DOI: 10.2436/20.1501.02.6

Endocrinologia molecular
(Jaume Reventós, ed.)

Treballs de la SCB. Vol. 56 (2005) 59-74

AN OVERVIEW OF THE PROACROSIN/ACROSIN SYSTEM IN HUMAN SPERMATOZOA

Mónica H. Vazquez-Levin,[1] Laura I. Furlong,[1,3] Carolina M. Veaute [1,4] and P. Daniel Ghiringhelli [2]

[1] *Instituto de Biología y Medicina Experimental. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Universidad de Buenos Aires.*
[2] *Laboratorio de Ingeniería Genética y Biología Celular y Molecular (LIGBCM), Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes.*
[3] *Current address: Research Unit on Biomedical Informatics (GRIB) IMIM/UPF.*
[4] *Current address: Cátedra de Inmunología Básica, Universidad Nacional del Litoral.*

Corresponding author: Mónica H. Vazquez-Levin. Instituto de Biología y Medicina Experimental. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Universidad de Buenos Aires. Vuelta de Obligado, 2490. 1428 Buenos Aires, Argentina. E-mail: *mhvaz@dna.uba.ar*.

RESUM

Entre totes les proteïnases espermàtiques semblants a la tripsina estudiades, l'acrosina (EC 3.4.21.10) ha estat identificada en totes les espècies estudiades, i ha estat associada amb el potencial fertilitzador de l'esperma. En aquesta breu revisió, volem presentar els principals aspectes cel·lulars, moleculars i bioquímics del sistema proacrosina/acrosina definits pel nostre grup de recerca i per molts altres equips de recerca del camp de la biologia de la reproducció. Com a resultat de tots aquests estudis, presentem el model putatiu de la interacció del sistema proacrosina/acrosina amb les glicoproteïnes de la zona pel·lúcida i la demostració de l'activació del proenzim i la seva activitat com a proteïnasa.

Paraules clau: humans, fertilització, esperma, acrosina, zona pel·lúcida.

SUMMARY

Among all of the sperm acrosomal trypsin-like proteinases studied, acrosin (EC 3.4.21.10) has been identified in all of the species evaluated and has been associated with human sperm fertility potential. In this report, a brief overview of cellular, biochemical, and molecular aspects related to the human proacrosin/acrosin system, has been compiled from studies performed by our research team, as well as contributions by numerous groups from the clinical and basic field of reproductive biology. As a result of these studies, a putative model for the interaction

between the proacrosin/acrosin system and the zona pellucida glycoproteins, in association with proenzyme activation and proteinase activity, is presented.

Keywords: human, fertilization, sperm, acrosin, zona pellucida.

Abbreviations: *Zona pellucida* (ZP); human preproacrosin (h-preproacrosin); human proacrosin (h-proacrosin); bp (base pair); mRNA (messenger ribonucleic acid); 5′UTR (5′ untranslated region); Domain I (DI); Domain II (DII); Domain III (DIII); amino *terminus (*N-*terminus),* carboxy *terminus* (C-*terminus*), *in vitro* fertilization and early embryo transfer (IVF-ET), enzyme-linked immunoassay (ELISA).

Sperm-egg interaction is a specialized cell adhesion process that leads to fertilization and the activation of development. Once spermatozoa reach the vicinity of the egg, they interact with the glycoproteins of the egg's extracellular coat, called the *zona pellucida* (ZP). Sperm-binding to the ZP triggers the exocytosis of the acrosomal granule (acrosomal exocytosis), wherein fusion of the sperm plasma membrane and the outer acrosomal membrane occurs, followed by the release of the acrosomal content; acrosome-reacted sperm penetrate the ZP and finally bind and fuse to the egg's plasma membrane (Yanagimachi, 1994; Wassarman *et al.*, 2001). Evidence of the participation of sperm's trypsin-like proteinases in the acrosomal exocytosis and ZP penetration first came from experiments showing a blockade on sperm penetration by the addition of trypsin inhibitors (Liu and Baker, 1993; Llanos *et al.*, 1993). Among all of the acrosomal trypsin-like proteinases studied *acrosin* (EC 3.4.21.10) has been associated with human sperm fertility potential (see below). In this report, a brief overview of cellular, biochemical, and molecular aspects related to the h-proacrosin/acrosin system, will be presented. Data has been compiled from studies performed by our research team, as well as contributions made by numerous groups from the clinical and basic field of reproductive biology.

MOLECULAR CLONING OF THE SEQUENCE ENCODING H-PROACROSIN

The cDNA encoding h-proacrosin (Baba *et al.*, 1989*a*; Adham *et al.*, 1990), as well as the human genomic sequence (Keime *et al.*, 1990; Vazquez-Levin *et al.*, 1992), have been previously reported. The h-proacrosin structural gene pattern (exon/intron) is very similar to that characterized in other members of the serine proteinases family: it is organized into five exons (exon 1: 77 bp from the ATG starting codon, exon 2: 204 bp, exon 3: 284 bp, exon 4: 146 bp, and exon 5: 555 bp), which are arranged in two clusters, separated by a long intron (Keime *et al.*, 1990). The amino acids of the acrosin active site are localized on exon 2 (His69), 3 (Asp123) and 5 (Ser221), and the substrate recognition site, Asp215, is located on exon 4 (Keime *et al.*, 1990; Vazquez-Levin *et al.*, 1992) (The nucleotide sequence reported for human proacrosin at the EMBL/GenBank can be found under the following accesion numbers: Y00970, Baba *et al.*, 1989; X17349, Keime *et al.*, 1990; M77378-M77381, Vazquez-Levin *et al.*, 1992.) In addition, the amino acids involved in proenzyme processing towards the activation to a mature enzyme (see below) are localized in exon 2 and 5. Moreover, exon 5 contains a Pro rich region, which is absent in other serine proteinases. The transcription initiation site was localized to the C residue at position –74, upstream from the translation initiation codon ATG (Keime *et al.*, 1990); sequence analysis of over 1 kbp nucleotides from the 5′UTR showed an absence

of an identifiable TATA and CCAAT boxes and the presence of a highly GC-rich region, as well as several regulatory elements identified in other testis specific genes (Keime *et al.*, 1990; Vazquez-Levin *et al.*, 1992; Vazquez-Levin *et al.*, 1996; Schulten *et al.*, 2001).

Although the h-pre-proacrosin gene was initially described as a single copy gene mapped to chromosome 22 (q13-qter) (Adham *et al.*, 1989), a report by Fan *et al.* (2002), identified the truncated copy of exons 4 and 5 of the h-proacrosin gene in chromosome 2, in a region called the 2qFUS region, which resulted from the fusion of duplications in the sub-telomeric regions of chromosomes 9p and 22q, now located in the 2q13-2q14.1 region.

EXPRESSION OF H-PROACROSIN DURING SPERMATOGENESIS

Expression of the pre-proacrosin gene is specific to male germ cells, finding an mRNA form of approximately 1.6 kbp in all of the species studied; however, evidence regarding the cell stage where transcription and translation occur, is still controversial. The human protein has been detected in pachytene spermatocytes (Escalier *et al.*, 1991), although regulation of mRNA transcription and translation in humans has not been reported. The expression of mouse acrosin mRNA and its functional association with polysomes was first detected in pachytene spermatocytes, and increased transcription and translation was found throughout spermiogenesis (Kashiwabara *et al.*, 1990). In the rat, transcription of the acrosin gene was found at day 19 of spermatogenesis, which does not contain haploid cells (Nayernia *et al.*, 1994), but proacrosin biosynthesis was first identified in early spermatids (Phi-van *et al.*, 1983); similarly, transgenic mice, carrying a construct with 2.3 kbp of the proacrosin 5′ flanking sequence and the CAT reporter, showed transcription of the CAT gene in pachytene spermatocytes, although the enzyme was first found in round spermatids (Nayernia *et al.*, 1992). Partial control of acrosin gene translation may result from the association of the DNA/RNA binding protein MSY2 to stored or translationally delayed acrosin transcripts (Yang *et al.*, 2005).

STRUCTURAL FEATURES OF H-PROACROSIN

The primary structure of h-proacrosin has been deduced from its cDNA sequence (Baba *et al.*, 1989*a*; Adham *et al.*, 1990). The proenzyme N-*terminus* is preceded by a highly hydrophobic, cleavable signal peptide of 19 residues. The protein sequence of proacrosin can be divided into three major domains: DI, which encodes the amino acids 1-23 of the light chain, DII or catalytic domain, which contains the residues of the heavy chain for the mature enzyme, and DIII or tail domain, which comprises the C-terminal region of the heavy chain (see figure 1*a*).

