BIOLOGY OF REPRODUCTION **59,** 1498–1505 (1998)

Developmental Profile of a Caltrin-Like Protease Inhibitor, P12, in Mouse Seminal Vesicle and Characterization of Its Binding Sites on Sperm Surface[1]

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ABSTRACT

We examined the developmental profile of a kazal-type trypsin inhibitor (P12) of M_r 6126 in mouse seminal vesicle, characterized its binding sites on the surface of sperm, and assessed its effect on Ca^{2+} uptake by spermatozoa. Among the genital tracts of adult mice, P12 was found only in the male accessory glands including seminal vesicle, coagulating gland, and prostate. It was immunolocalized on the luminal epithelium of the primary and secondary folds in both the seminal vesicle and coagulating gland, and on the folds projecting into the lumen of the glandular alveolus in the prostate. The protein and its RNA message in seminal vesicle did not appear in the prepubertal period, but expression coincided with maturation. Castration of adult mice resulted in cessation of P12 expression. Treatment of the castrated mice with testosterone propionate in corn oil restored the protein expression in the seminal vesicle. Spermatozoa collected from caudal epididymis were devoid of P12. Cytochemical study illustrated a P12-binding region on the anterior acrosomes of cells preincubated with P12. Analysis of equilibrium data from the binding assay using ^{125}I-P12 with a Scatchard plot showed a single type of P12-binding sites on sperm, with an apparent dissociation constant of 70.15 ± 5.25 nM and the capacity of 1.49 ± 0.06 × 10^6 binding sites/cell. The protein could serve as a calcium transport inhibitor to suppress a great extent of Ca^{2+} uptake by spermatozoa. The immunohistochemical staining patterns of testis revealed that the P12-binding sites appeared on postmeiotic cells such as spermatids and spermatozoa, but were absent in Leydig cells, Sertoli cells, spermatogonia, and spermatocytes in seminiferous tubules.

INTRODUCTION

Existence of protease inhibitors (PIs) in the sexual glands of mammals is well known [1–3]. They have physiological functions in addition to their inhibitory effect on protease activity [4]. It is believed that they are important for the protection of genital tract epithelium against proteolytic damage [5] and/or have a regulatory role in the fertilization process [6, 7]. The trypsin-like activity seems to involve the binding of mouse spermatozoa to the zona pellucida [8]. In rat seminal vesicle, one calcium transport inhibitor (caltrin), which is identical to pancreatic secretory trypsin inhibitor, is able to suppress Ca^{2+} uptake by spermatozoa to prevent a premature acrosome reaction far from the oviduct [9]. Hence, the study of PIs in the genital tract becomes an important subject of reproductive biology.

We have purified a PI of M_r 6126 from mouse seminal vesicle secretions (SVS) and identified it as a Kazal-type trypsin inhibitor with very strong affinity to trypsin [10]. It is a basic polypeptide of 57 amino acid residues with no glycoconjugate. Its primary structure is identical to P12 deduced from P12 cDNA that has been cloned from mouse ventral prostate by Mills et al. [11]. Recently, a recombinant P12 with full activity of the naturally occurring P12 was recovered from a chimerical polypeptide of glutathione-S-transferase and P12 (GST-P12) expressed in *Escherichia coli* [12].

The P12 RNA message is detectable in the male accessory sexual glands of adult mice while its expression is constitutive in pancreas [13]. Substantial progress has been made to establish its genomic structure. As a result, the DNA-binding sites for some transcription factors such as GC2 and SP1 have been identified in this gene [14, 15]. On the other hand, less progress has been made in studying the role of P12 in reproductive biology despite its presence in SVS. Accordingly, we conducted this work to better understand the characteristics of P12-sperm binding as well as the developmental profiles of P12 in the seminal vesicle and its binding sites during sperm generation in the testis. In addition, we assessed the ability of P12 to suppress Ca^{2+} uptake by spermatozoa.

MATERIALS AND METHODS

Preparation of P12 and Its Derivatives

The seminal vesicles of mature male mice (ICR, 8–12 wk; Charles River Laboratories, Wilmington, MA) killed by cervical dislocation were carefully dissected free of the adjacent coagulating glands. The secretions collected from 100 mice were expressed directly into 100 ml of ice-cold 5% acetic acid. P12 was purified from SVS according to our previous procedure [10]. A fusion protein of GST-P12 was prepared from the expression of a recombinant DNA in *E. coli* [12]. A recombinant P12 with the full activity of the naturally occurring P12, with regard to its inhibitory effect on trypsin activity, was recovered from the chimerical polypeptide.

Detection of P12 and Its mRNA in Reproductive Tracts

The protein in normal saline (1.0 mg/ml) was mixed with an equal volume of complete adjuvant. Rabbits received intrasplenic injection of 0.25 ml of the mixture. After 12 wk, the animals were treated in the same way with 100 μg of antigen in a mixture of normal saline and incomplete adjuvant (1:1 by volume). The antiserum was collected 2 wk later. The P12 antibody was partially purified by affinity chromatography on a protein A-Sepharose (Pharmacia, Uppsala, Sweden) column.

The protein extract of tissue homogenate was resolved on a 15% polyacrylamide gel slab (6.5 cm × 10.5 cm × 0.075 cm) by the method of Laemmli [16]. The proteins on the gel were stained with Coomassie brilliant blue dye or transferred to a nitrocellulose membrane. After transfer,

Accepted July 14, 1998.
Received May 13, 1998.
[1]This study was partially supported by a grant (NSC 87–2311-B-002–041) from National Science Council, Taiwan.
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protein blots were immunodetected by Western blot procedures, using the P12-induced antisera or the partially purified antibody as the primary antibody and goat anti-rabbit IgG conjugated with horseradish peroxidase as the secondary antibody.

