

Tyrosine Phosphorylation Events in Mouse Sperm Capacitation

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TYROSINE PHOSPHORYLATION EVENTS IN MOUSE SPERM CAPACITATION

A Dissertation Presented

by

ENID ARCELAY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Animal Biotechnology and Biomedical Sciences

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Approved as to style and content by:

Pablo E. Visconti, Chair

Rafael A. Fissore, Member

Sandra Peterson, Member

Samuel J. Black, Department Head Veterinary and Animal Science

DEDICATION

To my mother, Victoria,

for her unconditional help and to my sonshine Adam,

for making the New England winters fade away.

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I express my deepest gratitude to my advisor, Prof. Pablo E. Visconti, for giving me the opportunity to conduct this project in his laboratory. His mentorship has been instrumental in building up my scientific skills. I also wish to thank my committee members, Prof. Rafael A. Fissore and Prof. Sandra Peterson for their advice and guidance. I thank my friends and family for their immense support and encouragement.

ABSTRACT

TYROSINE PHOSPHORYLATION EVENTS IN MOUSE SPERM CAPACITATION

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ENID ARCELAY, B.Sc., UNIVERSITY OF PUERTO RICO M.Sc., UNIVERSITY OF PUERTO RICO Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST Directed by: Professor Pablo E. Visconti

TYROSINE PHOSPHORYLATION EVENTS IN MOUSE SPERM CAPACITATION

Mammalian sperm are not able to fertilize immediately upon ejaculation; they become fertilization-competent after undergoing changes in the female reproductive tract collectively termed capacitation. Although it has been established that capacitation is associated with an increase in tyrosine phosphorylation, little is known about the role of this event in sperm function. In this work we used a combination of two dimensional gel electrophoresis and mass spectrometry to identify proteins that undergo tyrosine phosphorylation during capacitation. Some of the identified proteins are the mouse orthologues of human sperm proteins known to undergo tyrosine phosphorylation. Among them we identified VDAC, tubulin, PDH E1 β chain, glutathione S-transferase, NADH dehydrogenase (ubiquinone) Fe-S protein 6, acrosin binding protein precursor (sp32), proteasome subunit alpha type 6b and cytochrome b-c1 complex. In addition to previously described proteins, we identified two testis-specific aldolases as substrates for tyrosine phosphorylation. Genomic and EST analyses suggest that these aldolases are retroposons expressed exclusively in the testis, as has been reported elsewhere. Because of the importance of glycolysis for sperm function, we hypothesize that tyrosine phosphorylation of these proteins can play a role in the regulation of glycolysis during capacitation. However, neither the Km nor the Vmax of aldolase changed as a function of capacitation when its enzymatic activity was assayed in vitro, suggesting other levels of regulation for aldolase function. Looking upstream the kinase cascade, the identity of the kinase (s) that brings about the phosphorylation of the tyrosine residues remains to be elucidated. It has been suggested that the non receptor tyrosine kinase Src family is involved in the capacitation associated phosphorylation cascade. Using an immunological approach we show that the only Src family member present in mouse sperm extract is Src. The capacitation associated tyrosine phosphorylation is greatly reduced in the presence of Src specific inhibitors (SU6656 and SKI606) in vivo. As a means of control for the activity of Src inhibitors in our system, parallel experiments assaying the activity of PKA both *in vivo* and *in vitro* were realized. Surprisingly, Src inhibitors down regulates the phosphorylation of serine/threonine residues that correlate on earlier events in the capacitation, as assayed by western blot with PKA substrates antibody. However, in vitro kinase activity of PKA showed no effect of Src inhibitors in the phosphorylation of the PKA specific substrate, kemptide.

TABLE OF CONTENTS

	P	'age
ACKN	OWLEDGMENTS	V
ABSTF	RACT	vi
LIST O	OF TABLES	X
LIST O	OF FIGURES	xi
CHAP	TER	
I.	MOUSE SPERM CAPACITATION SIGNALING	1
	Sperm Physiology	1
	Sperm Capacitation	2
	Phosphorylation of Tyrosine Residues Associated with Sperm Capacitation	3
II.	MATERIALS AND METHODS	8
	Materials	8
	Preparation of mouse sperm	9
	SDS-PAGE and Western blots	10
	Two-dimensional gel-electrophoresis	10
	Image analysis of 2D gels	11
	Multi tissue Western blot	12
	Indirect immunofluorescence localization	12
	Triton X-100 extraction	13
	Aldolase activity	14
	Immunoprecipitation Src kinase assay	15

	In vitro PKA activity assay	16
III.	IDENTIFICATION OF PROTEINS UNDERGOING TYROSINE	
	PHOSPHORYLATION UPON MOUSE SPERM CAPACITATION	17
	Introduction	17
	Results	19
	Characterization of Proteins that undergo tyrosine phosphorylation during capacitation	19
	Aldolase activity does not change during capacitation	26
	Discussion	29
IV.	SRC FAMILY INVOLVEMENT IN MOUSE SPERM CAPACITATION	35
	Introduction	35
	Src family kinases	35
	Results	37
	Src kinase family tyrosine inhibitors reduce the capacitation dependent tyrosine phosphorylation	37
	Presence of Src family kinases in mouse sperm	37
	Src activity in mouse sperm	40
	Specificity of Src inhibitors	41
	Discussion	43
V.	CONCLUSIONS AND FUTURE DIRECTIONS	47
	Conclusions	47
	Future Directions	49
BIBI	LIOGRAPHY	50

