An acrosomal protein, sp32, was completely purified from acid extracts of ejaculated porcine sperm. Purified sp32 gave a single 32-kDa protein band on SDS-polyacrylamide gel electrophoresis and was characterized as a binding protein specific for 55-, 53-, and 49-kDa forms of (pro)acrosin. This protein was not capable of binding a 43-kDa acrosin intermediate and 35-kDa mature acrosin. sp32 significantly accelerated autoactivation of proacrosin at a basic pH in vitro and affected the maturation pathway of proacrosin. In the presence of sp32, the 49-kDa acrosin intermediate from the 55- and 53-kDa proacrosins was accumulated, instead of the 43-kDa acrosin intermediate. These results suggest that sp32 interacts with both the amino- and carboxyl-terminal sequences of the 53-kDa proacrosin. The cDNA clones coding for porcine and guinea pig sp32 have been identified from testis cDNA libraries in A×11. The deduced amino acid sequence indicates that sp32 is initially synthesized as a 61-kDa precursor protein with a putative signal peptide at the amino terminus. The carboxyl-terminal half of the precursor molecule corresponds to the mature sp32. Thus, sp32 is produced by post-translational modification of the precursor. The binding of sp32 to proacrosin may be involved in packaging the acrosin zymogen into the acrosomal matrix.

Mammalian sperm possesses an exocytotic vesicle, the acrosome. As a consequence of the acrosome reaction, the components of the acrosome are released and interact with the zona pellucida surrounding the oocyte. Thus, the acrosomal components are essential for fertilization. Acrosin is an endoprotease with a trypsin-like cleavage specificity (for review, see Refs. 1–3). During meiosis of male germ cells, acrosin begins to be produced in the testis (4–7). Moreover, both acrosin and its zymogen have been reported in human sperm (8). During proacrosin maturation, acrosin is converted into a 35-kDa mature enzyme via several intermediate steps: cleavage of the peptide bond between Arg23 and Val16 in the amino-terminal region of the 55-kDa proacrosin, leading to the formation of light and heavy chains, and removal of proenzyme segments from the carboxyl terminus (11). A 32-kDa protein, termed sp32,1 is co-purified in a complex with proacrosin, when acid acrosomal extracts are treated with ammonium sulfate (12). Our preliminary study (12) has demonstrated that the process of proacrosin maturation is affected by the 32-kDa protein; the 49-kDa acrosin intermediate is notably accumulated, whereas only a small amount of the 43-kDa form is detectable during proacrosin maturation.

It has been suggested that a proacrosin-binding protein is present in the sperm acrosome (14–19). Hardy et al. (16) showed that a 28-kDa acid-soluble protein is co-localized with proacrosin in the acrosomal matrix. Since the matrix does not contain a visible lipid bilayer, it is likely that the 28-kDa protein as well as proacrosin is not directly bound to the inner acrosomal membrane of the sperm (16). On the basis of the co-localization with proacrosin in the acrosomal matrix, they (16) have suggested that the 28-kDa protein corresponds to a proacrosin-binding protein. Polakoski et al. (14, 17) also reported the presence of a proacrosin-binding protein with a molecular mass of 29 kDa in porcine sperm. An antiserum against the porcine 29-kDa protein recognizes two proteins with similar molecular sizes from sperm extracts of various mammalian species (18). However, the binding of the 29-kDa protein with proacrosin has only been assessed by gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS). Thus, it is essential to obtain more biochemical data from the proacrosin-binding protein, including the primary structure.

In this paper, we have established a purification method for sp32 from porcine ejaculated sperm. Characterization of the purified protein demonstrates that sp32 is a binding protein specific for the 55- and 53-kDa proacrosins and the 49-kDa

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1 In a previous paper (Ref. 12 in the text), we named a 32-kDa protein from porcine sperm "acrosinin." However, the name acrosinin is similar in spelling and sound to "acrosin." To avoid the confusion, we changed the name from acrosinin to "sp32."
acrosin intermediate and that sp32 affects the pathway of proacrosin maturation. Furthermore, it is specifically localized in the acrosome. The primary structures of porcine and guinea pig sp32 are also reported here.

