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Evaluation of the proacrosin/acrosin system and its mechanism of activation in human sperm extracts

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Abstract

Acrosin is an acrosomal protease synthesized as a proenzyme and activated into β-acrosin during the acrosome reaction. In the present study, a set of sensitive assays was developed to identify the proacrosin/acrosin system and to evaluate its activation pattern in human sperm extracts. Immunocytochemical analysis with monoclonal antibody (Mab) AcrC5F10 showed specific staining on the acrosome of permeabilized ejaculated and capacitated spermatozoa. Acrosome reaction was associated with a decrease in staining. AcrC5F10 specifically recognized a 55-kDa band (proacrosin) in Western immunoblots. Activation studies showed enzymatically active intermediates of 39 and 35 kDa after zymography. Immunoreactive bands of 52, 43, 34, 21–26 and 16 kDa were identified in the activation patterns developed with AcrC5F10. Activation was completely inhibited in the presence of 9 mM $CaCl_2$ or 100 mM benzamidine. A multiple sequence alignment revealed partial conservation of putative cleavage sites in the proacrosin sequence. The tests described allow the detection of human proacrosin in spermatozoa and sperm protein extracts, as well as the evaluation of the proenzyme activation pattern. They can be used to study the effect of

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inhibitors upon proenzyme activation. In addition, alterations in proacrosin activation in semen samples with abnormal acrosin enzymatic activity can be analyzed using these assays.

Keywords: Human spermatozoa; Proteases; Acrosin; Activation

1. Introduction

Acrosin (EC 3.4.21.10) is a trypsin-like endoprotease present in the acrosome of all mammalian spermatozoa studied (Urch, 1991). It has been involved in sperm–egg interaction by participating in the proteolysis of the zona pellucida (ZP) (Urch et al., 1985), by regulating the release of acrosomal components during the acrosome reaction (Hardy et al., 1991; Yamagata et al., 1998), and by recognizing homologous ZP glycoproteins (Urch and Patel, 1991; Jansen et al., 1995, 1998; Richardson and O'Rand, 1996; Crosby et al., 1998; Furlong et al., 2000).

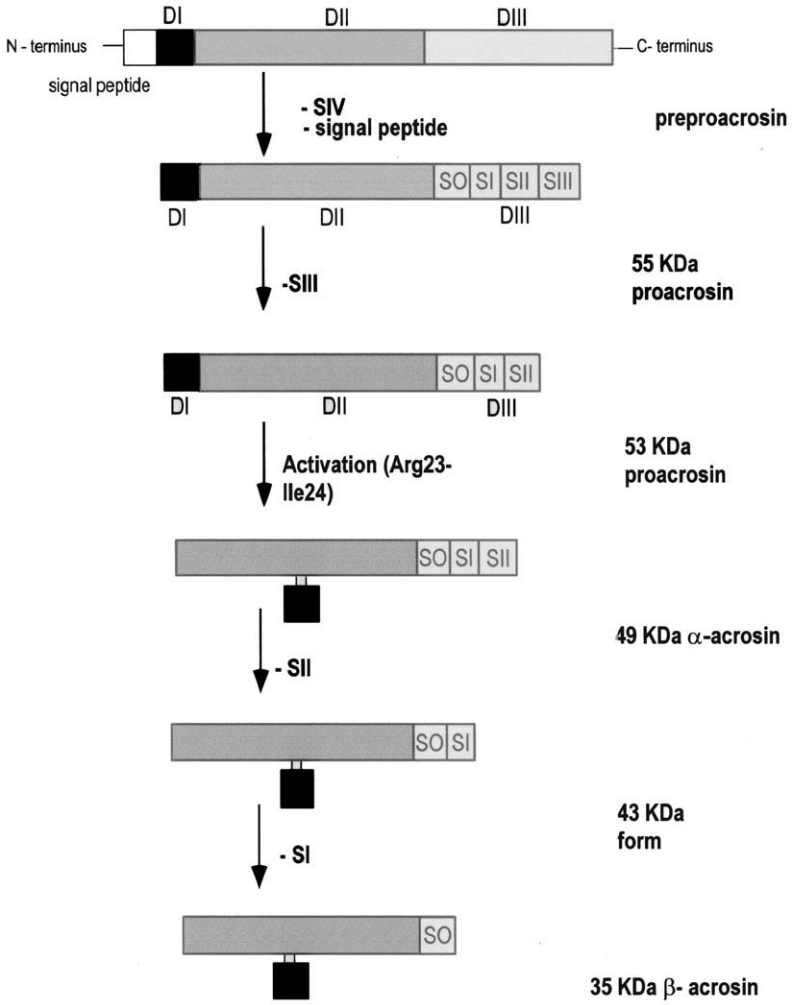
The primary structure of the proenzyme can be divided in three domains (see Scheme 1). The Domain I (DI) encodes the amino acids of the light chain; the Domain II (DII) comprises residues of the mature protein heavy chain; the Domain III (DIII) has the carboxyl terminal (C-terminal) region of the heavy chain; this region is not present in other members of the serine proteases super-family, and it is unique because of its high proline content (Baba et al., 1989a).

Acrosin is synthesized and stored in the sperm acrosome in its zymogen form, proacrosin. During the acrosome reaction, proacrosin is apparently converted into the mature enzyme and released (Tesarik et al., 1988, 1990; Nuzzo et al., 1990; Moos et al., 1993). The majority of the biochemical characterization on proacrosin activation has been obtained from studies using boar spermatozoa as the model system. The purified proenzyme (55 kDa) is autoactivated to the mature β-acrosin (35 kDa) in vitro at basic pH following a pathway in which it is converted to 53, 49, and 43 kDa forms (Polakoski and Parrish, 1977; Baba et al., 1989a,c) (see Scheme 1). Boar proacrosin activation studies in a cell free system have shown intermediate forms of 49, 36, 31 and 25 kDa (Moos et al., 1991). The 49 kDa unstable intermediate (α-acrosin) has been identified as the first enzymatically active form in the maturation cascade.

In human spermatozoa, more than 90% of the total acrosin is in the inactive form (Polakoski et al., 1977). The purified proenzyme appears as a doublet of 53 and 55 kDa. Autoactivation at pH 8.0 renders a 49 kDa form, followed by conversion into 30–40 kDa molecular mass forms (Siegel et al., 1986), and lower relative molecular weight (Mr) forms (12–15 kDa, Schleuning et al., 1976). Using the gelatin-SDS-PAGE system (zymography), two

proteolysis regions were reported, between 47–54 and 34–38 kDa (Siegel and Polakoski, 1985). This last evaluation shows enzymatically active forms, but does not allow detection of inactive and/or non-abundant intermediates.