LIST OF TABLES

Table			Page

1. Proteins that undergo tyrosine phosphorylation upon mouse sperm capacitation...... 22

LIST OF FIGURES

Figure		Page
1.	Sperm sub cellular structures	2
2.	Structure of the flagellar axoneme	2
3.	Slow and fast events involved in sperm capacitation	4
4.	Analysis of the capacitation-associated increase in protein tyrosine phosphorylation by 2-D gels	20
5.	Validation of anti-aldolase, anti-PDHE1ß and anti-VDAC antibodies using mouse sperm extract.	22
6.	Antibodies against aldolase, pyruvate dehydrogenase E1ß chain and VDAC matched the tyrosine phosphorylated spots in capacitated mouse sperm	23
7.	Immunolocalization of phosphorylated protein targets in mouse sperm	25
8.	Solubility of tyrosine phosphorylation targets in Triton X 100 and Western blot analysis of mouse tissues using α -aldolase antibodies	26
9.	Aldolase activity does not change during capacitation	28
10.	Concentration dependent effects of Src family inhibitors on the appearance of tyrosine phosphorylated proteins in capacitated mouse sperm	
11.	Src kinase is present in mouse sperm extract	
12.	Src kinase activity in non capacitated and capacitated mouse sperm	40
13.	Effects of Src family inhibitors on the appearance of phosphorylation of PKA substrates in capacitated mouse sperm	42
14.	Effects of Src family inhibitors on PKA activity in vitro	43

CHAPTER I

MOUSE SPERM CAPACITATION SIGNALING

Sperm physiology

The term sperm is derived from the Greek word $(\sigma \pi \epsilon \rho \mu \alpha)$ sperma (meaning "seed") and refers to the male reproductive cells. Sperm are very specialized, terminally differentiated cells with the unique function of protecting and delivering the genetic material to the next generation. This unique role is supported by the sperm degree of compartmelization and unique cellular structures. Like its female counterpart, the oocyte, sperm cell cycle remains arrested until the male achieves puberty. At this point the systemic testosterone levels trigger the resumption of the sperm cell cycle and maturation.

During sperm differentiation, named spermatogenesis, round germ cells undergoes a dramatic transformation (Yanagimachi 1994). The mature sperm contains the head, middle piece and the flagellum. The head contains the nucleus with densely condensed chromatin and the acrosome, anterior to the head (Fig 1). The acrosome is a specialized organelle that contains digestive enzymes that upon contact with the oocyte are released to break down the protective zona pellucida of the ovum. Posterior to the head is the mid piece, during spermatogenesis mitochondria are fused forming the mitochondrial sheat.

The sperm flagellum is a substructure that endows the sperm with motility capability. The flagellar axoneme (Fig 2.) consists of 9 double sets of microtubules surrounding a central double set of microtubules (9x2+2) arrangement, characteristics of

eukaryotic flagella. Dynein arms are attached to the outer microtubule doubles, providing the motor force to propel the sperm.





Figure 2 Structure of the flagellar axoneme

Figure 1 Sperm sub cellular structures

Sperm Capacitation

Upon ejaculation mammalian sperm lack fertilizing ability. In order to be able to fertilize sperm need to undergo a maturational process inside the female reproductive tract. It involves a series of biochemical and biophysical events that were first described by two independent laboratories at the beginning of the 1950's (Austin 1951; Chang 1951). These changes occur both in the head (preparation for acrosome reaction) and in the tail (change in pattern of motility from progressive to hyperactivated).

The foundation of assisted reproductive technologies was established by the pioneering work of Yanagimachi and Chang. In these two articles, using hamster sperm,

they successfully performed the first documented mammalian sperm capacitation *in vitro*. Since then, the signaling pathways that trigger sperm capacitation had been extensively researched, and although much had been learned, the mechanisms are still far from fully elucidated (Salicioni et al. 2007).

Capacitation occurs in a time dependent manner (Yanagimachi 1994), while the sperm travels through the female reproductive tract. The time requirements for this process are species specific. Defined media containing energy substrates as pyruvate, lactate and glucose, a cholesterol acceptor, and an ionic environment mainly constituted by NaHCO₃, Ca^{2+} , K^+ and Na²⁺ have been successfully used to mimic capacitation *in vitro*. The ability of sperm to fertilize an egg is the initial definition and still the most important evidence of capacitation. However, capacitation has also been described using other functional assays and endpoints related to physiological changes in both sperm head and tail. These events include hyperpolarization, a tight regulation in intracellular ion concentrations and the promotion of tyrosine residues phosphorylation of a subset of proteins (Visconti et al. 2002). Moreover, the sperm plasma membrane of the capacitating sperm surface has an increased disordering of the fatty acid chains of the phospholipids and enhanced lateral fluidity probably due to lose of membrane cholesterol (Harrison 1996; Gadella and Harrison 2000; Harrison et al. 2000; Visconti et al. 2002).