The amino acid sequence of h-proacrosin shows an overall high degree of similarity to those reported in other mammals: DI is highly conserved within all of the species (91-97% similarity), and the same is observed in DII (75-92%). In contrast, DIII shows a broad range of sequence conservation across the species (27% ascidian/mouse and 83% boar/human) and is not present in other members of the serine proteases superfamily. In most mammals, except the mouse and rat, this region is highly hydrophilic and unique because of its high Pro content; on the other hand, the ascidian proacrosin C-*terminus* is not Pro rich and has two CUB domains (Kodama *et al.*, 2001). Different functions have been proposed for the proenzyme DIII (Klemm *et al.*, 1991; Mori *et al.*, 1995; Kodama *et al.*, 2001; see below).

The molecular weight of h-proacrosin deduced from its amino acidic sequence is 43,860 Daltons, although a higher Mr (55-60 KDa)

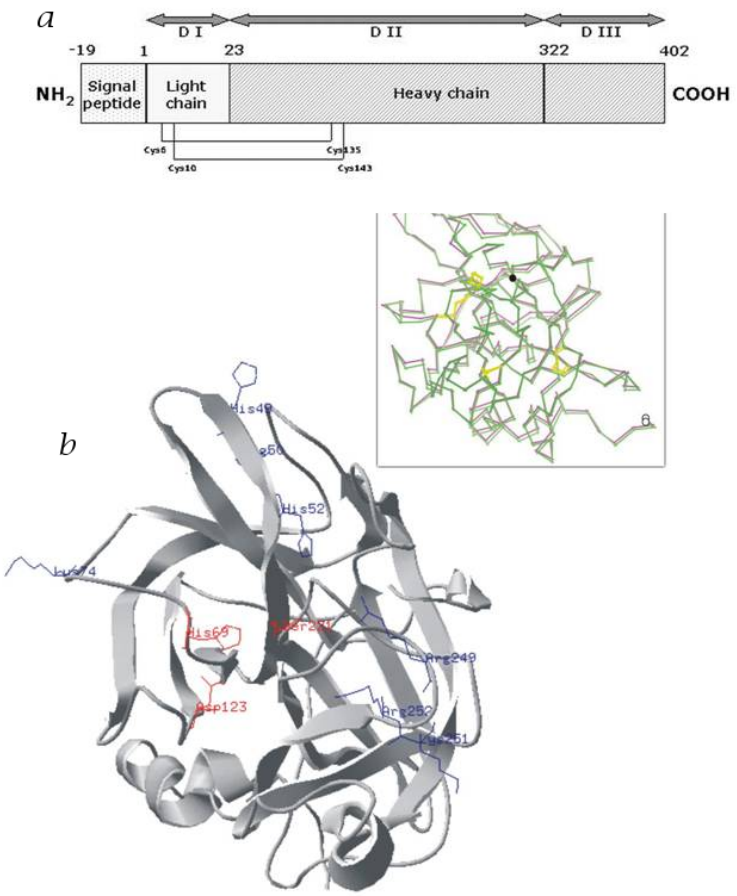

FIGURE 1. *a*) A schematic representation of the h-pre-proacrosin primary structure: signal peptide (19 amino acids), DI (light chain, residues 1-23), DI (heavy chain, residues 24-322) and DIII (heavy chain, residues 323-402). Cys residues involved in the inter-chain disulfide bonds are indicated. *b*) Inset: the super-imposed backbones of boar and human beta-acrosin structures. The 3D structure of h-acrosin (amino acid 24-282) was computationally predicted, using an automated protein modeling service (SWISS-MODEL 36.0002 and Swiss-Pdb Viewer v3.6b3 Expasy Proteomics Server, Swiss Institute of Bioinformatics, Switzerland) and by using the previously determined 3D structure of boar beta-acrosin as a model (Tranter *et al.*, 2000). The N- and C- ends are represented by black-filled and white-filled circles, respectively. Yellow lines represent intra-chain disulfide bridges. Main: A 3D-model of an h-acrosin heavy chain (amino acids 24-284), obtained as indicated in the Inset. Representation of the polypeptide chain (gray ribbons), where beta sheet (arrows) and alpha helix structures are shown. Red shaded Cα indicate protease active site residues: $_{64}$LTAAH$_{69}$, $_{118}$TEGND$_{123}$ and $_{217}$QGDS$_{221}$; blue shades Cα correspond to amino acids that may be involved in interaction with the ZP components: $_{47}$NSHR$_{50}$, $_{72}$VGKNN$_{76}$, and $_{249}$RAKR$_{251}$.

has been estimated by SDS-PAGE under reducing conditions; the discrepancy between both values has been attributed, at least in part, to the presence of sugar residues (see below). In this regard, the h-proacrosin sequence contains two N-linked glycosylation sites

(Asn-Xaa-Thr) in the light and heavy chains. In addition, several putative O-glycosylation sites are present, some of which are partially conserved in other species (data not shown). The ability of h-proacrosin to bind to Concanavalin A-Sepharose suggests its glycosylation (Schleuning *et al.*, 1976), although additional studies are needed to fully characterize its glycosylated status.

The light and heavy chains of proacrosin are connected through inter-chain disulphide bonds (see figure 1*a*), in addition to the presence of several intra-chain bonds. Biochemical identification of these residues was carried out on boar proacrosin (Töpfer-Petersen *et al.*, 1990); the arrangement of the disulfide bonds in h-proacrosin is probably the same as in boar, taking into account that the 12 Cys residues are highly conserved within the species (data not shown).

The 3D structure of h-proacrosin has not been determined yet; however, the crystal structures of mature beta-acrosin from both ram and boar have been solved in complex with p-aminobenzamidine (Tranter *et al.*, 2000). Using these 3D structures as templates (a boar-human sequence homology of DII: 74% and ram-human: 79%), our group obtained the predicted 3D structure of h-proacrosin DII (residues Ile 24-Thr282) (see figure 1*b*). In close correspondence to the findings obtained in other species (see inset), a high number of beta sheets and the alpha helix structures were found; moreover, the amino acids from the catalytic triad were localized in two beta sheet subdomains. The 3D-structure of DIII remains to be determined, and since no similarity to protein sequences deposited in the Proteins Data Bank (PDB) has yet been found, a model of the complete proacrosin structure cannot be obtained.

ACTIVATION OF H-PROACROSIN TO THE MATURE ACTIVE ENZYME BETA-ACROSIN

Acrosin is synthesized and stored mainly in its zymogen form, proacrosin (Siegel *et al.*, 1986; Hardy *et al.*, 1991), and is activated to the mature enzyme and released during acrosomal exocytosis (Tesarik *et al.*, 1990; Moos *et al.*, 1993). By immunocytochemical analysis, human sperm have shown the localization of proacrosin/acrosin to the acrosomal cap of ejaculated and capacitated cells, while the proteinase system has been associated with the equatorial segment or is absent after the sperm have undergone the acrosome reaction (Zahn *et al.*, 2002). Studies done on guinea pig sperm have found that proacrosin/acrosin is located in the acrosomal matrix (Noland *et al.*, 1989), as well as on the outer and inner acrosomal membrane (Urch, 1991); with regard to the human proenzyme system, even though it behaves as a membrane-associated protein, sequence analysis has not revealed any motifs that would indicate its association with sperm membranes (Zahn *et al.*, 2002).

The activation of h-proacrosin (55-60 KDa) to the mature active enzyme, beta-acrosin (34 KDa), can be achieved by raising the pH and involves processing of both the N- and C-terminal protein regions, with the generation of several enzymatically active intermediates (52, 43, 22-24, 16 KDa). Protein activation patterns obtained from purified proacrosin (Siegel *et al.*, 1986) or from whole human sperm extracts (Zahn *et al.*, 2002) have been reported, showing similarities to those described for the boar enzyme (Baba *et al.*, 1989*b*). Proacrosin activation can be modulated by synthetic compounds (Zahler and Polakoski, 1977), including sulphated polymers and phospholipids (Parrish *et al.*, 1978), DNA (Eberspaecher *et al.*, 1991), the acrosomal protein sp32 (Baba *et al.*, 1994*a*), and by natural inhibitors present in seminal plasma (Lee and Wei, 1994; Elisen *et al.*, 1998); with

regard to the latter, our group recently completed a study that described the inhibitory effect of caltrin (calcium trypsin inhibitor), a proteinase inhibitor purified from rat seminal vesicles, upon h-proacrosin activation in whole sperm extracts (Biancotti *et al.*, manuscript under consideration).