Some studies have described the use of specific antibodies towards human acrosin in the detection of the enzyme in whole cells and in protein sperm extracts (Gallo et al., 1991; Valdivia et al., 1994; Furlong et al., 2000). However, until the present time, there are no reports focused on the use of



Scheme 1. Schematic representation of the proposed mechanism for boar proacrosin activation (adapted from Baba et al., 1989a) Domain I (DI): light chain. Domain II (DII) and Domain III (DIII): heavy chain. SI–VI: Segment I–IV.

such antibodies to characterize the proenzyme activation pattern. The monoclonal antibody (Mab) AcrC5F10, developed towards human acrosin, recognizes a 53–55 kDa doublet acrosin form in human sperm extracts (Valdivia et al., 1994; Furlong et al., 2000), and the recombinant human proenzyme produced in bacteria (Furlong et al., 2000). Using a peptide library of overlapping hexapeptides (proacrosin residues 264–363) the epitope was mapped (residues 305–309; LPWYF of human proacrosin; see Scheme 1 and Fig. 6, Segment 0) (Furlong et al., 2000). Thus, this antibody could be used to detect the proenzyme and to characterize its activation pattern in protein sperm extracts.

This study was aimed at the development of a set of sensitive assays to evaluate the human proacrosin/acrosin system. The studies of proacrosin/acrosin involved the evaluation of whole spermatozoa by immunocytochemistry, and detection of the proenzyme and intermediate forms in activated extracts by zymography and Western immunoblotting. A multiple alignment analysis was performed with all reported proacrosin sequences to aid in the understanding of the mechanism of human proacrosin activation. These assays could be utilized to study the proacrosin/acrosin system in spermatozoa from semen samples of infertile patients with abnormal acrosin enzymatic activity, contributing to understand the cause of such decreased activity. In addition, they may be used to evaluate the modulatory effect of other proteins upon proacrosin activation.

2. Materials and methods

2.1. Materials

Unless specified, reagents were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA) and BioRad (Hercules, CA, USA). Monoclonal antibody AcrC5F10 was purchased from BIOSONDA (Santiago, Chile). Production of the antibody has been described elsewhere (Valdivia et al., 1994).

2.2. Immunocytochemistry of human spermatozoa

Human semen samples were obtained from eight donors with normal semen parameters according to the World Health Organization guidelines (World Health Organization, 1992). Spermatozoa freed of seminal plasma were fixed in 2% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. Fifty thousand sperm cells were loaded in each well of eight-spot polylysine coated microscope slides, and allowed to dry at

37 °C. Then, cells were washed in PBS for 5 min, permeabilized in methanol for 10 min at 4 °C, and incubated with a 1:1000 dilution of AcrC5F10 antibody in PBS with 0.02% Tween 20 for 1 h at room temperature in a humid chamber. Following three washes of 5 min in PBS, anti mouse IgG FITC in PBS supplemented with 4% bovine serum albumin (BSA) was added and incubated for 1 h at room temperature in the darkness. After three washes with PBS, the smears were mounted and observed in a Zeiss fluorescence microscope equipped with epiillumination.

The immunostaining procedure with AcrC5F10 was performed on: (a) ejaculated spermatozoa devoid of seminal plasma, (b) spermatozoa incubated for 18 h in capacitating conditions with Human Sperm Medium (HSM) (Suarez et al., 1986) containing 26 mg/ml BSA, and (c) spermatozoa subjected to acrosome reaction after incubation of capacitated cells with 10% human follicular fluid for 45 min at 37 °C and 5% CO_2 in air as described earlier (Calvo et al., 1989). Under the conditions assayed, the percentage of follicular fluid-acrosome reacted spermatozoa ranged between 25 and 35%.

To test for the presence of proacrosin/acrosin on the sperm plasma membrane, non capacitated motile spermatozoa were incubated in suspension with a 1:300 dilution of AcrC5F10 in culture medium supplemented with 3% BSA, followed by a 1:50 dilution of mouse IgG FITC in PBS with 4% BSA. Cells were placed onto microscope slides, mounted and analyzed as described before.

2.3. Preparation of human sperm extracts

Spermatozoa freed of seminal plasma were subjected to protein extraction using two procedures, as follows:

- For detergent extracts, proteins were extracted for 2 h on ice in buffer containing PBS with 1% Triton X-100, 1% sodium deoxycholate, and a cocktail of protease inhibitors (Jones, 1989).
- For preparation of pH 3.0 protein extracts, three methods were tested:
  Method I (Leyton et al., 1986): the sperm pellet was incubated for 1 h in 0.15 M NaCl at 37 °C, 5 min in the presence of 0.1% Triton X-100, and extracted in 1 mM HCl with 10% glycerol at pH 3 for 18 h.
  Method II (modified from Siegel et al., 1986): the sperm pellet was resuspended in 1 mM HCl, containing 10% glycerol and 50 mM benzamidine, incubated on ice for 18 h, and centrifuged at $25\,000 \times g$ for 10 min at 4 °C. The supernatant was recovered and dialyzed against 1 mM HCl for 48 h.
  Method III (Shimizu et al., 1997): the sperm pellet was extracted for 18 h at 4 °C in 2% acetic acid, 10% glycerol, 10 mM benzamidine. Protein extracts were dialyzed at 4 °C towards 1 mM HCl.

2.4. Activation of human proacrosin in pH 3.0 extracts

Human sperm pH 3.0 extracts prepared following Method II were used for human proacrosin activation studies. For each activation time assayed (5, 15 and 30 min; 1, 2, and 4 h), an aliquot of pH 3.0 extracts from 10 million sperm were brought to pH 8.0 with 0.1 M Tris buffer and incubated at 20 °C. The incubations were stopped by the addition of sample buffer and boiling for 5 min. Samples were analyzed by zymography or SDS-PAGE under non-reducing/reducing conditions followed by Western immunoblotting. Activation experiments were performed in the presence of 100 mM benzamidine or 9 mM $CaCl_2$ to assess their inhibitor capacity on proacrosin activation.

2.5. Electrophoresis and Western blotting

SDS-PAGE of protein sperm extracts was performed in 10 or 12% polyacrylamide gels containing 0.1% SDS (Laemmli, 1970), under reducing and non-reducing conditions.

For Western Blot analysis, proteins were electrotransferred as described earlier (Towbin et al., 1979) onto nitrocellulose membranes, and immunostained with AcrC5F10 in blocking solution (5% skim milk in PBS containing 0.02% Tween 20). Biotinylated goat anti mouse IgG (1:1000) was used as the secondary antibody. Blot immunostaining was performed at room temperature with constant shaking. Membranes were developed with avidin-peroxidase and diaminobenzamidine (DAB) as chromogen.