Phosphorylation Of Tyrosine Residues Associated

With Sperm Capacitation

As sperm becomes terminally differentiated, it loses the translational machinery; therefore, regulation of protein function relies on cell signaling events. Among these, tyrosine phosphorylation is one of the most characterized post translational modifications. The events that bring about sperm capacitation can be subdivided temporally into slow and fast events (Salicioni et al. 2007; Visconti 2009) (Fig. 3). Despite of the difference in dynamics, both slow and fast events are mediated through the cyclic AMP dependent serine/threonine kinase PKA. Upon contact with an isotonic solution containing HCO_3^- and Ca^{2+} , which coordinate the stimulation of a atypical soluble adenylyl cyclase (SACY), the flagellar movement show vigorous activation. It is believed that the transport is mediated by a Na⁺/HCO₃⁻ co transporter (NBC) and a sperm specific calcium channel (CatSper).



Figure 3 Slow and fast events involved in sperm capacitation. Fast events are triggered upon contact with bicarbonate and calcium. Longer incubation in a capacitation conducive media elicits the fertilizing ability that is correlated with an increase of tyrosine phosphorylated proteins.

Until recently, however, the majority of the research in sperm capacitation has been focused on slow occurring events (Salicioni et al. 2007). These slow events give rise to the verifiable endpoints of capacitation status. These endpoints are: 1) increase of tyrosine phosphorylation, 2) hyperactivation of sperm motility and, 3) readiness to undergo the acrosome reaction.

The phosphorylation of tyrosine residues have been shown to correlate with the capacitated state in several mammalian species (Visconti et al. 1995; Leclerc et al. 1996; Galantino-Homer et al. 1997; Kulanand and Shivaji 2001). Since the fertilizing competence of sperm involves the activation of kinase cascades, the characterization of proteins undergoing phosphorylation upon capacitation would help to assess how these changes promote the acquisition of fertilizing capacity. Some of the protein that have been identified as differentially phosphorylated in tyrosine residues upon capacitation in mammals are: two members of the PKA anchoring proteins, AKAP 3 (Ficarro et al. 2003), AKAP 4 (Jha and Shivaji 2002), FSP95, a 95 kD fibrous sheat protein (Mandal et al. 1999), dihydrolipoamide dehydrogenase (DLD) (Mitra and Shivaji 2004), heat shock protein 90 (Ecroyd et al. 2003), valosin containing protein (p97) (Ficarro et al. 2003), a calcium binding tyrosine phosphorylated protein from the fibrous sheat (CABYR) (Naaby-Hansen et al. 2002), the mitochondrial phospholipid hydroperoxidase glutathione peroxidase (Nagdas et al. 2005) and pyruvate dehydrogenase E1 α (Kumar et al. 2008) and E1 β (Ficarro et al. 2003), among others.

Looking upstream the signaling cascade, it has been shown that the capacitation dependent tyrosine phosphorylation is triggered by an increase of cyclic AMP. The best characterized effector of cAMP in sperm is PKA. This have been demonstrated using specific inhibitors of PKA which block the increase in tyrosine phosphorylation (Visconti et al. 1995) and more recently using a genetic-chemical switch approach to study the

temporal regulation of PKA (Morgan et al. 2008). The kinase(s) that mediate this signaling pathway, phosphorylating tyrosine residues downstream of the serine/threonine kinase PKA, still remain obscure.

Tyrosine kinases mediate very diverse signaling pathways including growth, differentiation, metabolism, adhesion, motility, death. They can be divided in two classes: receptor and non receptor (or cytoplasmic). Cytoplasmic tyrosine kinases are subdivided into 10 families. The Src family of tyrosine kinases has been implied as modulator of events associated with sperm capacitation by work from several laboratories (Baker et al. 2006; Lalancette et al. 2006b; Lalancette et al. 2006a; Lawson et al. 2008; Mitchell et al. 2008).

Of viral nature, Src was discovered as the agent causative of fibro sarcoma in chicken by Peyton Rous in 1911. The cellular analog, c-Src was the first proto-oncogenic tyrosine kinase discovered by J. Michael Bishop and Harold E. Varmus (Bishop et al. 1978). Src family members share the same three domains, an N-terminal SH3 domain, a central SH2 domain and a tyrosine kinase domain, SH1. c-Src is phosphorylated in a tyrosine residue on the c-terminus, rendering the kinase inactive. This changes the conformation of the protein producing a binding site for the SH2 domain which, when bound, facilitates binding of the SH3 domain the linker between the SH2 and SH1 domains. This SH3 binding, misaligns the residues of the kinase domain, causing inactivation of the enzyme (Ingley 2008).

The Src family in mammals is constituted by Src, Hck, Lck, Fyn, Lyn, Lck, Blk, Fgr and Yes (Ingley 2008). Src, Fyn and Yes are ubiquitous. They can vary in the level of expression or a spliced variant can be expressed in a cell specific manner. Blk, Fgr, Hck. Lck and Lyn are primarily found in hematopoietic cells (Thomas and Brugge 1997).

It has been reported in human sperm capacitation c-yes (Leclerc and Goupil 2002) and Src (Lawson et al. 2008) are modulated by calcium. This group has also reported the presence of hck in bovine sperm (Lalancette et al. 2006b; Lalancette et al. 2006a; Bordeleau and Leclerc 2008). Varano et al (2008) reported that in humans Src protein trigger the acrosome reaction and the capacitation. Recently, Baker et al (2006) proposed Src as a downstream effector of PKA. According to their model, PKA directly phosphorylates Src in serine 17 residue, activating the tyrosine kinase activity. They also suggest that PKA phosphorylates Csk, a known inhibitor of Src kinase activity. This PKA phosphorylation of Csk would indirectly cause the activation of Src.

