Physiological Non-equivalence of the Two Isoforms of Angiotensin-converting Enzyme

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Running Title: Physiological Non-equivalence of the Two ACE Isoforms
Summary

The structurally related somatic and germinal isoforms of angiotensin-converting enzyme (ACE) contain the same catalytic active center and are encoded by the same gene, whose disruption causes renal atrophy, hypotension and male sterility. The reason for the evolutionary conservation of both isozymes is an enigma, because, in vitro, they have very similar enzymatic properties. Despite the common enzymatic properties, discrete expression of both isoforms is maintained in alternate cell types. We have previously shown that sperm-specific expression of transgenic gACE in ACE -/- male mice restores fertility without curing their other abnormalities [Ramaraj, et al., (1998) J. Clin. Invest. 102, 371-378.]. In this report we tested the biological equivalence of somatic ACE and germinal ACE utilizing an in vivo isozymic substitution approach. Here we report that restoration of male fertility was not achieved by the transgenic expression of enzymatically active, somatic ACE in the sperm of ACE -/- mice. Therefore, the requisite physiological functions of the two tissue-specific isozymes of ACE are not interchangeable.
INTRODUCTION

The Angiotensin-converting enzyme (ACE) has long been regarded as a central player in the Renin-Angiotensin-System (RAS) through its action in converting angiotensin I (AngI) to the vasopressive peptide. ACE also degrades bradykinin as well as the vasodilating, apoptosis inducing peptide Ang (1-7) (1). While there are two isozymes of ACE in every mammal, both are transcribed in a tissue-specific fashion from distinct promoters within the same gene (2, 3). The 140kDa somatic (sACE) isoform is expressed in vascular endothelial cells, epithelial cells in the kidney proximal tubules, brain, intestinal brush border cells, monocytes, epididymis and Leydig cells of the testes (4-6). The 70kDa germinal (gACE) isoform is expressed exclusively in late pachytene spermatocytes (7). The overall sequence homology between isoforms across mammalian species is 80-90%.

Expression in such diverse tissue types evokes a broader, modern view of ACE as a physiological mediator of multiple regulatory processes. This concept was illustrated in ACE deficient mice which suffer from gross kidney structural abnormalities, altered vascular wall architecture, electrolyte imbalance and male infertility (8-10). The fertility defect was
due to poor sperm migration through the oviduct and failure to bind and penetrate the zona pellucida. However, there was no fertility defect in angiotensinogen knockout mice. Taken together, these results suggest that gACE may hydrolyze an oviduct or ovum-specific substrate (11). The absolute requirement for ACE expression was demonstrated in Drosophila in which two unique genes encode alternatively expressed ACE-like isozymes, AnCE and Acer (12). Two embryonic lethal mutants have mapped to the AnCE gene locus and heteroallelic combinations of these two mutations produced sterile male progeny (13).

Transcription initiations of gACE and sACE mRNAs occur at two alternative sites within the same gene. The gACE-specific sequences are spliced out of the mature sACE transcript while the gACE transcription initiates within an intron of the sACE transcription unit (2, 3, 6). As a result, the two proteins contain 665 common residues at their carboxyl termini whereas at the amino termini, sACE and gACE contain 664 unique residues and 72 unique residues, respectively (14). Both ACE isoforms are type I ectoproteins that are anchored in the plasma membrane through hydrophobic regions present near their carboxy termini. In addition, a soluble form of the sACE protein containing the extracellular domain is
also produced by the regulated action of a membrane-associated cleavage-secretion process (15-17).

Both isozymes share a common catalytic center located in the identical carboxyl terminal domains. As expected, the enzymatic properties of the gACE and carboxy domain of sACE are very similar owing to the common sequence shared by both isozymes (18-21). In contrast, sACE contains another catalytic center in its unique amino terminal domain (8, 19). Although both domains of sACE contain the same zinc-binding motif (His-Glu-X-Y-His), the amino-terminal active site cleaves LHRH thirty times faster and the hematopoietic peptide N-acetyl-Ser-Gly-Lys-Pro forty times faster than the C-terminal active site (18, 22). Similar substrate preferences were observed for the AnCE and Acer proteins (12, 23).

