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Increased Shedding of Angiotensin-converting Enzyme by a Mutation Identified in the Stalk Region*

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Angiotensin-converting enzyme (ACE), an enzyme that plays a major role in vasoactive peptide metabolism, is a type 1 ectoprotein, which is released from the plasma membrane by a proteolytic cleavage occurring in the stalk sequence adjacent to the membrane anchor. In this study, we have discovered the molecular mechanism underlying the marked increase of plasma ACE levels observed in three unrelated individuals. We have identified a $Pro^{1199} \rightarrow Leu$ mutation in the juxtamembrane stalk region. *In vitro* analysis revealed that the shedding of [Leu^{1199}]ACE was enhanced compared with wild-type ACE. The solubilization process of [Leu^{1199}]ACE was stimulated by phorbol esters and inhibited by compound 3, an inhibitor of ACE-secretase. The results of Western blot analysis were consistent with a cleavage at the major described site ($Arg^{1203} \downarrow Ser^{1204}$). Two-dimensional structural analysis of ACE showed that the mutated residue was critical for the positioning of a specific loop containing the cleavage site. We therefore propose that a local conformational modification caused by the $Pro^{1199} \rightarrow Leu$ mutation leads to more accessibility at the stalk region for ACE secretase and is responsible for the enhancement of the cleavage-secretion process. Our results show that different molecular mechanisms are responsible for the common genetic variation of plasma ACE and for its more rare familial elevation.

Angiotensin I-converting enzyme (DCP1, EC 3.4.15.1, ACE)[1] is a zinc metallopeptidase that plays an important role in blood pressure regulation by cleaving the inactive decapeptide angiotensin I to angiotensin II, a potent vasopressor octapeptide. It also inactivates bradykinin, a potent vasodilator peptide (1). There are two ACE isoforms transcribed from a single *ACE* gene by two alternate promoters (2). Somatic ACE (170 kDa) is synthesized by vascular endothelial cells as well as several types of epithelial cells, whereas testis ACE (110 kDa) is expressed exclusively by male germinal cells (3, 4).

Somatic ACE consists of two homologous catalytic domains, a juxtamembrane stalk region, a hydrophobic transmembrane domain of 17 amino acids, and a 30-residue C-terminal cytosolic domain (5). Thus ACE is primarily an integral membrane protein anchored to the plasma membrane by its C-terminal segment. But the membrane-bound enzyme can be fully solubilized *in vitro* by detergents or limited proteolytic cleavage (6). *In vivo*, a soluble form of the enzyme exists in plasma and other body fluids (7). Plasma ACE is known to be strongly modulated by a common polymorphism in linkage disequilibrium with an insertion/deletion polymorphism (8). Although this quantitative trait locus (QTL) was localized on the *ACE* gene itself (9), the functional variant has not yet been identified.

Human plasma ACE is derived from endothelial cells by post-translational cleavage. Several membrane-anchored proteins are solubilized by limited proteolysis with release of their extracellular domains. This common phenomenon, also referred to as "shedding" displays typical characteristics such as induction by phorbol esters, calcium ionophores, and unidentified serum factor; inhibition of the shedding protease by hydroxamate-based metalloprotease inhibitors; localization of the shedding event at the cell surface; and structural requirements of the juxtamembrane stalk that determine cleavage efficiency (10–12). Cleavage secretion of ACE is topologically constrained to an accessible stalk region of at least 11 residues in length and requires a minimum distance of 3 residues from the proximal extracellular domain and 8 residues from the membrane (10). Furthermore, mutational analysis of the stalk region has shown that conservation of the amino acid sequence was not essential for shedding, even though the secretase seems to have a weak preference for cleavage after Arg or Lys residues (10, 13, 14). Thus, the critical parameter, for cleavage efficiency seems to be the conformation of the stalk more than the amino acid sequence. In addition, experiments using the homologous stalk region of CD4 (which is not cleaved) to construct a chimeric ACE revealed that the distal extracellular domain of ACE also has a prominent role for determining the cleavage (14).

Here we report the identification of a $Pro^{1199} \rightarrow Leu$ muta-

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[1] The abbreviations used are: ACE, angiotensin-converting enzyme; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; HMEC, human microdermal endothelial cell; hEGF, human epidermal growth factor; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SV40 simian virus 40; QTL, quantitative trait loci; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; HCA, hydrophobic cluster analysis; WT, wild-type; PCR, polymerase chain reaction.

tion found associated with high plasma ACE levels in three unrelated Dutch individuals. *In vitro* study of this mutation was performed, and effects of this mutation on the solubilization process of ACE were investigated. We present experimental results showing that this single amino acid change in the stalk region is responsible for an enhancement of the cleavage and secretion of ACE in an ACE-secretase activity-dependent manner. A hypothesis implicating a modification of the ACE structure caused by the point mutation that leads to better accessibility for the ACE secretase is proposed.

EXPERIMENTAL PROCEDURES

Subjects and Biological Findings—Three unrelated individuals (HOL.2, HOL.10, and HOL.19) were identified through a screening of plasma ACE levels in a population of patients referred to the hospital for various causes. The study included the measurement of several clinical, paraclinical, and biological parameters. Plasma ACE enzymatic activity was measured by a commercial kit (ACEcolor, Fujizoki Pharmaceutical Co. Ltd., Tokyo, Japan) using Gly-His-Leu as substrate. The complete clinical report has been described in Kramers *et al.*[2]

Reagents—Phorbol 12-myristate 13-acetate (PMA), MCDB-131 medium, Dulbecco's modified Eagle's medium, CHAPS, and hydrocortisone were from Sigma. Fetal calf serum was from Valbiotech (Paris, France). Phosphate-buffered saline, L-glutamine, penicillin-streptomycin, and geneticin were from Life Technologies, Inc. Human recombinant epidermal growth factor (hEGF) and EXGEN 500 were from Euromedex (Souffelweyersheim, France). Compound 3 [*N*-[-D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl]-L-3-(tert-butyl-alanyl-L-alanine, 2- aminoethyl amide) was provided by Dr. Roy A. Black, Immunex Research and Development Corp. (Seattle, WA).