CHAPTER II

MATERIALS AND METHODS

Materials

The following antibodies were used: anti-phosphotyrosine antibody (clone 4G10; Upstate Biotechnology, Lake Placid, NY); anti-rabbit muscle aldolase (CHEMICON International); anti-pyruvate dehydrogenase E1 α -subunit (mouse IgG1, monoclonal 9H9); anti-pyruvate dehydrogenase E1β-subunit (mouse IgG1, monoclonal 17A5; Molecular Probes, Eugene, OR); anti-VDAC (PA1-954 rabbit polyclonal; Affinity Bio Reagents, Golden, CO); anti-Src (36D10; Cell Signaling Technology Inc, Danvers, MA); anti-Src (GD11; Upstate biotechnology, Lake Placid, NY); anti-β tubulin monoclonal antibody (Clone E7) developed by Chu and Klymkowsky (9) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institutes of Health, NICHD, and maintained by The University of Iowa Department of Biological Sciences, Iowa City, IA.; anti-fyn, anti-yes and anti- hck (BD Biosciences); anti-lck, (3A5), anti-lyn (H6), anti-blk (C-20) and anti-Fgr (D6) from Santa Cruz Biotechnology, Inc.; anti-phospho-PKA substrate (100G7) was purchased from Cell Signaling Technology Inc., (Danvers, MA). Horseradish peroxidase-conjugated antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA); horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare) and horseradish peroxidase-linked anti-goat antibody (Sigma-Aldrich, Saint Louis, MO). ECL and ECL plus chemiluminescence detection kits were from Amersham Biosciences (Uppsala, Sweden).

Protein G Sepharose was from GE Healthcare. PVDF membrane was from Millipore (Bedford, MA). Alexa 555-conjugated anti-rabbit antibody, Alexa 555-conjugated antimouse antibody, Texas Red-conjugated anti-goat antibody, Mitotracker Green and Alexa 488-labeled PNA were from Molecular Probes (Invitrogen). SU6656 was purchased from Calbiochem (La Jolla, CA). SKI606 was purchased from Husker Chemical Ltd. Cdc 2 was purchased from Upstate biotechnology (Lake Placid, NY). All other reagents were purchased from Sigma (St. Louis, MO).

Preparation Of Mouse Sperm

Caudal epididymal sperm were collected from CD1 retired breeder males (Charles River Laboratories, Wilmington, MA) sacrificed in accordance with IACUC guidelines. *Cauda* epididymis from each animal were placed in 1 ml of modified Krebs-Ringer medium (Whitten's-HEPES buffered medium) (WH) (Moore et al. 1994) containing 100 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 5.5 mM glucose, 1 mM pyruvic acid, 4.8 mM L(+) -lactic acid hemicalcium salt in 20 mM HEPES, pH 7.3. This medium, prepared in the absence of bovine serum albumin (BSA) and NaHCO3, does not support capacitation. Sperm released into the media during a 10 min time period were counted and collected by centrifugation at 800 x g for 10 min at room temperature. Sperm pellets were resuspended in WH medium (without BSA or NaHCO3) and then 1-2 x 106 sperm were incubated in 1 ml of the medium at 37°C for the indicated time periods. When the sperm were incubated under capacitating conditions, WH medium was supplemented with 5 mg/ml BSA plus 20 mM NaHCO3. In all cases, pH was maintained at 7.3. In some experiments, capacitating medium contained 2-hydroxypropyl-βcyclodextrin (2-OH-propyl-β-CD)(3mM) instead of BSA (Visconti et al. 1999), plus 20

mM NaHCO3, dbcAMP (1mM) and IBMX (100uM). To test the effect of SU6656 and SKI606 in capacitation, sperm were pre incubated with them for 30 min previous to the start of the capacitation. Sperm motility was checked in all the experiments and the percentage of motile sperm was over 80%.

Sds-Page And Western Blots

Phosphorylated proteins were analyzed in protein extracts from mouse sperm. Sperm pellets were washed in 1 ml of PBS, resuspended in Laemmli sample buffer (Laemmli 1970) without 2-mercaptoethanol and boiled for 5 min. After centrifugation, the supernatants were saved, and 2-mercaptoethanol was added to a final concentration of 5%. Samples were boiled for 5 min and subjected to SDS-PAGE using 8-10% mini-gels; protein extracts equivalent to 1-2 x 106 sperm were loaded per lane. Each gel contained dual-prestained molecular weight standard (Bio-Rad, Hercules, CA). Proteins were transferred to PVDF membranes and incubated in appropriate blocking solution for 1 h at the room temperature. Western blots were conducted as described and developed using ECL chemiluminescence reagents.

