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A Basic 18-Amino Acid Peptide Contains the Polysulfate-Binding Domain Responsible for Activation of the Boar Proacrosin/Acrosin System[1]

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ABSTRACT

Proacrosin is the zymogen of acrosin, a serine protease localized in the acrosomal matrix of mammalian sperm. Proacrosin/acrosin binds to solubilized zona pellucida glycoproteins (ZPGs) and various polysulfates in a non-enzymatic mechanism. In addition, both polysulfates and ZPGs induce proacrosin activation once they bind to the polysulfate-binding domain (PSBD) of the enzyme. We show here that the peptide ^{43}IFMYHNNRRYHTCGGILL^{60} inhibited the proacrosin activation induced by either fucoidan or ZPGs. In addition, the peptide was recognized by the monoclonal antibody C5F10, which is directed against the PSBD region. Our data suggest that the PSBD is composed of many ''subsites'' that may or may not interact with each other.

INTRODUCTION

Proenzyme activation is a highly controlled process that is regulated by protein-protein and/or cell-cell interaction [1–5]. It normally involves the interaction among the proenzyme, the substrate, and the regulatory molecule. Proacrosin is the zymogen form of acrosin (EC3.4.21.10) and is present in the acrosomal matrix of mammalian sperm [2, 6–10]. Proacrosin activation in vitro involves an autocatalytic cleavage between R23 and V24 at the amino terminus, resulting in a linked two-chain molecule. The newly generated 23 amino acid fragment (''light chain'') remains bridged by disulfide bonds to the rest of the molecule (''heavy chain'') [5]. This 49-kDa form is enzymatically active and represents α-acrosin [11–13]. The second and third cleavages take place at the carboxy-terminus between K363 and R367 and between R322 and P323, respectively. These cleavages lead to a sequential loss of 18- and 43-residue fragments [5, 14–16]. These last processing events result in the formation of a stable 36-kDa enzymatically active form named β-acrosin [11]. To our knowledge, proacrosin is the only member of the serine proteinase family to be autoactivated through processing at both the carboxy- and amino-terminal ends. Once proacrosin is activated, α- and then β-acrosin are released into the extracellular medium [17].

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Proacrosin and β-acrosin bind in vitro to solubilized zona pellucida glycoproteins (ZPGs) with equal affinity ($K_d = 10^{-8}$ M) [18–20]. This binding involves ionic bonds between basic residues on the surface of proacrosin/acrosin and polysulfate groups on ZPGs [13, 21–26]. Proacrosin and β-acrosin also bind with a similar affinity to dextran sulfate, polyvinylsulfate, fucoidan, and heparin [13, 14, 18–21, 27–29], but not to polyvinylphosphate, chondroitin sulfate, desulfated fucoidan, or dextran. Thus, it seems that the interaction between proacrosin/acrosin and these polysulfates involves stereospecific recognition of the sulfated side chains [18, 19, 23, 24, 30]. Many biochemical and mutagenesis-directed studies have shown that this polysulfate-binding domain (PSBD) encompasses H47, R50, and R51 in the boar and the analogous amino acids in the rabbit [30–32]. In addition, R250, K252, and R253 appear to be involved in a ''second'' PSBD in the same molecule [31]. Thus, the PSBD may be composed of multiple subsites that probably interact with the sulfate groups of ZPGs and polysulfate compounds [33]. In fact, we have shown that a monoclonal antibody against the PSBD (C5F10) prevents the binding of ZPGs, but not heparin, to boar proacrosin [9]. Fucoidan, but not soybean trypsin inhibitor, prevented the binding of the antibody C5F10 to either permeabilized human sperm or boar proacrosin [9]. Therefore, these results suggest that the PSBD is in the surface of the molecule but does not encompass the active site. Nonetheless, the binding of the PSBD with polysulfates may induce some conformational change in the active site of the enzyme.