In addition to the compounds mentioned above, ZP glycoproteins have been found to accelerate boar proacrosin activation (Töpfer-Petersen and Cechova, 1990), but they had no effect upon the activation of trypsinogen (Eberspaecher *et al.*, 1991). It is possible that, since proacrosin and other zymogens from the trypsin family mainly differ on their protein C-*termini* (DIII in proacrosin), this specific domain could be the target of ZP regulation of proacrosin activation. In this regard, the studies conducted on boar proacrosin first suggested that ZP binding sites would be present in the C-terminal region of proacrosin and would be lost during its activation (Mori *et al.*, 1995); in agreement with these findings, our group demonstrated the ability of hZPA to bind to this protein region in h-proacrosin (see below).

The regulation of proacrosin activation may also involve phosphorylation of Ser/Thr residues, a post-translational modification that has been shown to alter protein stability to intracellular proteolysis and/or to affect enzyme catalytic efficiency (Reddy *et al.*, 1996). A molecular analysis done on h-proacrosin by our group revealed the presence of several putative PKC phosphorylation sites (Ser48, Thr287, Thr356). In agreement with these findings, the enhancement of the ZP-induced acrosome reaction of human sperm involves activation of PKC (Liu and Baker, 1997); and, the detection of PKC alpha and beta II isoforms was reported in the sperm equatorial segment (Rotem *et al.*, 1992). Protein Tyr sulfation may also regulate proacrosin activation/activity; this post-translational modification occurs in secretory, lysosomal, and plasma membrane proteins, as well as in proteins of the trans Golgi network (Hille and Huttner, 1990), and alters protein sensitivity to proteolytic cleavage (i.e., *in vitro* chymotryptic cleavage does not occur at the C-*terminus* of sulphated tyrosine residues; Huttner, 1987). In h-proacrosin, a putative Tyr388 sulfation site was identified in our analysis and located within a region that participate in the initial steps of proenzyme processing (Zahn *et al.*, 2002). In addition, this protein region may participate in the interaction with sp32; biochemical studies will help to confirm these *in silico* findings.

H-PROACROSIN/ACROSIN FUNCTION(S) DURING MAMMALIAN FERTILIZATION

Trypsin-like proteinase activity

Biochemical studies have characterized acrosin as a serine proteinase (Urch, 1991). A BLAST analysis carried out on the whole sequence confirmed acrosin homology with other proteases from the same family, i.e., chymotrypsin, trypsin, plasminogen, kallikrein, and thrombin, as well as testicular TESP-1 and TESP-2 proteases. The catalytic triad (His69, Asp123 and Ser221) was found in all species at conserved positions; moreover, the Asp215 residue was conserved within the species and appeared to act as a recognition residue for specific hydrolysis of the Arg/Lys bonds in peptides. As acrosin is a trypsin-like serine proteinase, several methods have been described to assess its enzymatic activity, including spectrophotometry and gelatinolysis; among the spectrophotometric methods, a simple clinical assay for h-acrosin enzymatic activity was initially described by Kennedy and collaborators (1989) and has been widely used by many groups (see below).

For a long time, acrosin was thought to aid in sperm penetration by the limited proteolysis of ZP glycoproteins, facilitating the

passage of motile spermatozoa through the egg's extracellular matrix (Urch, 1991). However, its role as ZP proteinase has been challenged by studies using an homologous recombination model of male mice, carrying a disruptive mutation in the proacrosin gene leading to the deletion of a portion of exon 2 and the entire exon 3 (Baba *et al.*, 1994*b*; Adham *et al.*, 1997). Acrosin-deficient mice were found to be fertile, although acrosin −/− sperm exhibited delayed penetration of the ZP and fertilization at the early stages of insemination, when compared to Acr +/+ and Acr +/− (Baba *et al.*, 1994*b*); moreover, the incubation of oocytes with equal quantities of wild-type and acrosin-deficient sperm rendered only fertilized eggs carrying the Acr +/+ sperm (Adham *et al.*, 1997). Further studies have suggested that these findings could result from a delay in the release of the acrosomal components (Yamagata *et al.*, 1998), indicating that acrosin must play a role in their coordinated release. In a more recent study, the incubation of Acr −/− sperm along with eggs, treated to harden the ZP, rendered a significantly lower number of penetrated eggs, as compared to wild-type sperm, suggesting that the cells lacking acrosin were at a disadvantage in their ability to penetrate the ZP (Nayernia *et al.*, 2002). These results could imply the potential contribution of alterations in proacrosin/acrosin functions in the sperm fertilizing ability, in cases of multi-factorial infertility. In agreement with this hypothesis, there have been several reports that described an association between a decrease in sperm acrosin amidase activity and the abnormal fertilization of human oocytes *in vitro* (see below). Thus, further work is needed to clarify the "ZP lysin" role of acrosin in species other than the mouse.

Alterations in H-acrosin activity in infertile patients, and its impact upon male fertility

As mentioned before, total sperm acrosin enzymatic activity can be determined in ejaculated sperm by means of a spectrophotometric assay using an exogenous substrate (Kennedy *et al.*, 1989). Utilizing this methodology, an association between acrosin enzymatic levels and male fertility has been reported, finding lower acrosin levels in infertile men than in fertile controls (Gerhard *et al.*, 1989; Koukoulis *et al.*, 1989; Francavilla *et al.*, 1992; El-Segini *et al.*, 2002).

Treatment of male infertility by standard *in vitro* fertilization and early embryonic transfer (IVF-ET) has allowed the identification of an association between abnormal levels of acrosin enzymatic activity and the reduced ability of sperm to fertilize human oocytes (Tummon *et al.*, 1991; Sharma *et al.*, 1993; De Jonge *et al.*, 1993). A biochemical and molecular evaluation of the proacrosin/acrosin system was performed by our group on a subset of male patients with no apparent cause of infertility, undergoing treatment with standard IVF-ET (Marí *et al.*, 2003). Out of over 200 cases, a total of 27 patients were included in the study, finding an average acrosin activity within normal values (53 μIU/million spermatozoa). However, 5 of the 27 cases (19%) had an abnormal acrosin activity, and four of them (80%) had an FR lower than 50%; similar observations have been recently reported (Chaundhury *et al.*, 2005). Additional evaluations made in our study revealed the lack of sperm in the egg cytoplasm, in cases with abnormal acrosin activity. In addition, there were no decondensed paternal DNA in the ooplasm from all of the cases studied but one, indicating that a diminished acrosin enzymatic activity was associated with a sperm penetration failure in most of the cases. When testing whether the decreased enzymatic activity was the result of an abnormal expression of the protein in the male gamete,

no major differences in the signal were observed by immunocytochemical analysis of the sperm proacrosin/acrosin system, suggesting that alterations in the proenzyme activation and/or in the enzyme function, rather than a lack of enzyme, was responsible for the diminished activity. Western immunoblotting of protein sperm extracts, incubated to undergo proacrosin activation, revealed a significant decrease in the conversion to the mature enzymatic active form, the 34 KDa beta acrosin, which could explain the reduced activity. Altogether, these studies first described the association between an abnormal acrosin enzymatic activity and a diminished *in vitro* fertilization rate in patients with unexplained infertility, and they found abnormalities in the proenzyme activation in these cases, which could justify the abnormal enzymatic activity (Marí *et al.*, 2003).

A previous study (Shimizu *et al.*, 1997) reported a lack of reactivity in the human enzyme towards a boar anti-acrosin antibody and a change in the enzyme's peptidic map in protein samples from patients with an abnormal acrosin activity, suggesting that alterations in the activity could have resulted from changes in the proteinase amino acid sequence. To further investigate the molecular basis of the alterations identified in our study, an evaluation of the genomic sequence encoding proacrosin was carried out using Single Strand Conformation Polymorphism (SSCP) and nucleotide sequence analyses of PCR products, amplified from the genomic DNA sequence around the acrosin catalytic triad. No changes were found either in the nucleotide sequence encoding the active site residues or in the sequence around the light/heavy chain cleavage site (Marí *et al.*, 2003), although in some cases, two polymorphic points (A777 → G; Tyr → Cys; A902 → G; Met → Val) were identified in exon 5 (Falcinelli and Vazquez-Levin, unpublished data). Further studies will help in understanding the molecular basis of the abnormalities in the acrosin protease activity of infertile men.