The evolutionary conservation of the two isoforms of ACE indicates that both are functionally indispensable (6). Moreover, their tissue-specific expression suggests that the specific physiological function of an isoform requires its expression in the correct tissue, a concept that was experimentally supported by our observation that gACE expression in sperm alone is sufficient for maintaining male fertility (24). It remains unclear, however, given the similarities in their enzymatic properties in
vitro, why the two isoforms are needed. The conservation of gACE is especially puzzling because the C-terminal active site of sACE is both structurally and functionally identical to the single active site of gACE. Moreover, recombinant sACE C-domain can hydrolyze several sperm-specific proteins in vitro (20). Thus, one would expect that sACE should be able to carry out all physiological functions of gACE if expressed in the appropriate tissues. This idea was experimentally tested in the studies reported here. By creating transgenic mice that express sACE exclusively in ACE deficient sperm, we demonstrate that each isoform is functionally distinct. Enzymatically active, sperm surface bound sACE failed to restore ACE -/- male fertility. Though the level of sACE expression exceeded that of normal gACE expression, there was no fertility blockage in ACE +/- mice that also contained the sACE transgene. Our results explain the strict evolutionary requirement for the expression of both ACE isoforms. In addition, we demonstrate the applicability of an in vivo isozymic substitution approach to test the functional equivalence of any two or more isoforms which share very similar enzymatic properties.
EXPERIMENTAL PROCEDURES

Plasmid Construction- The full-length rabbit somatic ACE cDNA was assembled in pCDNA3 (Invitrogen) which contains the T7 and CMV promoters. The 177 bp Ncol-Xhol genomic GC rich fragment, was joined at the unique Xhol site to the remainder of the sACE cDNA amplified by HiFidelity RT-PCR (Roche) on rabbit lung mRNA template utilizing a sense primer rACEp5Xho1 (5'GCTACAACTCGAGCGCCGAGCAGG3' and antisense primer rACEpendAS (5'CCCGTCGACTCAGGAGTGTCTTAGCTCCACCTCG3')). The vector containing the pgk2-somatic ACE transgene (Ps), was then generated by substituting the sperm specific 515 bp human phosphoglycerate kinase II (pgk2) promoter for the T7 and CMV promoters (24). The 5246 bp (Ps) transgene, released by SpeI and AsnI digestion, was purified from agarose gel using a Gene Clean system (Bio101) prior to microinjection into FVB zygotes by the Cleveland Clinic Foundation Transgenic Core Facility utilizing standard techniques.

Expression of sACE in Vitro and in Vivo- Prior to transgene microinjection, we tested for expression of full-length, enzymatically active sACE protein in vitro. A coupled transcription/translation reaction with
\(^{35}\)S Met was performed according to instructions in the T7 TNT kit (Promega). The resulting 140 kDa rabbit sACE protein was visualized in an 8% SDS-PAGE gel and autoradiography for 16 hours. Expression of enzymatically active sACE in vivo was tested by transiently transfecting plasmid AP005 into the ACE-expressing opossum kidney cells (OPK) (ATCC# CRL-1840) and human fibrosarcoma cells (HT1080) (ATCC# CCL-121) using Fugene 6 Transfection Reagent according the manufacturer’s instructions (Roche). Cells were cultured as described previously (25). The standard ACE enzyme assay that measures the cleavage of His-Leu from Hip-His-Leu was performed on the cell extracts as described (19).