**EXPERIMENTAL PROCEDURES**

**Materials**—Freshly ejaculated porcine semen was kindly provided by Drs. T. Nagai and K. Kawakura at the National Institute of Animal Industry (Ibaraki, Japan). Proacrosin (a mixture of the 55- and 33-kDa forms) was purified from the sperm by the method of Polakoski and Parrish (10) with some modifications, as described previously (13). *Staphylococcus aureus* V8 protease and peroxidase-conjugated goat anti-rabbit IgG (H + L) were purchased from Wako Pure Chemicals (Osaka, Japan) and Jackson Immunoresearch Laboratories (West Grove, PA), respectively. Nitrocellulose and nylon (GeneScreen Plus) membranes were purchased from Amersham and DuPont NEN, respectively. Radiosotope, [32P]dCTP (3,000 Ci/mmol), was purchased from Bresatec (Adelaide, Australia). The cDNA libraries in Agt11 were prepared from polyadenylated RNAs of porcine and guinea pig testes, as described previously (11, 20). Commercial kits of cDNA synthesis, random-primer DNA labeling, and DNA sequencing were purchased from Pharmacia and LKB Biotechnology, Nippon Gene, and Takara Shuzo, respectively. All other reagents were of the highest purity available.

**Purification of sp32**—Acrosomal acid extracts were prepared from porcine sperm, as described by Polakoski and Parrish (10). The extracts were treated at pH 5.8 for 15 min and centrifuged at 10,000 *x* g for 10 min. The supernatant was re-adjusted to pH 3.0 with 6 m HCl and dialyzed thrice against 2% acetic acid. A portion (15 ml) of the dialyzed solution was filtered through a column (3.2 x 90 cm) of Sephadex G-200 that had previously been equilibrated with 2% acetic acid.

**Western Blot Analysis** of Crude Extracts from Porcine Testis and Epididymis—Using affinity-purified anti-sp32 antibody, showed no broad immunoreactive band of a protein with a molecular mass of 54 kDa was observed in both tissues. A broad immunoreactive band of a protein with a molecular mass of 29 kDa putative proacrosin-binding protein described by Yi was detected in the epididymis. The precipitates that contained sp32 in both tissues were probed by 32P-labeled DNA fragments. The conditions of hybridization and washing were the same as those described (11).

**Analytical Procedures**—Nucleotide sequence analysis was carried out by the dideoxy chain-termination method (28), using a BcaBEST dye terminator cycle sequencer (model 477A/120A) equipped with an on-line phenylthiohydantoin analyzer. Analysis of carboxyl-terminal amino acid sequence of sp32 was carried out as described previously (11).

**RESULTS**

When acrosomal acid extracts from porcine sperm were filtered through a Sephadex G-200 column, sp32 was completely separated from proacrosin by the gel filtration (Fig. 1A). Fractions containing sp32 were then dialyzed against 0.5 m NaCl in 1 mM HCl, 0.5 m NaCl solution, dissolved again in 6 m guanidine HCl, pH 2.5, and then applied onto a Toyopearl HW-55F column (1.6 x 85 cm) previously equilibrated with the same guanidine HCl solution. Fractions (1.6 ml) were collected at a flow rate of 6 ml/h and assayed for absorbance at 280 nm. Each of the fractions was dialyzed against 1 m HCl and analyzed by SDS-PAGE, as described above. The fractions containing sp32 were then stored at -20°C. The purification procedures were carried out at 0-4°C after the sperm pellet was collected from the ejaculated semen.

**Measurement of Acrosin Activity**—Proteolytic activity of acrosin was measured by 1-hydroxylation of benzoyl-l-arginine ethyl ester (Sigma) at 253 nm at 30°C, as described by Parrish and Polakoski (10, 22). One unit of the enzyme activity was defined as 1 μmol of N-benzoyl-l-arginine ethyl ester hydrolyzed per min under these conditions.

**Antibodies**—Purified sp32 (0.1 mg) was dissolved in 1 ml of phosphate-buffered saline, emulsified by sonication with 1 ml of Freund's complete adjuvant (Difco), and injected intradermally into female New Grove, PA, respectively. The blots were blocked with 1% skim milk, probed by affinity-purified antibody against sp32 or proacrosin, and then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate. The immunoactive bands were detected as described previously (12). After detection, the probes were removed by incubation of the blots in 50 m NaTris buffer containing 2% SDS, 1 x 2-mercaptoethanol at 50°C for 30 min. The blots were then re-probed, as described above.

**Immunohistochemical Analysis**—Porcine testicular tissues were fixed in 4% formaldehyde, phosphate-buffered saline at room temperature for two days, dehydrated through graded alcohol, and embedded in paraffin. The paraffin sections were placed on clear glass slides, dehydrated, and stained by the ABC method (24), using a Vector Stain Elite ABC kit (Vector Laboratories). The sections were probed by a 512-fold-diluted affinity-purified anti-body against sp32 or proacrosin, using 3,3'-diaminobenzidine as a chromogen. The sections were also counterstained with methyl green. For control experiments, the affinity-purified antibodies were replaced by a preimmune rabbit serum.