Binding to the ZP glycoproteins activity

Acrosome-reacted spermatozoa remain associated with the egg's extracellular matrix in a process called secondary binding. The molecular basis of this interaction is not fully known, at least in part, due to the difficulty in assessing such events; however, the identification of the sperm and ZP proteins involved in the early steps of gamete interaction, is of great relevance, especially considering the evidence which shows that a large proportion of the recorded fertilization failures is associated with sperm's inability to bind and penetrate the ZP (Liu and Baker, 2000).

The mammalian ZP is composed of three glycoproteins, initially characterized in the mouse and named ZP1, ZP2, and ZP3. The ZP glycoproteins result from the expression of ZPA, ZPB and ZPC genes (Rankin and Dean, 2000); in humans, each gene is translated to a glycoprotein with an estimated molecular mass of 90-110 (ZPA), 64-78 (ZPB) and 57-73 kDa (ZPC) (Bauskin *et al.*, 1999). By sequence analysis, it has been determined that human ZPA is the homologue of mouse ZP2 (mZP2, 57% amino acid similarity) and human ZPC of mouse ZP3 (mZP3, 67% similarity). The similarity between human ZPB and mouse ZP1 (mZP3) is only 33% (Spargo and Hope, 2003); in this regard, Hughes and Barratt (1999) first found a human genomic sequence, orthologous to the mouse ZP1 (67%) and paralogous to the human ZPB gene (Spargo and Hope, 2003). In addition, the human ZP1 protein was recently identified using tandem mass spectrometry, indicating that ZP is composed of at least four glycoproteins (Lefievre *et al.*, 2004). On the other hand, contrasting with the extensive information suggesting that oligosaccharides from mZP3 could participate in its lig-

and activity during primary sperm binding and in its ability to induce the acrosome reaction (Wassarman *et al.*, 2001), much less is known about the function of the other ZP components. Several reports have suggested that mZP2 could be involved in secondary sperm binding to the ZP (Bleil and Wassarman, 1986; Beil *et al.*, 1988; Keer *et al.*, 2002), while mZP1 could maintain the integrity of the ZP structure (Bleil and Wassarman, 1986). However, recent models have also proposed that mZP2 could regulate the supra-molecular structure of the ZP, required to support sperm binding (Rankin *et al.*, 2003).

Similar to the mouse, several pieces of evidence indicate that human ZP sugar moiety plays a major role in receptor sperm binding (Mori *et al.*, 1993; Benoff, 1997; Ozgur *et al.*, 1998), particularly fucose (Tesarik *et al.*, 1993; Lucas *et al.*, 1994) and mannose (Mori *et al.*, 1989; Benoff *et al.*, 1993; Chen *et al.*, 1995), as well as sulphated glycans (Oehninger *et al.*, 1990). Biochemical studies with native human ZP glycoproteins have been hampered by the scarcity of this biological material, although to overcome this limitation, several groups developed *in vitro* systems to produce recombinant human ZP proteins in mammalian cells (Van Duin *et al.*, 1994; Whitmarsh *et al.*, 1996; Harris *et al.*, 1999; Dong *et al.*, 2001). Further studies also demonstrated that recombinant ZPC was effective in inducing acrosomal exocytosis of human sperm (Van Duin *et al.*, 1994; Whitmarsh *et al.*, 1996; Dong *et al.*, 2001; Bray *et al.*, 2002).

With regard to the identification of sperm receptors for secondary binding to the ZP, studies conducted on animal models suggested the participation of proacrosin/acrosin. In those studies, it was found that specific antibodies for acrosin interfered with the sperms ability to interact with the ZP (Peknicova *et al.*, 2001), and biochemical studies showed the ability of animal proacrosin/acrosin to bind to homologous native ZP glycoproteins (Jansen *et al.*, 1995; Richardson *et al.*, 1996; Howes *et al.*, 2001; Kodama *et al.*, 2001). Finally, spermatozoa from acrosin-deficient mice were found to have decreased binding activity to mZP2, when compared to wild-type cells (Howes *et al.*, 2001). Our group first demonstrated the ability of h-proacrosin to recognize native human egg ZP glycoproteins (Furlong *et al.*, 2000). These studies were performed with whole solubilized ZP; however, the scarce material precluded the assessment of the participation of each ZP component in the interaction. In a later study, recombinant ZP glycoproteins were used with the aim of evaluating the interaction between each ZP component and h-proacrosin/acrosin; the results of those studies showed that the ZPA had the highest affinity to bind to h-proacrosin/acrosin. In addition, significantly lower affinity binding was obtained to ZPB and ZPC proteins (Furlong *et al.*, 2005*a* These results indicated that all of the ZP glycoproteins tested could be involved in the interaction of proacrosin/acrosin with the ZP, as had been suggested in other studies done on human (Koyama *et al.*, 1991; Rath *et al.*, 2002), porcine (Yurewicz *et al.*, 1998) and bovine models (Yonezawa *et al.*, 2001). In the study by Furlong *et al.* (2005*a*), competitive experiments indicated that h-acrosin binding to the ZPA could be mediated by ZP oligosaccharides; the ligand recognized by proacrosin/acrosin would have mannosyl and fucosyl residues and sulphated glycans, probably as part of a complex structure. Complementary experiments performed with BSA-mannose as a surrogate for the ZP glycoproteins, supported the evidence alleging the participation of mannose residues in the interaction (Furlong *et al.*, 2005*b*). In agreement with these findings, it has been reported that the mannose content of the human ZP is greater than in other mammals (Maymon *et al.*, 1994) and that mannose residues have been involved in the mechanism of secondary sperm binding/penetration of the human ZP (Chen *et*

al., 1995; Mori *et al.*, 1989). H-proacrosin binding sites were localized at both the N- and C-terminal regions of the protein and were mapped to residues 56-160 and 300-402; nevertheless, the binding of full-length proacrosin to ZP proteins was significantly higher than that observed in the N-terminal fragments. These data highlighted the relevance of the sequence between positions 300-402 (DIII) for interaction with the ZP components, since studies from the animal models tested truncated recombinant proteins (Furlong *et al.*, 2005*a*). The sequence KRLQQLIE, proposed to be involved in the binding of boar native proacrosin to the ZP glycoproteins (Urch and Patel, 1991; Mori *et al.*, 1995), was localized in this protein region (residues 367-374) and was highly conserved across the species (Zahn *et al.*, 2002); consequently, this motif could mediate, at least in part, h-proacrosin binding to the ZP glycoproteins through the interaction of its basic residues (arginine, lysine) with the ZP oligosaccharides.

In summary, our studies suggested that h-proacrosin/acrosin binding to the ZP was mainly a sugar-based interaction, residing primarily on the ZPA; the ligand recognized by h-proacrosin/acrosin had mannosyl and fucosyl residues and sulphated glycans. In addition, proacrosin/acrosin interaction with the ZP glycoproteins could be mediated by contact sites, present both at the proenzyme N- and C-protein ends.

Antibodies for H-proacrosin/acrosin: the incidence and impact upon protein functions and fertility. Clinical findings and development of an experimental model

The presence of anti-sperm antibodies (ASA) has been associated with 10-25% of the reported cases of infertility (Bronson, 1999). However, the pathophysiology of ASA-related infertility has not yet been established. In women consulting for infertility, ASA have been detected in serum, follicular fluid, and also in vaginal cervical secretions (Mazumdar and Levine, 1998). Some adverse effects have been attributed to sperm immobilization in the female tract, sperm agglutination and cytolysis, the impairment of sperm-egg interaction by interfering with the dispersion of the *cumulus* mass, in addition to sperm binding and ZP penetration, sperm fusion, and early embryonic development (Bronson *et al.*, 1982; Kamada *et al.*, 1985; Mahony *et al.*, 1991; Vazquez-Levin *et al.*, 1992, 1997; Bohring *et al.*, 2002; Taneichi *et al.*, 2002). However, the identity of the sperm antigens, recognized by these antibodies in most of the cases, is still unknown; in particular, the presence and incidence of anti-acrosin antibodies and their effect upon fertilization has not been reported. Several methods are routinely used in the assessment of ASA in cells and fluids; however, considering that the proacrosin/acrosin system is trapped in the acrosome until the acrosome reaction, the methods used worldwide (i.e., Immunobead Binding Test, IBT; MAR-Test) do not allow the identification of anti-acrosin antibodies, because they only detect surface anti-sperm antibodies.