Southern Blot Hybridization- Genomic DNA (15 µg) was digested overnight with Sac I as described (24). The DNA was electrophoresed in a 0.8% TBE agarose gel, transferred to Hybond N+ membrane (Amersham Pharmacia Biotech), pre-hybridized and hybridized in Church Buffer and washed as described (26). The 405bp-sACE probe used for founder identification was generated from plasmid AP005 by EcoR1 digestion. The 218bp mouse ACE exon13-14 probe was obtained by RT-PCR amplification from mouse testes total RNA and cloned into pBluescriptII (Stratagene) to yield mACE13-14/pBSII (2). The exon 13-14 probe, released by BamH1
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and HindIII digestion, was used for all subsequent genotyping since it hybridized to both mouse ACE and transgenic rabbit ACE genes. Probes were radiolabeled with $\alpha^{32}$P dCTP as indicated in the Random Prime Labeling Kit (Roche). After exposing to a Phosphorimager screen for 4 hours, each band was quantitated using ImageQuant analysis software. Copy number and transgene homozygosity was determined by normalizing the transgene signal to the corresponding mouse ACE signal.

Western Blot and Immunoprecipitated ACE Enzyme Assay - Tissues isolated from age-matched adult mice were homogenized in ACE lysis buffer as described (19). All Line A, C and D extracts (100 µg) were electrophoresed on an 8% SDS-PAGE then transferred to Immobilon-P (Millipore). Membranes were probed with the goat anti-rabbit ACE polyclonal antisera #447 (17) as described previously (24).

The immunoprecipitation ACE enzyme assay was performed by incubating 200 µg of tissue extract with antisera #447 diluted 1:150 in 150 µl ACE lysis buffer. Following rotation at 4°C for 1hr, 30 µl of ACE lysis buffer equilibrated rabbit anti-goat agarose beads (Sigma) was added. Incubation continued for an additional 1 hr at 4°C with rotation. Following extensive washing with ACE lysis buffer, the standard ACE
enzyme assay that measures the cleavage of His-Leu from Hip-His-Leu was performed on the rabbit ACE tethered to the agarose beads (19).

Quantitation of Rabbit ACE Expression- Un-fixed, adult testes were embedded in O.C.T. (Tissue-Tek) in dry ice/methanol bath and cryosectioned at 20 µm thickness. Slides were equilibrated at 25 C for 30 min in 50mM Tris, pH 7.4, 100 mM NaCl prior to probing with 800 pM $^{125}$I-351A (2176 Ci/mmol) Peptide Radiiodination Service Center, Washington State University, Pullman, WA) as described (27). All sections were quantitated with subtraction of background using ImageQuant software analysis of signals obtained by Molecular Dynamics Phosphorimager.

Histology and Immunohistochemistry- Age-matched, adult kidneys and testes were fixed in Histochoice (Sigma), paraffin embedded, cross-sectioned at 5µm thickness and hematoxylin and eosin stained by the Lerner Research Institute Histology Core (Cleveland, OH). Immunohistochemistry was performed following standard de-paraffinization procedures employing Clear-Rite and Flex solutions (Richard-Allan). Slides were twice boiled in 10mM sodium citrate pH 6.0
for 5 min and returned to PBS. The slides were blocked for 2hrs at 25 C in PBS + 10% horse serum + 0.3% Triton X-100 (blocking buffer). The primary antibody #447, diluted 1:1000 in blocking buffer was applied to the slides in a humid chamber for 16hrs at 4 C. Following washes in PBS + 0.3% Triton X-100 (PBST), anti-goat-FITC (1:200) (Santa Cruz) was applied to each section for 2 hrs in the dark at 25 C. Following washes in PBST, Vectashield (Vector Laboratories) diluted 1:1 in PBS was applied. All stained slides were visualized with a Leica digital fluorescent microscope and Adobe Photoshop software.

Mating Scheme- The C57Bl/6 strain ACE knockout mice were a kind gift from Oliver Smithies (9). To generate ACE knockout FVB strain mice, ACE +/- C57Bl/6 males were crossed with wildtype FVB females. Successive back-cross matings were continued for 8 generations with ACE +/- males and wild-type FVB females (Taconic). Since the previously reported transgenic mice, [pgk2-gACE-BGHpA, (Pg)] were FVB strain, generation of FVB strain ACE +/-, Pg +/- mice was performed by crossing FVB ACE +/- mice with Pg +/- - FVB strain mice. Mating ACE +/-, Pg +/- F1 sibs was performed to generate F2, ACE +/-, Pg +/- - sibs that were used as the control group for the fertility assay. Similarly, FVB pgk2-sACE-BGHpA (Ps)
transgenic founder mice were mated with ACE +/- FVB mice to generate ACE +/-, Ps +/- mice. Matings were then performed with all male and female genotypes to produce (ACE -/-, Ps +/-) and (ACE -/-, Ps +/-) experimental males. The number of pups sired from all of these matings was recorded.

