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INVOLVEMENT OF PROTEIN TYROSINE PHOSPHORYLATION OF HUMAN SPERM IN CAPACITATION/ACROSOME REACTION AND ZONA PELLUCIDA BINDING

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1. ABSTRACT

The aim of this article is to review the surface molecules that are involved in capacitation/acrosomal exocytosis and zona pellucida (ZP) binding in context of tyrosine phosphorylation leading to signal transduction in human sperm. During capacitation, at least 7 proteins (200, 112, 104, 48, 42, 31 and 25 kD) are phosphorylated as studied by the ^{32}P metabolic labeling assay, and 14 proteins (122, 105, 95, 89, 73, 62, 48, 46, 40, 33, 30, 28, 25 and 22 kD) are autophosphorylated as demonstrated in the *in vitro* kinase assay. Of the 7-14 proteins, two proteins of 95 and 51 kD molecular identities were phosphorylated at tyrosine residues. Treatment with T α1 enhanced and anti-FA-1 monoclonal antibody completely blocked phosphorylation of all the relevant proteins. Sperm proteins belonging to four molecular regions, namely 95 kD (double band), 63 kD (one band), 51 kD (one band) and 14-18 kD (three bands) were involved in ZP binding. Three of these, namely 95 kD, 51 kD and 14-18 kD proteins demonstrated the presence of tyrosine phosphorylation, and the 51 kD protein (that is FA-1 antigen) also showed autophosphorylating activity. These findings, along with the other available data, indicate a vital role of protein tyrosine phosphorylation in sperm capacitation, acrosomal exocytosis and zona pellucida binding in humans. Since tyrosine phosphorylation is a primary/even exclusive indication of signal transduction, it appears that a signal transduction pathway is involved in fertilizability of human sperm.

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2. INTRODUCTION

Protein phosphorylation, especially at the tyrosine residues, has been shown to have a definite role in the regulation of function of various receptors (1). It is the most prevalent form of post-translational modification in metazoan cell and along with allosteric modulation, is recognized as a universal mechanism for regulating function of proteins involved in many biological processes. Receptors for several growth factors are themselves tyrosine protein kinases that are activated by ligand binding (1). Protein tyrosine kinases regulate cell proliferation and differentiation, and tyrosine phosphorylation may be the primary, or even the exclusive, indication of signal transduction. Receptor tyrosine kinases participate in transmembrane signaling, whereas the intracellular tyrosine kinases take part in signal transduction within the cell including signal to nucleus (2).

All the receptor tyrosine kinases possess a large glycosylated extracellular ligand binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains tyrosine kinase catalytic domain (1-3). The tyrosine kinase catalytic domain is the most conserved portion and among other highly conserved sequences of unknown function, it contains a consensus sequence, GlyXGlyXXGlyX Lys, that functions as a part of the binding site for ATP (1, 2). The phosphate group can be incorporated into either the catalytic domain or the regulatory domain(s) of the

enzymes. Intra- or intermolecular autophosphorylation of site within the kinase holoenzyme structure is a common, and perhaps a universal theme of these proteins.

Fertilization is a complex process requiring the spermatozoon to undergo a cascade of events before it can fuse with the oocyte plasma membrane (4). Two crucial steps in the fertilization process that sperm undergo are: a) to attain the fertilizing capacity, the physiological process called capacitation, that is followed by acrosomal reaction (exocytosis), and, b) to attach to zona pellucida (ZP) of the ovum, that requires recognition and interaction between complementary molecules present on the spermatozoon and the ZP. The molecules and mechanisms involved in capacitation/acrosomal exocytosis, and ZP binding are not clearly understood (4). It needs to be elucidated whether there is a signal transduction pathway(s) involved in these crucial steps. The aims of this review article are: 1) to review the sperm surface molecules that are involved in these two processes namely capacitation/acrosomal exocytosis and ZP binding, and, 2) to examine evidence whether or not there is membrane tyrosine phosphorylation leading to signal transduction in the crucial steps of fertilization.