A study designed by our group determined the incidence of anti-acrosin antibodies in sera from women consulting for infertility, and it was also able to determine the protein region(s) recognized by the antibodies and the effect of those antibodies on the proacrosin/acrosin function(s). Using an ELISA with recombinant proacrosin (Rec-40) and N-terminal fragments (Rec-30, Rec-20, Rec-10) as antigen (Furlong *et al.*, 2000), 6 of 34 sera (18%) were found to recognize proacrosin/acrosin proteins, but only a few of them were considered to be immunopositive towards the sperm head by the clinical IBT. In all sera carrying anti-acrosin antibodies, it was noted that the antibodies only recognized the proacrosin C-*terminus* (a region found to participate in proacrosin binding to the ZP glycoproteins) and inter-

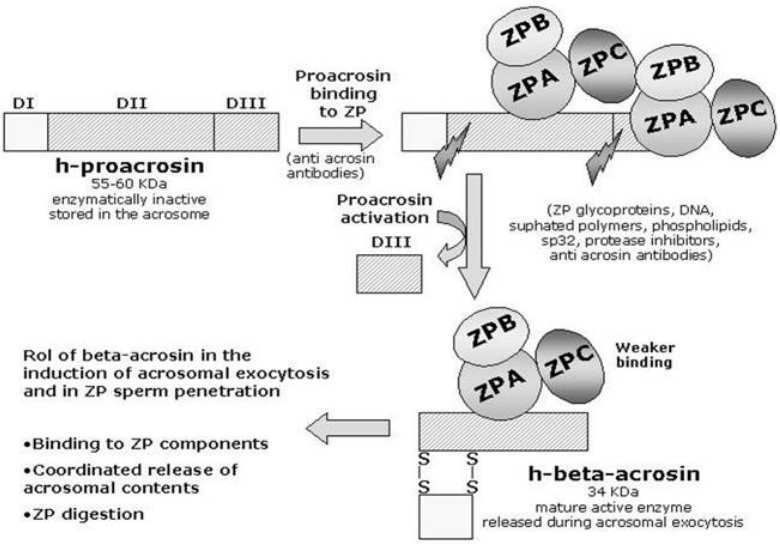

FIGURE 2. A putative model of h-proacrosin/acrosin interaction with the ZP glycoproteins, in association with proenzyme activation and proteinase activity: proacrosin would initiate contact with the ZP glycoproteins (mainly ZPA) during the early stages of acrosomal exocytosis through binding sites on domains DII and DIII of the proenzyme; the ZP-proacrosin interaction would accelerate proenzyme activation towards beta-acrosin (enzymatically active), and the contact sites present in DIII would be lost. Beta-acrosin could remain associated with the ZPA through contact points located on DII (weaker binding than that displayed by the proenzyme). Beta-acrosin would aid in the coordinated release of the acrosomal contents and the hydrolysis of the matrix. Proteinase inhibitors (i.e., caltrin, sp32, etc.), and in pathological conditions, anti-acrosin antibodies, would modulate proacrosin activation, affecting sperm detachment from the ZP, the process towards the mature enzyme, and consequently, sperm penetration.

ferred with h-proacrosin ability to interact with the recZPA and, in some cases, with its ability to undergo activation to the enzymatically active form. In a similar way, the monoclonal antibody AcrC5F10, that recognized the proacrosin C-*terminus* (Furlong, et al, 2000), inhibited h-proacrosin interaction with the ZPA and sperm ability to undergo the ZP-acrosome reaction. Altogether, the studies reported the incidence of anti-acrosin antibodies in sera from female patients undergoing infertility treatment, as well as its negative effect upon the proacrosin/acrosin functions and sperm performance. In general, these studies have proposed a potentially deleterious effect upon sperm-egg interaction (Veaute *et al.*, 2003*a*). In agreement with these observations, female mice carrying circulating anti-proacrosin/acrosin antibodies, generated in our group by genetic immunization with the sequence encoding h-proacrosin, showed significant levels of anti-acrosin antibodies and affected both the proacrosin/acrosin functions and the IVF rate, suggesting an inhibitory effect of the anti-acrosin antibodies upon sperm's fertilizing ability (Veaute *et al.*, 2001, 2003*b*).

A model for H-proacrosin/acrosin binding to the ZP, and proenzyme activation to mature active beta-acrosin

Based on the experimental evidence presented here, a simple, putative model of h-proacrosin/acrosin interaction with the ZP

glycoproteins, in association with proenzyme activation and proteinase activity, can be envisaged (see figure 2): proacrosin could initiate contact with the ZP glycoproteins during the early stages of acrosomal exocytosis, when acrosomal contents are exposed to the sperm surface, as also suggested for mouse sp56 (Kim and Gerton, 2003). Proacrosin interaction with the ZP components, mainly ZPA, would take place through binding sites, located on both domains DII and DIII of the proenzyme. ZP-proacrosin interaction would accelerate proenzyme activation towards the mature form, beta-acrosin (Töpfer-Petersen and Cechova, 1990), and the contact sites present in DIII would be lost. Beta-acrosin could remain associated with the ZPA through contact points located on DII, but this binding would be weaker than that displayed by the proenzyme. Enzymatically, active beta-acrosin would aid in the coordinated release of the acrosomal contents and in sperm progression through the ZP matrix, driven by sperm motility and the hydrolysis of the matrix. Several proteins, such as caltrin proteins and other proteinase inhibitors, acrosomal sp32 and, in pathological conditions, anti-acrosin antibodies, would modulate proacrosin activation, thus affecting sperm detachment from the ZP, the process towards the mature enzyme, and consequently, ZP sperm penetration.

Future studies will help us to understand the mechanisms that regulate h-proacrosin binding to the ZP glycoproteins, its activation during sperm acrosomal exocytosis, and its functions during ZP sperm penetration at a molecular level, and thus, we will be able to identify the alterations that negatively impact male fertility potential.

GRANT SUPPORT

Preparation of this article was supported by a grant from the Agencia Nacional de Promoción de la Ciencia y Tecnología (PICT 2000) to MHVL.

REFERENCES

Adham, I.; Klemm, U.; Maier, W.; Engel, W. (1990). «Molecular cloning of human preproacrosin cDNA». *Human Genet.*, 84: 125-128.

Adham, I. M.; Grzeschik, K. H.; Geurts Van Kessel, A. H., Engel, W. (1989). «The gene encoding the human preproacrosin (Acr) maps to the q13-qter region on chromosome 22». *Human Genet*., 84: 59-62.

Adham, I. M.; Nayernia, K.; Engel, W. (1997). «Spermatozoa lacking acrosin protein show delayed fertilization». *Mol. Reprod. Dev.*, 46: 370-376.

Baba, T.; Azuma, S.; Kashiwabara, S.; Toyoda, Y. (1994*a*). «Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization». *J. Biol. Chem*., 269: 31845-31849.

Baba, T.; Michikawa, Y.; Kawakura, K.; Arai, Y. (1989*a*). «Activation of boar proacrosin is effected by processing at both N- and C- terminal portions of the zymogen molecule». *FEBS Lett*., 244: 132-136.

Baba, T.; Niida, Y.; Michikawa, Y.; Kashiwabara, S.; Kodaira, K., Takenaka, M.; Kohno, N.; Gerton, G. L.; Arai, Y. (1994*b*). «An acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two proacrosins and an acrosin intermediate». *J. Biol. Chem*., 269: 10133-10140.

Baba, T.; Watanabe, K.; Kashiwabara, S.; Arai, Y. (1989*b*) «Primary structure of human proacrosin deduced from its cDNA sequence». *FEBS Lett.*, 244: 296-300.

Bauskin, A. R.; Franken, D.; Eberspaecher, U.; Donner, P. (1999). «Characterization of human zona pellucida glycoproteins». *Mol. Human Reprod*., 5: 534-540.

Benoff, S. (1997). «Carbohydrates and fertilization: an overview». *Mol. Human. Reprod.*, 3: 599-637.