Fertility testing of all four Line A experimental males was conducted by mating each with a total of six wild-type age-matched females (Jackson Labs) for 10 days. Females were observed for plugs. If no pups were produced within 22 days from male removal, the same females were mated with ACE +/- FVB males for 10 days. The number of pups per litter was noted. Control group fertility testing was performed with ACE -/-, Pg +/- sibs, age-matched to the Line A experimental group.
RESULTS

Construction of the sACE Transgene- The full length rabbit sACE cDNA was assembled in the pCDNA3 vector by joining a 177 bp ACE genomic fragment, (3), to a large fragment of the sACE cDNA that was generated by HiFidelity RT-PCR (Fig. 1A). Verification of full-length sACE expression was demonstrated by an in vitro transcription and translation assay (Fig. 1B). The in vitro translated sACE was enzymatically active in the standard ACE enzyme analysis that measures the cleavage of Hip-His-Leu to His-Leu (data not shown) (19). Expression of rabbit sACE in vivo was measured following transient transfection into HT1080 cells and sACE-expressing opossum proximal kidney tubule (OPK) cells. Again, 140 kDa, enzymatically active rabbit sACE was produced (Fig 1 C&D). Verification of the species specificity of the antisera was also observed. The goat anti-rabbit ACE antisera did not detect sACE in whole mouse lung (Fig. 1C).

With these results, the 515 bp human pgk2 promoter, previously shown to direct sperm specific transgene expression in mice (24), was substituted for the CMV promoter in the CMV-sACE-BGHpA construct to generate the pgk2-sACE-BGHpA (Ps) transgene (Fig. 2).
Generation of ACE -/-, Ps +/- Mice

For generating transgenic mice, the 5.25 kb SpeI to AsnI fragment of the pgk2-sACE-BGHpA expression vector, was micro-injected into fertilized FVB strain eggs. Out of twenty-one pups born, four had transgene integration. The founders were identified by southern blot hybridization with a probe isolated from the rabbit sACE cDNA (Fig. 2). For establishing lines from the four transgenic mice, they were mated with FVB mice of the ACE +/- genotype. It was important to assess the effects of the sACE transgene on mice with syngeneic backgrounds because physiological properties, such as blood pressure or litter size, are known to vary significantly among different strains (24).

Since our transgenic mice were of FVB strain, we transferred the ACE null genotype from the original C57Bl/6 background to the FVB background by repeated back-crossing for eight generations. Of the original four transgenic founders, only A (#5), C (#16) and D (#18) transmitted the transgene (Fig. 2). Founder B (#8) failed to transmit the transgene to any of 33 offspring analyzed (data not shown). Simultaneous verification of mouse ACE and Ps transgene genotype was performed using a mouse exon 13-14 probe. Hybridization of this probe to genomic DNA digested with Sacl resulted in an 8.4 kb ACE knockout allelic fragment carrying the neo' gene insertion, a 6.6 kb wild-type ACE fragment and a 3.7 kb sACE
transgene fragment (Fig. 2) (9). Further characterization of copy number and homozygosity was determined by Phosphorimager quantitation of the transgene normalized to the single copy mouse ACE gene. The copy number of line A, C and D was determined to be four, two and eight respectively. Sibling matings were then performed between ACE +/-, Ps +/- mice to generate the ACE -/-, Ps +/- male experimental mice used in the fertility analysis (Fig. 2).

