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Germinal Angiotensin I-Converting Enzyme Is Totally Shed from the Rodent Sperm Membrane During Epididymal Maturation[1]

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ABSTRACT

Acquisition of sperm fertilizing ability is due, in part, to the reorganization of plasma membrane proteins that occurs during epididymal sperm transit. Using polyclonal antibodies against angiotensin I-converting enzyme (ACE), we showed that this enzyme is immunolocalized mainly on the middle piece of rat and mouse testicular sperm and with less intensity along the initial part of the principal piece of the flagellum. In both species, only some sperm from the caput epididymis were still reactive, whereas no labeling was observed on cauda epididymal sperm. The 105- to 110-kDa germinal ACE was absent from the rat testicular fluid but appeared in the fluid of the anterior epididymis. Thereafter, its molecular weight shifted to 94 kDa in the corpus epididymal fluid and remained at this weight in the caudal region. The 105- to 110-kDa immunoreactive protein was present in testicular rat sperm extract but was completely absent from epididymal sperm extracts. Western blot analysis of testicular and epididymal tissue extracts from the rat and mouse also confirmed that the germinal enzyme was absent from the epididymal sperm cell. Our results demonstrated that the rodent germinal ACE is released from the testicular sperm membrane when sperm enter the epididymis, a process similar to that observed in domestic mammals. This result is discussed in view of the suggested role for this enzyme in sperm fertility.

epididymis, gamete biology, male reproductive tract, sperm maturation, testis

INTRODUCTION

Sperm fertilizing ability results from their modifications in response to changes in the surrounding epididymal fluid [1–3]. Among these modifications, the sperm membrane surface is extensively remodeled by the integration, modification, or removal of proteins from the sperm membrane during epididymal transit [1, 2, 4, 5]. We have recently shown that the germinal form of the angiotensin I-converting enzyme (gACE) is one of these epididymis-processed proteins. This enzyme is liberated from the sperm membrane as it passes through the caput epididymis in various domestic mammals (ram, boar, stallion, and bull) [6]. This 105-kDa protein is retrieved in the fluid of the caput region, shows a subsequent shift to 94 kDa during epididymal transit, and remains at this molecular weight in the caudal fluid and in semen. The polyclonal antibody raised against the caudal fluid ACE mainly immunolabeled the middle piece of the testicular and proximal caput of ram sperm. Only the acrosome was labeled on some sperm of the subsequent regions, indicating that part of the ACE remained inserted in the membrane in this species.

Several groups have obtained ACE-deficient transgenic mice in which both somatic ACE (sACE) and gACE were absent [7–9]. These animals had lowered blood pressure and renal defects, and the males showed greatly reduced fertility but normally motile sperm. More recently, it has been shown that the lack of germinal ACE is specifically responsible for this reduced male fertility [10, 11]. The precise stage at which the absence of ACE interferes with fertility has not been determined, but these sperm are unable to pass through the female genital tract or bind to the oocyte [7, 9]. A role has been suggested for ACE and angiotensin II in sperm capacitation and acrosome reaction processes [12–14], and the angiotensin AT1 receptor has been immunolocalized on the tail of rat and human sperm [15]. However, other results in human and domestic animals have indicated that this enzyme is not directly involved in the binding and fusion of the sperm with the oocyte [16–18]. We therefore extended our previous study on domestic mammals to laboratory models (mouse and rat) to determine whether germinal ACE is important for fertility within the male or female genital tract.

MATERIALS AND METHODS

Biological Materials

Epididymides were obtained from adult rats (Lewis; age, >6 mo) and mice (SV109; age, >3 mo). The organ was divided into five zones for rat fluids and sperm collection (Fig. 1) and into three zones (caput, corpus, and cauda) for rat and mouse tissue extraction. Rat testicular sperm were obtained after 24-h accumulation induced by ligature of the efferent duct [19]. The rat cauda epididymal fluid and spermatozoa were obtained from regions 4 and 5 by retroperfusion with PBS. The epididymal fluids and sperm from regions 1–3 were collected by gently extruding the epididymal content on a parafilm sheet. These fluids were mixed with an equal volume of PBS, and spermatozoa were separated by centrifugation (10 min, 15 000 $\times$ *g*). The supernatants were centrifuged again (10 min, 15 000 $\times$ *g*) and kept at −20°C until use. Sperm pellets were resuspended in PBS, washed by two cycles of centrifugation (10 min, 500 $\times$ *g*), and then extracted by mixing the last pellet with an equivalent volume of reducing sample buffer. After 5 min, the mixture was centrifuged (10 min, 15 000 $\times$ *g*), and the supernatant was then removed and heated for 5 min at 90°C and used directly or stored at −20°C.