PCR Amplification and Sequencing—Genomic DNA of all subjects was used as template for PCR amplification. Specific primers were designed to amplify each of the 26 exons of the *ACE* gene, according to our own sequence determination and to the published data (GenBank™/EBI accession numbers AC002345 and AF118569). The primer sequences are available upon request. Exon 25 amplification was performed with oligonucleotides 5′-CATGTTGAGCTACTTCAAGC-3′ (sense) and 5′-CCAGTGTTCCCATCCCAG-3′ (antisense), using the following cycling parameters: one denaturation step at 95 °C, 4 min; 31 cycles (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s); and one final elongation step at 72 °C, 10 min. Purified PCR products were submitted to direct sequencing using fluorescent dideoxyterminator (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, PE Biosystems) on an ABI 377 sequencer.

Site-directed Mutagenesis—A single Pro (CCG) to Leu (CTG) mutation at position 1199 was introduced into pACE-WT (or peACE), an expression vector containing the full-length somatic ACE cDNA controlled by the SV40 early promoter (15). An oligonucleotide-directed mutagenesis system (QuickChange site-directed mutagenesis kit, Stratagene) was used according to the manufacturer's recommendation. The following oligonucleotides containing the desired mutation were used: 5′-GCAGTACAACTGGACGCTGAACTCCGCTCGC-3′ (sense); 5′-GCGAGCGGAGTTCAGCGTCCAGTTGTACTGC-3′ (antisense). Mutant cDNAs were screened by DNA sequencing. One mutant containing the desired mutation was selected and designed as pACE(Leu^{1199}). The entire cDNA fragment of this construct was sequenced to ensure that no other mutations occurred. The same protocol was used to introduce the Pro^{1199} → Leu mutation into plasmid pACE-CF containing the coding sequence for the signal peptide, the C-terminal domain, and the transmembrane domain of human endothelial ACE (16). The mutant containing the desired mutation was designed as pACE-CF(Leu^{1199}).

Transient Expression of pACE-WT, pACE(Leu^{1199}), pACE-CF, and pACE-CF(Leu^{1199})—pACE-WT, pACE(Leu^{1199}), pACE-CF, and pACE-CF(Leu^{1199}) were expressed in COS-7 cells. Transient transfections were performed by using polyethyleneimine suspension in a commercially available solution (EXGEN 500). COS-7 cells (250 × 10^3/well) were plated on a 6-well plate and incubated 24 h in Dulbecco's modified Eagle's medium supplemented by 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin and then treated with a mix containing 2 μg of plasmid, 4 μl of EXGEN 500, and NaCl (150 mM) in a final volume of 100 μl. The same protocol was used to express pACE-WT and pACE(Leu^{1199}) in HMEC-1 (human microdermal endothelial cells transfected and immortalized with a pBR-322-based plasmid containing the coding region for the SV40 A gene product, large T antigen, Ref. 17) except that they were grown in MCDB-131 medium supplemented by 20% heat-nactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, 10 ng/ml hEGF, 1 μg/ml hydrocortisone, and 6 μl of EXGEN 500 were used for transfection. Cells were incubated overnight with the transfection mix.

Expression of pACE(Leu^{1199}) in CHO Cells—pACE(Leu^{1199}) was introduced by cotransfection with neomycin resistance plasmid pSV2neo into CHO cells. CHO cells (1 × 10^6) were plated on a 10-cm^2 Petri dish, incubated 24 h, then treated with a mix containing 2.95 μg of pACE-(Leu^{1199}), 500 ng of pSV2neo, 6 μl of EXGEN 500, and NaCl (150 mM) in a final volume of 100 μl. The transfected cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, and 750 μg/ml geneticin. Single colonies of primary G418-resistant transformants were assayed for the expression of pACE (Leu^{1199}) by Western blot analysis and enzymatic assays (see below). Cell lines expressing pACE(Leu^{1199}) were selected and purified by subcloning using limiting dilution. Establishment of a CHO cell line expressing pACE-WT has been previously described (15).

Metabolic Labeling and Immunoprecipitation—Wild-type and mutant ACE CHO cell lines with similar cellular contents of ACE activity were seeded in 60-mm dishes at a density of 2 × 10^6 cells/dish and grown to confluency. Metabolic labeling and immunoprecipitation were performed as previously described (18), except that immunoprecipitation was performed using antiserum HKE obtained from sheep immunized against pure human kidney ACE and protein G-Sepharose (Sigma). Proteins were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and revealed by autoradiography.

Preparation of Subcellular Fractions—CHO cells expressing wild-type ACE and ACE(Leu^{1199}) were scraped and suspended in buffer A containing 50 mM HEPES pH 7.5, 0.25 M sucrose, and 5 mM $MgCl_2$. All steps were performed at 4 °C. The cell suspension was homogenized using a glass-Teflon homogenizer for 30 s and then centrifuged at 1,000 × *g* for 10 min. The pellet was resuspended in buffer A and centrifuged at 10,000 × *g* for 10 min. The pellet was solubilized in CHAPS (8 mM) and conserved as a lysosomal fraction. The supernatant was centrifuged again at 105,000 × *g* for 1 h. The sedimented membranes were washed with buffer A and solubilized in CHAPS (8 mM), and the supernatant was conserved as a cytosolic fraction.

Enzymatic Characterization—Cells were scraped and washed with phosphate-buffered saline and then centrifuged at 1,200 × *g* for 10 min. The pellet was dissolved in CHAPS (8 mM). After stirring for 12 h to solubilize the membrane-bound ACE, the suspension was centrifuged at 12,000 × *g* for 10 min, and the supernatant was used for enzymatic assays.

Secreted ACE was obtained from the culture medium of transfected cells grown in serum free medium.

All enzymatic studies were performed using *p*-benzoyl-L-glycyl-L-histidyl-L-leucine (Hip-His-Leu, Bachem, Switzerland) as substrate. The detection and quantification of hippuric acid released from the Hip-His-Leu were performed by HPLC as previously described (15). Kinetic parameters for the hydrolysis of Hip-His-Leu were determined from Michaelis-Menten plots using ENZFITTER software. All enzymatic studies were performed under initial rate conditions.

Western Blot Analysis—Two different rabbit antisera were used to characterize expressed forms of ACE: antisera 3 and 4 (18) which were raised against synthetic peptides corresponding to sequences of the C-terminal ACE (see Fig. 1). Membrane-bound and secreted ACE were collected as described above and analyzed by SDS-PAGE on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane (Pall Gelman Sciences) in a buffer containing 25 mM Tris-HCl, pH 8.3, and 150 mM glycine using a semi-dry transfer system (Bio-Rad). Western blotting was performed in TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) with antiserum 3 (1:500 dilution) or antiserum 4 (1:500 dilution). The peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch) (1:5000 dilution) was used as secondary antibody. The detection step was carried out with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Computer-assisted Structural Analysis—Multiple alignment of domain 1 (N-terminal domain) and domain 2 (C-terminal domain) of several ACE orthologs was performed using ClustalW program (19). The sequence data used are available in the Swiss-Pro database under accession numbers P12821, P09470, and Q10751.