Testis-specific Expression of Enzymatically Active Transgenic sACE.- Successful transmission of the Ps transgene was observed in Lines A, C and D. A western blot screen was performed to verify testes-specific expression of the Ps transgene. Figure 3A illustrates the production of 140 kDa sACE in the testes of Lines A and C mice as detected by rabbit sACE-specific antisera. Line D, however, produced a larger ACE-like protein. The anti-rabbit ACE antisera did not cross-react with murine sACE or gACE present in the testes extracts of wild-type mice. Further tissue analysis revealed that expression of the transgene was confined to the testes. There was no expression in Line A (ACE +/-, Ps +/-) brain, blood, heart, intestine kidney, liver, lung or spleen. Verification of the species specificity of the antiserum was again observed. The goat
polyclonal anti-rabbit ACE antisera did not detect sACE in any somatic tissue (Fig. 3B).

Because all testes contain both gACE, expressed on the sperm and sACE, expressed in the vasculature and somatic Leydig cells, we needed to quickly determine which founders expressed enzymatically active, transgene encoded sACE in sperm. We devised an immunoprecipitation procedure in order to selectively immunoprecipitate the transgenic rabbit ACE away from the endogenous murine ACE. Equal quantities of whole testicular extracts from transgenic and non-transgenic, age-matched mice were incubated with the rabbit ACE specific antisera raised in goat prior to immunoprecipitation with anti-goat antibody conjugated latex beads. Following several washes, the standard ACE enzyme assay, measuring Hip-His-Leu cleavage, was performed on the rabbit ACE tethered to the beads. Mice from Line A and Line C both expressed enzymatically active sACE while Line D failed to express active ACE when compared to non-transgenic, age matched males (Fig. 4A). Though line D produced a protein which was recognized by the anti-ACE antisera (Fig. 3A), it was not of correct molecular mass nor was it enzymatically active. No further experimentation was performed with mice from Line D and Line C mice. Mice from Line A were used exclusively for all further analysis.
We wanted to compare the levels of gACE expression in the sperm of ACE +/- mice with the corresponding level of the transgenic sACE expression in the ACE -/-, Ps +/- mice to ensure that enough of the transgenic protein was being expressed. Because an ACE-antiserum that reacts with both rabbit and mouse ACE with equal avidity was not available, we used an in situ ligand binding assay (28). In this assay, a radio-labeled Ang I analog (351A) was bound to testis sections and bound radioactivity was quantitated by Phosphorimager analysis. The assay revealed that about 70-fold more probe was bound to a testis section of an ACE -/-,Ps +/- mouse than to a corresponding section of an ACE +/- mouse (Fig. 4B). Thus, the transgenic protein was expressed to a much higher level than the level of the natural proteins.

Histology of the Kidney and the Testis- As previously reported for C57Bl/6 mice, in FVB ACE -/- mice, there was no observable distortion or abnormality of the testes architecture as revealed by hematoxylin and eosin staining (not shown) (8,11). Consequently, the overall histology of a testes section from an ACE -/-, Ps +/- mouse was indistinguishable from that of an ACE +/- mouse (Fig. 5A 1&2). In contrast, the same was not true for the kidney. Compared to the kidney of a wild-type mouse, the
kidney structure of an ACE -/-, Ps +/- mouse was profoundly abnormal (Fig. 5A 3&4). Thus, as expected, transgene sACE expression in the sperm did not cure the kidney defects of the ACE -/- mouse.

To demonstrate the sperm specific sACE expression in the Ps transgenic testes, an immunohistochemical assay was performed. Testes cross-sections, probed with the anti-rabbit ACE antisera, displayed a cobble-stone staining pattern indicative of surface localization of sACE on the sperm (Fig. 5B 2&4). Sperm specific staining intensified coincident with sperm maturation. The greatest staining was observed on fully mature sperm in the lumen of the seminiferous tubules. No staining occurred in Leydig cells, Sertoli cells nor any of the blood vessels of the testes, indicating testicular expression was restricted to the sperm. An age-matched, ACE -/- testes did not exhibit staining with this antisera (Fig. 5B 1&3).