2.6. SDS-gelatin proteinase assay (zymography)

Proteolytic activity of proacrosin and acrosin activation intermediates was evaluated using non reducing SDS-PAGE containing 0.1% gelatin as described earlier (Siegel and Polakoski, 1985), with the exception that gels were incubated in 50 mM Tris buffer (pH 8.0) at 37 °C for 18 h.

2.7. Molecular analysis on the h-proacrosin sequence

2.7.1. Multiple sequence alignment

Localisation of protein sequences potentially involved in proacrosin activation were analyzed using a multiple sequence alignment done with the proacrosin sequences of different species publicly available (DDBJ/Embl/GenBank databases: human: M77378-M77381; boar: J04950; bovine: X68212; mouse: D00754; rat: X59254; rabbit: U05204; guinea pig: Z12153).

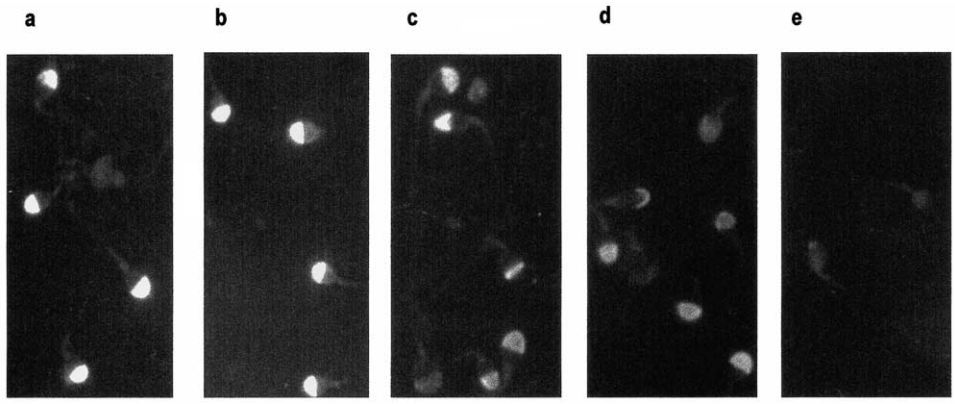


Fig. 1. Immunocytochemistry of human spermatozoa with AcrC5F10. Spermatozoa subjected to different experimental conditions were analyzed by immunocytochemistry with the Mab AcrC5F10. Panel a: Ejaculated spermatozoa. Panel b: Capacitated spermatozoa. Panel c and d: Follicular fluid-acrosome reacted spermatozoa. Panel e: Non-permeabilized ejaculated spermatozoa.

To perform the analysis, the CLUSTAL software was run (Thompson et al., 1997).

2.7.2. Sequence logos

Based on the multiple alignment of all proacrosin sequences included in this study, sequence logos were calculated. Logos were computed using alpro and makelogo (Schneider and Stephens, 1990), and generated using a WEB server (http://www.bio.cam.ac/seqlogo).

3. Results

3.1. Immunocytochemistry of human spermatozoa with AcrC5F10

In smears of fixed and permeabilized ejaculated human spermatozoa, over 90% of cells exposed to AcrC5F10 showed a bright fluorescence in the acrosomal cap, and a faint signal in the postacrosomal region, midpiece, and flagellum (Fig. 1, panel a), in agreement with an earlier report (Moreno et al., 1998). Omission of the first antibody resulted in the absence of fluorescence in the acrosome of the sperm cells (data not shown). Spermatozoa incubated under capacitating conditions showed the same pattern found in ejaculated cells (Fig. 1, panel b). Spermatozoa incubated with human follicular fluid under conditions to induce the acrosome reaction showed several patterns of staining (Fig. 1, panel c and d). As mentioned, when

using human follicular fluid to induce the acrosome reaction, around 35% of the spermatozoa undergo exocytosis of the acrosomal granule. Under these experimental conditions, over 60% of the cells showed an intense staining over the acrosomal region. In the other cells, different patterns of staining were observed: (1) a virtual loss of the acrosomal cap staining, (2) an heterogeneous spotty pattern of the acrosome, (3) a narrow band of fluorescence in the equatorial segment. In samples containing non-permeabilized motile spermatozoa, over 95% of the cells were unstained. (Fig. 1, panel e). This indicated that, in non-capacitated spermatozoa, the antibody does not detect proacrosin/acrosin on the sperm surface. Similar staining patterns were obtained when using the Mab AcrC2E5 (Biosonda, Chile) developed towards bovine acrosin (data not shown).

3.2. Identification of human proacrosin in protein sperm extracts with AcrC5F10

Using detergent extracts from 30 million spermatozoa, AcrC5F10 recognized several components of the sperm extract at both dilutions evaluated

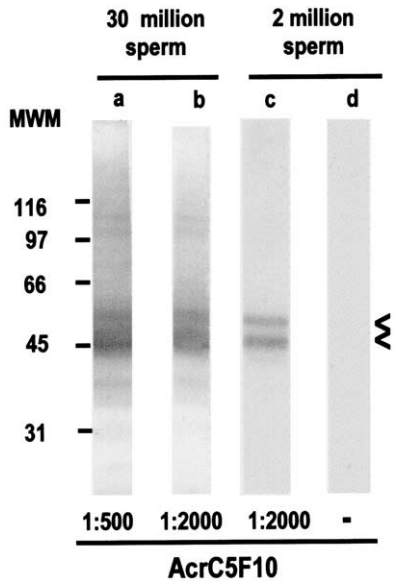


Fig. 2. Identification of human proacrosin in protein sperm detergent extracts. (A) Human sperm detergent extracts were subjected to non-reducing SDS-PAGE and analyzed by AcrC5F10 immunoblotting. Protein extracts corresponding to 30 (lanes a and b) and 2 (lanes c and d) million spermatozoa were analyzed. AcrC5F10 was tested at two dilutions: 1:500 (lane a), 1:2000 (lanes b and c). Control: first antibody omitted (lane d). A typical experiment is shown. This experiment was performed three times obtaining similar results.

(1:500; 1:2000) (Fig. 2A, lane a and b). The highest reactivity was associated to a band of Mr 55 and to a broad band of 49–53 kDa. In addition, the antibody recognized protein bands of higher (112, 105, 88, 83 kDa) and lower (40, 36, 28 and 22 kDa) molecular weight. When extracts from 2 million spermatozoa were tested, immunodetection was predominant in 55 and 50 kDa protein bands (Fig. 2A, lane c). Western blots of sperm extracts incubated in the absence of the first (Fig. 2A, lane d) or second (data not shown) antibodies exhibited no reactivity of the sperm proteins.