**Isolation of cDNA Clones**—Approximately 8 x 107 recombinant phage from a porcine testis cDNA library in Agt11 were plated on E. coli lawns and incubated at 42°C for 4 h. The plates were overlaid with nitrocellulose membranes that had been saturated with 10 m isopro- pyl-1-thio-p-p-galactopyranoside and incubated at 37°C for 6 h. The membranes were probed by affinity-purified anti-sp32 antibody, as described (11, 25). Screening of porcine and guinea pig testis cDNA libraries, using DNA fragments as probes, was also carried out by the plaque hybridization method (26). The DNA probes were labeled with [32P]dCTP by the random-primer labeling method (27). Hybridization was performed at 85°C, and the stringency employed for washing was the same as that described previously (11, 25). Positive clones were plaque-purified, and the cDNA inserts were subcloned into the EcoRI site of pUC19 or pUC119 for further characterization.

**Northern Blot Analysis**—Total cellular RNA (usually 10 μg) was glyoxylated, separated on 1.2% agarose gels, and blotted onto GeneScreen Plus or Hybond-N (Amersham) nylon membranes. The blots were probed by 32P-labeled DNA fragments. The conditions of hybridization and washing were the same as those described (11).

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autoactivation of proacrosin at pH 8.5. Aliquots (30 µl) of each fraction were precipitated by addition of ammonium sulfate (50% saturation), dissolved in 6 M guanidine HCl, pH 2.5, and dialyzed against 1 M NaCl. The resulting precipitate was collected by centrifugation, dissolved in 6 M guanidine HCl, and then applied onto a column (1.6 x 85 cm) of Toyopearl HW-55F equilibrated with the above guanidine HCl solution. Fractions (1.6 ml) were collected and assayed for absorbance at 280 nm (●) and acrosin activity (▲) after autoactivation of proacrosin at pH 8.5. Aliquots (30 µl) of each fraction were analyzed by SDS-PAGE, and fractions containing sp32 (horizontal bar) were combined. B, gel filtration of porcine sperm sp32 on Toyopearl HW-55F. Partially purified sp32, after gel filtration on Sephadex G-200, was precipitated by addition of ammonium sulfate (50% saturation), dissolved in 6 M guanidine HCl, pH 2.5, and dialyzed against 1 M NaCl. The resulting precipitate was collected by centrifugation, dissolved in 6 M guanidine HCl, and then applied onto a column (1.6 x 85 cm) of Toyopearl HW-55F equilibrated with the above guanidine HCl solution. Fractions (1.6 ml) were collected and assayed for absorbance at 280 nm (●). A horizontal bar indicates pooled fractions containing sp32.

al. (17) strongly suggests that sp32 is identical to the proacrosin-binding protein.

To examine the localization of sp32 in male germ cells, immunohistochemical studies were carried out using affinity-purified antibody against sp32 or proacrosin (Fig. 3). Both sp32 and proacrosin were localized in the acrosome of germ cells, but appeared to be present only in the round spermatids and elongating spermatids. Although our previous data (5) have demonstrated that the acrosin gene is already transcribed and translated in the pachytene spermatocytes, it is not certain at present whether sp32 is present in the pachytene spermatocytes as well as in the round spermatids. At any rate, sp32 was co-localized in the acrosome with proacrosin. No significant signal was observed when the preimmune serum was used as a probe (not shown).

The time course of proacrosin maturation in the presence of various amounts of sp32 was monitored by measuring enzyme activity as acrosin, and analyzed by SDS-PAGE (Fig. 4). In the absence of sp32, 55- and 53-kDa proacrosins were converted into a 49-kDa acrosin intermediate, which was quickly processed to a 43-kDa form and then to 35-kDa mature acrosin, as described previously (12, 13). Autoactivation of proacrosin was significantly stimulated by addition of sp32; the enzyme activity following the activation periods of 3 and 5 min in the absence of sp32 was much lower than those in the presence of various amounts of this protein. The 55- and 53-kDa proacrosins were still detectable after 3-min incubation in the absence of sp32, whereas these two proacrosins were hardly found in the presence of sp32. As the amount of sp32 increased, the 49-kDa acrosin intermediate was produced as a predominant form instead of the 43-kDa intermediate. Indeed, formation of the 43-kDa acrosin intermediate was decreased by the increas-