[2] C. Kramers, S. M. Danilov, J. Deinum, I. V. Balyasnikova, N. Scharenberg, M. Looman, F. Boomsma, M. H. De Keijzer, C. Van Duijn, S. Martin, F. Soubrier, and G. J. Adema, submitted manuscript.

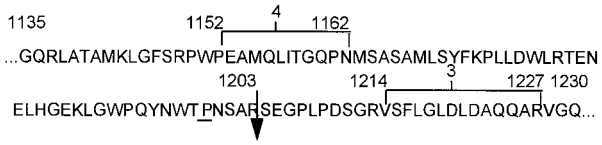

FIG. 1. **C-terminal sequence of wild-type ACE from Gly[1135] to Glu[1230].** The proline mutated into leucine in the three individuals is underlined. The cleavage site previously described is shown by an *arrow*. Sequences of synthetic peptides used for the production of antisera 4 and 3 are indicated.

Hydrophobic cluster analysis (HCA), a two-dimensional structure prediction method based on the detection and comparison of the structural segments constituting the hydrophobic core of globular protein has been described elsewhere (20, 21). ACE sequences were submitted to HCA analysis, and domain 1 and domain 2 of ACE orthologs were aligned according to hydrophobic clusters determined by HCA plots. Optimal alignment was performed using combined data from ClustalW and HCA analysis.

RESULTS

Biochemical Parameters—Three unrelated subjects with marked increases in plasma ACE levels were identified through a screening of patients referred to a general hospital (55.8, 83.8, and 86.6 units/liter). Using the colorimetric assay ACEcolor, normal values of plasma ACE fluctuate between 8.3 and 21.4 units/liter. Plasma ACE levels are 2.5–4-fold higher than highest levels found in the normal population. All other biological and paraclinical parameters measured were normal.

Sequencing of the ACE Gene Exons—A heterozygote point mutation was identified in exon 25 of the *ACE* gene. This point mutation is a C-T transition, introducing a leucine in position 1199 instead of a proline found normally at this position (Fig. 1). No other mutation was present in *ACE* gene exons from these subjects. Cotransmission of high plasma ACE levels and the Pro[1199] → Leu mutation was observed in the family, although this mutation was not found in a large sample of Dutch subjects (Kramers *et al.*).[2]

To elucidate the mechanism by which this mutation was responsible for the high plasma ACE levels observed in these individuals, an *in vitro* study using site-directed mutagenesis was undertaken.

Expression of Wild-type ACE and [Leu[1199]]ACE in COS-7 and HMEC-1 Cells—The expression plasmid pACE-WT (or peACE) was used to direct the synthesis of wild-type ACE (16). The expression plasmid pACE(Leu[1199]) is identical to pACE-WT except for a single Pro (CCG) to Leu (CTG) mutation at position 1199 of the coding sequence of somatic ACE and was used to direct the synthesis of [Leu[1199]]ACE.

The kinetic parameters for Hip-His-Leu hydrolysis of the wild-type ACE and of the [Leu[1199]]ACE, calculated from a Michaelis-Menten plot, were not significantly different (Fig. 2). The K_m and the V_{max} values of the [Leu[1199]]ACE were similar to that of the wild-type ACE (3.33 ± 0.11 mM *versus* 2.51 ± 0.12 mM and 0.35 ± 0.007 nmol/ml/min^{-1} *versus* 0.34 ± 0.008 nmol/ml/min^{-1}).

Both plasmids were transiently expressed in COS-7 cells for comparing the ability of cells to secrete [Leu[1199]]ACE with that of the wild-type ACE (Fig. 3, *A*, *C*, and *E*). Transfected cells were grown in serum-free medium for indicated times before the ACE enzymatic activity was measured with a Hip-His-Leu substrate by HPLC. No significant difference was observed in levels of cell-associated ACE between the wild-type form and [Leu[1199]]ACE during the time-course study (Fig. 3*A*). In contrast, levels of soluble [Leu[1199]]ACE activity were higher than wild-type ACE suggesting that the rate of release was enhanced (Fig. 3*C*). The percentage of solubilization, which was defined as the ratio of the enzymatic activity in the medium to the total enzymatic activity (medium + cells) was in average 1.5-fold higher for [Leu[1199]]ACE, as compared with wild-type ACE in COS-7 cells with a maximum difference of 2.5-fold at 24 h (Fig. 3*E*).

We also investigated the rate of secretion of [Leu[1199]]ACE in HMEC-1, an endothelial cell line, which does not express ACE, because endothelium is a major site of expression of ACE *in vivo* and the source for plasma ACE (Fig. 3; *B*, *D*, and *F*). Higher levels of soluble [Leu[1199]]ACE activity were observed, as compared with wild-type ACE, and lower cell associated ACE activity was observed. The rate of solubilization was in average 2.5-fold higher for [Leu[1199]]ACE compared with the wild-type form.

These data showed that a leucine in position 1199 instead of a proline in ACE protein leads to an enhancement of cleavage and secretion of ACE. Thus, both in an endothelial cell type and a nonendothelial cell type, differences between wild-type ACE and [Leu[1199]]ACE release observed *in vitro* are consistent with the plasma ACE increase observed in subjects bearing this mutation.

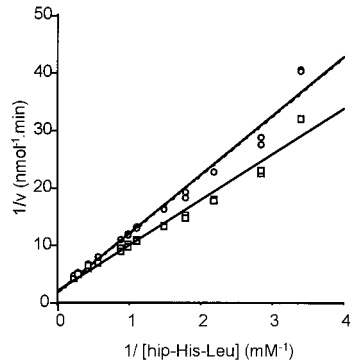

FIG. 2. **Lineweaver-Burk plots for hydrolysis of Hip-His-Leu by wild-type ACE and [Leu[1199]]ACE.** Wild-type ACE (□) was compared with [Leu[1199]]ACE (○). Assay was performed in a mix containing 100 mM potassium phosphate, pH 8.3, 300 mM NaCl, and 10 mM $ZnSO_4$ at 37 °C.