Does the PSBD have any function during the processes of the acrosome reaction and/or gamete interaction? Biochemical studies have shown that ZPGs, dextran sulfate, fucoidan, and heparin stimulate the conversion of proacrosin to β-acrosin [9, 22–37]. However, they also inhibit the enzyme activity of β-acrosin [12, 23]. Thus the interaction of polysulfates with proacrosin has different consequences from the interaction with β-acrosin. This finely tuned regulation of proacrosin activation and acrosin activity may help in gradual and continuous degradation of ZPGs during sperm penetration [38, 39]. A second function of acrosin is in the release of acrosomal proteins after the acrosome reaction [40]. A third proposed function for proacrosin in gamete interaction is its role as a ''secondary receptor'' during sperm penetration [28]. In the mouse paradigm, the molecule ZP3 induces the acrosome reaction [41–43]. Then, a secondary receptor associated with ZP2, and probably ZP1, retains acrosome-reacted sperm on the zona surface [44, 45]. The polysulfate-binding property of proacrosin may account for this role [46, 47]. In support of this, the monoclonal antibody C5F10, which is raised against the PSBD, inhibits the binding of human sperm to homologous zonae pellucidae [9]. In addition, fucoidan and heparin prevent the binding of human and mouse sperm to the zona pellucida [9, 25, 48–54].

Deletion and site-directed mutagenesis studies have defined the domains of interaction between proacrosin and

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polysulfates. However, three of four studies produced an inactive recombinant enzyme [30–32, 55]. Moreover, the only study with an enzymatically active enzyme assayed the binding of polysulfates to the recombinant proteins, and not the effects of this interaction on the enzyme activity [55]. We have taken into account all of this information about the structure and localization of the PSBD and generated an 18 amino acid length peptide encompassing the residues H47, R50, and R51 of boar proacrosin. Here we present data showing that this peptide is recognized by the monoclonal antibody C5F10. In addition, this peptide inhibited the proacrosin activation induced by either ZPGs or fucoidan, suggesting that a functional relationship between the PSBD and proacrosin activation exists.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, all chemicals and reagents were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). Synthetic peptides were purchased from Chiron Corporation (San Francisco, CA).

Dot Blot Assay

Various concentrations of peptide 1 and 2 were immobilized onto strips of nitrocellulose and blocked with PBS plus 5% BSA. Strips were rinsed in PBS and then incubated with the affinity-purified monoclonal antibody C5F10 at 1 mg/ml in PBS-BSA for 1 h at room temperature. After extensive washing with PBS plus 0.05% Tween 20, they were incubated with a rabbit anti-mouse IgG tagged with peroxidase. After incubation, the nitrocellulose membranes were washed in PBS-Tween, and then the immunoreactive bands were visualized using the ECL plus system (Amersham Pharmacia Biotech, Piscataway, NJ).

Proacrosin Extraction

Boar proacrosin was obtained with extraction as described previously [11]. Briefly, boar semen ejaculate was adjusted with 1 M benzamidine to a final concentration of 50 mM and filtered through Miracloth (Droguería Simon Bolivar, Santiago, Chile). Aliquots (20 ml) were carefully placed in 25 ml of 1 M sucrose, 50 mM benzamidine, and 0.02% sodium azide and were centrifuged for 30 min at 500 rpm in an IEC HNS clinical centrifuge (Damon/IEC Division, International Equipment Company, Needham Heights, MA) at room temperature. Boar acrosin was then extracted in an acid solution containing 1 mM HCl, 10% glycerol, 0.02% sodium azide, and 50 mM benzamidine at pH 3.0 and left at 4°C overnight. This suspension was then centrifuged at 27 000 $\times$ *g* for 30 min in a Sorvall (Newtown, CT) model RC2-B centrifuge, and the supernatant was stored at −20°C. Before the experiment, a 200-μl aliquot of frozen boar proacrosin was dialyzed overnight against 2 L of 1 mM HCl at pH 3.0.

Acrosin Activity Assay

Acrosin activity was measured spectrophotometrically at 25°C by following the hydrolysis of *N*-benzoyl-L-arginine-ethyl-ester (BAEE), with the addition of either 50 or 100 μl of the enzyme solution. The assays were performed in 3-ml volumes, using a substrate mixture of 50 mM Tris-HCl, 50 mM $CaCl_2$, and 500 μM BAEE at pH 8.0. A molar absorption coefficient of 1150 $M^{-1}\cdot cm^{-1}$ was used to convert changes in optical density to μmol of BAEE hydrolyzed [56]. One international unit was defined as the amount of acrosin hydrolyzing 1 μmol BAEE/min at 25°C.