![FIG. 1. Purification of sp32 from acid extracts of porcine ejaculated sperm. A, gel filtration of acid extracts following treatment at pH 5.8 on Sephadex G-200. To the acid extracts 1 M NaOH was added to give pH 5.8, and the mixture was allowed to stand for 15 min and centrifuged. The supernatant solution was dialyzed against 2% acetic acid. A portion (15 ml) of the dialyzed solution was then filtered through a column (3.2 x 90 cm) of Sephadex G-200 that had been previously equilibrated with 2% acetic acid. Fractions (7 ml) were collected and assayed for absorbance at 280 nm (●) and acrosin activity (▲) after autoactivation of proacrosin at pH 8.5. Aliquots (30 µl) of each fraction were analyzed by SDS-PAGE, and fractions containing sp32 (horizontal bar) were combined. B, gel filtration of porcine sperm sp32 on Toyopearl HW-55F. Partially purified sp32, after gel filtration on Sephadex G-200, was precipitated by addition of ammonium sulfate (50% saturation), dissolved in 6 M guanidine HCl, pH 2.5, and dialyzed against 1 M NaCl. The resulting precipitate was collected by centrifugation, dissolved in 6 M guanidine HCl, and then applied onto a column (1.6 x 85 cm) of Toyopearl HW-55F equilibrated with the above guanidine HCl solution. Fractions (1.6 ml) were collected and assayed for absorbance at 280 nm (●). A horizontal bar indicates pooled fractions containing sp32.](image)

![Fig. 2. SDS-PAGE of purified porcine sperm sp32 and Western blot analysis of crude extracts from porcine testis and epididymis. Protein samples of sperm acid extract (lane 1, approximately 200 µg of protein), reduced sp32 (lane 2, 10 µg), and native sp32 (lane 3, 10 µg) were subjected to SDS-PAGE (left panel). Protein bands were detected by staining with Coomassie Brilliant Blue. Lane M, molecular size standards. Testicular and epididymal tissues (lanes 4 and 5, respectively) were homogenized in a solution (10 ml) of 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1% SDS, and extracted at 4 °C for 12 h. After centrifugation, a portion (10 µl) of the supernatant was subjected to SDS-PAGE. Proteins were transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) and probed by affinity-purified antibody against sp32. The immunoreactive proteins were detected as described under "Experimental Procedures." Note that the testicular tissues contained 54- and 32-kDa immunoreactive protein bands (arrows), whereas only the 32-kDa band was detected in the epididymal tissues.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>sp32</th>
<th>Proacrosin-binding protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asn</td>
<td>26.1 (26)</td>
<td>23</td>
</tr>
<tr>
<td>Thr</td>
<td>18.3 (18)</td>
<td>24</td>
</tr>
<tr>
<td>Ser</td>
<td>21.6 (22)</td>
<td>25</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>36.6 (37)</td>
<td>31</td>
</tr>
<tr>
<td>Gly</td>
<td>19.3 (19)</td>
<td>17</td>
</tr>
<tr>
<td>Ala</td>
<td>16.6 (17)</td>
<td>17</td>
</tr>
<tr>
<td>Cys</td>
<td>13.2 (13)</td>
<td>11</td>
</tr>
<tr>
<td>Val</td>
<td>15.2 (15)</td>
<td>14</td>
</tr>
<tr>
<td>Met</td>
<td>8.9 (9)</td>
<td>7</td>
</tr>
<tr>
<td>Ile</td>
<td>9.1 (9)</td>
<td>8</td>
</tr>
<tr>
<td>Leu</td>
<td>31.4 (31)</td>
<td>33</td>
</tr>
<tr>
<td>Tyr</td>
<td>9.4 (9)</td>
<td>7</td>
</tr>
<tr>
<td>Phe</td>
<td>11.2 (11)</td>
<td>11</td>
</tr>
<tr>
<td>Lys</td>
<td>13.4 (13)</td>
<td>14</td>
</tr>
<tr>
<td>His</td>
<td>5.8 (6)</td>
<td>5</td>
</tr>
<tr>
<td>Arg</td>
<td>14.4 (14)</td>
<td>13</td>
</tr>
<tr>
<td>Pro</td>
<td>10.1 (10)</td>
<td>9</td>
</tr>
<tr>
<td>Trp</td>
<td>ND*</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* A proacrosin-binding protein from porcine sperm reported by Yi et al. (17).

b Values of 72-h acid hydrolysis.

c Not determined.
ing amounts of sp32 (0–30 μg/10 μg of proacrosin, data not shown). When a nonacrosomal protein, carbonic anhydrase, was added as a control to the reaction mixture, the proacrosin activation pattern was essentially identical to that in the absence of sp32.

Since the amino acid compositions of sp32 and a putative proacrosin-binding protein were similar (Table I), it was intriguing to examine the binding interaction of sp32 with proacrosin and its processed forms. Sp32 bound the 55-, 53-, and 49-kDa forms of (pro)acrosin, but was not capable of binding the 43-kDa acrosin intermediate and 35-kDa mature acrosin. It is important to note that sp32 bound to the 49-kDa acrosin intermediate, as judged by the intensity of the immunoreactive protein bands (Fig. 5A). These intensity of the immunoreactive protein bands (Fig. 5A). These 43-kDa acrosin intermediates were abundantly present in the samples at the incubation periods of 5, 15, and 60 min, whereas the 49- and 43-kDa acrosin intermediates were abundantly present in these three samples (see C and B in Figs. 4 and 5, respectively). However, sp32 bound to the 53-kDa proacrosin much more strongly than to the 49-kDa form (Fig. 5A).