3. DISCUSSION

3.1. Membrane tyrosine phosphorylation during spontaneous capacitation/acrosome reaction

Since the first discovery by Austin and Chang in 1957, the sperm capacitation, although extensively explored, has not been clearly understood (4). Capacitation is a physiological process during which the spermatozoon acquires the ability to fertilize an ovum. Capacitation is followed by acrosome reaction, that presumably takes place on ZP surface and/or can also occur in medium devoid of ZP (spontaneous acrosome reaction). The molecules and mechanisms involved in sperm capacitation/acrosome reaction are not clearly understood and also the exogenous stimulus that triggers the capacitation is not delineated. Also, it is not yet known whether there is a signal transduction pathway involved in the process. Since the protein tyrosine phosphorylation is a prerequisite for a signal transduction pathway, our laboratory investigated the phosphorylation pattern of human sperm during capacitation/acrosome exocytosis and its modulation by two molecules namely thymosin α1 (Tα1) and anti-FA-1 monoclonal antibody (mab).

Tα1 is a synthetic 28 amino acid peptide (3.108 kD) of thymic origin and anti-FA-1 mab is an antibody against the well-characterized human sperm membrane glycoprotein of 51 kD, designated fertilization antigen-1 (FA-1) (5-8). Tα1 increases up to 2-6 fold (9) and anti-FA-1 mab completely blocks human sperm capacitation acrosome reaction (5-8). The study was conducted using *in vitro* ^{32}P metabolic labeling technique, *in vitro* kinase assay, Western blot procedure and immunofluorescence assay (10, 11). The tyrosine phosphorylation was determined using anti-phosphotyrosine monoclonal antibody (PTA) (PY20) that specifically reacts with phosphotyrosine and not with phosphoserine and phosphothreoine residues.

3.1.1 Effect of Tα1

In metabolic labeling experiments, ^{32}P was incorporated into at least 7 proteins (200, 112, 104, 48, 42, 31 and 25 kD) predominantly belonging to four molecular regions (190, 97, 43 and 29 kD) (10, 11). Treatment with Tα1 enhanced phosphorylation of all these proteins in a concentration-dependent manner (11). In *in vitro* kinase assay, 14 proteins (122, 105, 95, 89, 73, 62, 48,46 40, 33, 30, 28, 25 and 22 kD), belonging to similar four regions, were autophosphorylated during capacitation. Of the 7-14 proteins, two proteins, namely 95 and 51 kD, respectively, were phosphorylated at tyrosine residues. Treatment with Tα1 enhanced phosphorylation of all these proteins in a concentration-dependent manner. The exact mechanism involved in stimulation of phosphorylation is not clear at the present time, since receptor for Tα1 has not been delineated on sperm or in any other somatic/immune/non-immune cell/cancer cell line. It appears that sperm cell membrane has a specific receptor for Tα1 that after ligand binding subsequently phosphorylates other relevant membrane proteins through signal transduction pathway.

3.1.2 Effect of anti-FA-1 monoclonal antibody

Treatment with anti-FA-1 mAb blocked phosphorylation/ autophosphorylation of the relevant proteins of the four molecules regions, and also blocked tyrosine phosphorylation of 95 kD and 51 kD proteins during capacitation (10, 11). Anti-FA-1 mAb specifically binds only to a single protein band of 51 ± 2 kD (corresponding to dimeric form of FA-1 antigen) on immunoblot involving human sperm membrane-solubilized proteins (5-8). Besides blocking phosphorylation/autophosphorylation/tyrosine phosphorylation of 51 ± 2 kD protein, anti-FA-1 mAb also reduced/blocked phosphorylation of other proteins including tyrosine phosphorylation of 95 kD protein.