For tissue extracts, the testes, kidneys, and various epididymal regions were carefully dissected from fat pad and gross blood vessels and rinsed in Dulbecco modified Eagle medium before grinding at 4°C in the same medium with 1% (w/v) SDS, a cocktail of protease inhibitors (Sigma, St. Quentin Fallavier, France), and 1 mM EDTA. The extract was then centrifuged (5 min, 2000 $\times$ *g*, and the supernatant was carefully removed and centrifuged twice at 15 000 $\times$ *g* (5 min). The final supernatant was diluted (1:1 v/v) with sample buffer, boiled, and either used immediately or stored at −80°C. The quantity of protein for each extract was equilibrated visu-

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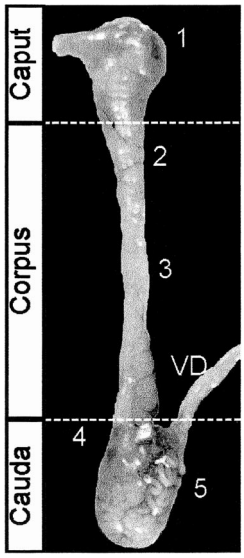

FIG. 1. Rat epididymis. Fluids and spermatozoa were collected at different sites of the epididymis: caput (zone 1), corpus (zones 2 and 3), and cauda (zones 4 and 5).

ally on a Coomassie blue-stained gel to be approximately 100 μg of protein per lane.

Gel Electrophoresis and Protein Blot Analysis

The sample preparation and electrophoretic methods used were as previously described [20]. Semidry transfer of proteins to nitrocellulose was performed over 2 h at 0.8 mA/cm^2. The blots were blocked with Tris-buffer saline-Tween containing 5% goat serum or nonfat dried milk. Two different rabbit polyclonal antibodies made in the laboratory against the gACE purified from the ram cauda epididymides were used in the present study either separately or together (final dilution, 1:5000) [6, 18]. These sera gave the same results on Western blot analysis. The polyclonal antibodies directed against intracellular C-terminal sequence were from the 28 series (28C8 and 28B8 [21]) and were a gift from Dr Alhenc-Gelas (U367, INSERM, Paris, France). They recognized only the complete membrane protein and the short sequence of approximately 10–15 kDa, which remains inserted in the membrane after ACE release from the cell [21]. They were used at a 1:2000 dilution. The secondary antibody was a goat anti-rabbit antibody conjugated with peroxidase (dilution, 1:5000; Sigma), was revealed with a chemoluminescent method (ECL+; Amersham Pharmacia Biotech, Uppsala, Sweden), and was visualized on film or by an imaging device. The second antibody used alone did not react on Western blots of the different samples.

Immunolocalization

Immunolocalization was performed as previously described [6, 22] on PBS-washed spermatozoa obtained either by collection after ligature of testes or epididymal perfusion (rats) or from cut pieces of testis and epididymis (rats and mice). The cells were fixed and washed before the incubation with anti-ACE polyclonal antibodies (dilution, 1:500) and revealed with a goat anti-rabbit antibody labeled with fluorescein isothiocyanate (dilution, 1:100; Sigma). The fluorescence was photographed using a 400 ASA black-and-white film (Kodak, Rochester, NY). The second antibody used alone did not react significantly.

ACE Activity Measurement

The ACE activity was measured using rat caudal fluid as described previously [18, 23] with the furanacryloyl-L-phenylalanylglycylglycine substrate (FAPGG; Sigma). The ACE-inhibitor captopril was diluted from a 10^{-3} M stock solution in ethanol:dimethyl sulfoxide (1:1).

Chemicals

Molecular weight standards for electrophoresis were from Amersham Pharmacia Biotech. Reagents were of the best available grade from Sigma and Prolabo (Fontenay-sous-Bois, France).