Biosynthesis and Secretion of Metabolically Labeled Wild-type ACE and [Leu[1199]]ACE—CHO cells stably transfected with the expression plasmid pACE-WT or pACE(Leu[1199]) were used to investigate the role of the Pro[1199] → Leu mutation on ACE secretion. Cells were labeled for 30 min with [^{35}S]methionine/cysteine and chased in serum-free medium for the indicated periods of time (Fig. 4). Affinity precipitates of cell lysates of transfected cells revealed two bands of immunoreactive ACE around 170 kDa. The lower band, which disappeared after 24 h of chase, corresponds to the underglycosylated form of ACE, and the upper band, which appeared at 1 h of chase, corresponds to the glycosylated form of ACE. The secreted [Leu[1199]]ACE appeared earlier than soluble wild-type ACE at 4 h of chase (Fig. 4*B*), whereas only traces of soluble wild-type ACE were visible after 8 h of chase (Fig. 4*A*). Furthermore, soluble [Leu[1199]]ACE increased rapidly in contrast with wild-type ACE, which was difficult to detect in the medium even after 16 h of chase.

The metabolic labeling results were consistent with the kinetic experiments based on ACE activity, indicating that [Leu[1199]]ACE was released more efficiently from the cell surface than wild-type ACE.

Subcellular Localization of [Leu[1199]]ACE—To test whether the Pro[1199] → Leu mutation influences the intracellular trafficking of ACE, distribution of ACE was determined in various compartments of CHO cells stably transfected with either wild-type ACE or [Leu[1199]]ACE grown for 3 or 24 h in serum-free medium. Medium was then collected, and lysosomal, cytosolic, and membranous compartments were separated by successive centrifugations. ACE activity was measured in each fraction and expressed as percentage of total ACE activity (Fig. 5). We determined that total ACE activity was equivalent for wild-type ACE and [Leu[1199]]ACE.

ACE activity measured in the cytosol for wild-type ACE and [Leu[1199]]ACE was very low, indicating that these proteins are rapidly addressed to the membrane. A higher proportion of total ACE (38.6 ± 8.5% for wild-type ACE and 24.9 ± 9.7% for [Leu[1199]]ACE at 3 h) was detected in lysosomal fraction. At 3 h (Fig. 5*A*) only 6% of total [Leu[1199]]ACE was detected in membranous compartment, whereas high levels of [Leu[1199]]ACE (34% of total ACE) was observed in the medium. At the same time, wild-type ACE levels were similar in membranous compartment and medium (17 and 16% of total ACE respectively). This difference between [Leu[1199]]ACE and wild-type ACE was more pronounced at 24 h (Fig. 5*B*).

Immunological Characterization of Membrane-bound and Soluble [Leu[1199]]ACE—To map the cleavage site of [Leu[1199]]ACE, Western blot analyses using specific antibodies were performed (Fig. 6). Antiserum 3 was raised against sequences from the C-terminal side of the Arg[1203] ↓ Ser[1204] cleavage site (22) and antiserum 4 was directed against a synthetic peptide located at the N-terminal side of this cleavage site (Fig. 1).

CHO cells stably transfected with either wild-type ACE and [Leu[1199]]ACE were grown to subconfluence and then incubated in serum-free medium for 48 h. Medium and CHAPS-solubilized cell lysates were analyzed on SDS-PAGE gel followed by Western blotting. Antiserum 3 failed to recognize wild-type or [Leu[1199]]ACE secreted in the medium, indicating that cleavage occurred before amino acid 1214. In contrast antiserum 4 was able to recognize the soluble form of wild-type and [Leu[1199]]ACE, indicating that cleavage occurred after amino acid