Benoff, S.; Cooper, G. W.; Hurley, I.; Napolitano, B.; Rosenfeld, D. L.; Scholl, G. M.; Rosenfeld, D. L. (1993). «Human sperm fertilizing potential in vitro is correlated with differential expression of a head-specific mannose- ligand receptor». *Fertil. Steril*., 59: 854-862.

Bleil, J. D.; Greve, J. M.; Wassarman, P. M. (1988). «Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs». *Dev. Biol.*, 128: 376-386.

Bleil, J. D.; Wassarman, P. M. (1986). «Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm». *J. Cell Biol*., 102: 1363-1371.

Bohring, C.; Skrzypek, J.; Krause, W. (2001). «Influence of antisperm antibodies on the acrosome reaction as determined by flow cytometry». *Fertil. Steril*., 76: 275-80.

Bray, C.; Son, J. H.; Kumar, P.; Harris, J. D.; Meizel, S. (2002). «A role for the human sperm glycine receptor/Cl^-

channel in the acrosome reaction initiated by recombinant ZP3». *Biol. Reprod.*, 66: 91-97.

Bronson, R. A. (1999). «Antisperm antibodies: a critical evaluation and clinical guidelines». *J. Reprod. Immunol.*, 45: 159-183.

Bronson, R. A.; Cooper, G. W.; Rosenfeld, D. L. (1982). «Sperm-specific iso- and auto-antibodies inhibit binding of human sperm to the human zona pellucida». *Fertil. Steril.*, 38: 724-729.

Chaundhury, K.; Das, T.; Chakravarty, B.; Bhattacharyya, A. K. (2005). «Acrosin activity as a potential marker for sperm membrane characteristics in unexplained male infertility». *Fertil. Steril.*, 83: 104-109.

Chen, J-S.; Doncel, G. F.; Alvarez, C.; Acosta, A. A. (1995). «Expression of mannose-binding sites on human spermatozoa and their role in sperm-zona pellucida binding». *J. Androl.*, 16: 55-63.

De Jonge, C.; Tarchala, S. M.; Rawlins, R. G.; Binor, Z.; Radwanska, E. (1993). «Acrosin activity in human spermatozoa in relation to semen quality and in vitro fertilization». *Human Reprod.*, 8: 253-257.

Dong, K. W.; Chi, T. F.; Juan, Y. W.; Chen, C. W.; Lin, Z.; Xiang, X. Q.; Mahony, M.; Gibbons, W. E.; Oehninger, S. (2001). «Characterization of the biologic activities of a recombinant human zona pellucida protein 3 expressed in human ovarian teratocarcinoma (PA-1) cells». *Am. J. Obstet. Gynecol.*, 184: 835-843.

Duin, M. van; Polman, J. E. M.; Breet, I. T. M. de; Ginneken, K. van; Bunschoten, H.; Grootenhuis, A.; Brindle, J; Aitken, R. J. (1994). «Recombinant human zona pellucida protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion». *Biol. Reprod.*, 51: 607-617.

Eberspaecher, U.; Gerwien, J.; Habenicht, U.-F.; Schleuning, W.-D.; Donner, P. (1991). «Activation and subsequent degradation of proacrosin is mediated by zona pellucida glycoproteins, negatively charged polysaccharides, and DNA». *Mol. Reprod. Dev.*, 30: 164-170.

Elisen, M. G.; Van Kooij, R. J.; Nolte, M. A.; Marquart, J. A.; Lock, T. M.; Bouma, B. N.; Meijers, J. C. (1998). «Protein C inhibitor may modulate human sperm-oocyte interactions». *Biol. Reprod.*, 58: 670-677.

El-Segini, Y.; Schill, W. B.; Kohn, F. M.; Zeid, S. A.; Kamshushy, A. A.; Marzouk, S. (2002). «Assessment of sperm functions in infertile patiens with varicoceles». *Andrologia*, 34: 291-295.

Escalier, D.; Gallo, J. M.; Albert, M.; Meduri, G.; Bermudez, D.; David, G.; Schrevel, J. (1991). «Human acrosome biogenesis: inmunodetection of proacrosin in primary spermatocytes and of its partitioning pattern during meiosis». *Development*, 113: 779-788.

Fan, Y.; Newman, T.; Linaedopoulou, E.; Trask, B. J. (2002). «Gene content and function of the ancestral chromosome fusion site in human chromosome 2q13-2q14.1 and paralogous regions». *Genome Res.*, 12: 1663-1672.

Francavilla, F.; Palermo, G.; Gabriele, A.; Cordeschi, G., Poccia, G. (1992). «Sperm acrosin activity and fluorescence microscopic assessment of proacrosin/acrosin in ejaculates of infertile and fertile men». *Fertil. Steril.*, 57: 1311-1316.

Furlong, L. I.; Harris, J. D.; Vazquez-Levin, M. H. (2005*a*). «Binding of recombinant human proacrosin/acrosin to zona pellucida (ZP) glycoproteins. I. Studies with recombinant human ZPA, ZPB, and ZPC». *Fertil. Steril.*, 83: 1780-1790.

Furlong, L. I.; Hellman, U.; Krimer, A.; Tezón, J. G.; Charreau, E. H.; Vazquez-Levin, M. H. (2000). «Expression of human proacrosin in *Escherichia coli* and binding to zona pellucida». *Biol. Reprod.*, 62: 606-615.

Furlong, L. I.; Veaute, C.; Vazquez-Levin, M. H. (2005*b*). «Binding of recombinant human proacrosin/acrosin to zona pellucida (ZP) glycoproteins. II. Participation of mannose residues in the interaction». *Fertil. Steril.*, 83: 1791-1796.

Gerhard, I.; Fröhlich, E.; Eggert-Kruse, W.; Klinga, K.; Runnebaum, B. (1989). «Relationship of sperm acrosin activity to semen and clinical parametres in infertile patients». *Andrologia*, 21: 146-154.

Hardy, D. M.; Oda, M. N.; Friend, D. S.; Huang, T. T. F. (1991). «A mechanism for differential release of acrosomal enzymes during the acrosome reaction». *Biochem. J.*, 275: 759-766.

Harris, J. D.; Seid, C. A.; Fontenot, G. K.; Liu, H. F. (1999) «Expression and purification of recombinant human zona pellucida proteins». *Protein Expr. Purif.*, 16: 298-307.

Hille, A.; Huttner, W. B. (1990). «Occurrence of tyrosine sulfate in protein—a balance sheet. 2. Membrane proteins». *Eur. J. Biochem.*, 188: 587-596.

Howes, E.; Pascall, J. C., Engel, W.; Jones, R. (2001). «Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization». *J. Cell Sci.*, 114: 4127-4136.

Hughes, D. C.; Barratt, C. L. R. (1999). «Identification of the true human orthologue of the mouse ZP1 gene: evidence for a greater complexity in the mammalian zona pellucida?». *Biochim. Biophys. Acta*, 1447: 303-306.

Huttner, W. B. (1987). «Protein tyrosine sulfation». *TIBS*, 12: 361-363.

Jansen, S.; Quigley, M.; Reik, W.; Jones, R. (1995). «Analysis of polysulfate-binding domains in porcine proacrosin, a putative zona adhesion protein from mammalian spermatozoa». *Int. J. Dev. Biol.*, 39: 501-510.

Kamada, M.; Daitoh, T.; Hasebe, H.; Irahara, M.; Yamano, S.; Mori, T. (1985). «Blocking of human fertilization in vitro by sera with sperm-immobilizing antibodies». *Am. J. Obstet. Gynecol.*, 153: 328-31.

Kashiwabara, S.; Arai, Y.; Kodaira, K.; Baba, T. (1990). «Acrosin biosynthesis in meiotic and postmeiotic spermatogenic cell». *Biochem. Biophys. Res. Comm.*, 173: 240-245.

Keime, S.; Adham, I.; Engel, W. (1990). «Nucleotide sequence and exon-intron organization of the human proacrosin gene». *Eur. J. Biochem.*, 190: 195-200.

Kennedy, W. P.; Kamiski, J. M.; Ven, H. H. van der; Jeyendran, R. S.; Reid, D. S.; Blackwell, J.; Bielfield, P.; Zaneveld, L. J. D. (1989). «A simple, clinical assay to evaluate the acrosin activity of human spermatozoa». *J. Androl.*, 10: 221-231.