With the aim to analyze the activation pattern of human proacrosin in human sperm pH 3.0 extracts, it was crucial to prepare an extract in which acrosin is obtained in the proenzyme form. Processing human semen samples for proacrosin extraction with different protocols results in protein extracts containing variable proportions of the proenzyme as well as several activated forms (Leyton et al., 1986; Siegel et al., 1986; Shimizu et al., 1997). Then, it was decided to test different protocols to select the one that, in our hands, would preserve acrosin mainly as a zymogen. Extracts prepared following Methods I, II, and III (see Section 2) were analyzed by non-reducing SDS-PAGE and Western immunoblotting. In all cases, proacrosin/

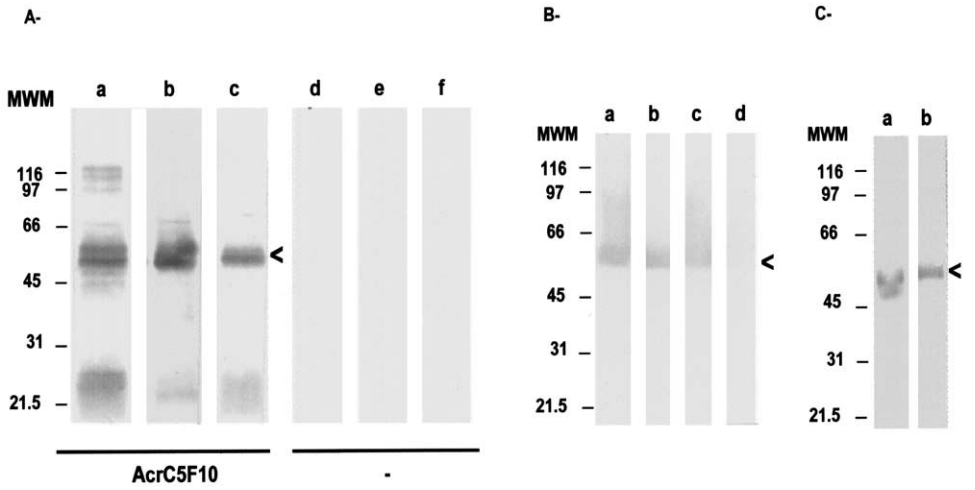


Fig. 3. Identification of human proacrosin in pH3 extracts. (A) Acid sperm extracts (pH3 extracts), obtained following different methodologies, were evaluated by AcrC5F10 immunoblotting. Lane a and d: Method I. Lane b and e: Method II. Lane c and f: Method III. Control: first antibody omitted (lanes d–f). (B) Differential partitioning of human proacrosin in semen fractions obtained using extraction Method II. Lane a: total sperm pH3 extract. Lane b: pH3 extract soluble fraction. Lane c: pH3 extract insoluble fraction. Lane d: seminal plasma. (C) SDS-PAGE analysis of the proacrosin doublet band under non-reducing (lane a) and reducing (lane b) conditions. A typical experiment is shown. This experiment was performed three times obtaining similar results.

acrosin forms of Mr 48-56 were identified with AcrC5F10 (Fig. 3A, lanes a–c). In extracts prepared using Method I, immunoreactivity was also detected in proteins of higher (124, 110 and 102 kDa) and lower Mr (22–30 kDa) (Fig. 3A, lane a). Using Method II, immunoreactivity to AcrC5F10 was predominant in acrosin forms of 48–56 kDa, and a faint band at 22–25 kDa was also developed (Fig. 3A, lane b). Using Method III, in addition to the proenzyme form, immunoreactivity was associated to a smear between 21 and 29 kDa (Fig. 3A, lane c). Immunostaining was specific, since omission of the first (Fig. 3A, lanes d–f) or second antibody (not shown) showed no staining. From these results, Method II was selected for proacrosin activation studies.

After sperm protein extraction using Method II, immunoreactivity to proacrosin/acrosin forms was found in both the soluble (Fig. 3B, lane b) and insoluble fractions (Fig. 3B, lane c), indicating that part of the protein remains in the particulate fraction after the extraction procedure. No proteins from the seminal plasma were reactive to AcrC5F10 (Fig. 3B, lane d).

Immunodetection with AcrC5F10 of h-proacrosin in pH 3.0 extracts gave, in all cases, a 48–56 kDa broad band (see above). Proacrosin autoactivation involves conversion of the single chain 55 kDa proenzyme to a 53 kDa form, followed by cleavage into a 49 kDa double chain acrosin intermediate, in which both chains are connected by disulfide bonds (see Scheme 1) (Baba et al., 1989a; Moos et al., 1993). To determine whether the Mr 49 band recognized by AcrC5F10 was a single chain or a double chain (active) form, pH 3.0 extracts were run in SDS-PAGE under different conditions. As shown before, in the absence of the reducing agent (Fig. 3C, lane a), AcrC5F10 recognized proacrosin/acrosin forms of 48–56 kDa. In an aliquot of the same extract processed under reducing conditions (Fig. 3C, lane b), the signal shifted towards forms with lower mobility (Mr of 57–63), that would indicate that acrosin forms present in the extracts are mainly single chain polypeptides.

3.3. Activation of proacrosin from pH 3.0 extracts

A zymographic analysis was first utilized to identify proacrosin activation intermediates in human sperm extracts. For that, pH 3.0 extracts were incubated under conditions for proacrosin activation, and proteins were analyzed using a zymography. Prior to activation, a strong 52–57 kDa digestion area was detected, which would represent the proenzyme form. In addition, a faint signal was detected in a 28 kDa band (Fig. 4, lane a). After activation, a decrease in the proenzyme digestion region was accompanied by the presence of several well-defined digestion bands of lower Mr (15 min:

56–59, 39, 34–35, 28 kDa; 1 h: 56–59, 39, 34–35 kDa; Fig. 4, lane b and c, respectively).

To confirm whether the digestion bands were proacrosin activation intermediates, pH 3.0 extracts incubated under conditions for activation were subjected to non reducing SDS-PAGE and Western immunoblotting with AcrC5F10 (Fig. 5). At 0 min activation time, most of the immunostaining was associated to a broad band of 48–57 kDa (Fig. 5A, lane a), that could correspond to the Mr 56–59 form detected in the zymography (Fig. 4). Activation resulted in an intensification of the 57 kDa band during the first hour and permanence of the signal until 4 h incubation (Fig. 5A, lane a–g). An additional band of 61 kDa was detected at 5 and 15 min activation time points (Fig. 5A, lane b and c). A 43–45 kDa doublet was detected from 5 min to 4 h, showing a decrease in the signal throughout the incubation time (Fig. 5A, lane b–g). A 34 kDa protein band was first detected at 5 min, showing a weak signal up to 1 h incubation (Fig. 5A, lane b–e), with the same Mr as the active form (Fig. 4). Finally, a broad band of low Mr (20–24) showed a strong signal from 5 min to 4 h of activation (Fig. 5, lane b–g).