The total RNA of fresh tissue was prepared by the acid guanidium thiocyanate/phenol/chloroform method [17]. RNA samples were analyzed by separation on a 1.0% agarose/formaldehyde-containing gel [18], followed by capillary transfer to a nylon membrane, and were hybridized to ^{32}P-labeled nucleic acid probes [19]. ^{32}P-Labeled random-primed probe was prepared with a Promega random-priming kit (Promega, Madison, WI) using a template of P12 cDNA (369 base pairs [bp]) [12] or a cDNA segment of mouse β-actin gene inserted into a pGEM4Z vector.

Preparation of Spermatozoa

Cells were extruded from the distal portion of the caudal epididymis of adult male mice in a modified Tyrode's solution containing 125 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 4.5 mM glucose, 0.09 mM pyruvate, 8.9 mM lactate, 0.5 mM taurine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at pH 7.4 [20]. The cell suspension was gently filtered through two layers of nylon gauze, layered on top of a linear gradient of 10–90% Percoll (Pharmacia), and centrifuged at 150 × *g* for 10 min. Two distinct cell layers were formed. The lower layer, which contained more than 95% of viable cells with progressive motility [21, 22], was diluted with three volumes of modified Tyrode's solution and centrifuged at 150 × *g* to remove Percoll solution. The cells were resuspended in modified Tyrode's solution (1.0×10^7 cells/ml) and used for the study. Throughout this study we used only the preparation in which more than 70% of cells were motile.

Histological Studies

Tissues were fixed in freshly prepared Bouin's solution (0.2% picric acid and 2% formaldehyde in PBS) overnight, dehydrated in ethanol, infiltrated, and embedded in paraffin (Paraplast m.p. 56–57; Curtin Matheson Scientific Co., Houston, TX). Each tissue section (7 μm) was mounted on a slide that had been precoated with a solution containing 0.25% gelatin (Gibco, Grand Island, NY) and 0.025% chromium potassium sulfate. Sections were dried at 45°C, deparaffinized in xylene, and rehydrated through a gradient from alcohol to distilled water. The rehydrated sections were placed in a saturated lead thiocyanate solution and heated according to the method of von Wasielewski et al. [23]. After the slides had been cooled at room temperature for 15 min, they were rinsed in distilled water and PBS each for 5 min. The slides were immersed in a blocking solution (5% nonfat skim milk in PBS) in a moisture chamber at 25°C for 1 h and washed with PBST (0.05% Tween in PBS) four times, each for 15 min. The testis sections on slides were preincubated with 3.0 μM P12 in PBS at 25°C for 1 h, and the slides were washed with PBST as above. The slides prepared from each tissue were incubated for 2 h in the presence of the P12-induced antiserum diluted 1:250 in blocking solution. After the slides had been gently agitated in four changes of PBST for 15 min each, the antibody to P12 were immunodetected with alkaline phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:1000 in the blocking solution. The sections were covered with 50% glycerol in PBS and photographed with a microscope (AH3-RFCA; Olympus, Tokyo, Japan) after they had been washed with three changes of PBS for 15 min each.

Spermatozoa were air-dried on a glass slide and washed twice with PBS before cytochemical staining. The slides were immersed in 3.5% perchloric acid containing 0.04% Coomassie G-250 to manifest the intact acrosomes of spermatozoa according to previously described methods [24, 25]. To examine P12-binding region on spermatozoa, the slides were incubated with 3.0 μM P12 for 40 min, washed with PBS, and incubated with the P12-induced antiserum diluted 1:250 in the blocking solution for 30 min. The slides were washed three times with PBS to remove excess antibody before they were incubated with fluorescein-conjugated goat anti-rabbit IgG (Sigma) diluted 1:100 in the blocking solution for 50 min. All of the slides were rinsed with PBS and covered with PBS:glycerol (1:1 by volume) before observation under a microscope equipped with epifluorescence (AH3-RFCA).

$^{45}Ca^{2+}$ Uptake by Spermatozoa

Ca^{2+} uptake by epididymal spermatozoa was measured according to the method of Coronel and Lardy [26]. Before this measurement, the spermatozoa in the modified Tyrode's solution (1.0×10^7 cells/ml) were preincubated with 1.5 μM P12 or recombinant P12 at 37°C for 10 or 60 min. Then they were incubated in the presence of 200 μM $CaCl_2$ labeled with $^{45}Ca^{2+}$ (10–40 μCi/mg calcium; Amersham, Arlington Heights, IL) at 37°C for 10 min, and the cells were collected by vacuum filtration under 0.16 atm through a cellulose acetate filter. The cells on the filters were washed with six volumes of modified Tyrode's solution. The filters were dried and transferred into scintillation vials containing 5.0 ml of scintillation counting cocktail (Merck, Darmstadt, Germany). Radioactivity was counted by a liquid scintillation counter (Parker; LKB, Stockholm, Sweden).

Assay of P12 Binding to Spermatozoa

P12 was labeled with ^{125}I-Na in the presence of Iodobead (Pierce, Rockford, IL) according to previously described method [27]. The radiolabeled P12 was used to characterize the P12-binding site on sperm. Epididymal spermatozoa were incubated with ^{125}I-P12 at 25°C. The cells were collected on a Whatman (Clifton, NJ) GF/C glass microfiber filter by rapid filtration at a pressure of 0.5 atm. The filters had been blocked with 1% nonfat skim milk in 50 mM Tris buffer at pH 7.4 for 30 min and washed with ice-cold buffer before they were used. The filter was washed with six changes of 0.2 ml of ice-cold PBS and air-dried on a filter paper. Radioactivity of the filter was counted by a γ-counter. The nonspecific binding of ^{125}I-P12 to cells was measured by addition of a 100-fold excess of nonradioactive ligand. Each assay was performed in triplicate, and specific binding was the difference between the averages of the total binding and the nonspecific binding.