Two-Dimensional Gel-Electrophoresis

Two dimensional gel electrophoresis (2D-PAGE) was conducted as described (Jha *et al.*, 2006); briefly, after incubation in media that support or not capacitation, sperm were centrifuged and resuspended in a modified Celis extraction/rehydration buffer (5 mM urea, 2 mM thiourea, 2% CHAPS, 0.2% ampholytes pH 3-10, 50mM DTT, 0.0002% Bromophenol blue, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 30 mM β - glyceraldehyde, 2 mM EGTA and protease inhibitors), vortexed for 2 min and kept on ice for 30 min. After centrifugation at 12,000 x g for 5 min, extracted proteins

(equivalent to 16 x 10⁶ sperm per strip) were loaded passively onto IPG strips (pH 3-10) and incubated overnight at room temperature. Isoelectric focusing (IEF) was performed using Protean IEF Cell apparatus (BioRad, Richmond, CA) starting with 15 minutes at 250 V and rapid increase to 4000 V overnight to a final 20,000 Vh. After IEF, IPG strips were equilibrated in equilibration buffer (6M urea, 2% SDS, 0.05 M Tris/HCl, 2% DTT, 20% glycerol) at room temperature for 10 min, followed by incubation on a second equilibration buffer in which DTT was replaced with 2.5% iodoacetamide, for 10 min at room temperature. Second dimensional gel-electrophoresis was performed on 10% SDSPAGE gels. Proteins were transferred onto PVDF membranes and analyzed by Western blotting as described above or developed using silver stain as previously described (Ficarro et al. 2003).

Image Analysis Of 2 D Gels

Gel electrophoresis was performed concurrently to ensure equivalent electrophoretic conditions. Gels were stained with silver (Ficarro et al. 2003) or transferred to PVDF membrane and probed with α PY (clone 4G10, UBI). The silver stained gel and X-ray films (short and long exposure of ECL's) were scanned at 300 dpi using a desktop HP scanner. Digitized images were overlaid in Adobe Photoshop 7.0 using different % of transparency. Using known "landmarks", such as hexokinase and tubulins, the silver-image was aligned with the ECL-images. After marking all reactive spots on the ECL-image with arrows, the ECL image was hidden and the arrows identified corresponding silver stained spots. These spots were then cored from the silver stained gel and submitted for mass spectrometry analysis, performed at the Proteomic

Mass Spectrometry Facility, University of Massachusetts Medical School, Worcester, MA, USA.

Multi Tissue Western Blot

Tissues from CD1 male retired breeders, or from females, were collected and proteins were extracted using RIPA buffer (10 mM Tris pH 7.2, 150 mM NaCl, 0.1 % SDS, 1% Triton X-100, 1 % deoxycholate, 5 mM EDTA, protease and phosphatase inhibitors). Tissues were resuspended in the buffer, set on ice for 30 minutes and spun down for 20 minutes. Protein concentration was assayed using the BCA (bicinchoninic acid) kit from Pierce, using bovine serum albumin (BSA) as standards. The absorbance was measured at 562 nm using a DYNEX plate reader and DYNEX Revelation software 4.02 (DYNEX Technologies, Worthing, UK). In each lane 10 µg of total protein was loaded in an SDS-PAGE 10 % gel. Proteins were transferred onto PVDF membranes and Western blotting was performed using anti-aldolase antibody.

Indirect Immunofluorescence Localization

Sperm obtained by the swim-up method in WH medium (without BSA or NaHCO₃), were washed once, resuspended in PBS at a concentration of $1-2 \times 10^5$ sperm/ml and seeded on 8-well glass slides. After air-dried, sperm were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS (4 washes each for 5 min) and permeabilized with 0.5% Triton X-100 for 5 min. Following permeabilization, sperm were treated with 10% BSA in PBS for 1 h at room temperature, and then incubated either with the respective primary antibody (1:50-1:250) diluted in PBS containing 1% BSA, or with the same concentration of the

corresponding normal purified IgG; incubations were carried out at 4°C overnight. After incubation, sperm were washed thoroughly with PBS and incubated with the corresponding Alexa 555-conjugated secondary antibody (1:200) diluted in PBS containing 1% BSA for 1 h at room temperature; these solutions also contained Alexa 488-conjugated PNA (1:100) for staining acrosomes, and Mitotracker (1:1000) for detecting mitochondria. Incubation with the secondary antibody was followed by 4 washes in PBS, mounting using Slow-Fade Light reagents (Molecular Probes, Eugene, OR) and observation by epifluorescence microscopy using a Zeiss Axiophot microscope (magnification 60x) (Carl Zeiss, Inc. Thornwood, NY). DIC images were taken in parallel, and served as control for sperm morphology. Negative controls using secondary antibody alone were also used to check for antibody specificity (not shown).

Triton X-100 Extraction

For the separation of the Triton soluble and Triton insoluble (assumed to be the cytoskeletal) fractions, after incubation in media supportive or not of capacitation, mouse sperm samples were pelleted and resuspended in a Triton extraction buffer (1 % Triton X-100, 1 mM EGTA, 5 mM EDTA, protease and phosphatase inhibitors in PBS) and kept on ice for 30 min. After the incubation samples were spun at 10,000g for 2 min and supernatant (soluble fraction) was kept on ice. The remaining pellet (non soluble fraction) was resuspended in Triton extraction buffer and vortexed briefly. Sample buffer was added and samples were boiled for 5 min. After centrifugation, the supernatants were saved, and 2-mercaptoethanol was added to a final concentration of 5%. Samples were boiled for 5 min and subjected to SDS-PAGE using 10% mini-gels as described in the

SDS-PAGE and Western blot methodology and developed with the antibodies indicated in the respective figure. Each lane contains equivalent sperm amounts (~ 2×10^6 cells).