A better resolution of the protein bands was obtained when samples were evaluated under reducing conditions (Fig. 5B). Prior to activation, a sharp 61 kDa band was present, in addition to a faint staining at lower Mr

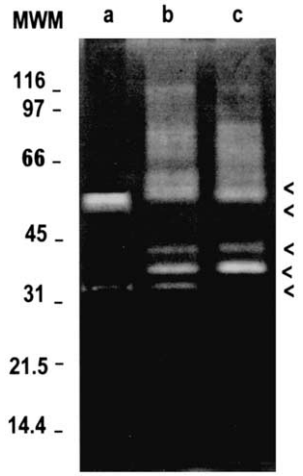


Fig. 4. Analysis of human proacrosin activation in pH3 extracts by zymographic assay. Acid sperm extracts obtained by Method II were incubated under proacrosin activation conditions and analyzed by gelatin SDS-PAGE (zymography). Samples were analyzed at different times of activation: zero time (lane a), 30 min (lane b), 1 h (lane c). Proacrosin and activation intermediates are indicated. A typical experiment is shown. This experiment was performed three times obtaining similar results.

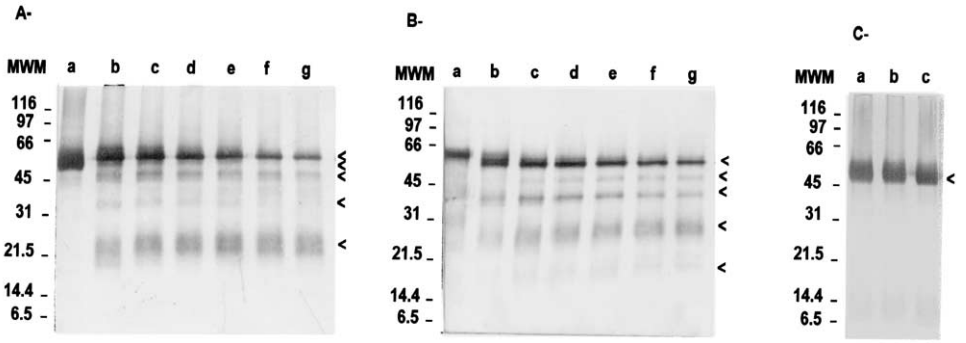


Fig. 5. Analysis of human proacrosin activation in pH 3 extracts by immunoblotting. Acid sperm extracts obtained by Method II were incubated under proacrosin activation conditions and analyzed by Western immunoblotting with AcrC5F10. (A) SDS-PAGE under non-reducing conditions. (B) SDS-PAGE under reducing conditions. (C) Activation performed in the presence of 100 mM benzamidine. In (A) and (B), activation patterns at 0 (zero) time (lane a), 5 min (lane b), 15 min (lane c), 30 min (lane d), 1 h (lane e), 2 h (lane f) and 4 h (lane g) are shown. In (C), patterns from extracts incubated for activation in the presence of 100 mM benzamidine at 0 (zero) time (lane a), 1 h (lane b), and 4 h (lane c) are shown. Proacrosin and activation intermediates are indicated. A typical experiment is shown. This experiment was performed three times obtaining similar results.

proteins (Fig. 5B, lane a). After 5 min of activation, three well defined forms of 61, 58, and 52 kDa showed a strong signal (Fig. 5B, lane b). The signal on the 52kDa protein band persisted over 4 h activation. A 43 kDa band was first detected at 30 min activation, and remained at all other times tested (Fig. 5B, lanes d–g). A 34 kDa protein was first seen at 5 min and gave the strongest signal at 30 min and 1 h (Fig. 5B, lane b–g). In addition, a broad signal was seen in 20–24 kDa forms after 5 min activation, and increased with time (Fig. 5B, lane b–g). There was an additional 16 kDa form seen between 30 min and 4 h activation (Fig. 5B, lanes d–g). The addition of 100 mM benzamidine, a known competitive serine proteinase inhibitor, completely inhibited human proacrosin conversion to mature

Fig. 6. Multiple sequence alignment of proacrosin from several species. A multiple alignment was done with the CLUSTAL software on proacrosin sequences to analyze conservation of putative activation cleavage sites. The regions comprising Domain I, Segments 0 (zero), I, II and III are indicated. The epitope recognized by the Mab AcrC5F10 (LPWYF) located within residues 305–309 is underlined (see Segment 0). As a result of the PROSITE analysis (release 16) (Bucher and Bairoch, 1994; Hofmann et al., 1999), a sequence similar to the pattern for the prokaryotic membrane lipoprotein lipid attachment site (Pattern ID: PROKAR LIPOPROTEIN PS00013 PDOC00013) was found. The patterns are shown with the black boxes. The target residue for the attachment to the membrane is a charged (or polar) amino acid located at least six residues to the N-terminus of the eleven residues with high sequence conservation.

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                  Signal peptide  |     Light chain(DI)    |     Heavy chain
Boar      ---MLPTAVLLVLAVSVAARDNATCDGPCGLRFRQKLESGMRVVGGMSAEPGAWPWMVSL
Human     MVEMLPTAILLVLAVSVVAKDNATCDGPCGLRFRQNPQGGVRIVGGKAAQHGAWPWMVSL
Bovine    ---MLPTAILLVLAVSVVTRDNTTCEGPCGTRFRQNRQGGMRIIGGQDAAHGSWPWMVSL
G. Pig    --EMLPTAALLILAVSVIARDNTTCDGPCGLRFRQNLQGSVRIIGGQTAQPGAWPWMVSL
Mouse1    ---MLPT-AFWSVKVSAGAKDNATCFGPCGLRTRQNSQAGTRIVSGQSAHVGAWPWMVSL
Mouse2    -VEMLPTVAVLVLAVSVVAKDNTTCDGPCGLRFRQNSQAGTRIVSGQSAQLGAWPWMVSL
Mouse3    MVEMLPTVAVLVLAVSVVAKDNTTCDGPCGLRFRQNSQAGTRIVSGQSAQLGAWPWMVSL
Rabbit    ---MLPTAVLLVLVVSVVAKDNATCDGPCGLRFRQNPQGGFRVVGGQAAQQGAWPWMVSL
Ram       --------------------DNTTCDGPCGVRFRQNRQGGVRIIGGQDAAHGAWPWMVSL
Rat       MVEMLPTVVALVLAVSVVAKDNTTCDGPCGLRFRQNPQAGIRIVGGQTSSRWAWPWMVSL
                              **:** **** * **: :.. *::.*  :   :*******