Gel Electrophoresis and Western Blotting

The molecular forms of proacrosin and acrosin were analyzed by one-dimensional SDS-PAGE. Samples for analysis were run in a 10% SDS-PAGE under reducing and denaturant conditions, then transferred to nitrocellulose sheets at 100 V (200 mA) for 2 h as described previously [9]. Nitrocellulose was blocked with 2% BSA, PBS, pH 7.4 (PBS-BSA). Since the monoclonal antibodies we had were raised against human and not boar acrosin, we decided to use a mixture of them, i.e., C5F10, A8C10, and C2B10 (30 mg/ml of each), in order to increase the efficiency of detection of the various acrosin isoforms [57, 58]. After extensive washing with PBS plus 0.05% Tween 20, incubation was performed with a rabbit anti-mouse IgG tagged with peroxidase. After incubation, the nitrocellulose membranes were washed in PBS-Tween, and the immunoreactive bands were then visualized using the ECL plus system (Amersham Pharmacia Biotech).

Antibodies

Mice of the Balb/c strain were immunized with human purified human acrosin, and hybridoma cell lines secreting monoclonal antibodies were produced as described previously [57]. Monoclonal antibodies A8C10, C2B10, and C5F10 were purified from ascites through G protein Sepharose chromatography [57, 58].

Helical Wheel Representations

The three-dimensional structure of α-helices was represented by two-dimensional projections called helical wheels [59]. The helical wheel representation allows visualization of the possible side-chain interactions, and in the case of heparin (fucoidan)-binding consensus sequences, allows the detection of basic residue clusters [60, 61].

RESULTS

Design of an Inhibitory Peptide to Polysulfate-Induced Proacrosin Activation

In a search for the presence of consensus sequences for heparin (polysulfate) recognition on the heavy chain of β-acrosin in boar sperm, we have found only one sequence homologous to the type BBXB or BBBXXB (where X represents any hydropathic amino acid and B any basic amino acid) [60]. This sequence is located at the N-terminus and corresponds to residues 48–54 (N-**R**-**R**-Y-**H**-T) (Fig. 1A). We synthesized a peptide containing residues 48–54 for two reasons: 1) previous biochemical and mutagenesis studies have shown that the main portion of the PSBD is in the N-terminal region of the heavy chain [21, 62], and 2) we found another H residue near the first sequence and thought that this cluster of amino acids may have a higher binding affinity than the other region. Thus, we decided to select a peptide 18 residues in length in order to get the closest conformation to the native protein, ^{43}IFMYHNNRRY-HTCGGILL^{60} (peptide 1) (Fig. 1A). When the sequence of peptide 1 was compared with the protein sequences listed in the National Center for Biotechnological Information (NCBI), it was discovered that this peptide had significant homology with regions in proacrosin/acrosin and acrosin-

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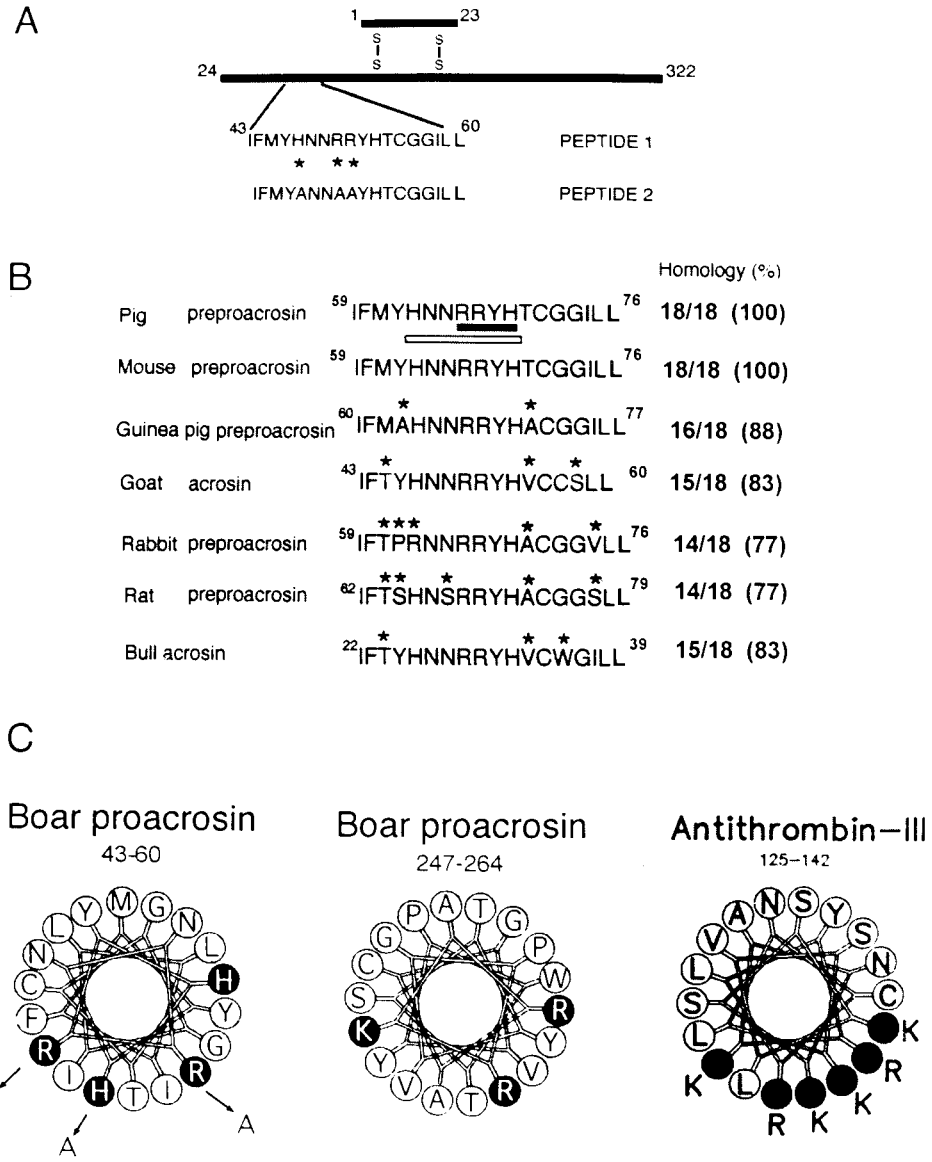