Effect of Transgene Expression on the Fertility Status of Male Mice-

Previously, we reported that the pgk2 promoter driven, sperm-specific expression of rabbit gACE restored fertility in an ACE -/- mouse. However, the mice from that study were of mixed background due to the crossing of the C57Bl/6 strain, harboring the ACE -/- allele, with the FVB strain,
harboring the pgk2-gACE transgene (24). To verify that the same results were true for pure FVB strain, we assessed the fertility of male ACE -/-, Pg +/ - mice in a syngeneic FVB background (Fig. 6). Fertility was restored to the ACE -/- male that expresses rabbit gACE on the sperm. Thus, the FVB background could be used for testing the fertility effects of sACE expression in sperm.

Having established that sufficient levels of enzymatically active, surface bound sACE were produced by Ps +/- sperm, a mating scheme was employed to test the ability of the transgene to complement the ACE -/- mutant phenotype. Figure 6 presents mating outcomes from three adult males (ACE -/-, Ps +/-) and one adult male (ACE -/-, Ps +/- +). All were Line A males mated with six separate wild-type adult females. The mice were mated for 10 days or the equivalent of two estrous cycles. All females were plugged within the 10 day mating period but produced no offspring. Following a twenty-two day gestational waiting period, the same females were mated for another 10 days with an ACE +/-, non-transgenic age-matched male to prove female fertility. In all of the ACE deficient mice expressing sACE on the sperm, none sired pups demonstrating the lack of ability of sACE to provide fertility functions. The expression of the sACE transgene did not interfere with the function of endogenous gACE. Males
(ACE +/-, Ps +/-), (ACE +/-, Ps +/-), (ACE +/+, Ps +/-) and (ACE +/+, Ps +/-) were mated with females with various ACE and Ps genotypes to generate the experimental ACE -/-, Ps +/- male mice. The data revealed no abnormalities due to the expression of sACE on the surface of the sperm, even at levels 70-fold above the level expressed off of the native allele of an ACE +/- mouse (Fig.6).
DISCUSSION

Genetic dissection is a common approach used to determine the specific roles of individual components of complex physiological pathways. Recent advances in gene ablation and transgene expression techniques have enabled investigators to apply this approach to mice and evaluate the contributions of different gene products in mediating multi-factorial diseases such as hypertension. Thus, genes for each component of RAS, renin, angiotensinogen, ACE and AII receptors have been knocked-out individually (29). As anticipated, blood pressure regulation was perturbed in mice lacking any of the above genes. The same approach has also been used to determine the effects of gene dosage on some of the components of RAS (30). These studies have, in addition, produced some unexpected results. For example, mice deficient in ACE exhibit, in addition to low blood pressure, dramatic renal atrophy, vascular wall thickening, electrolyte imbalance and male infertility. The last phenotype was traced to the inability of sperm of ACE -/- mouse to migrate within the oviduct and bind to zona pellucida (11). That ACE expression in sperm alone is sufficient for imparting male fertility, was demonstrated by combining the powers of transgenic and knock-out technology. Using a sperm-specific promoter, gACE was expressed solely in developing
sperm of transgenic mice. By appropriate matings of the transgenic strain with ACE -/- mice, experimental mice expressing gACE only in sperm were produced. Those male mice were fertile but they still maintained the other defects of the ACE -/- mice (24).

In the current study, we have exploited the above experimental approach to examine the physiological reciprocity between the two isozymes of ACE. Because the genetic background of a mouse influences its physiological properties, such as blood pressure and litter size, we decided to conduct this series of experiments with mice of syngeneic background. For this purpose, the ACE -/- genotype was transferred from the C57Bl/6 strain to FVB strain, the strain of choice, by repeated back-crossing. The transgenic mice were also generated in the FVB background. As a result, mating of the transgenic and the knockout mice produced offspring of the same genetic background. The above experimental system was used in the current study to inquire whether sACE, if exclusively expressed in sperm, can substitute for gACE in supporting male fertility.