As shown in Fig. 4, sp32 itself was proteolyzed by enzymatically active form(s) of acrosin during proacrosin maturation. To examine how sp32 was proteolytically processed, proacrosin was incubated at pH 8.5 in the presence of a 2-fold amount of sp32. The resulting samples were subjected to Western blot analysis using affinity-purified antibody against sp32 (Fig. 6). Peptide fragments with molecular masses of 30, 20, 17, 12.5, and 11 kDa were produced in the early stages of proacrosin maturation, and the 17- and 12.5-kDa fragments were found to be predominantly proteolyzed forms after the 15-min incubation. Edman degradation analysis identified the amino-terminal amino acid sequence of Phe-Tyr-Gly-Asp-Leu-Tyr for the 12.5-kDa peptide fragment. No amino-terminal sequence was obtained for the 30- and 17-kDa fragments.

To isolate cDNA clones coding for porcine sp32, affinity-purified antibody against this protein was used to screen approximately 8 × 10⁶ recombinant phage from a porcine testis cDNA library in Agt11. Six positive clones were finally obtained. Restriction mapping and Southern blot analysis of these cDNA inserts demonstrated that they were all related (not shown). Since two cDNA clones, termed PN12 and PN22, possessed the longest inserts with an approximate size of 1.8 kilobase pairs among these six clones, we selected these two clones for further characterization. The composite nucleotide sequence of the overlapping cDNA inserts encoded a 1,613-nucleotide open reading frame that was flanked by a 3'-untranslated region of 197 nucleotides (Fig. 7A). There was a putative polyadenylation signal, CATAAAA, which was similar to a consensus sequence of AAATAAA, 13 nucleotides upstream of a consecutive adenine sequence at the 3'-end. The deduced amino acid sequences at residues 292–297 and 422–428 matched the amino-terminal sequences of the peptide fragments that were obtained by hydrolysis of sp32 with S. aureus V8 protease and
acrosin, respectively. Moreover, the carboxyl-terminal sequence, Ala-Gly, was consistent with the sequence determined experimentally by carboxypeptidase Y digestion. These results clearly confirmed that the isolated cDNA clones coded for porcine sp32. However, the cDNA sequence lacked a 5’-untranslated region and an initiator methionine. Rescreening of other porcine testis cDNA libraries, using a 295-nucleotide EcoRII/TagI fragment from the 5’-end of the PN12 insert as a probe, failed to reveal any longer inserts carrying the 5’-untranslated region.

The PN12 cDNA insert encoding porcine sperm sp32 was then used as a probe to screen approximately 5 x 10^5 plaques from a guinea pig testis cDNA library. Five positive clones, termed GN1, GN2, GN3, GN5, and GN10, were isolated. GN10 was selected for complete sequence analysis, and the inserts of other cDNA clones were partially sequenced. The nucleotide sequence encoded a 1,629-nucleotide open reading frame (Fig. 7B). The translation initiation site was assigned to an ATG initiator codon at nucleotides 50–52 because of the presence of an in-frame stop codon, TGA, 13 nucleotides upstream from the ATG codon. A CATAAA sequence similar to the polyadenylation signal was located at nucleotides 1,862–1,867, as found in the porcine sequence (Fig. 7A).

Northern blot analysis of total cellular RNAs from a variety of tissues demonstrated the presence of a single mRNA with an approximate size of 2.2 kilobases only in porcine and guinea pig testes (Fig. 8). Thus, the sp32 gene was specifically expressed in the testis, probably in the male germ cell. Moreover, the sizes of the cDNAs encoding porcine and guinea pig sp32 were close to the full lengths of the transcripts.

Comparison of the deduced amino acid sequences between porcine and guinea pig sp32 suggested that the porcine sequence lacked only 2 amino acids at the amino terminus, including the initiator methionine (Fig. 9). Since the amino-ter-

Fig. 4. Time course of proacrosin autoactivation in the presence of sp32. Porcine sperm proacrosin (a mixture of 55- and 53-kDa forms, 10 ng) was autoactivated at room temperature in 0.1 M Tris/HCl, pH 8.5, containing 0 (C, A), 5 (B, B), 10 (A, C), and 20 ng (D, D) of sp32. At the time intervals indicated, aliquots were taken out from the incubation mixture and assayed for acrosin activity and yeast 3-phosphoglycerate kinase with molecular masses of 31, 30, and 20 kDa on SDS-PAGE, respectively, and transferred onto a polyvinylidine difluoride membrane. The blot was probed by purified sp32 in 0.1 M Tris/HCl, pH 7.5, at the concentration of 10 ng/ml for 1 h at room temperature. Bound sp32 was detected as described in Fig. 2, using affinity-purified anti-sp32 antibody (A). After detection, the blot was treated at 50 °C for 30 min in 50 mM Tris/HCl, pH 6.8, containing 2% SDS and 0.1 M 2-mercaptoethanol to remove the probes and then re-probed by affinity-purified anti-proacrosin antibody (B).