RESULTS

Distribution of P12 in Mouse Reproductive Tracts

Hypodermic injection of P12 to rabbits poorly induced the production of P12 antibody. Instead, we immunized the animals by intrasplenic injection of P12. The antiserum prepared from this method showed strong immunoaffinity to P12 as well as to GST-P12 but did not cross-react with GST (cf. lanes 1–3 of Fig. 1). Among the protein components

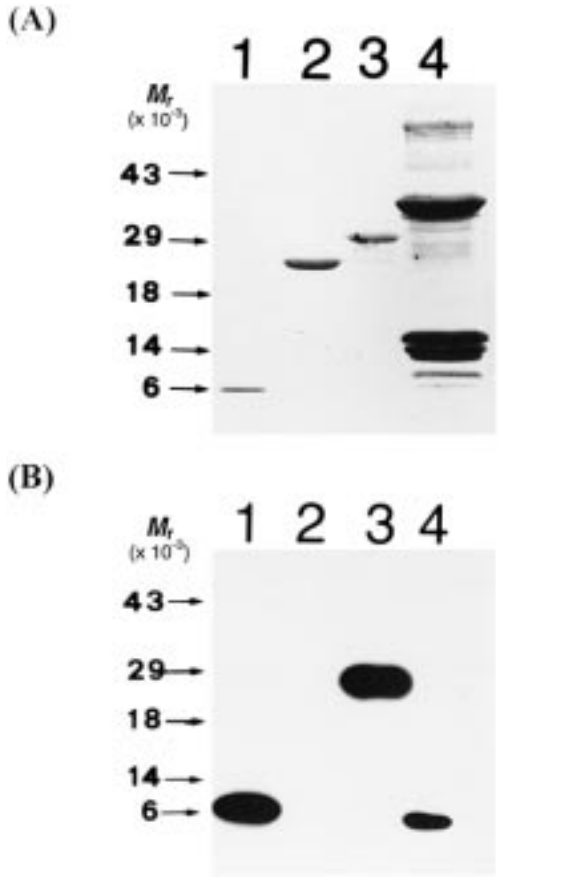

FIG. 1. Specificity of the P12-induced antiserum. The proteins were resolved by SDS-PAGE on a 15% polyacrylamide gel slab: lane 1, 2.0 μg of P12; lane 2, 4.0 μg of GST; lane 3, 4.0 μg of GST-P12; lane 4, 30 μg of the saline extract of mouse SVS. **A**) The proteins were stained with Coomassie brilliant blue dye. **B**) The proteins in the gel were transferred to nitrocellulose membranes and immunodetected by Western blot with the partially purified P12 antibody in the blocking solution (0.5 μg/ml) (see text for details).

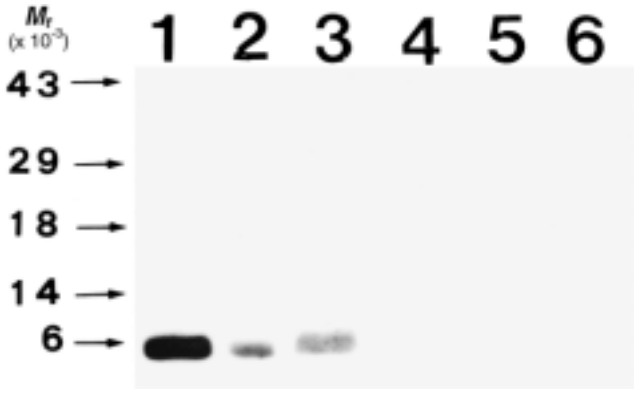

FIG. 2. Tissue distribution of P12 in sexual organs of adult male mice. Total protein (30 μg) prepared from the tissue homogenate of mice (12 wk) was run on a 15% polyacrylamide gel by SDS-PAGE, transferred to a nitrocellulose membrane, and immunodetected with the P12 antiserum. Tissues examined are seminal vesicle (lane 1), coagulating gland (lane 2), prostate (lane 3), testis (lane 4), epididymis (lane 5), and vas deferens (lane 6).

of SVS, which contained a trace amount of P12 (Fig. 1A, lane 4), the antiserum immunoreacted only with P12 (Fig. 1B, lane 4), showing the high specificity of the P12 antibody in the antiserum. Therefore, we used the antiserum for the immunodetection of P12 throughout this study.

We examined the distribution of P12 in the genital tracts of adult mice by Western blot analysis. In the male reproductive tracts, P12 was demonstrated in the homogenates of seminal vesicle, coagulating gland, and prostate, but it was not detectable in the homogenates of other sexual organs such as testis, epididymis, and vas deferens (Fig. 2). This is in agreement with the previous examination of the distribution of P12 mRNA in the male sexual organs [13]. The protein was not found in the female reproductive tracts including ovary, oviduct, uterus, and vagina. The existence of this protein in the three accessory sexual glands of adult male mice was also confirmed in the immunohistochemical staining patterns of P12 in the tissue sections shown in Figure 3. Abundant P12 appeared in the concretion of the glandular alveolar in the prostate and in the fluid in the lumen of both the seminal vesicle and coagulating gland. In the seminal vesicle, P12 was immunolocalized on the luminal epithelium of primary and secondary mucosa folds, but it was absent in smooth muscle cells, indicative of the secretion of P12 from the epithelium of mucosa folds to lumen. The secretion of P12 from the epithelium of mucosa folds was also noted in the coagulating gland. In the prostate, the secretion of P12 from the fold projecting into the lumen of glandular alveolus was obvious.