Aldolase Activity

The catalytic activity of aldolase towards the substrate fructose-1,6- biphosphate (FBP) was determined fluorometrically by measuring the decrease in β -NADH fluorescence over time in а coupled assay using αglycerol-phosphate dehydrogenase/triose phosphate isomerase (aGDH/TPI) and FBP as substrates (Racker 1947; Bergmeyer 1974). All solutions were made up in 100 mM Tris-HCl buffer, pH 7.4. The assay media contained the following: 30 mM FBP, 130 nM of β -NADH and 2.5 UI of α -Glycerophosphate Dehydrogenase/Triosephosphate Isomerase Enzyme Solution (Sigma G-6755). To set the parameters for fluorescent acquisition, the emission spectra for 1 mM β -NAD (Sigma N-1511) and β - NADH (Sigma N-8129) were obtained in a 300 to 580 nm wavelength range, exciting from 300 to 412 nm wavelengths. A Jobin Yvon Horiba Fluorolog-3 fluorometer, equipped with DataMax V2.2 software was utilized for acquisition. From the resulting spectra the 367/460 data nm wavelength excitation/emission pair for β -NADH was chosen. Using this combination of wavelengths, β -NAD has no fluorescent emission (see Fig. 6 A in Results). To determine the activity of sperm aldolase, cauda epididymal sperm was obtained as previously described and placed in either capacitating or non capacitating conditions for 1 hour. After incubation sperm aldolase Triton extraction was carried out. Briefly, sperm were sequentially centrifuged twice at 10,000 g for 30 second and supernatants were discarded. The pellet was resuspended in 100 mM Tris-HCl, pH 7.4, 0.5 % Triton, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 30 mM β -glyceraldehyde, 2 mM EGTA, protease

inhibitors and incubated on ice for 30 minutes. This extract was considered to be total sperm extracts. To obtain the triton soluble and particulate fractions, the sample was spun at 10,000g for 2 minutes. The remaining pellet was resuspended in extraction buffer and vortexed briefly. All the fractions were assayed; for each measurement, data was transformed from count per second (cps) to velocity, expressed in nM/sec, considering that the maximum fluorescence was obtained at 130 nM of NADH (initial concentration) and the minimal fluorescence obtainable coincided with 0 nM of β -NADH. From the graph of concentration versus time, the initial velocity of the enzymatic reaction (V0) was calculated as the slope of the lineal phase of the curve, using an in-lab, custom-written program in Igor software (Wavemetrics, Inc. Lake Oswego, OR), and expressed as nM β -NADH consumed per second (nM/sec). To determine kinetic parameters (Km and Vmax) Prism4 software (GraphPad software, Inc) was used adjusting the V0 *vs.* substrate concentration curve to the Michaelis-Menten equation: Y=Vmax X/ (Km+X), where X is the substrate concentration (mM) and Y is the enzyme velocity (mM/sec).

Immunoprecipitation Src Kinase Assay

The activity of the Src tyrosine kinase was evaluated by immunoprecipitation of Src using the anti-Src antibody (4 μ g/ ~ 10 x 10⁶ cells) mouse monoclonal (clone GD11) and anti-tubulin (clone E7) as a negative control. After capacitation samples were centrifugated at 1700 x g for 1 min. The resulting pellet was dissolved in RIPA buffer (10 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.1 % SDS, 1%(v/v) Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate and protease inhibitors), kept on ice for 30 min and centrifuged at 2500 x g at 4° for 5 min. Supernatants were incubated with antibodies at 4°C for 2 hours with constant rocking. After the addition of protein G Sepharose beads,

the reactions were incubated in the same conditions for 1 hour. The isolated Src protein was subjected to a kinase reaction.

As a substrate for Src kinase the synthetic peptide cdc2 (KVEKIGEGTYGVVYK) was used at a final concentration of 100 μ M. The final concentration of the reaction buffer was 25 mM HEPES, 1% (v/v) Triton X-100, 0.5 mM DTT, 1 mg/ml BSA, 0.5 mM EGTA, 40 μ M ATP, 1 μ Ci ³²P, 10 μ M aprotinin, 10 μ M leupeptin, 100 μ M sodium orthovanadate, 5 mM p-Nitrophenyl Phosphate (PNPP) and 40 mM β -glycerophosphate, 10 mM MnCl₂. Reactions were then incubated for 15 min at 30 °C. The reactions were stopped by adding 20 ml 20% TCA, cooled on ice for 20 by min, and centrifuged at room temperature for 3 min at 10,000*g*. Thirty microliters of the resultant supernatant was then spotted onto phosphocellulose papers (2 x 2 cm) (Whatman P81). The phosphocellulose papers were washed 5 x 5 min in 5 mM phosphoric acid with agitation, dried, placed in vials with 2.5 ml of scintillation fluid (ICN; EcoLite), and subjected to liquid scintillation counting. The experiment was repeated at least three times and was performed in triplicate; the means ± SE are shown from a representative experiment.

In Vitro Pka Activity Assay

PKA activity assay was performed as previously described (Visconti et al. 1997); briefly, sperm cells (~10⁵) were incubated in the presence of 1 mM db-cAMP, 100 μ M IBMX, 1 % Triton (v/v) in kinase buffer containing 40 μ M ATP, 1 μ Ci ³²P ATP, 10 mM MgCl, 100 μ M Kemptide and inhibitors as specified, over a period of 30 min at 30 °C. The reactions were stopped and processed as described for the immunoprecipitation Src kinase assay.