Fig. 5. sp32 is a binding protein specific for 55- and 53-kDa proacrosins and a 49-kDa acrosin intermediate. Proacrosin (10 ng) was autoactivated at room temperature at pH 8.5 for 1, 5, 15, and 60 min (lanes 1, 2, 3, and 4, respectively) in the presence of 10 ng of sp32 (also see Fig. 4C). Proteins were separated by SDS-PAGE under reducing conditions, and transferred onto a polyvinylidine difluoride membrane. The blot was probed by purified sp32 in 0.1 M Tris/HCl, pH 7.5, at the concentration of 10 ng/ml for 1 h at room temperature. Bound sp32 was detected as described in Fig. 2, using affinity-purified anti-sp32 antibody (A). After detection, the blot was treated at 50 °C for 30 min in 50 mM Tris/HCl, pH 6.8, containing 2% SDS and 0.1 M 2-mercaptoethanol to remove the probes and then re-probed by affinity-purified anti-proacrosin antibody (B).

Fig. 6. Proteolysis of sp32 during proacrosin maturation. Proacrosin was autoactivated at pH 8.5 in the presence of 2-fold amount of sp32, as described in Fig. 4. After incubation at room temperature for 3, 5, 10, and 15 min, proteins were analyzed by SDS-PAGE under reducing conditions followed by Western blotting using affinity-purified antibody against sp32 (WTN). Protein bands were detected by Coomassie staining (CBB). Note that sp32 was proteolyzed during proacrosin maturation, and peptide fragments with molecular masses of 30, 20, 17, 12.5, and 11 kDa were produced. Of these peptides, the 30-, 17-, and 12.5-kDa fragments were analyzed by Edman degradation. The amino-terminal amino acid sequence of the 12.5-kDa peptide fragment was found to be Phe-Tyr-Gly-Asp-Leu-%, whereas no amino-terminal sequence was obtained for the 30- and 17-kDa fragments. Northern blot analysis of total cellular RNAs from a variety of tissues demonstrated the presence of a single mRNA with an approximate size of 2.2 kilobases only in porcine and guinea pig testes (Fig. 8). Thus, the sp32 gene was specifically expressed in the testis, probably in the male germ cell. Moreover, the sizes of the cDNAs encoding porcine and guinea pig sp32 were close to the full lengths of the transcripts.

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minal 25-residue sequence in guinea pig sp32 was highly hydrophobic, as analyzed by the method of Kyte and Doolittle (32), this sequence probably acts as a signal peptide required for translocation of a newly synthesized protein across a membrane (33). It is therefore conceivable that porcine and guinea pig sp32 contain 514 and 518 amino acids with calculated molecular masses of 58,287 and 58,733 Da, respectively. The cal-
Sp32 from Mammalian Sperm Acrosome

Fig. 8. The gene encoding sp32 is specifically expressed in testis. Total cellular RNAs (10 μg) from various tissues of porcine and guinea pig derivation were glyoxylated, separated on 1.2%-agarose gels and blotted onto GeneScreen Plus (Hybond-N for guinea pig) nylon membranes. The blots were probed by the 32P-labeled cDNA insert of PN12 (GN10 for guinea pig).

The calculated molecular size is almost identical to the molecular mass (54 kDa) of a testicular protein, which immunoreacts with affinity-purified antibody against sp32, on SDS-PAGE (Fig. 2). Thus, the 54-kDa protein appears to be the precursor of sp32. Furthermore, Edman degradation of the peptide fragments obtained by proteolysis with S. aureus V8 protease and acrosin (Fig. 6) indicates that the carboxyl-terminal half of the precursor molecule corresponds to sp32. If Valflut or Glu*~* corresponds to the amino-terminal residue of sp32, the calculated molecular mass (31,025 or 30,770 Da) and predicted amino acid composition are consistent with those determined experimentally (Fig. 2 and Table I). Thus, it is likely at present that Gluflut corresponds to the amino terminus of sp32 and that this residue has changed into pyroglutamic acid.