FIG. 1. **A**) Schematic diagram of β-acrosin showing the light-chain (residues 1–23) linked to the heavy chain (residues 24–322) by disulfide bonds (S-S). The sequence of the synthetic peptide (peptide 1) is shown with its approximate localization in the heavy chain (residues 43–59). The mutant peptide (peptide 2) in which three basic residues were changed to A (asterisks) is also shown. **B**) Comparison of sequence from peptide 1 with acrosin from different species. The numbers flanking the sequences represent the relative position of the amino acid residues in the protein. Asterisks represent the changes in residues in the sequence. Black lines represent the heparin-binding consensus domain BBXB; white lines represent a conserved sequence among proacrosin BXXBBXB. **C**) Helical wheel representations of the peptide ^{43}IFMYHNNRRYHTCGGILL^{60} and ^{247}WGVGCARAKRPGVYTSTW^{264} of boar proacrosin. For comparison, we show the sequence corresponding to residues 125–142 of antithrombin III. Black circles indicate basic residues, and arrows indicate the substitutions made in the mutant peptide 2 (see **A**).

like enzymes isolated from other species (Fig. 1B). Interestingly, no other sequence matched peptide 1 using the BLAST algorithm. Thus, it seems that this region is unique to proacrosin/acrosin. This result prompted us to study the structures uniquely characteristic of this sequence in the proacrosin molecule. Possible side-chain interaction, as well as the central configuration of α-helices, can be more readily visualized if the sequence is plotted on two-dimensional figures called helical wheels. These wheels are projections of the amino acid side chains onto a plane perpendicular to the axis of the helix [59]. The helical wheel representation of the acrosin peptide 1 (Fig. 1C) indicates a clustering of basic residues at one hand of the helix. A similar three-dimensional conformation is shared by residues 125–142 of antithrombin, a well-characterized heparin-binding protein (Fig. 1C) [60, 61].

To study the relationship between the heparin (polysulfate)-binding consensus sequence of peptide 1 and its ability to inhibit fucoidan proacrosin activation, we designed a mutant peptide (Fig. 1, A and C). In this peptide the three central amino acids, **H**-N-N-**R**-**R,** were changed to A residues. We expected that such a change would completely eliminate the positively charged cluster of animo acids and therefore the ability to bind to polysulfates (Fig. 1C).

Peptide 1 Was Part of the Epitope Recognized by Antibody C5F10

In order to assess whether peptide 1 belongs to the PSBD, we decided to evaluate the interaction of this peptide with monoclonal antibody C5F10. We have previously shown that this antibody recognizes an epitope belonging to the binding domain [9]. In a dot-blot assay, the antibody C5F10 bound to the sequence of peptide 1 and gave a clear signal with use of as little as 15 μg of peptide (Fig. 2). In contrast, the antibody did not bind to the mutant sequence (peptide 2). This result suggests that peptide 1 might be involved in forming the PSBD recognized by monoclonal antibody C5F10.