Structurally, sACE contains all of gACE except the 72 residues at its amino terminus (14). Thus, the carboxy terminal active center is completely conserved between the two proteins and in vitro, they cleave
many substrates, including Ang I, with equal efficiency \((6, 20)\). In contrast, several studies have indicated that the amino terminal active center, present only in sACE, has enzymatic properties that are distinct from those of the shared active center \((22)\). Therefore, it is conceivable that, in vivo, the two active centers are designed to act upon two sets of different substrates: both isozymes cleaving a common set of peptides and only sACE cleaving a second set. If this scenario were true, sACE should be able to carry out all physiological functions of gACE, a hypothesis tested in our investigation. Isozyme-specific physiological functions of ACE have been tested before by us and others. As mentioned previously, by mating transgenic and knock-out mice, we have shown that gACE expression in sperm is sufficient for maintaining male fertility \((24)\). Similar conclusions were reached by Hagaman, et al. 1998, who used mice carrying an isozyme-specific sACE gene knock-out. In another study, attempts to produce isozyme-specific gACE knock-outs generated mice which expressed no gACE and sACE but a truncated sACE containing the amino-terminal active center only. Because the truncated protein contained no membrane-anchoring domain, it was totally secreted and accumulated in the serum. Those mice exhibited all of the defects of ACE -/- mice \((8)\).
Results reported here clearly demonstrate the potential use (or the first successful use) of this experimental design to test the physiological equivalence of any two or more alternatively expressed isozymes which share similar enzymatic properties. In our study, sACE cannot functionally fulfill the role of gACE in sperm functions. The experiments were designed to ensure that both transgenic proteins are expressed in the same cells of the same tissue at the same developmental stage.

Quantitation of the levels of expression of the proteins by several methods established that transgenic sACE was expressed at a level higher than that of endogenous gACE and comparable to that expressed in transgenic gACE mice. Moreover, the expressed transgenic sACE was enzymatically active when tested in vitro. Why, then, could it not restore the sperm functions as transgenic gACE could? It is reasonable to speculate that it was probably due to the failure of sACE to recognize and bind the relevant putative substrate present in the oviduct whose cleavage allows the sperm transport. Given the structural differences of the two isoforms, the unique 72 residues of gACE, not present in sACE, may be required for recognition of this substrate. Conversely, the extra amino-terminal half of sACE, not present in gACE, may prevent access of the substrate to the binding site. We think that the binding of the
putative oviduct or zona pellucida substrate to sACE is defective, not its cleavage by the active center. The same carboxy terminal active center is present in both isozymes and neither 351 A binding nor cleavage of Hip-His-Leu was affected. Moreover, expression of sACE in large excess, did not block the function of gACE in the ACE +/-, Ps +/- mice, indicating that sACE does not act as a dominant inhibitor by competing for binding to the same substrate as gACE. Further experimental mice designed to exclusively express the sACE carboxy terminal domain and target it to the surface of ACE deficient sperm may be required to resolve this matter while the search for the putative oviduct/ ovum ACE substrate continues.
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REFERENCES


FOOTNOTES:

1 The abbreviations used are: ACE, Angiotensin-converting enzyme; Ang, angiotensin; sACE, somatic ACE; gACE, germinal ACE; pgk2, phosphoglycerate kinase 2; Ps, pgk2-somatic ACE; Pg, pgk2-germinal ACE
FIGURE LEGENDS

Fig.1. **sACE cloning and expression.**  (A) The sACE cDNA was positioned downstream of the CMV and T7 promoters in pCDNA3. (B) Autoradiograph of T7 promoter driven sACE in an in vitro transcription and translation reaction incorporating $^{35}$S-Met. (C) Autoradiograph of a western blot from HT1080 cells transiently transfected with CMV-sACE or pCDNA3. The 140 kDa rabbit sACE is detected by the goat polyclonal, anti-rabbit sACE antisera #447, secondary anti-goat HRP conjugate and ECL TM reagent. Mouse lung extract serves as control. All lanes contain 20 µg protein. (D) ACE enzyme assay on cell extracts obtained from HT1080 and OPK cells transiently transfected with CMV-sACE.

