin-(1–9). This activity was completely inhibited by 10 mM EDTA (Fig. 4C), but activity was unaffected by 10 μM lisinopril (Fig. 4D), enalaprilat, or captopril (data not shown). In addition to angiotensin I, ACEH was also able to hydrolyze angiotensin II (Fig. 4E) to give products that co-migrated with angiotensin-(1–7) and phenylalanine but was unable to cleave bradykinin (Fig. 4F) or Hip-His-Leu (data not shown). Identical products were obtained by incubation of angiotensins I or II with carboxypeptidase A. The carboxypeptidase A inhibitor, benzylsuccinate, did not inhibit the hydrolysis of angiotensin I by ACEH under conditions that abolished the hydrolysis by carboxypeptidase A. Hydrolysis of bradykinin or Hip-His-Leu, by ACEH, did not occur even following overnight incubation (data not shown). Overnight incubation of ACEH with the typical carboxypeptidase A substrate, Hip-Phe, resulted in approximately 20% hydrolysis.

Genomic Sequence Analysis of the ACEH Gene—Searches of the GenBank™ data base with the cDNA-encoding ACEH revealed the corresponding genomic sequence. This was located in a sequence submitted to the data base by the Human Genome Sequencing Center, Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX. The sequence, obtained from Genome Systems Human BAC library, was defined as Homo sapiens Xp22. BAC GS-594A7 and has accession number AC003669. The ACEH gene contains 18 exons, interspersed with 17 introns, spans approximately 40 kb, and is localized to chromosome X, position p22. All the intron-exon junction sequences (Table I) follow the GT/AG rule of consensus sequence, whereas exon 8 contains the first HE zinc metalloprotease consensus sequence, whereas exon 8 contains the first HXXHX motif in the genomic sequence of ACE.

DISCUSSION

Following the identification of a novel zinc metalloprotease from an EST data base, we have determined its cDNA sequence, expressed it as a soluble protein, and determined its activity toward potentially important physiological substrates. As a single domain enzyme, ACEH is similar to AnCE and ACEr, the insect members of the ACE family. When compared with the human ACE isoforms, ACEH shares considerable homology which is particularly marked around the HXXHX zinc-binding domain. This sequence (HEMGH) is identical in

![Fig. 3. Analysis of ACEH protein expression. CHO cells were transiently transfected with a truncated, Myc-tagged ACEH construct (pSTMyc-TM7) as described under "Experimental Procedures." Total protein (30 μg) from concentrated medium was either treated with PNGase F (lane 1) or untreated (lane 2) and subjected to Western blotting as described. The blot was probed with an anti-Myc-horseradish peroxidase antibody (Invitrogen).](image)

![Fig. 4. HPLC analysis of the hydrolysis of peptide substrates by ACEH. Potential substrates were incubated with 30 μg of protein from the medium of either untransfected CHO cells (ACEH−) or from CHO cells expressing ACEH (ACEH+). Hydrolysis products were resolved by HPLC as described under "Experimental Procedures." A, 100 μM angiotensin I, ACEH−; B, 100 μM angiotensin I, ACEH+; C, 100 μM angiotensin I, ACEH+; D, 10 mM EDTA; D, 100 μM angiotensin I, ACEH+; E, 10 mM lisinopril; F, 100 μM angiotensin II, ACEH+; and F, 100 μM bradykinin, ACEH+. Absorbance peaks at 214 nm are as follows: I, angiotensin I; 2, angiotensin-(1–9); 3, EDTA; 4, angiotensin II; 5, angiotensin-(1–7); 6, 1-phenylalanine; and 7, bradykinin.](image)
ACE and ACEH. A conserved glutamic acid residue, 24 amino acids downstream of the HEXXH motif in ACEH, aligns with the critical glutamate necessary for the catalytic activity of ACE (24). This glutamate serves as the third zinc coordinating ligand. ACEH also contains 8 cysteine residues 6 of which are conserved in the N- and C-terminal domains of endothelial ACE and of testicular ACE. ACEH contains 7 potential N-linked glycosylation sites (compared with 10 and 7 in the N- and C-terminal domains of endothelial ACE, respectively) and is therefore likely to be glycosylated. This is further reinforced by the molecular mass of truncated, expressed ACEH that migrates at 120 kDa compared with the deglycosylated polypeptide that migrates at 85 kDa.

There is a putative transmembrane domain of 22 amino acids near the C terminus followed by a cluster of charged residues that are likely to constitute a stop-transfer sequence. In contrast, the transmembrane sequence of ACE is predicted to be only 17 amino acids, which is a minimal requirement for a membrane-spanning region. Together with the 17-amino acid signal sequence at the N terminus of ACEH, the enzyme has all the features of a type I integral membrane protein, like ACE. We have shown that a soluble form of ACEH, lacking the transmembrane and cytosolic domains, is secreted from CHO cells and that this form is catalytically active. Surprisingly, ACEH appears to be acting specifically as a carboxypeptidase, rather than as a peptidyl dipeptidase, as it is able to cleave exclusively the C-terminal residues from both angiotensin I and angiotensin II. Bradykinin, which has a C-terminal arginyl residue, is not hydrolyzed, suggesting a carboxypeptidase A-like specificity for ACEH. However, ACEH does not have a typical carboxypeptidase A-like zinc-binding motif (27). Several ACE inhibitors (lisinopril, captopril, and enalaprilat) were not able to inhibit the cleavage of angiotensin I by ACEH, although the metal-chelating agent, EDTA, was an effective inhibitor, showing complete inhibition at 10 mM. This reinforces the proposition that ACEH is a metalloprotease, but with a distinct substrate and inhibitor specificity from ACE. It is perhaps not unexpected that the typical ACE inhibitors do not inhibit ACEH as they have been designed to compete with peptides that are hydrolyzed to release C-terminal dipeptides. Hence, positioning of the inhibitors in the active site should not be in the correct conformation to affect the cleavage of a single amino acid from the C terminus of the substrate.

The high expression of ACEH mRNA in heart and kidney is of interest as these organs are important contributors to blood pressure homeostasis. The highest expression of ACEH mRNA, however, is in the testis. Testicular ACE is known to play a key role in fertility (28, 29), and ACEH may also therefore have reproductive functions. The tissue distribution of ACE mRNA is more widespread than ACEH, with both 3.5- and 4.2-kb species present in most of the tissues examined.

The genomic sequence of ACEH holds many similarities to the structure of the ACE gene. The sizes of many of the exons are identical. There is, however, a discrepancy at exons 5 and 6.
of ACEH, which together appear to correspond with exon 5 of ACE, suggesting a fusion of two exons. The HEXXH motif is therefore located in exon 9 of ACEH but exon 8 of the ACE gene.

The ACE gene is located on the X chromosome (Xp22) which is similar to the location (Xp22.1) of another membrane metalloproteinase, the product of the PEX gene associated with X-linked hypophosphatemic rickets (30). The Pex protein is a member of the nepriylsin (NEP) family (31).

Taken together, these findings indicate that ACEH is a metalloproteinase that may have a significant role not only in cardiovascular homeostasis but also in fertility. However, before the physiological roles of ACEH can be elucidated, further enzyme characterization is needed to identify selective inhibitors and the key residues that distinguish its activity from that of ACE itself.

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