RESULTS

gACE and sACE in Rat and Mouse Epididymis

Proteins from the rat rete testicular fluid and from the fluid of the five different epididymal zones (Fig. 2) were separated by SDS-PAGE and transferred to nitrocellulose. The antibodies obtained against ram gACE (Fig. 2A) only reacted with a protein in the fluid from the epididymis. This protein of approximately 105–110 kDa in the proximal caput fluid (zone 1) showed a shift toward 94 kDa in the distal caput and corpus regions (zones 2 and 3). The reactive protein remained at this size in the cauda epididymides, although its concentration appeared to decrease (zones 4 and 5) and a carboxypeptidase activity of 6.4×10^{-2} μmoles FAPGG/(min and mg of protein) was found in this caudal fluid. This activity was totally inhibited by 10^{-4} M captopril, a specific and irreversible inhibitor of ACE. This activity copurified with the 94-kDa immunoreactive protein on gel filtration and ion-exchange chromatography (not shown). The immunoreactive protein was never observed in the rat rete testicular fluid collected from three different animals, and no carboxypeptidase activity was measured in these testicular fluids.

The same anti-ram gACE antibodies reacted specifically with a 105- to 110-kDa compound with the rat testicular sperm extracts and, with much less intensity, on sperm extract from zone 1 (Fig. 2B). No reaction was observed in the sperm extract from the subsequent epididymal regions (Fig. 2B). The same sperm extracts were also probed with polyclonal antibodies (28C8 and 28B8) directed against the C-terminal sequence of the sACE, which recognize the

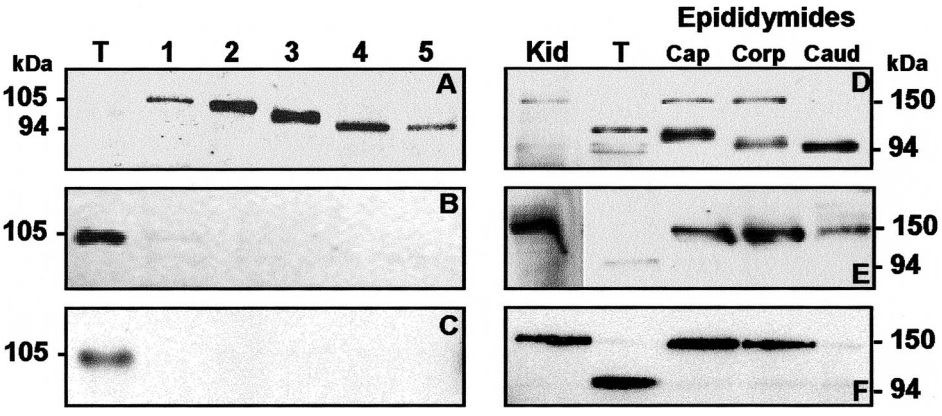

FIG. 2. ACE reactivity in epididymis. Rat epididymal fluids (**A**) and sperm plasma membrane extracts (**B** and **C**) from the testis (T) and different epididymal zones (1–5) as well as rat (**D** and **E**) and mouse (**F**) proteins extracted from kidney (Kid) and testis, caput, corpus, and cauda epididymal regions were separated on 6%–16% SDS-PAGE and subjected to Western blot analysis. The blots were incubated either with ram anti-gACE polyclonal antibodies (**A**, **B**, and **D**) or with anti-C-terminal ACE antibodies (**C**, **E**, and **F**).