Peptide 1, but Not Peptide 2, Inhibited Fucoidan-Induced Proacrosin Activation

The rate of autoactivation of proacrosin to acrosin at pH 8.0 is known to be stimulated significantly by calcium chlo-

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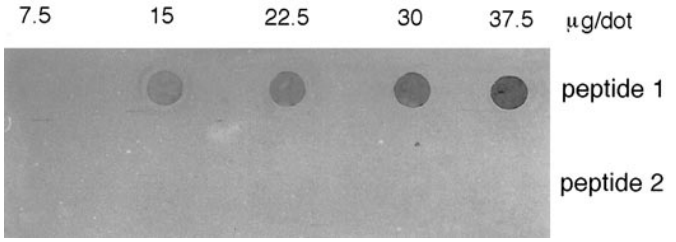

FIG. 2. Monoclonal antibody C5F10 recognized the sequence [43]IFMYHNNRRYHTCGGILL[60] of peptide 1. Various amounts (from 7.5 to 37.5 μg) of peptide 1 and peptide 2 were blotted onto nitrocellulose paper. Then the membrane was incubated with monoclonal antibody C5F10 against the PSBD of human acrosin. This antibody recognized only the native, not the mutant, peptide.

ride; all subsequent experiments were carried out in Ca^{2+}-free medium [11].

In the present work we confirm previous data showing that both ZPGs and fucoidan induce boar proacrosin activation (Fig. 3). Preincubation of proacrosin in the presence of either ZPGs or fucoidan plus peptide 1 prevented activation by these polysulfates (Fig. 3). On the other hand, the mutant peptide—peptide 2—did not prevent polysulfate-induced proacrosin activation (Fig. 3).

The experiments described, however, do not distinguish between a possible effect of the peptide and polysulfates on proenzyme autoactivation and a direct effect on enzyme activity. Therefore, we decided to monitor the formation of α- and β-acrosin by SDS-PAGE followed by Western blotting. Within 10 min of activation at 37°C, we detected two strong bands corresponding to the 53/55-kDa proacrosin and a third close band of α-proacrosin (Fig. 4A, lane 1, arrowhead). Increasing concentrations of fucoidan resulted in a barely detectable increase in the amount of β-acrosin with 1 or 10 μg/ml of fucoidan (Fig. 4A, lanes 2–3, arrow). However, there was a sharp increase in the amount of the 36 (β-acrosin) and 32 kDa at 100 μg/ml of fucoidan (Fig. 4A, lane 4). We also noticed that at the highest concentration of fucoidan (100 μg/ml), fucoidan and ZPGs (data not shown) induced the formation of an additional band of 32 kDa. This component was not present in our controls and might be due to a conformational change exposing an additional processing site [23]. Preincubation of proacrosin and fucoidan in the presence of peptide 1 prevented the formation of β-acrosin (36 kDa) in a concentration-dependent manner (Fig. 4A, lanes 5–7; Fig. 5). There was not a clear effect on the inhibition of α-acrosin (49 kDa) production (Fig. 4A, lanes 5–7), even though peptide 1 prevented proacrosin activation (Fig. 3). Under the same conditions, the mutant peptide 2 did not have effect on the formation of α (49 kDa)- or β (36 kDa)-acrosin (Fig. 4B, lanes 4–6; Fig. 5). We could also detect the formation of the 32-kDa band in the presence of peptide 2. ZPGs also induced proacrosin activation, which was inhibited in the presence of peptide 1 (data not shown).

Peptide 1, but Not Peptide 2, Inhibited β-Acrosin Activity

In order to determine the effect of peptide 1 in fucoidan inhibition of β-acrosin activity, the enzyme was mixed with either ZPGs or fucoidan in the presence of peptide 1, and the acrosin activity was assayed after 30 min. Results indicated that both ZPGs and fucoidan inhibited β-acrosin activity (Fig. 5). This inhibition could be blocked in the presence of peptide 1, but not using the mutant peptide 2.