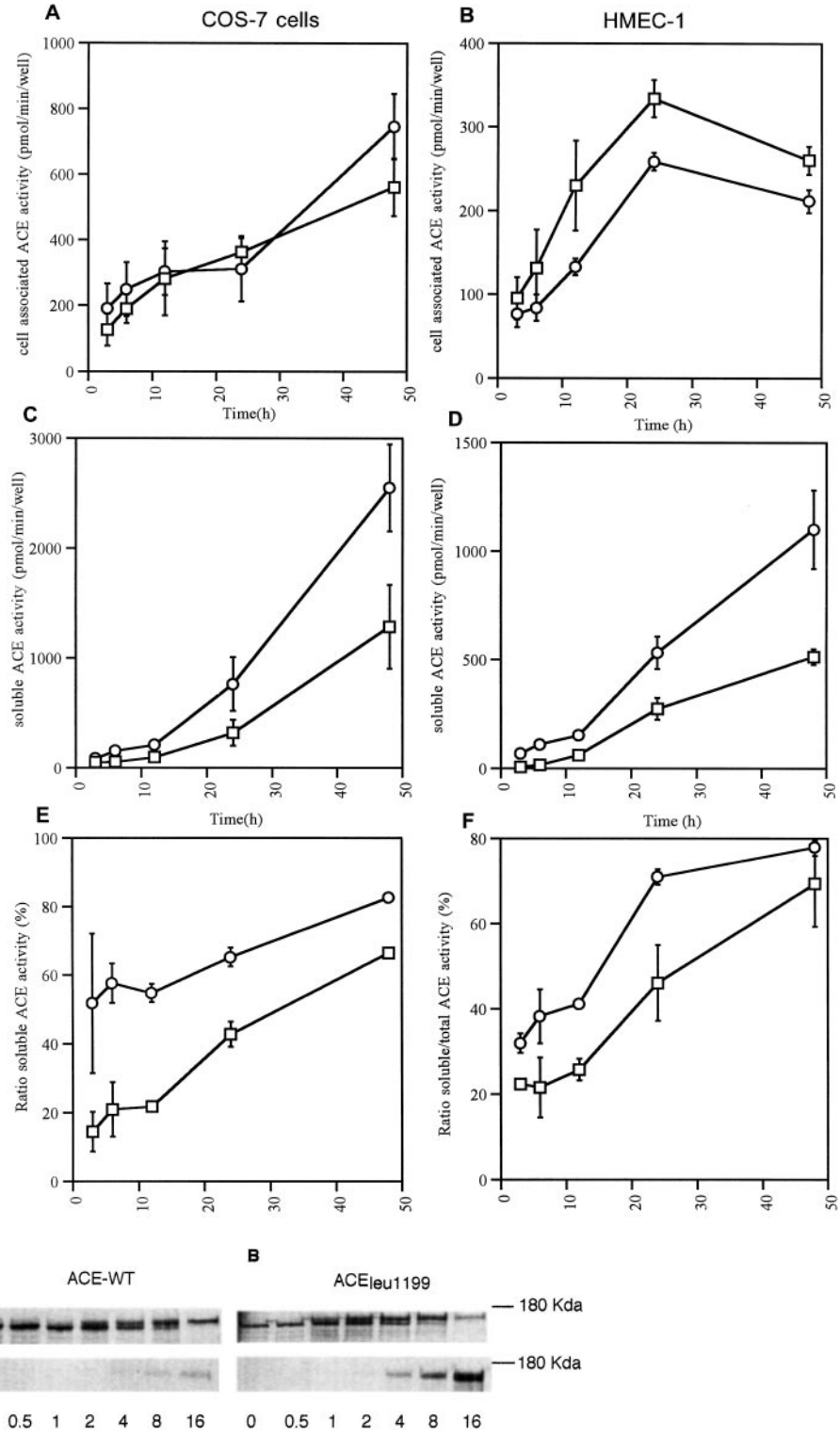

FIG. 3. **Time course of wild-type and [Leu^{1199}]ACE secretion.** Enzymatic activity of wild-type ACE (□) and [Leu^{1199}]ACE (○) transiently expressed in COS-7 cells (*A*, *C*, *E*) or in HMEC-1 (*B*, *D*, *F*) was measured for 48 h in serum-free medium. Medium samples (*C*, *D*) and detergent lysates (*A*, *B*) were collected at indicated time points and assayed with ACE substrate Hip-His-Leu. *E* and *F* are representations of the time course of ACE secretion, respectively, in COS-7 and HMEC-1 cells. The results are mean ± S.E. of duplicate determinations of two independent experiments. Where *error bars* are not evident, S.E. is less than the figure resolution.

FIG. 4. **Pulse-chase analysis of the wild-type ACE and [Leu^{1199}]ACE.** CHO cells expressing wild-type ACE (*A*) or [Leu^{1199}]ACE (*B*) were pulse-labeled with [^{35}S]methionine/cysteine for 30 min and then chased with serum-free medium from 0 to 16 h. An aliquot of cell lysates (200 μl) and medium (1 ml) were immunoprecipitated with HKE antiserum (3 μl).

1162. Both antisera recognized wild-type and [Leu^{1199}]membrane-bound ACE.

Effects of PMA and Compound 3 on [Leu^{1199}]ACE Secretion—To document the involvement of the already characterized ACE secretase in increased shedding of [Leu^{1199}]ACE, we investigated the effects of compound 3 on the [Leu^{1199}]ACE solubilization process. Transfected cells were grown in a serum-free medium containing (or not) compound 3 (50 μM), and the time course of secretion was investigated over a 48 h period (Fig. 7*A*). After 3 h, 31% of [Leu^{1199}]ACE was secreted from the untreated cells, whereas in compound 3-treated cells, only 4% of [Leu^{1199}]ACE was secreted at the same time. This effect was still observed after 48 h of treatment. So compound 3 inhibited [Leu^{1199}]ACE to the same extent that it inhibited wild-type ACE secretion.

COS-7 cells were transfected with wild-type ACE and [Leu^{1199}]ACE and were thereafter grown in serum-free medium containing (or not) PMA (500 nM). The time course of secretion was investigated over a 3-h period (Fig. 7*B*). PMA enhanced the rate of [Leu^{1199}]ACE cleavage secretion considerably. For example, after 3 h, 55% of [Leu^{1199}]ACE was secreted from the PMA-treated cells, whereas, in untreated cells, only

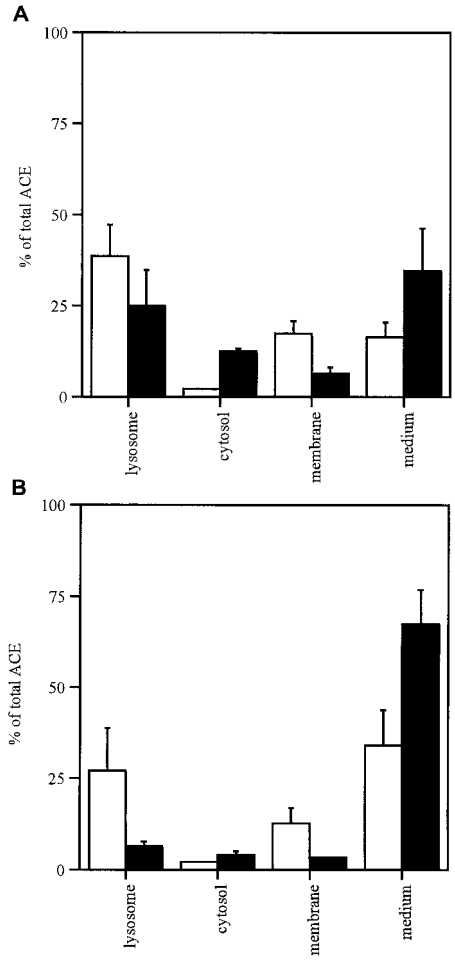

FIG. 5. **Subcellular localization of wild-type and [Leu^{1199}]ACE.** Enzymatic activity of wild-type ACE (*open bar*) or [Leu^{1199}]ACE (*filled bar*) was measured in subcellular compartments of CHO cells. The results are mean ± S.E. of three independent experiments. *A*, subcellular localization of wild-type and [Leu^{1199}]ACE in CHO cells grown 3 h in serum-free medium. *B*, subcellular localization of wild-type and [Leu^{1199}]ACE in CHO cells grown 24 h in serum-free medium.

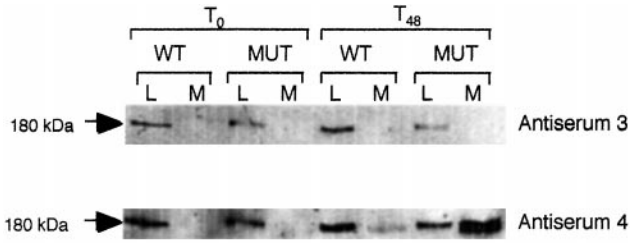

FIG. 6. **Western blot analysis of wild-type and [Leu^{1199}]ACE.** Wild-type ACE (*WT*) or [Leu^{1199}]ACE (*MUT*) expressed in CHO cells were analyzed on SDS-PAGE gel. Medium (*M*) and cell lysates (*L*) were collected immediately (T_o) or 48 h (T_{48}) after a switch to serum-free medium. Western blot analysis was performed using antisera 3 and 4.