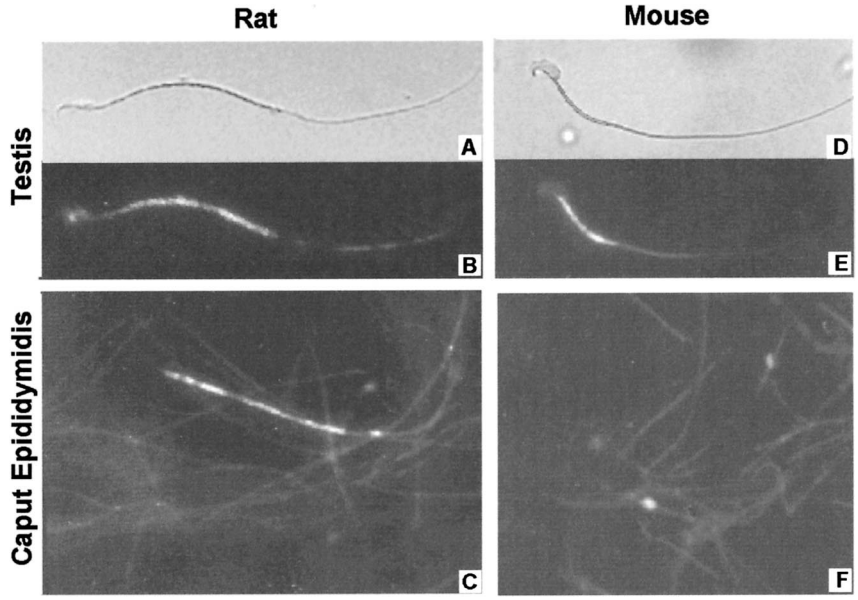

FIG. 3. ACE immunolocalization on rat and mouse sperm. Rat and mouse testicular and caput epididymal sperm were incubated with 28B8 anti-C-terminal ACE antibody and revealed with a secondary fluorescent antibody. **A** and **D**) Same as **B** and **E** in bright field. **B**, **C**, **E**, and **F**) Fluorescence. Scale: 12 mm = 10 μm.

complete (i.e., uncleaved) ACE. Both antibodies gave the same results (Fig. 2C); that is, they recognized a band at 105–110 kDa only in the testicular sperm extract representing the complete gACE (Fig. 2C). No reaction occurred with the sperm extracts from the other epididymal regions.

We also probed tissue extracts obtained from the kidney, testis, and the three main regions of the epididymis either with the polyclonal antibodies against ram gACE or the C-terminal region (Fig. 2, D and E, respectively). The anti-ram gACE antibodies reacted with the 150- to 160-kDa sACE present in the kidney extract (Fig. 2D). The 105- to 110-kDa band, representing gACE, was clearly reactive in the testicular extract, and in some cases, a faint band could also be detected at 150–160 kDa. A lower molecular weight band at approximately 90 kDa was also visible and could represent the intracellular nonglycosylated form of the gACE. Both the somatic 150- to 160-kDa form and the germinal form, showing the shift from 105–110 to 94 kDa, were reactive in the caput and corpus epididymal extracts, whereas mainly the germinal form was visible in the caudal extract. The same tissue extracts were probed with anti-C-terminal antibodies to determine the membrane-bound form of ACE (Fig. 2E). Only the somatic 150- to 160-kDa form reacted in the kidney and the three epididymal regions extracts, whereas it was mainly the 105- to 110-kDa form in the testicular extract. This confirmed that the gACE had lost its C-terminal anchor and was transported as a soluble enzyme in the epididymis.

Using the C-terminal antibody 28B8, which was the most reactive antibody in the mouse, we probed the tissue extracts obtained from mouse kidney, testis, and epididymis (Fig. 2F). The antibody recognized the 150- to 160-kDa sACE form in the kidney, caput, and corpus epididymis and the 105- to 110-kDa germinal form in the testis. No membrane-bound germinal form could be observed in the epididymal extracts, suggesting that the enzyme disappeared from the sperm membrane as previously observed in the rat. Unfortunately, our anti-ram gACE did not cross-react with the mouse sACE or gACE.

Immunolocalization of gACE on Rat and Mouse Sperm

We used the anti-C-terminal antibody 28B8 to immunolocalize gACE on rat and mouse sperm membranes (Fig. 3). In both species, the antibody clearly labeled the midpiece of the testicular sperm and, with less intensity, the beginning of the principal piece of the flagellum (Fig. 3, B and E). In the caput epididymis, only few sperm (estimated at $<5\%$) showed the same labeling (Fig. 3C). When the entire caput was used to retrieve sperm in mouse, almost no reactive sperm could be found (Fig. 3F). Only when sperm were taken from the proximal caput were some of them reactive (not shown). No labeling was observed with sperm from the cauda epididymis in either species.

DISCUSSION

In the male, two isoforms of ACE are derived from the same gene [24]. One is a somatic isoform that is constituted by two homologous N- and C-catalytic domains and has a molecular weight of 150–180 kDa. This isoform is involved in the regulation of blood pressure and is widely distributed in many tissues in the body [24, 25]. The other is a germinal isoform (also known as testicular) that is found exclusively on the haploid germ cell in the testis [26]. This germinal form of approximately 110 kDa is restricted to the C-terminus domain of the somatic enzyme due to the presence of a specific testis-promoter within the 12th intron of the gene. This germinal isoform is also characterized by a species-specific N-terminal [24]. The common C-terminal domain of these two isoforms contains a hydrophobic amino acid sequence allowing cellular membrane insertion. The somatic cell-bound ACE can become a free, blood-circulating enzyme after proteolytic cleavage of this anchor (for review, see [27]).