                                        Heavy chain
Boar      QIFMYHNNRRYHTCGGILLNSHWVLTAAHCFKNKKKVTDWRLIFGANEVVWGSNKPVKPP
Human     QIFT-YNSHRYHTCGGSLLNSRWVLTAAHCFVGKNNVHDWRLVFGAKEITYGNNKPVKAP
Bovine    QIFTYHNNRRYHVCWGLLLNAHWLLTAAHCFRIKKKVTDWRLIFGAKEVEWGSNKPVKPP
G. Pig    QIFMAHNNRRYHACGGILLNSHWVLTAAHCFDSKKKVYDWRLVFGAEEIEYGNNKPVRAP
Mouse1    QIFTSHNSRRYHACGGSLLNSHWVLTAAHCFDNKKKVYDWRLVFGAQEIEYGRNKPVKEP
Mouse2    QIFTSHNSRRYHACGGSLLNSHWVLTAAHCFDNKKKVYDWRLVFGAQEIEYGRNKPVKEP
Mouse3    QIFTSHNSRRYHACGGSLLNSHWVLTAAHCFDNKKKVYDWRLVFGAQEIEYGRNKPVKEP
Rabbit    QIFTPRNNRRYHACGGVLLNAHWVLTAAHCFNNKQKVYEWRMVFGAQEIEYGTDKPVRPP
Ram       QIFTYHNNRRYHVCGGSLLNSQWLLTAAHCFRIKKKVTDWRLIFGAKEVEWGTNKPVKPP
Rat       QIFTSHNSRRYHACGGSLLNSHWVLTAAHCFDNKKKVYDWRLVFGAHEIEYGRNKPVKEP
          ***   *.:***.* * ***::*:*******  *::* :**::***.*: :* :***: *

                                        Heavy chain
Boar      LQERFVEEIIIHEKYVSGLEINDIALIKITPPVPCGPFIGPGCLPQFKAGPP-RAPQTCW
Human     VQERYVEKIIIHEKYNSATEGNDIALVEITPPISCGRFIGPGCLPHLKAGLP-RGSQSCW
Bovine    LQERYVEKIIIHEKYSASSEANDIALIKITPPVICGHFIGPGCLPQFRAGPP-RVPQTCW
G. Pig    LQERYVEKIVIHEKYNIVNEGNDIALLKITPPVSCGPFIGPGCLPTFRAGPP-KIPQTCY
Mouse1    QEERYVQKIVIHEKYNVVTEGNDIALLKVTPPVTCGNFIGPCCLPHFKAGPPRKIPHTCY
Mouse2    QEERYVQKIVIHEKYNVVTEGNDIALLKVTPPVTCGNFIEPCCLPHFKAGPP-QIPHTCY
Mouse3    QQERYVQKIVIHEKYNVVTEGNDIALLKITPPVTCGNFIGPCCLPHFKAGPP-QIPHTCY
Rabbit    LQERYVEKVVTHDQYNYMTEGNDIALLKITPPVPCGPFIGPGCLPNSKAGPP-KAAQTCY
Ram       LQERYVEKIIIHEKYSASSEANDIALMKITPPVTCGHFIGPGCLPQFRAGPP-RVPQTCW
Rat       QQERYVQKIVIHEKYNAVTEGNDIALLKVTPPVTCGDFVGPGCLPHFKSGPP-RIPHTCY
           :**:*:::: *::*    * *****:::***: ** *: * ***  ::* * : .::*:

                                        Heavy chain
Boar      VTGWGYLKEKGPRTSPTLQEARVALIDLELCNSTRWYNGRIRSTNVCAGYPRGKIDTCQG
Human     VAGWGYIEEKAPRPSSILMEARVDLIDLDLCNSTQWYNGRVQPTNVCAGYPVGKIDTCQG
Bovine    VAGWGFLRENARRTSPVLQEAHVDLIDLDLCNSTRWYNGRIRSTNVCAGYPEGKIDTCQG
G. Pig    VAGWGYIREKAPRPSPVLLEARVELIDLDLCNSTQWYNGRVMSTNVCAGYPEGKIDTCQG
Mouse1    VTGWGYIKREAPRPSPVLMEARVDLIDLDLCNSTQWYNGRVTSTNVCAGYPEGKIDTCQG
Mouse2    VTGWGYIKEKAPRPSPVLMEARVDLIDLDLCNSTQWYNGRVTSTNVCAGYPEGKIDTCQG
Mouse3    VTGWGYIKEKAPRPSPVLMEARVDLIDLDLCNSTQWYNGRVTSTNVCAGYPEGKIDTCQG
Rabbit    VAGWGYVKENAPRPSPTLMEARVDLINLELCNSTQWYNGRITASNLCAGYPSGKIDTCQG
Ram       VAGWGFLQENARRTSPMLQEARVDLIDLGLCNSTRWYNGRIRSTNVCAGYPEGKIDTCQG
Rat       VTGWGYIKDNAPRPSPVLMEARVDLIDLDLCNSTQWYNGRVTSTNVCAGYPEGKIDTCQG
          *:***::. :. *.*. * **:* **:* *****:*****: .:*:***** ********