Kerr, C. L.; Hanna, W. F.; Shaper, J. H.; Wright, W. W. (2002). «Characterization of zona pellucida glycoprotein 3 (ZP3) and ZP2 binding sites on acrosome-intact mouse sperm». *Biol. Reprod.*, 66: 1585-1595.

Kim, K. S.; Gerton, G. L. (2003). «Differential release of soluble and matrix components: evidence for intermediate states of secretion during spontaneous acrosomal exocytosis in mouse sperm». *Dev. Biol.*, 264: 141-152.

Klemm, U.; Muller-Esterl, W.; Engel, W. (1991). «Acrosin, the peculiar sperm-specific serine protease». *Hum. Genet.*, 87: 635-641.

Kodama, E.; Baba, T.; Yokosawa, H.; Sawada, H. (2001). «cDNA cloning and functional analysis of ascidian sperm acrosin». *J. Biol. Chem.*, 276: 24594-600.

Koukoulis, G. N.; Vantman, D.; Dennison, L.; Banks, S. M.; Sherins, R. J. (1989). «Low acrosin activity in a subgroup of men with idiopathic infertility does not correlate with sperm density, percent motility, curvilinear velocity, or linearity». *Fertil. Steril.*, 52: 120-127.

Koyama, K.; Hasegawa, A.; Inoue, M.; Isojima, S. (1991). «Blocking of human sperm-zona interaction by monoclonal antibodies to a glycoprotein family (ZP4) of porcine zona pellucida». *Biol. Reprod.*, 45: 727-735.

Lee, S. L.; Wei, Y. H. (1994). «The involvement of extracellular proteinases and proteinase inhibitors in mammalian fertilization». *Biotechnol. Appl. Biochem.*, 19: 31-40.

Lefievre, L.; Conner, S. J.; Salpekar, A.; Olufowobi, O.; Ashton, P.; Pavlovic, B.; Lenton, W.; Afnan, M.; Brewis, I. A.; Monk, M.; Hughes, D. C.; Barratt, C. L. (2004). «Four zona pellucida glycoproteins are expressed in the human». *Human Reprod.*, 19: 1580-1586.

Liu, D. Y.; Baker, H. W. G. (1993). «Inhibition of acrosin activity with a trypsin inhibitor blocks human sperm penetration of the zona pellucida». *Biol. Reprod.*, 48: 340-348.

— (1997). «Protein kinase C plays an important role in the human zona pellucida-induced acrosome reaction». *Mol. Human Reprod.*, 3: 1037-1043.

— (2000). «Defective sperm-zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization». *Human Reprod.*, 15: 702-708.

Llanos, M.; Vigil, P.; Salgado, A. M.; Morales, P. (1993). «Inhibition of the acrosome reaction by trypsin inhibitors and prevention of penetration of spermatozoa through the human zona pellucida». *J. Reprod. Fertil.*, 97: 173-178.

Lucas, H.; Bercegeay, S.; Le Pendu, J.; Jean, M.; Mirallie, S.; Barriere, P. (1994). «A fucose-containing epitope potentially involved in gamete interaction on the human zona pellucida». *Human Reprod.*, 9: 1532-1538.

Mahony, M. C.; Blackmore, P. F.; Alexander, N. J.; Bronson, R. A. (1991). «Inhibition of human sperm zona pellucida tight binding in the presence of antisperm antibody positive polyclonal patient sera». *J. Reprod. Immunol.*, 19: 287-290.

Marí, S. I.; Rawe, V.; Biancotti, J. C.; Charreau, E.; Dain, L.; Vazquez-Levin, M. H. (2003). «Biochemical and molecular studies of the proacrosin/acrosin system in patiens with unexplained infertility». *Fertil. Steril.*, 79: 1676-1678.

Maymon, B. B. S.; Maymon, R.; Ben-Nun, I.; Ghetler, Y.; Shalgi, R.; Skutelsky, E. (1994). «Distribution of carbohydrates in the zona pellucida of human oocytes». *J. Reprod. Fertil.*, 102: 81-86.

Mazumdar, S.; Levine, A. S. (1998). «Antisperm antibodies: etiology, pathogenesis, diagnosis and treatment». *Fertil. Steril.*, 70: 799-810.

Moos, J.; Peknicova, J.; Tesarik, J. (1993). «Protein-protein interactions controlling acrosin release and solubilization during the boar sperm acrosome reaction». *Biol. Reprod.* 49: 408-415.

Mori, E.; Kashiwabara, S.; Baba, T.; Inagaki, Y.; Mori, T. (1995). «Amino acid sequences of porcine Sp38 and proacrosin required for binding to the zona pellucida». *Dev. Biol.*, 168: 575-583.

Mori, K.; Daitoh, T., Irahara, M.; Kamada, M.; Aono, T. (1989). «Significance of D-mannose as a sperm receptor site on the zona pellucida in human fertilization». *Am. J. Obstet. Gynecol.*, 161: 207-211.

Mori, K.; Daitoh, T.; Kamada, M.; Maeda, N.; Maegawa, M.; Hirano, K.; Irahara, M.; Aono, T. (1993). «Blocking of human fertilization by carbohydrates». *Hum. Reprod.*, 8: 1729-1732.

Nayernia, K.; Adham, I.; Shamsadin, R.; Muller, C.; Sancken, U.; Engel, W. (2002). «Proacrosin-deficient mice and zona pellucida modifications in an experimental model of multifactorial infertility». *Molec. Hum. Reprod.*, 8: 434-440.

Nayernia, K.; Burkhardt, E.; Beimesche, S.; Keime, S.; Engel, W. (1992). «Germ cell-specific expression of a proacrosin-Cat fusion gene in transgenic mouse testis». *Mol. Reprod. Dev.*, 31: 241-248.

Nayernia, K.; Reim, K.; Oberwinkler, H.; Engel, W. (1994). «Diploid expression and translational regulation of rat acrosin gene». *Biochem. Biophys. Res. Comm.*, 202: 88-93.

Noland, T. D.; Davis, L. S.; Olson, G. (1989). «Regulation of proacrosin conversion in isolated guinea pig sperm acrosomal apical segments». *J. Biol. Chem.*, 264: 13586-13590.

Oehninger, S.; Acosta, A.; Hodgen, G. D. (1990). «Antagonistic and agonistic properties of saccharide moieties in the hemizona assay». *Fertil. Steril.*, 53: 143-149.

Ozgur, K.; Patankar, M. S.; Oehninger, S.; Clark, G. F. (1998). «Direct evidence for the involvement of car-

bohydrate sequences in human sperm-zona pellucida binding». *Mol. Human Reprod*., 4: 318-324.

Parrish, R. F.; Straus, J. W.; Polakoski, K. L.; Dombrose, F. A. (1978). «Phospholipid vesicle stimulation of proacrosin activation». *Proc. Nat. Acad. Sci. USA*, 75: 149-152.

Peknicova, J.; Capkova, J.; Geussova, G.; Ivanova, M.; Mollova, M. (2001). «Monoclonal antibodies to intra-acrosomal proteins inhibit gamete binding in vitro». *Theriogenology*, 56: 211-223.

Phi-Van, L.; Muller-Esterl, W.; Florke, S.; Schmid, M.; Engel, W. (1983). «Proacrosin and the differentiation of the spermatozoa». *Biol. Reprod*., 29: 479-486.

Rankin, T.; Dean J. (2000) «The zona pellucida: using molecular genetics to study the mammalian egg coat». *Reviews of Reprod*., 3: 114-121.

Rankin, T. L.; Coleman, J. S.; Epifano, O.; Hoodbhoy, T.; Turner, S. G.; Castle, P. E.; Lee, E.; Gore-Langton, R.; Dean, J. (2003). «Fertility and taxon-specific sperm binding persist after replacement of mouse sperm receptors with human homologs». *Dev. Cell*, 5: 33-43.

Rath, A.; Choudhury, S.; Hasegawa, A.; Koyama, K.; Gupta, S. K. (2002). «Antibodies generated in response to plasmid DNA encoding zona pellucida glycoprotein-B inhibit in vitro human sperm-egg binding». *Mol. Reprod. Dev*., 62: 525-533.

Reddy, S. G.; McIlheran, S. M.; Cochran, B. J.; Worth, L. L.; Bishop, L. A.; Brown, P. J.; Knutson, V. P.; Haddox, M. K. (1996). «Multisite phosphorylation of ornithine decarboxylase in transformed macrophages results in increased intracellular enzyme stability and catalytic efficiency». *J. Biol. Chem*. 271: 24945-24953.