Immunofluorescence of fixed human sperm indicates that the capacitation and ZP exposure increases the degree of tyrosine phosphorylation per sperm, and the number of spermatozoa that are tyrosine phosphorylated (10). There is also a shift in the site of phosphotyrosine-specific fluorescence from the tail regions of non-capacitated sperm to the acrosomal region of capacitated/ZP-exposed sperm. These changes are enhanced by Tα1 and reduced/blocked by anti-FA-1 mab. Using other systems, there are reports indicating a similar shift in subcellular localization of various proteins after tyrosine phosphorylation (12). Since acrosomal/postacrosomal region of the spermatozoon is involved in interaction with ZP, the shift in

Table 1: Molecular Identities of Human Sperm Proteins that Bind Human Zona Pellucida

Molecular Identity*	Bands	Tyrosine Phosphorylation	Autophosphorylating Activity
95 kD	Double Band	Yes	No/Yes**
63 kD	One Band	No	No
51 kD (FA-1 Antigen)	One Band	Yes	Yes
14-18 kD	3-4 Bands	Yes	No

* In few samples, two additional bands of ~120 kD and 34 kD, respectively were also seen.
** In sperm of 8 fertile men, 3 were positive and remaining 5 of the autophosphorylating activity was not observed.

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phosphotyrosine-specific fluorescence site may have a physiological significance.

It has been proposed that phosphorylation plays a role in the regulation of the function of various potassium and calcium channels (13). Since Ca^{2+} is required for capacitation/acrosome reaction of human sperm, the tyrosine phosphorylation may regulate the fertilizability through modulation of Ca^{2+} (and/or possibly other ions) influx (4). Some of the sperm surface proteins that are tyrosine phosphorylated during capacitation/acrosome reaction are also involved in ZP binding, and serve as substrates for tyrosine kinase activity.

3.2. Membrane tyrosine phosphorylation involved in zona pellucida binding

The glycoprotein composition the ZP of several mammalian species has been relatively well elucidated (4,7). However, the molecular identities and biochemical characteristics including tyrosine phosphorylation activity of the sperm surface molecules that are involved in ZP binding in humans are not yet defined (7). In contrast to mouse sperm that presumably undergo acrosome reaction on the ZP surface, the sperm of other mammalian species including human sperm can be induced to undergo acrosome reaction in response to various stimuli including ZP (4). We conducted several studies to investigate: 1) the molecular identities of various sperm and ZP proteins that are involved in binding, and, 2) whether these proteins that are involved in sperm-ZP binding have phosphotyrosine residues and/or tyrosine kinase activity (14).

3.2.1 Molecular identities and tyrosine phosphorylation of sperm proteins

The sperm proteins that reacted with ZP proteins were a 95 kD (double band), 63 kD (one band), 51 kD (one band) and 14-18 kD (three bands) with the 63 kD and 51 kD proteins being the most prominent proteins (14). Another 34 kD band was seen in some (two out of five) experiments. The ZP that reacted most strongly with the sperm proteins had a molecular weight of 55 kD (ZP3). The 95 kD, 51 kD and 14-18 kD proteins, but not the 63 kD protein, demonstrated the presence of phosphotyrosine residues. The 51 kD protein also showed the autophosphorylating activity in the *in vitro* kinase assay. Interestingly, ZP proteins of 55 kD (ZP3) and 220 kD (ZP1/ZP2) that bind to sperm proteins also demonstrated autophosphorylating activity. These results are summarized in Table 1.

Tyrosine phosphorylation of sperm proteins that bind ZP seems to play a vital role in the sperm-ZP interaction/binding. Treatment with solubilized human ZP increased the tyrosine phosphorylation of the 95 kD sperm protein (10, 14). Also, treatment of human sperm with PTA that predominantly recognizes the two sperm proteins of 95 kD and 51 kD on the Western blots, involving membrane capacitation involving human sperm preparation also inhibited (completely blocked) sperm binding to the ZP in the hemizona assay (15). Treatment of human sperm with PTA also reduced sperm penetration in SPA (10), indicating an additional effect on capacitation/acrosome reaction. It would appear that binding between the sperm and ZP proteins is of enzyme-substrate type, involving hydrophobic and ionic interactions through o-phospho-L-tyrosine residues of the interacting epitope.