Developmental Profile of P12 Expression in the Seminal Vesicle

It is well known that accumulation of SVS, which contains a group of proteins that constitute the major protein components of seminal plasma, becomes prominent in the postpubertal period. We compared the levels of P12 and its message in the seminal vesicle at different ages of male mice in order to assess whether the P12 expression coincided with the development of this accessory sexual gland. The P12 RNA message was undetectable in the mice younger than 3 wk old, increased remarkably when they were 4 wk old, and remained at that level thereafter (Fig. 4A). The protein level corresponding to this change was examined by Western blot analysis, shown in Figure 4B. The protein did not appear in mice younger than 4 wk old, but it increased progressively with age after 5 wk. Apparently, P12 is synthesized by the translation at a constant level of P12 mRNA in the epithelium of mucosa folds, and the successive secretion from the epithelial cell results in its accumulation in the lumen. The positive correlation of P12 and its RNA message with the development of the seminal vesicle prompted us to assess the effect of androgen on P12 expression in the accessory sexual gland. Figure 5 displays the Western blot analyses of P12 in the tissue homogenates of seminal vesicles from mice (14 wk) treated in several ways. The protein disappeared in the tissue of mice that had been castrated 3 wk earlier (Fig. 5, lane 2). The protein was not detectable in the tissues of castrated mice that had received a daily injection of corn oil only (Fig. 5, lane 3). The protein reappeared in the tissues of castrated mice after they had received daily injections of testosterone propionate in corn oil for 8 consecutive days (s.c., 5 mg/kg of body weight per day; Fig. 5, lane 4). As compared with the P12 level in seminal vesicles of normal adults (Fig. 5, lane 1), the absence of P12 in the control castrated mice and its recovery soon after the hormonal administration revealed androgenic stimulation of P12 expression in the seminal vesicle.

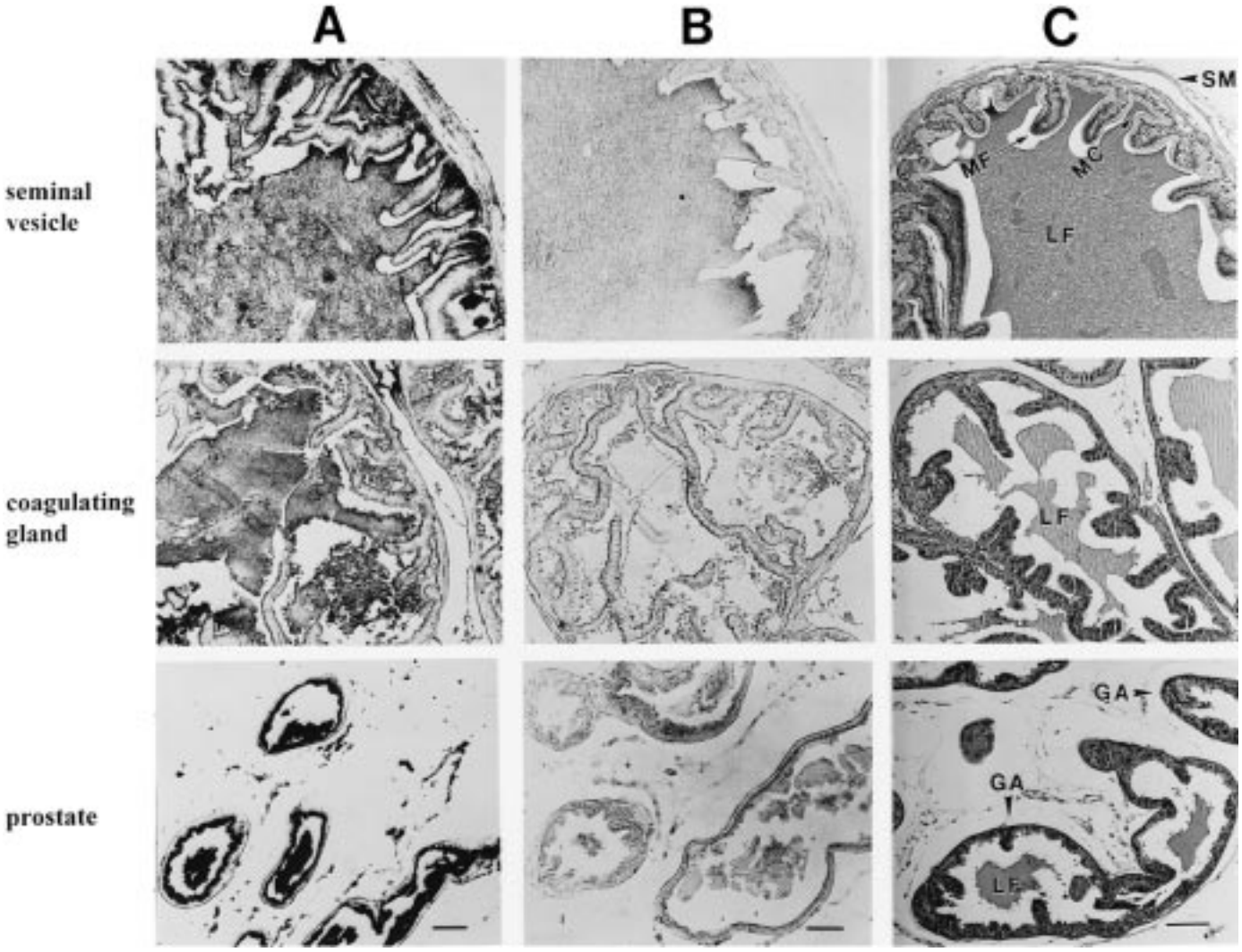

FIG. 3. Histochemical staining patterns of P12 on sections of reproductive tracts of adult male mice. Tissue slices of seminal vesicle, coagulating gland, and prostate were histochemically stained for P12 with the P12 antiserum and the anti-rabbit IgG conjugated with alkaline phosphatase. **A**) P12 was immunolocalized on the epithelium and the cavity of each tissue. **B**) The specimens were stained as in **A** except that the P12 antiserum was replaced by normal serum. **C**) The specimens were stained with hematoxylin and eosin to reveal the morphology. MF, mucosa fold; SM, smooth muscle; MC, mucosa crypt; LF, luminal fluid; GA, glandular alveolus. Bar = 100 μm.