The deduced amino acid sequence of the porcine sp32 precursor shares a high degree of identity (78%) with the guinea pig sequence (Fig. 9). The locations of 20 cysteine residues and two potential sites of asparagine-linked glycosylation were completely conserved. The sp32 precursors contained a domain rich in glutamic acid and glutamine (EQ-rich domain) at residues 187-233, and three domains rich in cysteine residues at residues 58-121, 323-390, and 437-503 in the porcine sequence.

DISCUSSION

This study clearly demonstrates that sp32 is a binding protein specific for the 55- and 53-kDa proacrosins and the 49-kDa acrosin intermediate. Sp32 is completely purified as a 32-kDa protein from acid extracts of ejaculated porcine sperm by the present purification procedures, including selective precipitation in 0.5 M NaCl (Figs. 1 and 2). Western blot analysis indicates the presence of a precursor protein of sp32 in testicular tissue (Fig. 2). The cDNA sequences encoding porcine and guinea pig sp32 reveals that this protein is initially synthesized as a 61-kDa precursor, including a putative signal peptide of 25 amino acids (Fig. 7). Thus, sp32 is produced by post-translational modification of the precursor protein probably during sperm (germ cell) maturation in the testis and/or epididymis. However, the trigger and catalytic protein for the post-translational modification are not clear at present. The amino acid composition of sp32 agrees well with that of a 29-kDa protein acrosin (Fig. 6) are underlined with wavy lines. Potential sites of N-glycosylation at asparagine and of cleavage for producing the precursor and mature proteins of sp32 are indicated by asterisks and arrows, respectively. A putative polyadenylation signal, CATATAA similar to the consensus sequence (AATAAA), is underlined.
Sp32 from Mammalian Sperm Acrosome

![Fig. 9. Sequence alignment of porcine (P) and guinea pig (GP) sp32. Amino acids are represented by standard single-letter codes, and identical residues are indicated by asterisks. Dashes represent gaps introduced to maximize the alignment. Potential cleavage sites for producing the precursor and mature proteins of sp32 are shown by closed arrows. The split site of porcine sp32 during proacrosin maturation, leading to formation of the 12.5-kDa fragment (Fig. 6), is indicated by an open arrow. A domain rich in glutamic acid and glutamine (EQ-rich domain) is boxed. The locations of 20 cysteine residues (closed circle) and two potential N-glycosylation sites (open circle) are completely conserved between the porcine and guinea pig sequences. Moreover, a 25-residue sequence at residues 275-299 in the porcine sequence (a 22-residue sequence at residues 284-305 in the guinea pig sequence) is characterized by an abundance of acidic amino acids (shown underlines).](image-url)

reported as a proacrosin-binding protein (17), although there is a slight difference in the molecular size (3 kDa) between these two proteins (Fig. 2 and Table 1). Sp32 exhibits no enzymatic activity as a protease, glycosidase, phosphatase, or sulfatase (12). Also, this protein does not prevent proacrosin autoactivation, but rather accelerates the enzyme activation (Fig. 4). This observation appears to be a specific effect of sp32, since the purified protein treated with diisopropyl fluorophosphate, which inactivates contaminating serine proteases such as acrosin, still accelerates the proacrosin activation (data not shown).

Our previous studies (11, 12) show that the 53-kDa porcine proacrosin is structurally distinguished from the 49-kDa acrosin intermediate by the presence of an amino-terminal extension of 23 amino acids, corresponding to a light chain in the 49-kDa form. The 53-kDa proacrosin possesses the same carboxyl terminus as the heavy chain of the 49-kDa form (11, 12). Moreover, the carboxyl-terminal 18-residue segment of the heavy chain of the 49-kDa form is removed during its conversion into the 43-kDa form (11). Our present results may suggest that the 18-residue sequence contributes to the interaction with sp32 (Fig. 5). Alignment of the carboxyl-terminal sequences of proacrosins reveals a highly conserved sequence of 15 amino acids among porcine, human, mouse, and rat (data not shown, see Ref. 34). The 15-residue sequence largely overlaps with the 18-residue cleavable sequence. Thus, the 15-residue sequence is probably correlated with the interaction with sp32. This possibility seems to explain the reason why the 49-kDa acrosin intermediate, instead of the 43-kDa intermediate, is accumulated during proacrosin maturation (Fig. 4); sp32 blocks the cleavage site for conversion of the 49-kDa acrosin intermediate to the 43-kDa form by binding to the 15-residue sequence, and hence, this conversion is prevented.

As shown in Fig. 5, sp32 binds to the 53-kDa proacrosin more strongly than to the 49-kDa acrosin intermediate. Since the reduced form of the 49-kDa acrosin intermediate was actually used for the binding assay with sp32 (Fig. 5), this result must be interpreted as that sp32 binding to the 53-kDa proacrosin is stronger than that to the heavy chain of the 49-kDa acrosin intermediate. Again, the 53-kDa proacrosin is distinguished from the heavy chain of the 49-kDa form solely by the presence of the light chain sequence at the amino terminus (11, 12). Therefore, this uncleaved sequence appears to be also essential to form a stable binding complex between sp32 and proacrosin.