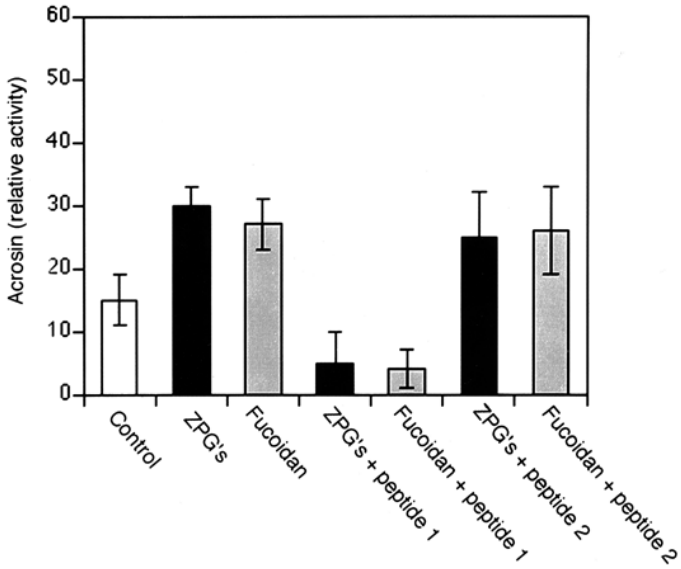

FIG. 3. Peptide 1, but not peptide 2, inhibited the interaction of fucoidan and ZPGs with proacrosin/acrosin. Boar acid extract was incubated in 1 mM Tris-HCl, pH 8.0, at 37°C for 30 min alone (white bar), in the presence of ZPGs (black bars), or with fucoidan (gray bars). In some cases either peptide 1 or peptide 2 was added to the reaction mixture at the start. Bars: mean ± SD.

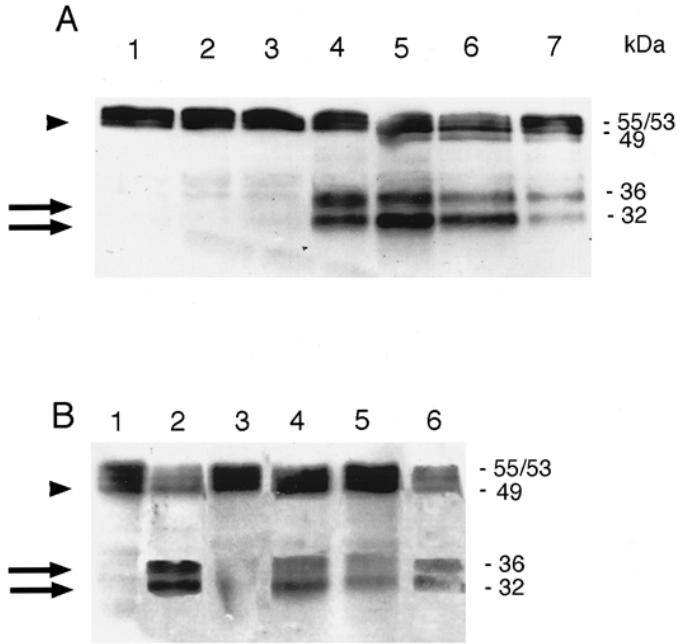

FIG. 4. SDS-PAGE analysis of the effect of fucoidan on the autoactivation of boar proacrosin in the presence of peptide 1 or 2. Samples of boar proacrosin were taken after 10 min of autoactivation in the presence of various concentrations of fucoidan, with or without peptide 1. The samples were blotted using a mixture of monoclonal antibodies against human acrosin. **A**) Lane 1, control; lane 2: fucoidan (1 μg/ml); lane 3, fucoidan (10 μg/ml); lane 4, fucoidan (100 μg/ml); lane 5, fucoidan (100 μg/ml) + 0.375 mg/ml peptide 1; lane 6, fucoidan (100 μg/ml) + 0.75 mg/ml peptide 1; lane 7, fucoidan (100 μg/ml) + 1.2 mg/ml peptide 1. **B**) Lane 1, control; lane 2, proacrosin + fucoidan (100 μg/ml); lane 3, fucoidan (100 μg/ml) + one 1.2 mg/ml peptide; lane 4; fucoidan (100 μg/ml) + 0.375 mg/ml peptide 2; lane 5, fucoidan (100 μg/ml) + 0.75 mg/ml peptide 2; lane 6, fucoidan (100 μg/ml) + 1.2 mg/ml peptide 2. The migration of α-acrosin, the 32-kDa band, and β-acrosin is indicated by arrows and arrowhead, respectively.