Fig. 2. **Southern hybridization and mating scheme.**

Autoradiograph of a Southern blot hybridization utilizing the 405 bp sACE probe to analyze 15 µg of Sac I digested mouse genomic tail DNA. Four out of 21 FVB pups generated by micro-injection received the pgk2-sACE-BGHpA transgene (Ps). Each founder was mated with ACE +/- FVB mice for transgene transmission. Founder and F$_1$ generation Sac I digested DNA was hybridized with the murine exon 13-14 probe to
determine the ACE and Ps genotype of each mouse based on the presence of a 6.6 kb (wild-type ACE allele), 8.4kb (ACE knockout allele) and 3.7 kb transgene fragment. Line A (ACE -/-, Ps +/-) mice were produced by mating ACE +/-, Ps +/- sibs.

Fig. 3. **Tissue-specific expression of sACE in transgenic mice.** (A) Autoradiograph of a western blot on 80 µg total testes extracts from Line A, C, and D (ACE +/-, Ps +/-), Line A (ACE -/-,Ps +/-) and wildtype (ACE +/- +) mice. Rabbit sACE (arrow) was detected with the goat anti-rabbit ACE antisera #447 (1:1000), anti-goat HRP conjugate (1:3000) and ECL TM reagent. The genotype of each mouse is listed. (B) Autoradiograph of a western blot on 100 µg total protein from a Line A (ACE +/-, Ps +/-) male. Transgene expressed sACE was visualized as described in (A) above. Bl, blood; Br, brain; H, heart; K, kidney; Li, liver; Lu, lung; Sp, spleen; T, testes.

Fig. 4. **Enzymatic analysis of transgene expressed sACE.** (A) Testis extracts from Line A, C, D (ACE +/-, Ps +/-) and wildtype (ACE +/- +) mice were incubated with anti-rabbit ACE specific goat antisera #447 and anti-goat conjugated latex beads. Following washes, the standard in vitro ACE
assay was performed on sACE tethered to the beads. The activity level of the wildtype mouse testes (2.0) has been subtracted from all samples. (B) Quantitation of sACE expressed on adult mouse sperm was measured by binding $^{125}$I-351A to 20$\mu$m thick testis cryosections. Triplicate slides for each genotype are represented. Slides were exposed to a phosphor screen and quantitated by Phosphorimager analysis with background correction.

**Fig. 5.** **Histology and immunohistochemistry.** (A) Hematoxylin and eosin stained sections of wildtype adult mouse testes (1), and kidney (3), compared with age-matched (ACE -/-, Ps +/-) mouse testes (2) and kidney (4). (B) Immunohistochemistry on sperm from (ACE -/-) and (ACE -/-, Ps +/-) testes. Sperm specific expression in the 5 $\mu$m section was detected with goat anti-rabbit ACE antisera #447 (1:1000) and anti-goat FITC conjugate (1:200). (1&2) and (3&4) are 10X and 40X magnification, respectively.

**Fig 6.** **Fertility testing of sACE expressing mice.** Three Line A, (ACE -/-, Ps +/-) and one Line A (ACE -/-, Ps +/-) mice were each mated with six different wild-type females. After failure to sire pups, the same females were mated with ACE +/- males and the number of pups/litter are
shown (•). The reported average for FVB strain is denoted (♦). Fertility of all genotype males mated with various genotype females is also noted. The control mating is between (ACE -/-, Pg +/-) sibs of FVB strain.
ACE +/- X

Line A

ACE +/+ +/− −/− −/− −/−

Line B

ACE +/+ +/− −/− −/− −/−

Line C

ACE +/+ +/− −/− −/− −/−

Line D

ACE +/+ +/− −/− −/− −/−
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| ACE   | ++ | ++ | ++ | ++ | -- |
| Ps:   | +/-| +/-| +/-| -- | +/-|

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140 kDa
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