20% of [Leu^{1199}]ACE was secreted at the same time. We observed a significant difference in solubilization of PMA-treated cells compared with untreated cells as early as 15 min after treatment. No similar effect was observed with wild-type ACE secretion.

Role of the N-terminal Domain: Expression of [Leu^{1199}]ACE-CF in COS-7 Cells and Effect of PMA on Secretion—The possible effect of the N-terminal domain on [Leu^{1199}]ACE solubilization was investigated using expression plasmid pACE-CF containing full-length somatic ACE cDNA except the coding sequence for the N-terminal domain (16). pACE-CF and pACE-CF(Leu^{1199}) were transiently expressed in COS-7 cells (Fig. 8*A*). At 6 h, nearly 50% of ACE-CF was solubilized in medium whereas at the same time only 20% of wild-type somatic ACE was secreted (compare Figs. 3*E* and 8*A*), confirming the lower solubilization

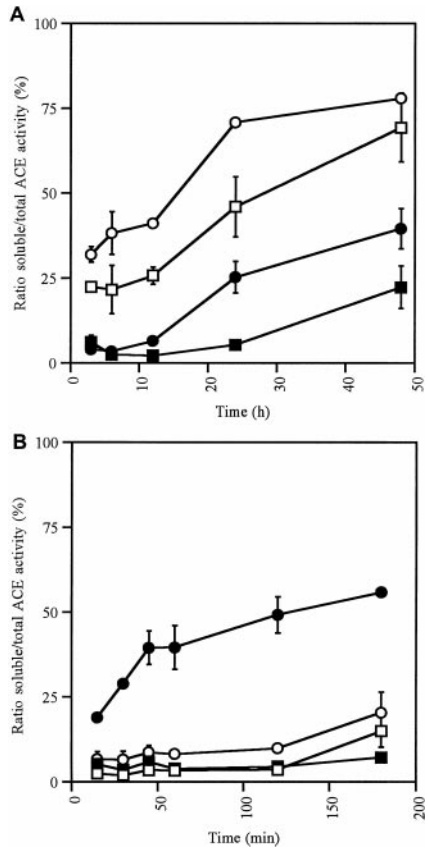

FIG. 7. **Effects of PMA and compound 3 on wild-type and [Leu^{1199}]ACE solubilization.** COS-7 cells transiently expressing wild-type ACE (*squares*) or [Leu^{1199}]ACE (*circles*) were grown in a serum-free medium with (*black symbols*) or without (*white symbols*) pharmacological reagents for indicated times. The solubilization was estimated by the ratio of enzymatic activity in the medium over total enzymatic activity (medium + cells). Results, expressed in percentage, are mean ± S.E. of duplicate determinations of two independent experiments. *A*, effect of compound 3 (50 μM) on ACE solubilization. Time course was performed from 3 to 48 h. *B*, effect of PMA (500 nM) on ACE solubilization. Time course was performed from 15 to 180 min.

of the two domain-containing enzyme. We observed a significantly higher rate of release for [Leu^{1199}]ACE-CF as compared with ACE-CF after 1 h (31 *versus* 7%, respectively). The percentage of solubilization was in average 1.8-fold higher for [Leu^{1199}]ACE-CF as compared with ACE-CF in COS-7 cells.

We also investigated the effect of PMA on ACE-CF and [Leu^{1199}]ACE-CF solubilization. (Fig. 8*B*). PMA enhanced the rate of solubilization of ACE-CF and [Leu^{1199}]ACE-CF. After 15 min, 14% of ACE-CF and 62% of [Leu^{1199}]ACE-CF were secreted from PMA-treated cells, whereas in untreated cells only 5% of ACE-CF and 20% of [Leu^{1199}]ACE-CF were solubilized at the same time. A PMA effect was more pronounced on [Leu^{1199}]ACE-CF solubilization in the early times of a kinetic analysis, but the difference was less evident between ACE-CF and [Leu^{1199}]ACE-CF after 2 h.

Structural Analysis—The HCA plots of domain 1 (N-terminal domain) and domain 2 (C-terminal domain) of several ACE orthologs were compared to produce an optimal alignment. Fig. 9 shows this alignment for the region of interest, *i.e.* close to proline 1199. HCA plots revealed that the shape of the hydrophobic cluster immediately upstream of this proline is typical of a β-strand, which would presumably end at that particular proline. An insertion of seven residues (NSARSEG in human ACE) is readily detectable before the next downstream pattern of hydrophobic residues. This insertion contains the cleavage site after Arg^{1203} of domain 2 except in the case of chicken domain 2 whose amino acid sequence differs at that site (Fig. 9). No hydrophobic residue was found within this insertion, thus suggesting that it corresponds to a loop. Interestingly, proline 1199 is located at the N-terminal side of this putative loop. Following the insertion, the shape of the hydrophobic cluster, although it presents an ambivalent character, might indicate the presence of an α-helix in domain 2 compatible with the constraint of linking the first extracellular domain to the membrane anchor.

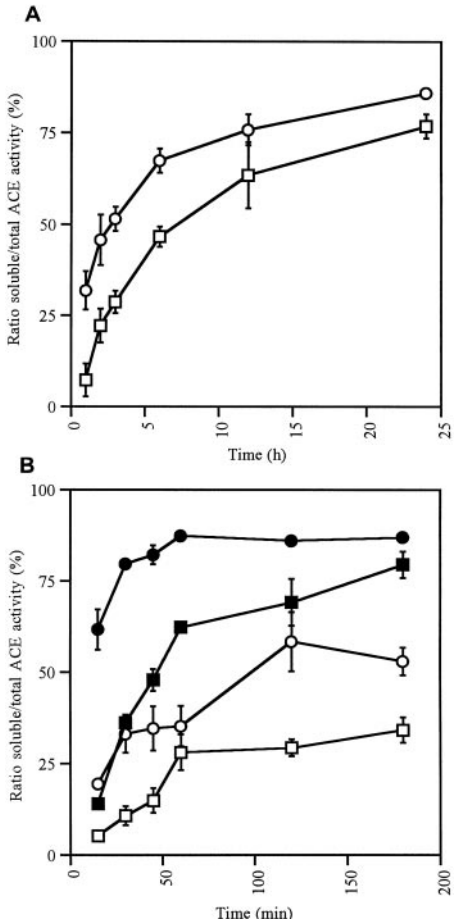

FIG. 8. **Time course of ACE-CF and ACE-CF(Leu^{1199}) secretion and effect of PMA.** *A*, rate of solubilization of ACE-CF (□) and ACE-CF(Leu^{1199}) (○) transiently expressed in COS-7 cells. Medium samples and detergent lysates were collected at indicated time points and assayed with ACE substrate Hip-His-Leu. The results are mean ± S.E. of duplicate determinations of two independent experiments. *B*, effect of PMA (500 nM) on ACE-CF and ACE-CF(Leu^{1199}) solubilization. COS-7 cells transiently expressing ACE-CF (*squares*) or ACE-CF(Leu^{1199}) (*circles*) were grown in a serum-free medium with (*black symbols*) or without (*white symbols*) pharmacological reagents for indicated times. Results, expressed in percentage, are mean ± S.E. of duplicate determinations of two independent experiments.