                                        Heavy chain
Boar      DSGGPLMCRDRAENTFVVVGITSWGVGCARAKRPGVYTSTWPYLNWIASKIGSNALQMVQ
Human     DSGGPLMCKDSKESAYVVVGITSWGVGCARAKRPGIYTATWPYLNWIASKIGSNALRMIQ
Bovine    DSGGPLMCKDSVENSYVVVGITSWGVGCSRAKRPGVYTSTWSYLNWIASKIGSNTVHMIQ
G. Pig    DSGGPLMCRDNANSPFVVVGITSWGVGCARAKRPGIYTATWDYLDWIASKIGPSALHAIQ
Mouse1    DSGGPLMCRDNARQPFVVVGITSWGVGCARAKRPGVYTATWDYLDWIASKIGPNALHLIP
Mouse2    DSGGPLMCRDNVDSPLCGRGDHELGGRLCRAKRPGVYTATWDYLDWIASKIGPNALHLIQ
```

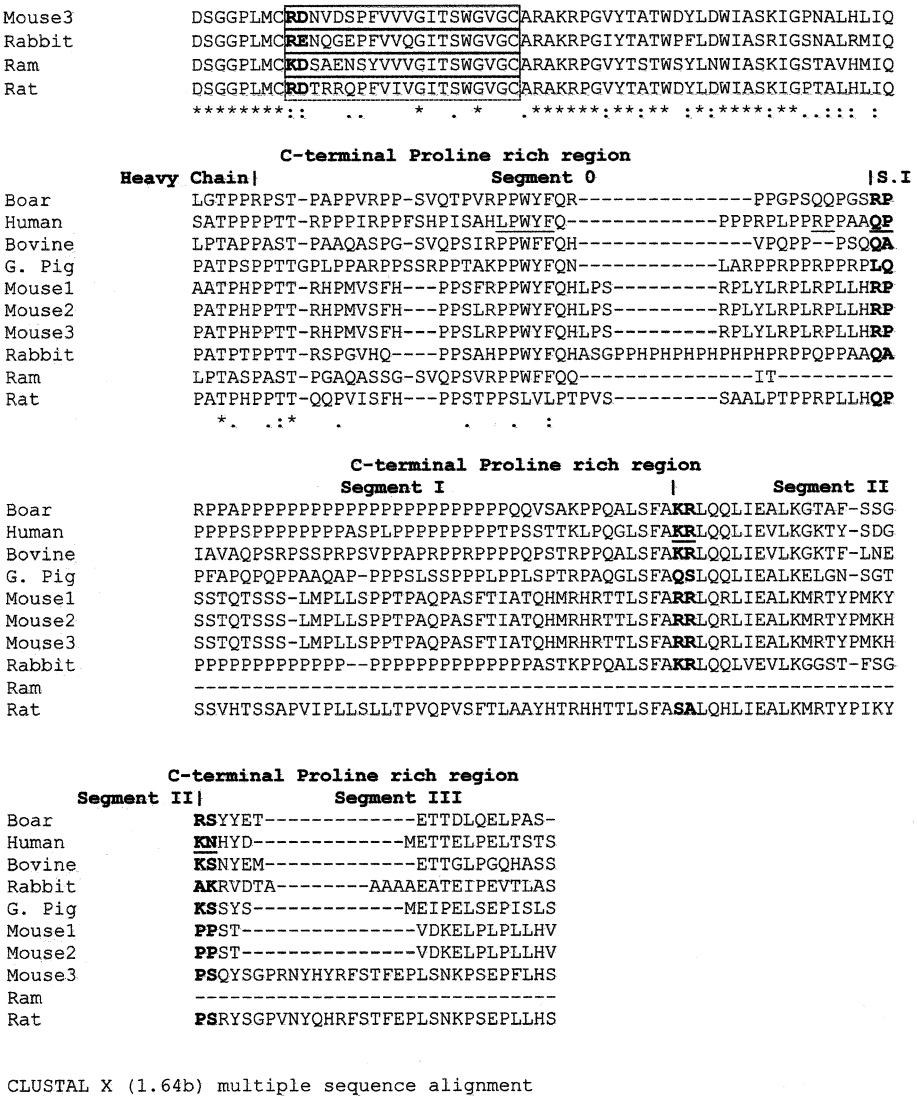

Fig. 6. (*Continued*)

forms (Fig. 5C). A similar inhibitory effect was obtained when 9 mM $CaCl_2$ was added to the incubation buffer (data not shown).

3.4. Molecular analysis on the h-proacrosin sequence in relation to its activation

Since biochemical characterization of activation intermediates had been

earlier reported for boar proacrosin (Baba et al., 1989a,c), the cleavage sites suggested from those studies (junction between C-terminus of earlier segment and N-terminus of the cleaved segment; see Scheme 1) were used to analyze their conservation. A multiple alignment analysis was done with the sequences encoding proacrosin from several species (Fig. 6). In addition, a graphic representation of this analysis (sequence logos) is depicted in Fig. 7.

As earlier suggested (Baba et al., 1989a), proacrosin activation would begin with the loss of a small segment from the C-terminus (Segment III). The first cleavage site in h-proacrosin could be located on Lys385–Asn386. This site is conserved in boar, bovine and guinea pig, while in mouse and rat an alternative site could be located two residues towards the N-terminus (Figs. 6 and 7panel a). The sequence involved in the light chain processing (Arg23–Val24 in boar) was found to be conserved in all species (in human Arg23–Ile24) (Baba et al., 1989b; Adham et al., 1990) (Figs. 6 and 7, panel b). The cleavage at this site produces the first double-chain active intermediate (α-acrosin). In Segment I–II cleavage site, the sequence Lys365–Arg366 proposed by Baba and collaborators was conserved in all species but guinea pig. Finally, formation of mature β-acrosin involves removal of Segment I (see Scheme 1). In the multiple alignment analysis, the proposed Segment I

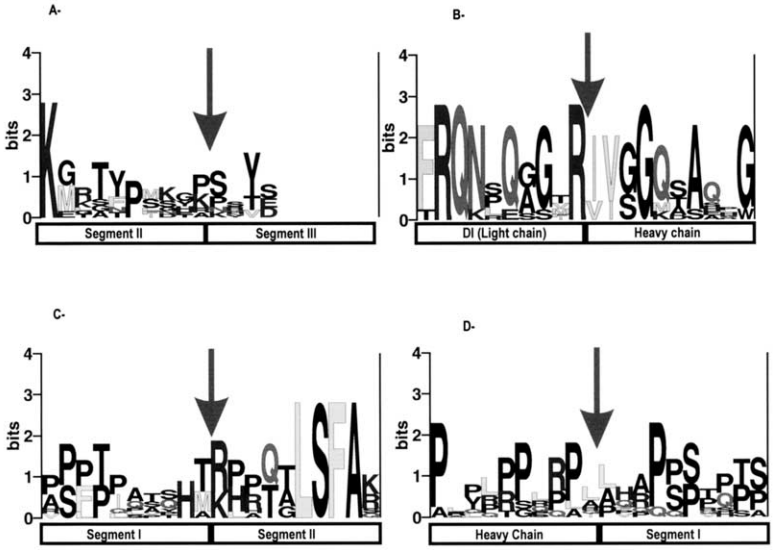

Fig. 7. Sequence logo for proacrosin activation. Sequence conservation, measured in bits of information, is shown by the height of a stack of letters for each position. The relative heights of the letters within a stack are proportional to their frequencies. Sequences between -10 and $+10$ of each cleavage site are depicted. The arrows indicate the location of the cleavage site. Segments comprise the sequences shown in Fig. 6. Panel a: Segment II–Segment III cleavage. Panel b: Light chain–Heavy chain. Panel c: Segment I–Segment II. Panel d: Segment 0 (zero)–Segment I.

cleavage site at residues Arg322–Pro323 was only present in the boar and mouse sequences (Figs. 6 and 7 panel d). In human proacrosin, a putative Arg–Pro site was found three residues towards the N-terminus of the proposed site for the boar proenzyme (Fig. 6 underlined).

A recent report (Tranter et al., 2000) has characterized a boar β-acrosin form lacking 38 amino acids (Segment 0) from the C-terminus (Fig. 6). As suggested by the authors, the loss of Segment 0 would result from hydrolysis during protein crystallization. The multiple alignment included conservation analysis of the cleavage sites that would give raise to this segment (Fig. 6).

4. Discussion

Acrosin is a serine proteinase synthesized as a zymogen and activated during the acrosome reaction, rendering an active enzyme attributed to have several roles during fertilization. The present study was done to develop a set of sensitive assays for the evaluation of the human proacrosin/acrosin system. These included sperm immunocytochemistry, preparation of protein extracts preserving acrosin in the proenzyme form, and analysis of the activation pattern using zymography and Western immunoblotting with a specific antibody towards human acrosin.

The antibody AcrC5F10 was utilized to localize the proacrosin/acrosin system in spermatozoa prior and after the acrosome reaction. In ejaculated spermatozoa acrosin staining was distributed over the acrosomal cap, in agreement with other reports (Gallo et al., 1991; Moreno et al., 1998; Furlong et al., 2000). The presence of a similar pattern of staining in capacitated cells would suggest no redistribution of the protein in the acrosomal vesicle, although minor changes would not be detected under the conditions assayed. The strong signal over the equatorial segment in some of the acrosome reacted cells would indicate that part of the protein remains in that region. These last observations are in agreement with a study using electron microscopy of calcium ionophore acrosome reacted spermatozoa (Tesarik et al., 1988), and reinforces a role proposed in sperm fusion to the oolema (Van der Ven et al., 1985; Takano et al., 1993) and/or oocyte activation (Smith et al., 2000).