CHAPTER III

IDENTIFICATION OF PROTEINS UNDERGOING TYROSINE PHOSPHORYLATION UPON MOUSE SPERM CAPACITATION

Introduction

Mammalian sperm are not ready to fertilize the egg immediately after ejaculation; they need to undergo a series of biochemical and physiological changes in the female reproductive tract collectively known as capacitation (Yanagimachi 1994). Although capacitation was discovered in the 1950s (Austin 1951; Chang 1951), the signaling pathways that regulate this process are not well established. Over the years, capacitation has been described using sperm functional assays and a series of endpoints have been considered to be part of this process. Among them, the ability of the sperm to fertilize the egg is the initial definition of capacitation and still the most important evidence that sperm are capacitated. More recently several authors have used two other functional correlates of capacitation. First, it is considered that only capacitated sperm can undergo an agonist-induced acrosome reaction (AR). Second, because sperm hyperactivation is necessary for fertilization, this change in motility pattern is also considered part of the capacitation process. Therefore, when one attempts to understand capacitation at the molecular level, it is necessary to consider events occurring both in the head (i.e., preparation for the AR) and in the tail (i.e., motility changes).

During the last ten years, at the molecular level, work from different laboratories in several mammalian species has demonstrated that capacitation is correlated with a cAMP-dependent increase in tyrosine phosphorylation (Visconti et al. 1995; Leclerc et al. 1996; Galantino-Homer et al. 1997; Kulanand and Shivaji 2001). Most of these studies are focused on determining how these posttranslational changes are regulated by different compounds in the capacitation medium known to be essential for capacitation(Salicioni et al. 2007). However, little is known about the role of the proteins that undergo the capacitation-associated increase in tyrosine phosphorylation. Recent work in humans, hamster and boar has started to identify some of the proteins that serve as substrates for tyrosine phosphorylation. The use of 2-dimensional (2D) gel electrophoresis followed by tandem mass spectrometry (MS/MS) provides a comprehensive approach to the analysis of proteins involved in cell signaling (Blomberg 1997). Specifically, changes in tyrosine phosphorylation can be monitored using 2D gel electrophoresis followed by Western blot analysis with anti- phosphotyrosine (α PY) antibodies (Naaby-Hansen et al. 1997; Ficarro et al. 2003). Proteins that undergo changes in tyrosine phosphorylation during cellular processes can be then isolated from a complementary gel and sequenced by MS/MS. In the present study, we have used this approach to identify sperm proteins that undergo tyrosine phosphorylation during mouse sperm capacitation. Our findings are consistent with the ones obtained for human sperm (Ficarro et al. 2003). Similar to that analysis, we have found that in mouse sperm, tubulin, VDAC, PDH E1 β chain, glutathione Stransferase, NADH dehydrogenase (ubiquinone) Fe-S protein 6, acrosin binding protein precursor, proteasome subunit alpha type 6b, and cytochrome b-c1 complex are phosphorylated in tyrosine residues. Interestingly, among the proteins phosphorylated on tyrosine residues we have identified two recently described testis-specific aldolases, (Vemuganti et al. 2007). Aldolase is the fourth enzyme of glycolysis; in mouse sperm, this metabolic process is the main pathway for ATP production as was demonstrated

using null mutants of other glycolytic enzymes (Miki et al. 2004). Because capacitation is correlated with hyperactivation, we hypothesize that tyrosine phosphorylation of aldolase during sperm capacitation could be involved in the regulation of the glycolytic pathway during this process. In addition to the localization of aldolase in the sperm principal piece, similar to hexokinase, aldolase was also found in the sperm anterior head suggesting that glycolysis could have additional functions during the preparation of the sperm for the acrosome reaction.

Results

Characterization of proteins that undergo tyrosine phosphorylation during capacitation

To identify those proteins that undergo tyrosine phosphorylation upon capacitation, mouse sperm were incubated for 90 min. under conditions that support (+ β CD, + NaHCO3) or not (- β CD,- NaHCO3) capacitation. Aliquots of each treatment were extracted in sample buffer and analyzed by Western blot using α PY antibodies (Fig. 4A). As expected, in conditions that support capacitation, an increase in protein tyrosine phosphorylation was observed. Samples from each treatment were extracted with Celis buffer and separated using 2D PAGE. For each experimental condition (NCap and Cap), 2 gels were run in parallel; one series of gels were silver stained with while the other was transferred and probed with α PY. Analysis for capacitated sperm is shown in Fig. 4 B, D; NCap samples probed in parallel using α PY served as controls (Fig. 4 C. Both the silver stained gel and the Western blot were scanned and compared. Several protein spots showing α PY staining were excised, digested, sequenced by MS/ MS (Table I) and the predicted localization of each spot is shown in Fig.4B,D. Among the mouse proteins identified in this search, VDAC, glutathione S-transferase, proacrosin binding protein (sp32), pyruvate dehydrogenase (E1 beta subunit),



Fig.4. Analysis of the capacitation-associated increase in protein tyrosine phosphorylation by 2-D gels. Mouse sperm were incubated for 90 min in media that do not support (minus 2-OH-p- β CD, minus HCO3 -) or support capacitation (plus 2-OH-p- β CD, plus HCO3 -). After this incubation period, aliquots from each condition were extracted either with SDS sample buffer for monodimensional α -pY Western blot (A) or with Celis buffer for 2D PAGE analysis (B,C,D) . 2D gels were then developed by α -pY Western blots (A,C,D) or with silver stain (B) . 2D images from capacitated sperm were overlaid using AdobePhotoshop and the silver stained proteins that exhibited tyrosine phosphorylation (shown by black arrows panel (D) were cored (shown by white arrows in panel (B) and sequenced by MS/MS. The following proteins were identified: 1,2, Alodart1, Aldoart2; 3, glutathione S-transferase; 4, NADH dehydrogenase (Ubiquinone); 5, acrosin binding protein (sp32); 6, proteasome subunit alpha type 6b; 7, PDH β ; 8, tubulin β ; 9, cytochrome b-c1 complex subunit 1; 10, VDAC, numbered in the same order as in Table I.