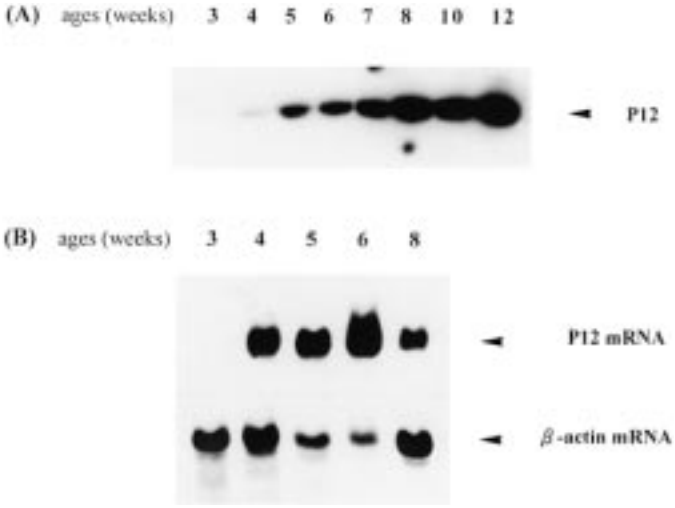

FIG. 4. Synthesis of P12 and its mRNA in mouse seminal vesicle at different ages. **A**) Western blot analysis for P12 in the tissue protein extract (30 μg); **B**) Northern blot analysis for P12 mRNA in the total RNA (40 μg), at ages shown. β-Actin mRNA of **B** was used as an internal control.

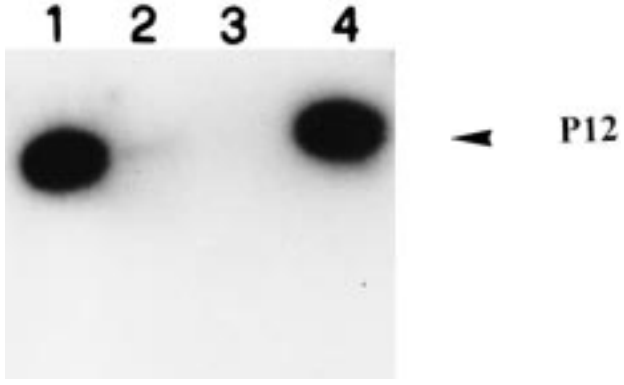

FIG. 5. Androgen dependence of P12 synthesis in seminal vesicle of adult mice. Western blot analysis of protein extract from mouse seminal vesicles of normal adults (lane 1), adults castrated 3 wk previously (lane 2) and receiving corn oil for 8 consecutive days (lane 3), and adults castrated 3 wk previously and receiving testosterone propionate in corn oil for 8 consecutive days (lane 4). Total protein extract (10 μg) was used for each experiment. Detection of P12 protein is described in Figure 2 legend.

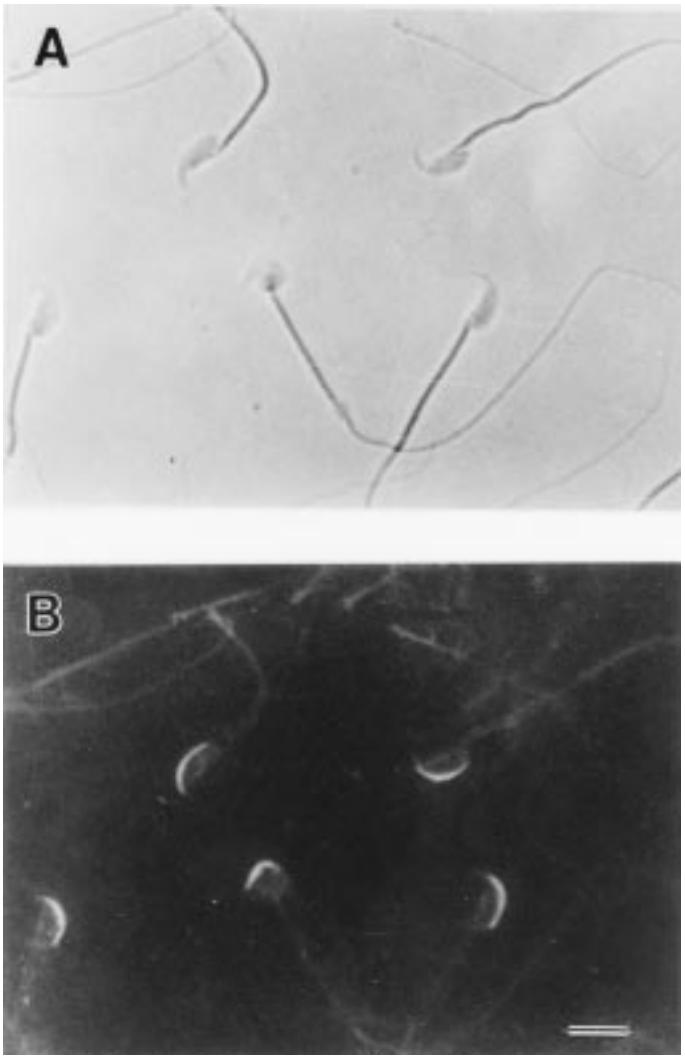

FIG. 6. Immunolocalization of P12-binding zone on the spermatozoa. Fresh cells were dried on glass slides. Slides were incubated in PBS in the presence of P12 for 30 min. The P12-binding zone on the cell was immunolocalized by the indirect fluorescence method with P12 antiserum and fluorescein-conjugated anti-rabbit IgG. The slides were observed by a light microscope (**A**) or a fluorescence microscope (**B**). Bar = 10 μm.

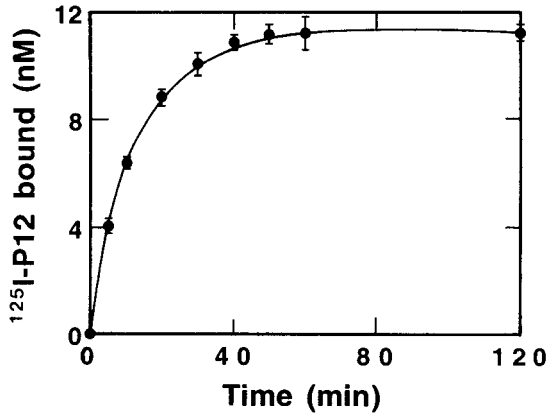

FIG. 7. Equilibrium binding of ^{125}I-P12 to fixed mouse sperm. Mouse sperm (1.0×10^7 cells/ml) in 50 mM Tris, pH 7.4, were incubated in the presence of 100 nM ^{125}I-P12 at 25°C for various times. The ^{125}I-P12 sperm binding at each incubation time was measured. ^{125}I-P12 reached equilibrium binding with a half-time of approximately 8 min. This data set shows results of one representative determination.