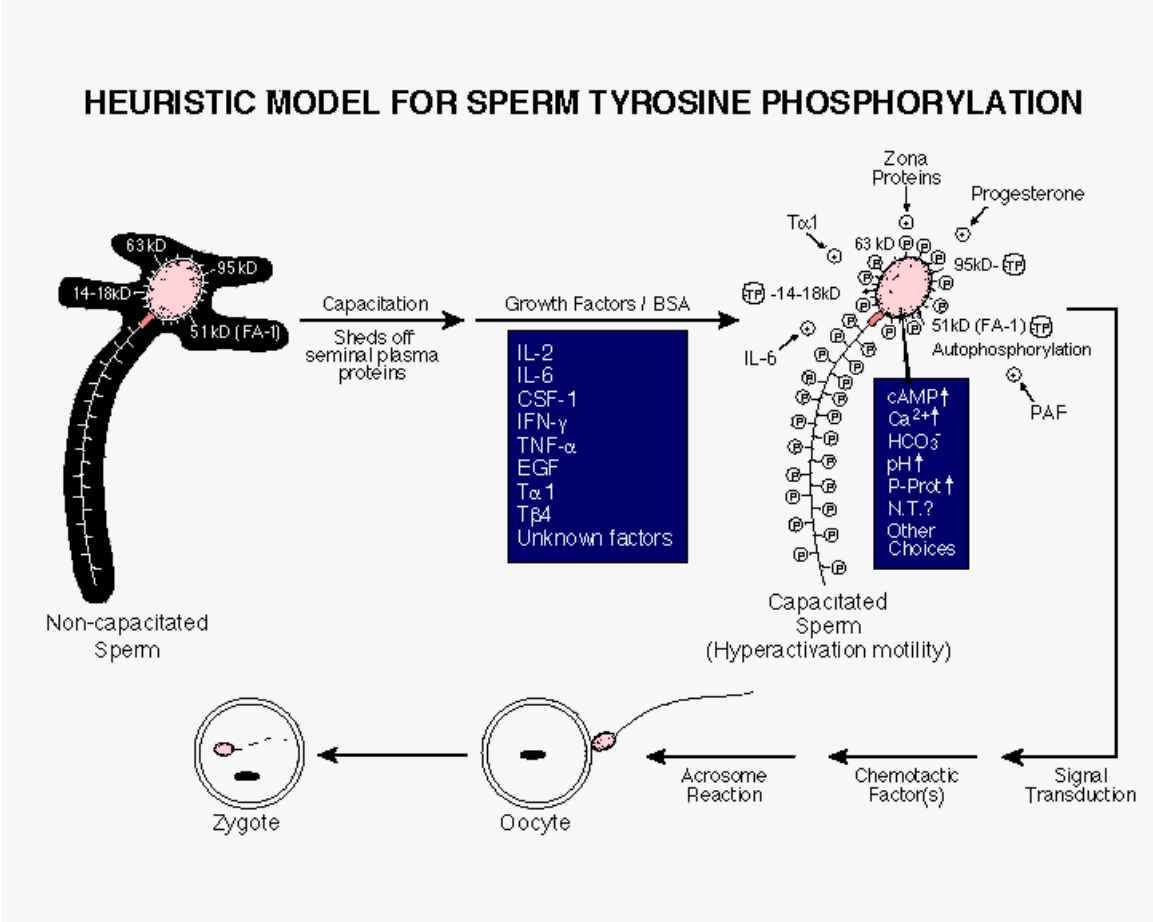

Figure 1. A heuristic model depicting the tyrosine phosphorylation of human sperm during capacitation/acrosome reaction and ZP binding. During capacitation, spermatozoa shed off seminal plasma that contains various growth factor/cytokines which include:interleukin-2 (IL-2) (31,32,34), interleukin-6 (IL-6) (35,36), interleukin-8 (IL-8) (33), colony stimulating factor-1 (CSF-1) (30), interferon-γ (IFN-γ) (35), tumor necrosis factor-α (TNF-α) (34), epidermal growth factor (EGF) (37), thymosin α1 (Tα1) (9), thymosin ß4 (Tß4) (9); and several unidentified factors (29).