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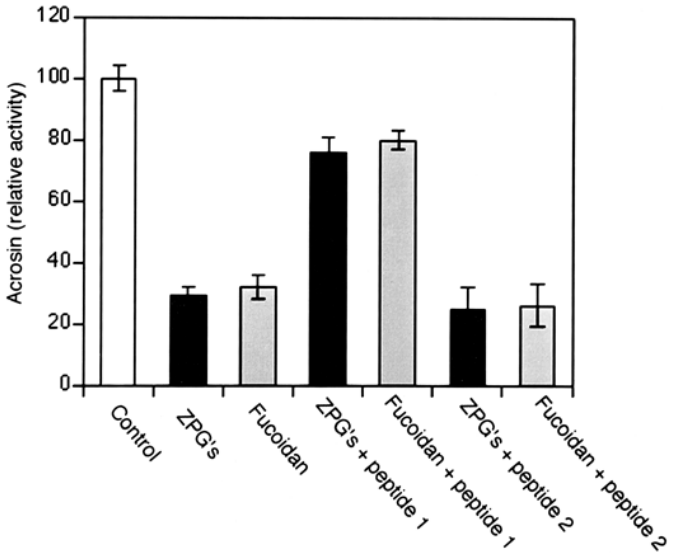

FIG. 5. Peptide 1, but not peptide 2, inhibited the interaction of fucoidan and ZPGs with proacrosin/acrosin. Boar β-acrosin was incubated in 1 mM Tris-HCl, pH 8.0, at 37°C for 30 min alone (white bar), in the presence of ZPGs (black bars), or with fucoidan (grey bars). In some cases either peptide 1 or peptide 2 was added to the reaction mixture at the start. Bars: mean ± SD.

DISCUSSION

Proacrosin activation is a highly regulated process during sperm penetration following the acrosome reaction [2, 11, 16, 23, 63]. Previous studies using modified fragmentation analysis, group-specific modifying agents, and deletion recombinants have shown that the PSBD on proacrosin is formed by basic residues on the surface of the molecule [32]. These residues display a specific orientation that is complementary to the negatively charged sulfate groups on ZPGs. However, there is no information about the exact nature of this ''specific orientation'' of the amino acid residues on proacrosin, or about how the interaction between the PSBD and ZPGs or other polysulfates affects the rate of proacrosin activation and/or β-acrosin activity.

Both proacrosin and its active form, β-acrosin, bind to different polysulfates such as fucoidan, glycosaminoglycans, and ZPGs with similar high affinity [18–20, 23–25, 29]. The data in the literature suggest that the binding of polysulfates to the PSBD of proacrosin stimulates the rate of proacrosin activation. On the other hand, the binding of polysulfates to the PSBD of β-acrosin induces inhibition of the enzyme activity [12, 23, 64, 65]. This evidence prompted us to search in the proacrosin sequence for a heparin-binding consensus sequence of the type BBBXXB or BBXB (where X represents any hydropathic amino acid and B any basic amino acid) [60]. This kind of sequence is present in many heparin- and dermatan sulfate-binding proteins such as antithrombin III, apolipoprotein B, and heparin cofactor II [60, 61, 66, 67]. Many of the natural sequences conforming to these consensus motifs show prominent amphipathic periodicities having both α-helical and β-strand conformations [60, 61]. It is believed that these consensus sequence elements form potential nucleation sites for the recognition of polyanions in proteins [60, 61]. A search for this kind of consensus sequence in boar proacrosin showed a region near the N-terminus of the protein. This result agrees with previous biochemical studies showing that the main PSBD of proacrosin is the N-terminus of the heavy chain [21, 55]. It is worth noting that the sequence R-R-Y-H is conserved among all acrosins, showing no substitutions of any kind. Studies in other proteins indicate that K is the most common residue at position −2 and −1, whereas R predominates at +2 and His appears infrequently [60]. Here we show that R, and not K, is the preferred amino acid in positions −2 and −1 in the acrosin PSBD and that H is found instead of R at +2. This unusual amino acid usage may be related to the polysulfate-binding specificity of the molecule. Since the natural ligand for proacrosin/acrosin seems to be the ZPGs and not heparin, the PSBD has a special arrangement of basic residues in order to fit with ZPG sulfates under low-stringency conditions [18, 19]. In this way, an interesting example is heparin cofactor II (HC-II). HC-II is an inhibitor of thrombin that binds both heparin and dermatan sulfate [68]. Recombinant forms or natural mutants of this inhibitor, in which the amino acid R189 has been replaced by H, bind heparin but not dermatan sulfate [68]. Thus, a single conservative substitution in the protein sequence may change the binding affinity of the molecule toward its ligand.