Richardson, R. T.; O'Rand, M. G. (1996). «Site-directed mutagenesis of rabbit proacrosin. Identification of residues involved in zona pellucida binding». *J. Biol. Chem*., 271: 24069-24074.

Rotem, R.; Paz, G. F.; Homonnai, Z. T.; Kalina, M.; Lax, J.; Breibart, H.; Naor, Z. (1992). «Ca^{2+}-independent induction of acrosome reaction by protein kinase C in human sperm». *Endocrinology*, 131: 2235-2243.

Schleuning, W. D.; Hell, R.; Fritz, H. (1976). «Multiple forms of human acrosin: Isolation and properties». *Hoppe Seyler's. Physiol. Chem*., 357: 855-865.

Schulten, H. J.; Nayernia, K.; Reim, K.; Engel, W.; Burfeind, P. (2001). «Assessment of promoter elements of the germ cell-specific proacrosin gene». *J. Cell Biochem*., 83: 155-162.

Sharma, R.; Hogg, J.; Bromham, D. R. (1993). «Is spermatozoan acrosin a predictor of fertilization and embryo quality in the human?» *Fertil. Steril.,* 60: 881-887.

Shimizu, Y.; Kodama, H.; Fukuda, J.; Tanaka, T. (1997). «Evidence of proacrosin molecule abnormality as a possible cause of low acrosin activity and unexplained failure of fertilization *in vitro*». *J. Androl*., 18: 281-288.

Siegel, M. S.; Bechtold, D. S.; Kopta, C. I.; Polakoski, K. L. (1986). «The rapid purification and partial characterization of human sperm proacrosin using an automated fast protein liquid chromatography (FPLC) system». *Biochim. Biophys. Acta*, 883: 567-573.

Spargo, S. C.; Hope, R. M. (2003). «Evolution and nomenclature of the zona pellucida gene family». *Biol. Reprod*., 68: 358-362.

Taneichi, A.; Shibahara, H.; Hirano, Y.; Suzuki, T.; Obara, H.; Fujiwara, H.; Takamizawa, S.; Sato, I. (2002). «Sperm immobilizing antibodies in the sera of infertile women cause low fertilization rates and poor embryo quality in vitro». *Am. J. Reprod. Immunol*., 47: 46-51.

Tesarik, J.; Drahorad, J.; Testart, J.; Mendoza, C. (1990). «Acrosin activation follows its surface exposure and precedes membrane fusion in human sperm acrosome reaction». *Development*, 110: 391-400.

Tesarik, J.; Mendoza, C.; Ramirez, J. P.; Moos, J. (1993). «Solubilized human zona pellucida competes with a fucosylated neoglycoprotein for binding sites on the human sperm surface». *Fertil. Steril*., 60: 344-350.

Töpfer-Petersen, E.; Calvete, J.; Schafer, W.; Henschen, A. (1990). «Complete localization of the disulfide bridges and glycosylation sites in boar sperm acrosin». *FEBS Lett*., 275: 139-142.

Töpfer-Petersen, E.; Cechova, D. (1990). «Zona pellucida induces conversion of proacrosin to acrosin». *Intern. J. Androl*., 13: 190-196.

Tranter, R.; Read, J. A.; Jones, R.; Brady, R. L. (2000). «Effector sites in the three-dimensional structure of mammalian sperm beta-acrosin». *Structure Fold Des*., 8: 1179-1188.

Tummon, I. S.; Yuszpe, A. A.; Daniel, S. A.; Deutsch, A. (1991) «Total acrosin activity correlates with fertility potential after fertilization in vitro». *Fertil. Steril*., 56: 933-938.

Urch, U. A. (1991). «Biochemistry and function of acrosin». In: Wassarman, P. *Elements of mammalian fertilization*. Boca Raton, Florida, US: CRC Press, p. 233-248.

Urch, U. A.; Patel, H. (1991). «The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides». *Development*, 111: 1165-1172.

Vazquez-Levin, M. H.; Ghiringhelli, P. D.; Zahn, A.; Rivarola, V.; Charreau, E. H. (1996). «Identification and localization of molecular sequences associated with regulation of human acrosin expression during spermatogenenis». 52nd Annual Meeting ASRM, Boston, EUA.

Vazquez-Levin, M. H.; Guzman I.; Kaplan, P.; Grunfeld, L.; Garrisi, G.; Navot, D. (1991). «The effect of female antisperm antibodies upon fertilization, early embryonic development and pregnancy outcome». *Fertil. Steril*., 56: 84-88.

Vazquez-Levin, M. H.; Notrica, J. A.; Polak De Fried, E. (1997). «Male immunological infertility: sperm performance on in vitro fertilization». *Fertil. Steril.*, 68: 675-681.

Vazquez-Levin, M. H.; Reventos, J.; Gordon, J. (1992).

«Molecular cloning, sequencing and restriction mapping of the genomic sequence encoding human proacrosin». *Eur. J. Biochem*., 207: 23-26.

Veaute, C.; Fontana, V.; Choren M.; Vazquez-Levin, M.; Cameo, M. (2003*b*). «Inmunización génica con proacrosina: efectos sobre fertilidad y desarrollo embrionario temprano». [Abstract no. 116] XVIII Reunión Bienal de la Asociación Latinoamericana de Investigadores en Reproducción Humana (ALIRH) (Varadero, Cuba). *Rev. Cubana de Salud Pública,* 29 (suppl. 1).

Veaute, C.; Furlong, L. I.; Biancotti, J. C.; Vazquez-Levin, M. H. (2001) «Development of antibodies towards human acrosin by gene immunization». [Abstract no. 097] VII International Congress of Andrology (Montreal, Canada). *J. Androl.*, 22 (suppl.): 142.

Veaute, C.; Furlong, L. I.; Bronson, R.; Vazquez-Levin, M. H. (2003*a*). «Antibodies towards proacrosin/acrosin in infertile women». [Abstract no. O24]. 59th Annual Meeting ASRM. (San Antonio, TX, US). *Fertil. Steril.*, 80 (suppl. 3): S10.

Wassarman, P. M.; Jovine, L.; Litscher, E. S. (2001). «A profile of fertilization in mammals». *Nat. Cell Biol.*, 3: 59-64.

Whitmarsh, A. J.; Woolnough, M. J.; Moore, H. D. M.; Hornby, D. P.; Barratt, C. L. R. (1996). «Biological activity of recombinant human ZP3 produced in vitro: potential for a sperm function test». *Mol. Human Reprod.*, 2: 911-919.

Yamagata, K.; Murayama, K.; Okabe, M.; Toshimori, K.; Nakanishi, T.; Kashiwabara, S.; Baba, T. «Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction». *J. Biol. Chem*., 273: 10470-10474.

Yanagimachi, R. (1994). «Mammalian fertilization». In: Knobil, E.; Neill, J. D. *The physiology of Reproduction*. New York: Raven Press, p. 189-317.

Yang, J.; Medvedev, S.; Reddi, P. P.; Schultz, R. M.; Hecht, N. B. (2005). «The DNA/RNA-binding protein MSY2 marks specific transcripts for cytoplasmic storage in mouse male germ cells». *Proc. Natl. Acad. Sci. USA*, 102: 1513-1518.

Yonezawa, N.; Fukui, Y.; Kuno, M.; Shinohara, H.; Golan, A.; Mitsui, S.; Nakano, M. (2001). «Molecular cloning of bovine zona pellucida glycoproteins ZPA and ZPB and analysis for sperm-binding component of the zona». *Eur. J. Biochem.*, 268: 3587-3594.

Yurewicz, E. C.; Sacco, A. G.; Gupta, S. K.; Xu, N.; Gage, D. A. (1998) «Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles». *J. Biol. Chem*., 273: 7488-7494.

Zahler, W. L.; Polakoski, K. L. (1977). «Benzamidine as an inhibitor of proacrosin activation in bull sperm». *Biochim. Biophys. Acta*, 480: 461-468.

Zahn, A.; Furlong, L. I.; Biancotti, J. C.; Ghiringhelli, P. D.; Marin-Briggiler, C. I.; Vazquez-Levin, M. H. (2002). «Evaluation of the proacrosin/acrosin system and its mechanism of activation in human sperm extracts». *J. Reprod. Immunol*., 54: 43-63.