Some of these cytokines are also present in cervical mucus of women (38, 39), through which the spermatozoa have to pass before interacting with the ovum after sexual intercourse. These molecules as well as albumin (HSA/BSA), that is used in the medium to capacitate sperm, have been known to have growth factor-like activity (41) These molecules after binding to sperm membrane could trigger the phosphorylation of the various membrane proteins/receptors. There are at least 7-14 proteins (prot), with approximate molecular weights of 190, 97, 43, and 29 kD, predominating in various domains of the sperm, that are phosphorylated (P) during capacitation. Among these, four proteins, namely the 95 kD (double band), 63 kD, 51 kD (FA-1 antigen) and 14-18 kD, respectively, that are involved in ZP binding, are also phosphorylated. Three (95 kD, FA-1 antigen and 14-18 kD proteins) of these proteins are phosphorylated at tyrosine residues (TP), and the FA-1 antigen also has autophosphorylating activity. Phosphorylation/tyrosine phosphorylation is enhanced by treatment with T α1 (11), progesterone (23), IL-6 (36), and platelet aggregation factor (PAF) (22).

Both acrosome-intact and acrosome-reacted sperm can bind to ZP of the human oocyte (4), through probable involvement of chemotactic factors attracting sperm to the oocyte. The spermatozoon undergoes acrosome reaction, penetrates the oocyte, and leads to formation of zygote that subsequently cleaves and forms a viable embryo/fetus.

3.2.2. FA-1 antigen is a sperm receptor for zona pellucida

Among the sperm proteins that bind ZP, there was a protein of 51 kD that had phosphotyrosine residues and autophosphorylating activity, and the binding of this protein with ZP was inhibited by the synthetic o-phospho-L-tyrosine. We examined (14) whether or not the 51 kD was the FA-1 antigen that our laboratory has been extensively investigating for several years (5-8). As shown by Western blotting, only the 51 kD among the four ZP binding sperm proteins reacted with by the anti-FA-1 mab in the Western blot