TABLE 1. Correlation of intact acrosome and the P12-binding zone on the sperm head treated under several conditions.[a]

Incubation time (min)	Additional incubation in P12[b] (min)	Percentage of cells with intact acrosome	Percentage of cells with P12-binding zone
0	—	86 ± 4	85 ± 3
60	—	77 ± 8	76 ± 7
70	—	74 ± 7	75 ± 6
120	—	68 ± 6	70 ± 5
60	10	72 ± 6	75 ± 7
60	60	67 ± 5	69 ± 6

[a] Fresh spermatozoa from caudal epididymis were incubated in modified Tyrode's solution at 37 °C for cell capacitation (see text for details).
[b] The protein was added to the cell suspension at a final concentration of 1.5 μM.

P12 Binding Site on Sperm Surface

No fluorescence appeared on the epididymal spermatozoa after they had been immunoreacted successively with the P12 antiserum and the fluorescein-conjugated anti-rabbit IgG, manifesting the lack of P12 on the cell surface. When the cells were preincubated with P12 before cytochemical staining, a crescent fluorescence zone on the anterior region of the sperm acrosome was visible (Fig. 6), indicating P12-binding sites on the acrosomal region. The proportion of epididymal spermatozoa having intact acrosomes was compared to the proportion of epididymal sperm with the P12-binding zone after they had been incubated in a modified Tyrode's solution according to the previously mentioned study [26] (Table 1). Around 86% and 85% of the fresh cells from caudal epididymis had an intact acrosome and P12-binding zone, respectively. The population of cells with an intact acrosome decreased progressively as long as the incubation proceeded. This was parallel to the decrease in the cell population with the P12-binding zone. At any incubation time, the population of cells with an intact acrosome was nearly equal to that with the P12-binding zone, and the cell morphology showed no visible change in the presence of P12 in the cell incubation, indicating P12-binding sites on intact acrosome.

^{125}I-P12 was used for the quantitative characterization of sperm-P12 binding. To minimize the nonspecific binding of P12 to the glass microfiber filters used in the binding assay, the filters had been blocked with 1.0% skim milk for 30 min. This resulted in low background binding of the radiolabeled P12 to the filters. Consequently, the nonspecific binding of ligand to the cells was sufficiently low to allow the measurement of specific binding. Figure 7 displays the data from one representative determination from the cell incubation (1.0×10^7 cells/ml) in 50 mM Tris-1.0% skim milk (pH 7.4) in the presence of 100 nM ^{125}I-P12. The equilibrium binding of ^{125}I-P12 to sperm reached a plateau after a 45-min incubation. Therefore, a fixed number of sperm cells (1.0×10^7 cells/ml) were incubated with an increasing ligand concentration for 60 min to determine the steady-state ligand saturation (Fig. 8). The nonspecific binding of ^{125}I-P12 to the cells was determined by addition of a 100-molar excess of unlabeled P12. The amount of ^{125}I-P12 bound to the sperm surface increased with increasing ligand concentration until the available binding sites were saturated. The isotherm saturation suggested 1.2×10^6 P12-binding sites per sperm cell. A Scatchard plot of the data on equilibrium binding due to specific binding yielded a linear plot (Fig. 8 inset) indicating that a single type of binding site on sperm is most likely for P12. The

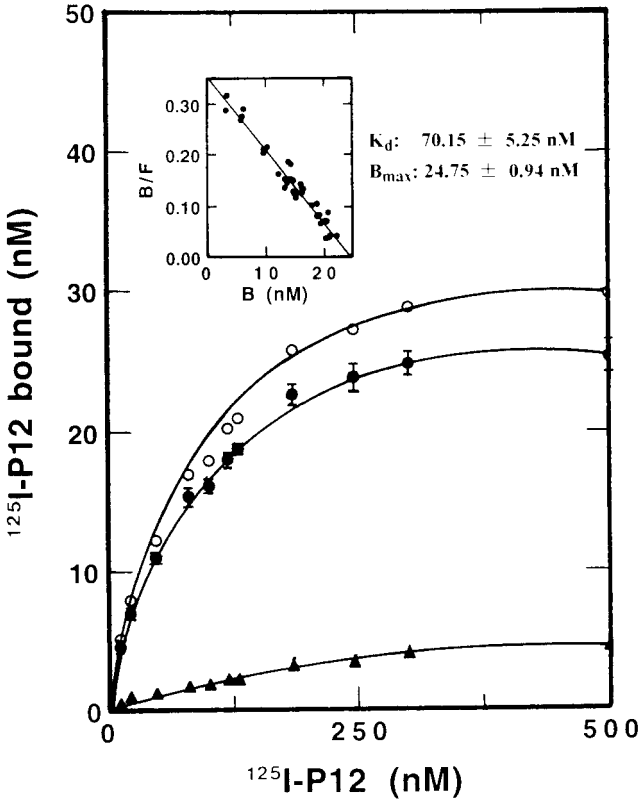

FIG. 8. The characteristics of the binding of P12 to sperm. A fixed number of spermatozoa (1.0×10^7 cells/ml) were incubated with various concentrations of ^{125}I-P12 in 50 mM Tris at pH 7.4 at 25°C for 60 min. The total bound ligand (open circles), specifically bound ligand (solid circles), and nonspecifically bound ligand (solid triangles) on the cells were measured as described in the text. A Scatchard plot of data from each ligand concentration is displayed in inset, where B denotes the total amount of specifically bound ligand (nM) and F the concentration of free ligand. The dissociation constant (K_d) and the maximum binding capacity (B_{max}) were estimated and listed. Points are means from four determinations. The correlation coefficient for the linear regression fitting of Scatchard plot was calculated to be more than 0.95.

apparent dissociation constant (K_d) was determined to be 70.15 ± 5.25 nM, and 1.49 ± 0.06 × 10^6 binding sites/cell was estimated from the maximum binding capacity (B_{max}). The latter is comparable to that estimated from the isotherm saturation.