A second interesting finding is that acrosins share another, possibly new, consensus sequence domain. This consensus sequence (BXXBBXB) is located within the heavy chain and encompasses the BBXB heparin-binding domain (Fig. 1C). Interestingly, five of the seven acrosins showed 100% homology with the sequence **H**-N-N-**R**-**R**-Y-**H.** This amino acid sequence is not present in any of the heparin-binding proteins as evidenced by a search in the NCBI using the BLAST program (data not shown). Perhaps this finding represents a unique feature of the PSBD of proacrosin that is related to both the binding and activation of the enzyme. In fact, helical wheel representations of this peptide showed a cluster of basic amino acids to one side of the helices (Fig. 1C). This three-dimensional pattern is similar to that of many other heparin-binding proteins and is directly related to the binding affinity of the molecule. Therefore, we interpreted our results as suggesting that the inhibition of proacrosin activation by fucoidan or ZPGs in the presence of peptide 1 is due to a competition between this peptide and its homologous region in the proacrosin chain. Moreover, since the effect of this peptide was also observed on β-acrosin, we proposed that the conformation of this portion of the PSBD does not change after proacrosin activation. A major point in favor of this view came from the results using a mutant peptide. In this peptide, three of the four basic amino acids in the putative binding domain were replaced by A residues. Under these circumstances, the density charge, along with the number of possible electrostatic interactions with the sulfated groups of either fucoidan or ZPGs, is significantly reduced, making the interactions less stable and nonspecific. This same kind of approach has been successfully used to determine the heparin-binding domain in the asymmetric form of acetylcholinesterase, apolipoprotein B, extracellular matrix proteins, and antithrombin III (Fig. 1C) [60, 66, 67, 69, 70].

Although there are no available data about the crystal structure of either proacrosin or β-acrosin, modeling of the latter using thrombin as a reference protein has been very useful in elucidating the putative interactions of the PSBD with its ligand. Modeling of boar β-acrosin reveals that the residues shown to be important for binding ZPGs are located on loops projecting from one face of the protein surrounding the catalytic site [31]. One of these loops, loop 1, contains the residues ^{43}IFMYHNNRRYHTCGGILL^{60} of peptide 1 used in the present study. Recently, it has been

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reported that another region of proacrosin encompasses residues R250, K252, and R253. The residues are crucial because their deletion or replacement reduces the affinity of the molecule toward ZPGs [30, 31, 71]. Molecular modeling has shown that these residues are in loop 3 of β-acrosin, with an approximate distance of 20–25 Å from loop 1 [30, 31, 71]. Helical wheel representations of this portion of the enzyme showed that loop 3 contains a lower charge density and that the basic residues are more spread out than those in peptide 1 (Fig. 1C). The molecular structure of proacrosin resembles that of HC-II, where the binding site for heparin and dermatan sulfate appears to be overlapping but not identical [72]. Moreover, we have shown previously that the monoclonal antibody C5F10, directed against human acrosin, prevents the binding of fucoidan and ZPGs, but not heparin, to boar acrosin [9, 33]. Therefore, it seems that the PSBD is composed of at least two different subsites that interact with different polysulfates. These subsites are far apart in the amino acid sequence, but they are brought together in the tertiary conformation; a similar case is reported for antithrombin III [70, 73, 74]. Whether these putative subsites have different roles or interactions during the normal activation pathway of proacrosin remains to be resolved. In this regard it is interesting to note that fucoidan may act as a coagulant interacting with HC-II and not antithrombin III [75]. Thus, while the two molecules have similar affinities for heparin, only HC-II binds fucoidan in an ex vivo assay.

A second possible function of the PSBD, besides proacrosin activation, is assisting in sperm penetration [6, 28]. The binding mechanism is essentially ionic, involving probably the same PSBD used for proacrosin activation. The density and orientation of the sulfate groups on ZPGs appear to be critical parameters in mediating the recognition and high binding affinity, and variations in their stereochemistry could account for their species specificity in fertilization [18, 20, 24, 28, 29]. It has been suggested that this mechanism permits cycles of hydrolysis and binding that help spermatozoa penetrate through the zona pellucida [10, 30]. In fact, fucoidan, heparin, and monoclonal antibodies raised against the active site and the PSBD in human acrosin inhibit sperm penetration [6, 9]. Thus, it seems that the same PSBD involved in proacrosin activation is involved in the ''secondary binding.'' Whether the antibodies against peptide 1 or recombinant proteins that contain this sequence inhibit the binding to and/or penetration through mammalian zona pellucida by mammalian spermatozoa remains to be resolved.

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