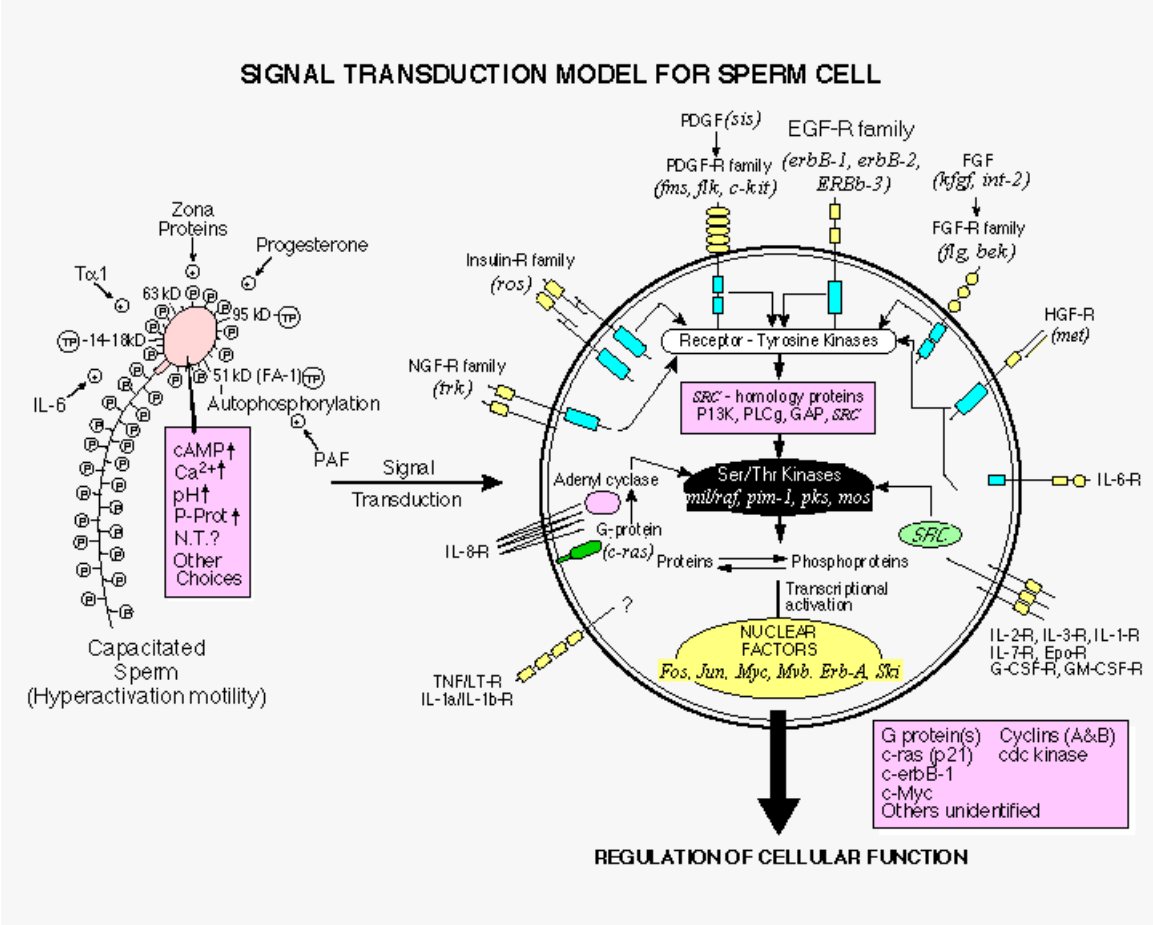

Figure 2 A schematic model for signal transduction in human sperm cell. In a somatic cell, shown in the right circle, the various steps of signal transduction are relatively more defined. Binding of a growth factor/cytokine to sperm leads to phosphorylation/tyrosine phosphorylation of the receptor that activates a cascade of intracellular events leading to elevation of cAMP/cGMP and Ca^{2+} levels. This is followed by induction of transcription and activation/posttranslational modifications of various proteins involved in regulation of cellular function (1-3, 29) The lines with arrows may involve several steps. Hatched rectangles and open circles in the extracellular region of the receptors indicate cysteine-rich and immunoglobulin-like domains, respectively. Filled rectangles in the intracellular region of the receptors show receptor-associated tyrosine kinase domains.

Capacitation involves phosphorylation (P)/ tyrosine phosphorylation (TP) of several proteins (prot), probably through binding of a cytokine/growth factor to its receptor. This can subsequently induce a rise in intracellular cAMP, Ca^{2+}, bicarbonate (HCO_3^-), pH and other yet unidentified changes (4, 29) Although nuclear transcription (NT) has not been clearly defined in human sperm cell, its potential role cannot be totally ruled out (42, 43).

Seminal plasma and cervical mucus have several cytokines/growth factors that through binding with the appropriate receptor can trigger the first event in signal transduction, that is phosphorylation/tyrosine phosphorylation. Several receptor tyrosine kinases and components of the signal transduction pathway including protein kinases (44), adenyl cyclase, components of phosphoinositol metabolism (44, 45), G proteins (45), c-ras (p21) (46), c-erbß-1 proto-oncogene product (40), c-Myc protein (47), cyclins (A and B) (48) and cdc^2 kinase (48) have been shown to be present in human sperm (lower right rectangle).

procedure. Also, the unlabeled FA-1 antigen, purified by immunoaffinity chromatography using anti-FA-1 mab, competed with the ^{125}I-labeled 51 kD protein for binding with the ZP in a concentration dependent manner. These findings confirmed that the 51 kD protein, was indeed the FA-1 antigen. FA-1 antigen as well as anti-FA-1 antibodies (both monoclonal and polyclonal) have been shown to inhibit sperm-ZP binding in a variety of species including humans (15-17).