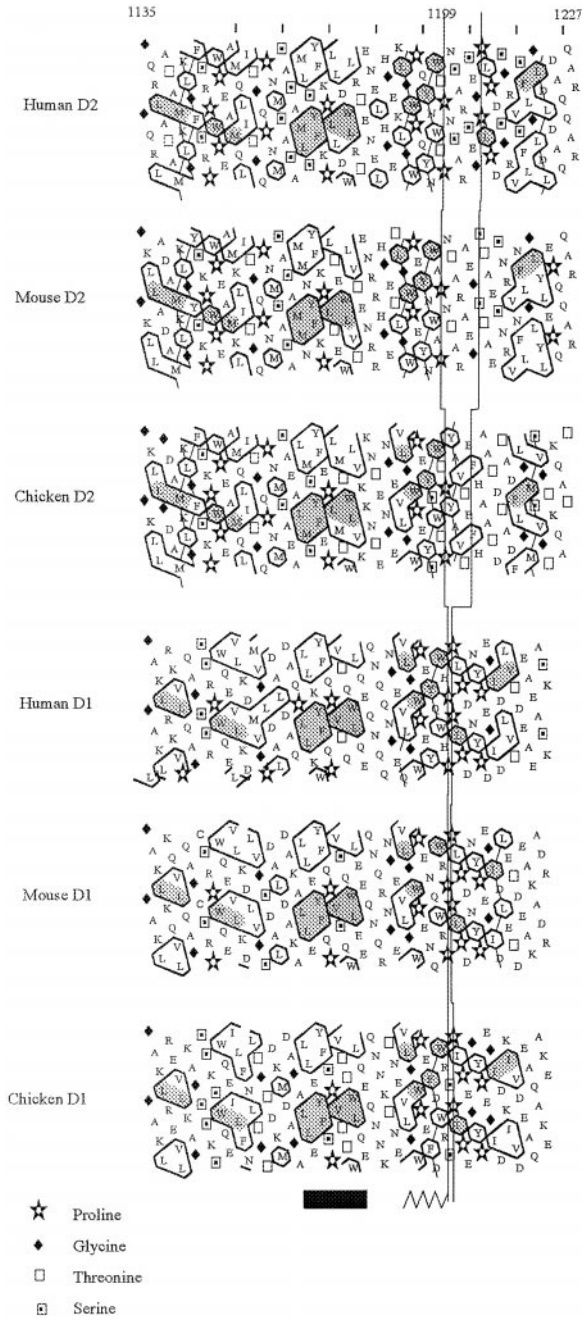

FIG. 9. **HCA plots of the N-terminal (*D1*) and C-terminal (*D2*) domains of somatic ACE.** This figure is limited to the C-terminal ends of both domains, *i.e.* when considering the human sequence between 1135 and 1227 for D2 and 537 and 622 for D1. On these plots, the sequence, which is duplicated to keep neighbors at the boundaries of the plot, is written following the *parallel* and nearly *vertical lines*. Four residues are represented by symbols as indicated at the *bottom* of the figure. Hydrophobic clusters are drawn when two hydrophobic residues (FILMVWY) are neighbors in this picture, and a cluster is broken by the presence of one proline inside it. It has been statistically demonstrated (31) that these clusters fit with secondary structure elements. *Gray shaded areas* correspond to conserved parts of the clusters among sequences. The last cluster before Pro^{1199} is clearly a β-strand element. The cluster following this proline does not have a conserved shape that allows elucidation of the nature of the corresponding secondary structure. The last two secondary structures before Pro^{1199} are indicated by *solid bar* and *zigzag symbols below* the figure.

DISCUSSION

We report a mutation of the *ACE* gene, which was found in three apparently healthy unrelated individuals from the same ethnic origin and selected for having very high plasma ACE levels. Pathological causes of high plasma levels could be eliminated in these individuals. Moreover, the genetic origin of the very high plasma ACE levels was supported by the Mendelian transmission of the trait in the families of these subjects (Kramers *et al.*).[2] Familial elevations of plasma ACE levels were already described in Japan and Italy, but the molecular basis has not been elucidated (23, 24).

In view of its large effect, the genetic increase of plasma ACE levels, which was observed in these subjects could not be attributed to the common polymorphism of the *ACE* gene, which explains 30% of plasma ACE variance in the Caucasian population and which is in linkage disequilibrium with an insertion/deletion polymorphism located within intron 16 of the *ACE* gene (8, 25).

The complete sequencing of the exons of the *ACE* gene was performed to detect a mutation specific to the three individuals. DNA sequencing revealed a mutation in exon 25 leading to a Pro^{1199} → Leu mutation of the somatic form of ACE. This mutation was not found in a large series of Caucasian subjects, and no other mutation was found in the coding sequence of the three selected subjects.

Because the Pro^{1199} → Leu mutation was located in the stalk region, which links the transmembrane domain to the first

extracellular domain, near the identified cleavage site (22), it was considered as a good candidate, and its functional effect was further investigated. We introduced the $Pro^{1199} \rightarrow Leu$ mutation into a somatic ACE expression vector, and we observed, as expected, no difference in enzymatic properties between the [Leu^{1199}]ACE and the wild-type ACE. The cleavage secretion process was therefore the most likely to be affected by the mutation. We investigated the release rate in two different cell lines, the COS-7 cells already used in previous studies, and the HMEC-1, an endothelial dermal microvessel cell line, which does not synthesize ACE. In both cell lines, an increased secretion rate of [Leu^{1199}]ACE was observed, as compared with wild-type ACE. The effect was however more pronounced in endothelial cells with a 2.5-fold increased secretion observed in average during the time-course study. Metabolic labeling experiments confirmed that [Leu^{1199}]ACE is secreted more efficiently than wild-type ACE. Subcellular localization of [Leu^{1199}]ACE in CHO cells revealed that this effect is associated with a slight but selective decrease of membranous ACE together with an increase in ACE concentration of the medium, indicating that a global increase of ACE production cannot explain the phenomenon observed. It is remarkable that an important part of total ACE is retrieved in the lysosomal fraction, indicating that a high proportion of both mutated and wild-type ACE is degraded before it is addressed to the membrane or that it is recycled from the membrane.