Different molecular weights have been described in the literature for human proacrosin, from 75 to 12 kDa (see Siegel et al., 1986). The extracts prepared in this study were enriched in the proenzyme form with an Mr of 48–57, as determined after zymographic analysis and Western immunoblotting. This estimated molecular weight is similar to that earlier reported by other groups (Siegel and Polakoski, 1985; Tesarik et al., 1990; Gallo et al., 1991; Valdivia et al., 1994).

Using pH 3.0 extracts, proacrosin activation was evaluated using zymography. The Mr of the activation intermediates was similar to that earlier described (Siegel and Polakoski, 1985). The use of Western immunoblotting revealed some similarities with the zymography and with the activation patterns obtained using purified human and boar proacrosin. The Mr 58–61 band could correspond to the 53–55 proacrosin form. The 52 kDa intermediate could be α-acrosin, and the 43 and 34 kDa forms were found to have a similar Mr to those described in boar (Baba et al., 1989a,c). In the conditions assayed in our study, the 52 kDa intermediate (α-acrosin) appeared to be stable throughout the activation time. These results are similar to those described in boar sperm extracts (Moos et al., 1991), and in cells incubated with calcium ionophore to undergo the AR (Moos et al., 1993). In contrast, using purified boar proacrosin, the α-acrosin intermediate seems to have a short half life-time (Baba et al., 1989c). Detection of these forms in the sperm extracts could result from protein stabilization by interaction with other acrosomal components, as described for the association of boar proacrosin and α-acrosin with the acrosomal protein sp32 (Baba et al., 1994).

Low Mr forms (22–24 and 16 kDa) were only identified using Western immunoblotting. These proteins could be enzymatically inactive, or in low quantities to be detected using zymography. In earlier studies, a 25 kDa form (γ-acrosin) was found to show enzymatic activity (Schleuning et al., 1976). Moreover, in the same study, low Mr forms (12 and 15 kDa) were identified. Since these forms are immunoreactive to AcrC5F10, they most likely are proacrosin fragments that comprise the region where the epitope is localized (residues 305–309). Limitations in the amount of these proteins hindered their characterization.

Activation of h-proacrosin in pH 3.0 extracts was completely inhibited by the presence of benzamidine, in agreement with earlier reports (Polakoski et al., 1977). These results indicate the involvement of serine proteinases in the process of proacrosin activation. Although self activation of purified human proacrosin has been earlier demonstrated (Siegel et al. 1986), whether this is the mechanism occurring in the presence of other acrosomal proteins remains to be determined. Proacrosin activation was also inhibited in the presence of calcium ions. These results are in agreement with earlier studies using the enzyme from human (Siegel et al., 1986) and boar (Brown and Harrison, 1978).

In addition to ions and protease inhibitors, acrosomal proteins (Srivastava and Ninjoor, 1982; Baba et al., 1994), DNA, ZP glycoproteins and sulfated polymers (Eberspaecher et al., 1991) have been found to modulate proacrosin activation and acrosin activity in several species. Moreover, anionic phospholipids vesicles were described to stimulate boar proacrosin

conversion in vitro, by a mechanism that would involve zymogen binding to the surface of the vesicle, promoting a conformational change leading to proenzyme activation (Parrish et al., 1978). Supporting this hypothesis, a lipid attachment site was identified in the proacrosin sequence (Fig. 6). Further studies are required to evaluate the biological significance of this site.

Proacrosin activation was triggered in vitro by only a raise in the pH, as earlier reported in several species (Meizel and Mukerji, 1976; Polakoski and Parrish, 1977; Siegel et al., 1986). An increase in the acrosomal pH has been described during the acrosome reaction (Working and Meizel, 1983; Florman et al., 1989). Thus, in vivo activation of proacrosin may be modulated by this change, inducing conformational change(s) in the proenzyme that ultimately would lead to its activation. Alternatively, a pH raise could affect the function of other acrosomal proteins that in turn may regulate proacrosin activation.

A significant correlation between alterations in acrosin proteinase activity and abnormal fertilization rate in human IVF-ET procedures has been reported (Kennedy et al., 1989; Tummon et al., 1991; Sharma et al., 1993; Senn et al., 1992; De Jonge et al., 1993; Menkveld et al., 1996; Shimizu et al., 1997). However, whether these alterations are associated to a lack of the enzyme or reflect abnormalities in proacrosin activation is yet unknown. The set of assays here described constitute a valid model to investigate this issue, as they allow the detection of proacrosin/acrosin in whole spermatozoa, and the identification of the proenzyme as well as its activation intermediates in sperm extracts, without the need of protein purification. In addition, this model will be useful in studying the effect of seminal plasma factors, as protease inhibitors, on proacrosin activation.

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