After most of sp32 is hydrolyzed during proacrosin maturation, the proacrosin processing is still affected; the 49-kDa acrosin intermediate is not readily converted into the 43-kDa form (Fig. 6). This result implies that a peptide fragment(s) generated by acrosin hydrolysis still possesses the ability to bind to the 49-kDa form. Thus, a relatively restricted region of sp32 may be responsible for the binding interaction with two proacrosins and the 49-kDa form. Interestingly, only the 25-residue sequence in the amino-terminal region of the mature sp32 is characterized by an abundance of acidic amino acids (Fig. 9), whereas the above 15-residue carboxyl-terminal sequence of proacrosin, highly conserved among species (34), is rich in basic amino acids. Therefore, the acidic region of sp32 may be a candidate for ionic binding with the carboxyl-terminal region of proacrosin.

Although sp32 binds two proacrosins and an acrosin intermediate, the biological roles of sp32, including its precursor, in acrosome biogenesis, sperm maturation, or fertilization are still unclear at present. The function of sp32 must be specific in the sperm acrosome, since the sp32 gene is expressed only in the testis (Fig. 8). In some eukaryotic cells, such as exocrine, endocrine, and neuronal cells, proteins are secreted by two distinct types of secretory pathway: the constitutive and regulated secretory pathways (for review see Ref. 35). In a proposed selective condensation mechanism (36, 37), regulated secretory proteins are condensed between the rough endoplasmic reticulum and trans-Golgi network, packaged into secretory storage granules, transported to the apical cytoplasm, and then released from cells by an external stimulus. It has also been reported that acidic polymers may play an important role in the condensation of basic proteins by ionic interactions (38-40).

The sperm acrosome is formed by fusion of proacrosomal vesicles and granules originating from the Golgi apparatus (6, 41-44). Thus, the acrosome can be considered to be a type of...
regulated secretory vesicle, since the components in the acrosome are released in response to a specific stimulus from the oocyte. Hardy et al. (16) have recently demonstrated that acrosomal components in guinea pig epididymal sperm are segregated into soluble and matrix compartments; proacrosin and a 28-kDa putative proacrosin-binding protein are the major proteins among the relatively few components in the acrosomal matrix. Since the acrosome contains a large amount of proacrosin (1), the acrosin zymogen must be tightly packaged and condensed in the acrosome matrix. Proacrosin is a basic protein (45, 46), whereas the sp32 precursor has an abundance of acidic amino acids, including those in the EQ-rich domain (Figs. 7 and 9). This fact is suggestive of an ionic interaction between these two proteins. Thus, it is interesting to suppose that both sp32 and its precursor protein functions as a protein required for aggregation with proacrosin to package and condense the acrosin zymogen in the acrosome matrix.

Our results also raise several questions concerning the implication of sp32 in (pro)acrosin function during fertilization. Two striking effects of sp32 on proacrosin activation/processing, the acceleration of proacrosin activation and simultaneous preservation of the 49-kDa form of acrosin (Figs. 4 and 6), may keep the protease activity associated with the acrosomal matrix during penetration of sperm into the zona pellucida of the oocyte. However, this possibility seems unlikely, because the 49-kDa acrosin intermediate as well as the 43-kDa form is the transient form of the mature acrosin (11). Also, the 49-kDa form exhibits protease activity probably as much as the mature acrosin (Fig. 4 and Ref. 12). In guinea pig sperm, Green (47) found the delay of acrosin release from sperm at the onset of the acrosome reaction; both the acrosome reaction and proacrosin activation are almost 50% complete in less than 3 min after the induction of the acrosome reaction with ionophore A23187. On the basis of this finding (47), Hardy et al. (16) suggested that acrosin may control its own release from the acrosome via proteolysis of the component(s) in the acrosomal matrix and that one or both of a putative proacrosin-binding protein and a 48-kDa protein in the matrix may be the physiologically relevant substrate for the release of acrosin from the sperm. Therefore, the acceleration of proacrosin activation by sp32 suggests promotion of acrosin release from the acrosomal matrix of the acrosome-reacted sperm.

After this paper was submitted for publication, a paper concerning an acrosomal matrix antigen, ACR-4, from porcine sperm appeared (48). A monoclonal antibody against ACR-4 recognizes a 28-kDa protein that binds proacrosin and a 49-kDa form of acrosin. It is likely that the 28-kDa protein is identical to sp32 represented here. Moreover, their conclusion for the function of the 28-kDa sperm protein (48) appears to be consistent with those of Hardy et al. (16) and us.