3.2.3 The 95 kD and 51 kD (FA-1 antigen) sperm proteins

The sperm proteins with the molecular weight of 95 kD and 51 kD have recently drawn special attention. These two proteins seem to have a significant role in both capacitation/acrosome reaction and ZP binding. We have isolated a 95 kD protein, designated FA-2 antigen, from human sperm using a sperm-specific mAb that is involved in capacitation/acrosome reaction (18). A similar 95 kD protein(s) has been shown to be involved in sperm-ZP binding and capacitation/acrosome reaction in mouse (19-21) and capacitation/acrosome reaction in humans (22, 23). The 51 kD protein is FA-1 antigen, that has been purified from sperm/testes of a variety of species including man and mouse, shows increased tyrosine phosphorylation after treatment with homologous ZP, progesterone, platelet aggregation factor (PAF), Tα1 and Ca^{2+} (10, 11, 14, and unpublished data). Also, the 95 kD protein has tyrosine kinase activity and autophosphorylates in response to homologous ZP and after capacitation/acrosome reaction (19-23). It appears that these two sperm membrane proteins are evolutionarily conserved across species (mouse and man), and can be activated/tyrosine phosphorylated by various exogenous stimuli (including Tα1, PAF and progesterone). However, among the various sperm proteins that bind ZP, the FA-1 antigen (51 kD protein) has the strongest binding in human sperm (14).

4. CONCLUSIONS

The sperm proteins that bind to 55 kD ZP3, have molecular weights of 95 kD, 63 kD, 51 kD and 14-18 kD. Three of these, the 95 kD, 51 kD and 14-18 kD proteins, have phosphotyrosine residues and involve the o-phospho-L-tyrosine epitope in sperm-ZP interaction. The 51 kD protein (FA-1 antigen) also has autophosphorylating activity. Interestingly, the 55 kD ZP3 also demonstrates autophosphorylating activity in the *in vitro* kinase (14). At least in the *in vitro* system the forces involved in the sperm-ZP interaction are mainly of hydrophobic and ionic in nature. Many of these sperm proteins that participate in binding are also involved in capacitation/acrosome reaction.

Proteins with similar molecular weights have been isolated and characterized from sperm/testes in various mammalian species. The 95 ± 3 kD-proteins are involved in sperm capacitation, acrosome reaction and ZP binding in mouse (19-21) and man (18,22,23). A 63 ± 2 kD-protein has been characterized from rabbit testes (24), human sperm (25) and guinea pig testes (26), and the 51 kD protein is indeed FA-1 antigen (14). The 14-18 kD-antigens seem to correspond to proteins designated as RSA antigens (27). cDNAs encoding for some of these antigens have been cloned and sequenced. However, there is minimal sequence homology among these antigens and no common epitope has been delineated that is involved in sperm-ZP interaction/binding (28).

A vital role of protein tyrosine phosphorylation and tyrosine receptor kinases in sperm capacitation, acrosome reaction and sperm-ZP binding in humans is indicated. Since the tyrosine phosphorylation is the primary, even exclusive, indication of a signal transduction pathway, it appears that a signal transduction pathway is involved in sperm capacitation, acrosome reaction and ZP binding. However, the exact mechanism/cascade involved in signal transduction requires further study. Modulation of phosphorylation of all the proteins by a ligand (Tα1)/antibody (anti-FA-1 mab) specifically directed against a single membrane protein (FA-1 antigen), suggests a cross-talk among these molecules. The ligand/molecule(s) that triggers the tyrosine phosphorylation, leading to capacitation and acrosome reaction is not yet identified. However, various cytokines/growth factors are present in seminal plasma (9, 29-37) and genital tract secretions of women (38,39). These proteins can potentially trigger the tyrosine phosphorylation. Presence of functional receptors (*e.g.*EGF receptor) for some of these ligands on human sperm membrane has been demonstrated (40). Figures 1 and 2 are heuristic models constructed based on these findings.

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