We examined the development of P-12 binding sites during sperm generation in the testis. In contrast to the cell morphology in the tissue (Fig. 9A), no P12 appeared in the seminiferous tubules or Leydig cells (Fig. 9C). P12 could be immunolocalized (Fig. 9B) in spermatids and spermatozoa but remained undetectable in Leydig cells, Sertoli cells, and precursor cells such as spermatogonia and spermatocytes in the tissue slices preincubated with P12 (Fig. 9B).

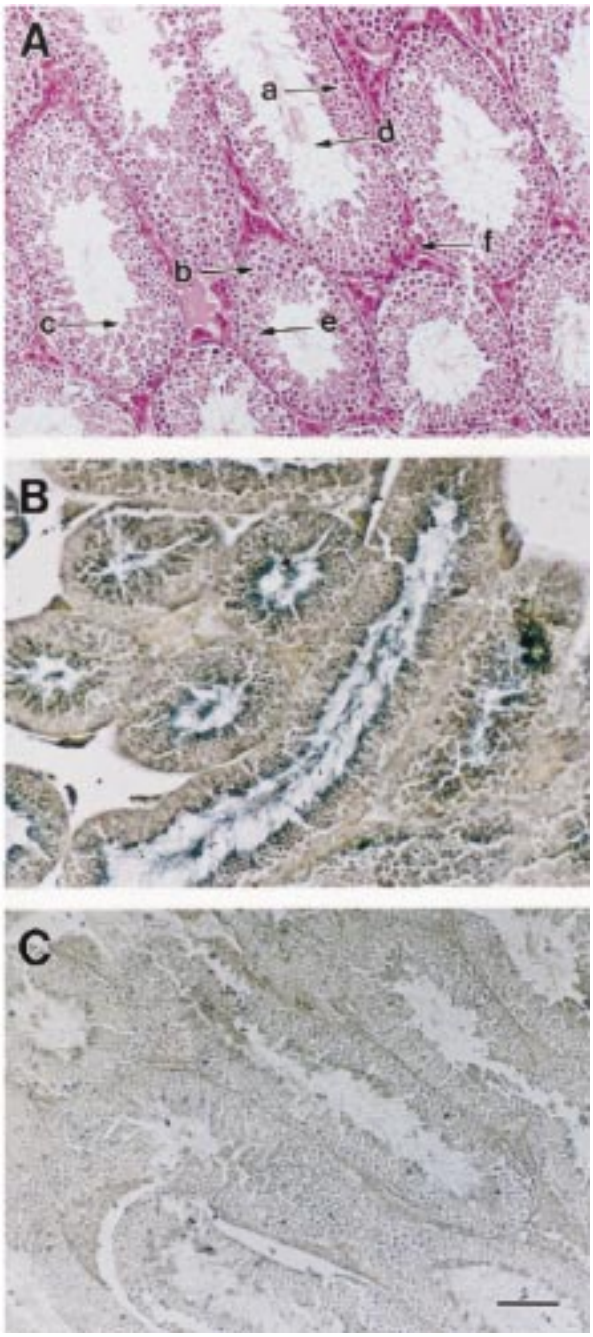

FIG. 9. Demonstration of the P12-binding cells in the seminiferous tubules of testis. The slices from testis of mice (8 wk) were histochemically stained. **A**) The slices were stained with hematoxylin and eosin to reveal the morphology of seminiferous tubules: a, spermatogonia; b, spermatocytes; c, spermatids; d, spermatozoa; e, Sertoli cells; f, Leydig cells. **B**) The slices were incubated in order with P12, P12 antiserum, and anti-rabbit IgG conjugated with alkaline phosphatase. The activity staining for alkaline phosphatase was performed. **C**) The slices were treated as described in **B** except not incubated with P12. Bar = 100 μm.

Suppression of Calcium Transport into Spermatozoa by P12

We measured $^{45}Ca^{2+}$ uptake by epididymal mouse spermatozoa incubated in modified Tyrode's solution (1.0×10^7 cells/ml) in the absence or presence of 1.5 μM P12 or recombinant P12 at 37°C. On the basis of the study by Coronel and Lardy [26], cell surface binding was represented by the uptake of $^{45}Ca^{2+}$ by fresh spermatozoa without cell capacitation. Thus, the induced $^{45}Ca^{2+}$ uptake, which reflected the calcium transport into cells, was estimated by subtraction of the cell surface binding from the total $^{45}Ca^{2+}$ uptake. At any incubation time, the induced $^{45}Ca^{2+}$ uptake by cells incubated in the presence of P12 was less than by those incubated in the absence of P12 (Table 2). About 50% of Ca^{2+} transport to the control cells during a 60-min incubation could be suppressed by P12. Recombinant P12 was as strong as the naturally occurring P12 in suppressing

TABLE 2. The inhibitory effect of P12 on Ca^{2+} uptake by the capacitated spermatozoa.[a]

	$^{45}Ca^{2+}$ uptake		Induced uptake[b]		Inhibition (%)[c]	
Inhibitor	I	II	I	II	I	II
None	8.4	14.5	3.9	10.0		
P12	8.1	9.5	3.6	5.0	8 ± 4	50 ± 4
Recombinant P12	7.9	9.4	3.4	4.9	3 ± 5	51 ± 9

[a] I, 10 min incubation; II, 60 min incubation. The data in terms of nanomoles of $^{45}CaCl_2$ per 10^8 cells were the average of results of triplicate experiments.
[b] Estimated by subtraction of cell-surface binding, which was determined to be 4.5 nmol $^{45}CaCl_2/10^8$ cell from fresh cells without preincubation, from the value obtained at a specified condition.
[c] The inhibitory effect of P12 or recombinant P12 on $^{45}Ca^{2+}$ uptake by spermatozoa was represented by percentage of $^{45}Ca^{2+}$ uptake of the controlled cells; data were calculated from three experiments.

the incorporation of Ca^{2+} by spermatozoa. These data support the identification of P12 as a caltrin.