Using biochemistry and immunochemistry, we showed in the present study that gACE was present only on the membrane of mouse and rat testicular sperm and that the protein was completely released as the sperm passed through the proximal caput of the epididymis. The gACE was immunolocalized on the middle piece and, to a lesser extent, on the principal piece of the flagellum, a location identical to that described in the ram. Similar immunolocalization was obtained using the anti-ram gACE polyclonal antibodies on rat testicular sperm, although these antibodies also labeled the acrosome (not shown).

This removal of gACE from the sperm membrane is very similar to that observed in domestic mammals, where

it also occurs in the proximal part of the caput epididymis, suggesting the need for a specific environment for this process. In the reproductive tract, several sperm membrane proteins have been shown to be processed proteolytically in the caput epididymis during transit [28–30], and several proteases have been described in the fluid [31, 32]. However, to our knowledge, the protease involved in the precise mechanism of ACE liberation has not yet been identified.

Once liberated, the gACE remained as far as the rat cauda epididymal fluid, but a change in its molecular weight from 105 to 94 kDa occurred in the corpus, a change that has also been reported in domestic mammals [6]. This could be due to variations in postsecretory modifications, because as revealed by the presence of several spots with pIs between 6.0 and 8.0 on two-dimensional separation (not shown), the rat caudal fluid gACE is certainly glycosylated. We attempted to purify the germinal ACE of the rat cauda epididymal fluid, but the quantity of protein obtained was very low (not shown) and was partly masked by the 97-kDa β-D-galactosidase [33], which copurified with the ACE.

It has been suggested that male mice ''knocked out'' for ACE have very low fertility because their sperm are unable to pass properly through the female genital tract (although they are perfectly motile) and they cannot bind to the oocyte during in vitro fertilization [7, 9]. Our findings suggest that the sperm physiological changes may not relate to a direct role of ACE in capacitation, acrosomal reaction, or sperm-oocyte binding, because ejaculated mouse sperm may have no remaining ACE. The hypothesis that sperm ACE is not involved in postejaculatory fertilization stages is also supported by the fact that oocyte fertilization in other mammals is not altered by the presence of antibodies against this enzyme [16] or by the presence of the ACE-inhibitor captopril [17, 18].

It is interesting to note that the male mice knocked out for fertilin alpha and cyritestin, which are also testicular sperm proteins processed during epididymal maturation, and for calmegin, which is a chaperone involved in the processing of membrane protein (for review, see [34]), have almost the same phenotype as ACE knock-out mice: a decrease in the number of sperm able to pass through the female genital tract and/or sperm binding to the oocyte, without alteration in sperm production or motility. Moreover, recent results have indicated that knock-out of fertilin and cyritestin induced a decrease in or disappearance of several other membrane components [35]. Considered together, these findings suggest that changes in one of the components of the sperm membrane produce a nonspecific phenotype and may be due to an impairment of maturation of the membrane domains during spermiogenesis or epididymal transit. The absence of ACE from the testicular sperm therefore may, in fact, produce slight changes in the sperm maturation process without affecting sperm motility. However, we cannot completely exclude the possibility that the ACE knockout phenotype may also be related to the enzymatic function of this protein in the epididymis [36]. However, the fact that sACE cannot restore fertility of the sperm of gACE−/− mice does not support this last hypothesis [11]. Moreover, angiotensin II does not seem to be involved in the gACE−/− sperm phenotype, because male mice without angiotensinogen and, thus, angiotensin apparently remain fertile [37]. It is also of note that in this species, gACE is released in an epididymal area, where the tissue is already very rich in sACE protein (see *Results*).

In conclusion, we have demonstrated in the rat and mouse that the germ cell membrane-bound ACE is completely released under an active form and that this release occurs in a very precise epididymal area, a process similar to that observed in other mammalian species. The exact role played by this enzyme in the sperm reproductive function has yet to be elucidated, but the present findings suggest that its absence may impair the building or remodeling of the sperm membrane domains.

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