Two antibodies, raised against peptides flanking the cleavage site determined for both human testicular and somatic ACE (26), were used to determine whether [Leu^{1199}]ACE was cleaved at the same location as the wild-type enzyme. Indeed, [Leu^{1199}]ACE is cleaved between amino acid 1162 and 1214, a result consistent with the cleavage site determined for the *in vitro* expressed wild-type somatic and testicular enzymes, and for the seminal plasma ACE (26). Thus, our results do not favor the hypothesis of another cleavage site created by the mutation but suggest a more active cleavage rate at the major site. However we cannot exclude that a small fraction of secreted ACE, not detected with antiserum 4, is cleaved at a secondary cleavage site, such as the one described between Arg^{1137} and Leu^{1138} for human plasma ACE (13).

The cleavage secretion of ACE is an enzymatic process which can be modulated by pharmacological agents. A hydroxamic acid-based inhibitor of metalloproteases, compound 3, was shown to block very effectively the cleavage-processing activity of testicular ACE in a mouse epithelial cell line (ACE 89) stably transfected with the rabbit testicular ACE (27). Our data show that compound 3 completely abolished [Leu^{1199}]ACE secretion as well as wild-type ACE over a prolonged period of time (12 h), after which a progressive degradation of the inhibitor likely enabled secretion to rise again. This clearly indicates that the increased cleavage-secretion process observed for [Leu^{1199}]ACE results from the similar enzymatic process to the wild-type and not from cleavage because of another type of enzyme or from a nonspecific leakage from the membrane.

Phorbol esters, which activate protein kinase C, were shown to enhance the rate of ACE cleavage-secretion, with a marked difference between the somatic and testicular isoforms, the latter being more strongly solubilized than the first, even after a short period of incubation (26). The phorbol ester PMA markedly and rapidly increased [Leu^{1199}]ACE solubilization (4–5-fold), in contrast to wild-type ACE whose secretion was not significantly increased by PMA.

The cleavage secretion of somatic ACE is lower compared with testicular ACE, and it has been proposed that the N-terminal domain could have an inhibitory effect on its secretion. Because both the basal and phorbol ester-induced cleavage secretion of [Leu^{1199}]ACE resembled what is observed for the testicular isoform of ACE, we investigated the hypothesis that the mutation could suppress an effect of the N-terminal domain on solubilization. This was achieved by introducing the mutation in an ACE expression vector lacking the N-domain of ACE (pACE-CF) (16). At the basal level, secretion of [Leu^{1199}]ACE-CF was increased 4-fold as compared with the wild-type ACE-CF in COS-7 cells. After treatment with PMA, an early 4-fold increase of [Leu^{1199}]ACE-CF secretion rate was observed as compared with the wild-type ACE-CF. Two kinds of arguments drawn from our results indicate that the mutation is not acting through the suppression of an inhibitory effect of the N-terminal domain on the secretion rate. First, cleavage-secretion of somatic [Leu^{1199}]ACE is lower compared with C-terminal domain [Leu^{1199}]ACE. Second, the effect of the $Pro^{1199} \rightarrow Leu$ mutation on secretion increase is also observed with the recombinant ACE molecule lacking the N-terminal domain.

It was recently shown that, in the ACE89 cell line, testicular ACE can make a complex with protein kinase C subunits, and this complex dissociates in the presence of phorbol ester (28). A subsequent enhanced effect of ACE secretase was proposed to explain the phorbol ester-induced cleavage-secretion. In the cases of both somatic and C-terminal domain ACE (ACE-CF) with the $Pro^{1199} \rightarrow Leu$ mutation, the preservation of a higher solubilization rate under PMA indicates that the increase in speed of shedding induced by the putative PMA-induced complex dissociation can still be enhanced by a change in conformation.

Because no data are available on the ACE three-dimensional structure, we performed multiple alignment deduced from HCA plots of the N-terminal and the C-terminal domains of several ACE orthologs to predict secondary structures around proline 1199. It is remarkable that proline 1199 is highly conserved among ACE family sequences. Furthermore, this amino acid is the last common residue of the two domains preceding a loop specific for the C-terminal domain and which contains the cleavage site. The insertion of a fairly long loop between two secondary structures might be an indication of a better accessibility for proline 1199 by the C-terminal domain compared with the homologous proline 601 in the N-terminal domain. Proline is known to induce conformational constraints by creating twists and turns in peptidic sequences. We proposed that the presence of a leucine in position 1199 instead of a proline confers more flexibility in this region leading to better accessibility and action of ACE secretase.

Thus, even though it was shown that the cleavage-secretion process is not constrained by a specific amino acid sequence but is much more dependent on topological parameters (10), we show here that an amino acid sequence important for the conformational state of the juxtamembrane stalk region is critical for ACE release. More investigation in the structural conformation of the stalk region would provide interesting data for the study of the regulated shedding of ACE and other shedded membrane-associated proteins.

The elucidation of the molecular basis of a QTL affecting the plasma concentration of ACE has implications that go beyond the *ACE* gene itself. First, it illustrates that a QTL is not necessarily acting by modifying the expression level of the gene affected by the QTL, but conformational changes can modify its secretion rate and, hence, its concentration in a particular biological fluid where it can be measured in clinical practice. Second, it shows that the plasma concentration is a biological phenotype, which can be of limited value, because it does not necessarily reflect the level of expression at the cellular level. In the case described here, the membranous concentration of

ACE is even slightly decreased, and the high plasma concentration is not associated with any detectable pathological effect. In contrast, it has been shown that the above mentioned common polymorphism of ACE also affects the cellular level of the enzyme, where it can cause potential deleterious effects (29, 30). Finally, because the mutation described here cannot explain interindividual variations of plasma ACE associated with an insertion/deletion polymorphism of the *ACE* gene, our results clearly demonstrate that different molecular mechanisms account for strong genetic influences on plasma ACE.

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PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:

Increased Shedding of Angiotensin-converting Enzyme by a Mutation Identified in the Stalk Region

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