DISCUSSION

Protease inhibitors are ubiquitous in the genital tracts of mammals. Previously, Nicholson et al. [28] identified a 6.4-kDa PI from mouse seminal vesicle by SDS-PAGE. Since its amino acid sequences have not been reported yet, it is assumed to be identical to P12 for the time being, considering that they are secreted from the same organ and have similar molecular size. Among the male and female genital tracts, P12 was found only in the seminal vesicle, coagulating gland, and prostate of male adults. In each of the accessory sexual glands, P12 is predominantly secreted from the luminal epithelium of mucosa folds to the lumen, where the secretion is accumulated. Apparently, P12-sperm binding to affect sperm activity should take place after ejaculation, and the removal of P12 from the cells may proceed in the uterus, according to the study of Irwin et al. [29].

The synthesis of some secretory proteins from male sexual glands and several aspects associated with the growth of genital tracks are androgen-dependent as are some differentiation processes. As shown, the castration of male adults results in the disappearance of P12 mRNA in the seminal vesicle, and the gene expression is restored in the sexual gland after administration of testosterone to the castrated males. This is consistent with the lack of P12 as well as its RNA message in the seminal vesicle before puberty, when serum androgen is at a low level (Fig. 5), representing a positive correlation of the androgen-dependent P12 expression in the accessory sexual gland with the animal's maturation.

The P12-binding sites are on the anterior region of the acrosome of mouse sperm. Corresponding to the histological localization of the cells developed during spermatogenesis and spermiogenesis in the testis, they are present in the cell layers closely restricted to the lumen of seminiferous tubules. Their absence in the spermatogenic cells such as spermatogonia and spermatocytes indicates that the P12-binding sites are synthesized predominantly in the postmeiotic cells such as spermatids and spermatozoa. Thus, the biosynthesis of P12-binding sites is likely to start in the early haploid spermatids during sperm generation.

The study of Boettger-Tong et al. [30] shows the inhibitory effect of a PI (P12) on the binding of ZP_3 to sperm. The recent study of Thaler and Cardullo [31] indicates the presence of 3.0×10^4 ZP_3-binding sites/sperm cell and the complexity of ZP_3-sperm binding, which is attributed to the cooperative interaction between a low-affinity ZP_3-binding site with a K_d value of 50 nM and a high-affinity ZP_3-binding site with a K_d value of 0.72 nM. They suggest the involvement of multiple receptors on the sperm surface and/or multiple ligand moieties. This work illustrates a single type of P12-binding site ($[1.49 \pm 0.06] \times 10^6$ sites/cell) on the sperm surface with a K_d value of 70.1 ± 5.3 nM. Apparently, the ZP_3-binding sites cannot account for the capacity and characteristics of the P12-binding sites, even though the binding strength of P12-sperm binding sites is as strong as that of the low-affinity ZP_3-binding sites.

Trypsin shows a strong affinity to P12, with a K_d value of 0.15 nM [10]. Since acrosin is a serine protease of the trypsin superfamily, this raises the question of whether acrosin or/and its zymogen form (proacrosin) is involved in the P12-binding sites in the acrosomal region. No active acrosin is present in intact spermatozoa [32], and it is released from the cell once proacrosin is activated during acrosomal exocytosis at the fertilization sites in the oviduct. Thus, no acrosin but proacrosin exists in spermatozoa before their passage to the oviduct. Since proacrosin is not exposed on the sperm surface but is located in the acrosomal matrix of intact sperm [33], P12 is unlikely to interact with the zymogen unless P12 penetrates into the acrosomal region. In fact, there is one protein component, which is distinct from acrosin/proacrosin but is capable of binding a PI of seminal vesicle origin, in the supernatant of frozen-thawed murine epididymal sperm suspension [34].

As stated by Barros [35], the fertile condition of spermatozoa is not a terminal condition but rather a transient one. It is well recognized that mammalian sperm from epididymis should undergo some Ca^{2+}-dependent modifications before fertilization. In the reproductive tract, the Ca^{2+} concentration is sufficient to elevate intracellular Ca^{2+} in the induction of these cell modifications at any time earlier than the sperm-egg encounter. However, these modifications far from the oviduct would cause spermatozoa to become infertile. Thus, the calcium movement across the membrane of spermatozoa should be prohibited at ejaculation until the cells reach the oviduct. In this regard, caltrin is believed to play a most important role in the inhibition of Ca^{2+} uptake by sperm. This kind of protein was identified and purified originally from bovine seminal plasma and subsequently from seminal vesicle fluid of the guinea pig [36]. Our results support the idea that P12 is a caltrin-like protease inhibitor. The P12-sperm binding leading to the suppression of Ca^{2+} movement across the cell membrane sheds some light into the function of the P12-binding sites. The primary structure of P12 shows a high degree of similarity to that of a caltrin containing 56 amino acid residues in rat seminal vesicle fluid [9]. A caltrin consisting of 75 amino acid residues in mouse seminal vesicle fluid has been characterized by Coronel et al. [9]. This caltrin and P12, though they coexist in seminal vesicle fluid, show no appreciable sequence similarity. It is possible that they exhibit different mechanisms in the regulation of calcium movement across the membranes of sperm. Future study is needed to elucidate how the dual roles of P12 as a protease inhibitor and as a caltrin interplay in the regulation of sperm activity.

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