-	Protein Attrives & secondary	NCBI woomation	MW, pl	Reptides 6	equence Coverage (%)
-	Alf obter A, retropro Min 1	1000001	45.0, 60.9	N. ANGERTRICHA N. AGGGERTRICHA N. TGATAGEGLIFERMANY	0001
04	Aldokos A, refrograðin 2	AB/00000	384, 7.34	K, ACDGRIPT PP CIN, S M, SP PP A, TP CONK, E K, VT PS NES CA ANGECLE SIMMAY	14,20
	Glui Méricon a Francisco, mu su C.R.A. Bortami	1010802	202, 7, 10	KORELILIAK KETYA PADELI KEAMLOODER PKI KEAMLOODER PKI	000
4	NADII dahydro ganwa (u biquah ora) Fa-5 problin 3	A4107270	302, 6.70	K DIPPLI OWELAY RIVALIPVILACITRIK RISOVINGOLSAFICEY VARUACIY	10401
60	Acrosin binding protein presumor (spilit)	001140	611, 409	K ARTILS FOR COPPTICE A ORIVORIMMELY DOGWELS	5.92
0	Professionne suburit alpha ype (b	0250703	279, 7.10	RUTTEREDWELVALAERD RUTTEREDWELVALAERD	15.00
•	Pyruvske dehydrogeneses El bela	A4100100	34.0, 5.72	K DO ECOMPLAT RUMCOMMENDAR WILV K TY MUSACOPYINTELO K THALVITVEOOMYONO VOADICAELI	10.45
•	Tubulit, bein 5	\$2000444	49.7, 459	RIVLTVAVIR. G REPOZLANCIAN RISCOTANCIAN RAMJOURODIAS RAMJOURODIAS	1921
a	Cylochrone b-cl complex, advuid 1	000210	52.0, 59.4	RISTITIGRA RUGRVOMLAD RUGRVOMLAD RUGRVOMLAD	14.27
₽	Voltage-dependent an bruch an rel 2	162004W	31.7, 7.45	RISNEAGYELT RIDITANGE GROUNCL KUTTED TERMET GANS KUMMERLIGNOVT GRUER	21.00

PROTEINS THAT UNDERGO TYROSINE PHOSPHORYLATION UPON MOUSE SPERM CAPACITATION

TABLE 1

Protein were cut out from their stated 2-D get and recrossepanced and MSMS. Peptide sequenced is and more are shown together with the perdicted MW and pt. The experimental and the related values agree that causes, except for the account briding protein proteins protein from the protein. In addition, the percentage conserge of the protein and the MCBI accession number are shown.

tubulin, NADH dehydrogenase (ubiquinone) and the proteasome subunit alpha type 6 were previously detected as substrates for tyrosine phosphorylation in human sperm undergoing capacitation (Ficarro *et al.*, 2003). Interestingly, two of the proteins that have not been yet described as tyrosine phosphorylated substrates were testis specific isoforms of the aldolase family recently cloned (Vemuganti *et al.*, 2007).

To confirm these findings and analyze the localization of some of the identified proteins, three of the sequenced proteins including the aldolase isoforms were further analyzed using commercial antibodies. Antibodies against aldolase from rabbit muscle, anti-pyruvate dehydrogenase E1 β (PDH β) chain and anti-voltage dependent anion channel (VDAC) were used to probe Western blots of total mouse sperm extracts (Fig. 5).



Mouse whole sperm protein extracts

Fig. 5. Validation of anti-aldolase, anti-PDHE1 β and anti-VDAC antibodies using mouse sperm extracts. Total mouse sperm extracts (~ 2 x 10⁶ cells) were lysed in sample buffer and separated by 10 % SDS-PAGE and transferred to Inmobilon P. Western blots were performed using anti-aldolase, anti-pyruvate dehydrogenase E1 β chain and anti-VDAC antibodies. Experiments shown are representative of experiments performed at least three times with similar results.

Each of these antibodies recognized proteins at the appropriate molecular weight.

As expected from our two-dimensional sequencing as well as from the recently published

work from Vemuganti et al. (Vemuganti et al., 2007), anti-aldolase antibodies recognized



Fig. 6. Antibodies against aldolase, pyruvate dehydrogenase E1 β chain and VDAC matched the tyrosine phosphorylated spots in capacitated mouse sperm. Mouse sperm were incubated in capacitation-supporting medium for 90 minutes. After this incubation period, proteins were extracted in Celis buffer and separated by 2 D PAGE. Parallel immunoblots were probed using α PY antibodies (A), anti-aldolase (B), anti- pyruvate dehydrogenase E1 β chain (C), and anti-VDAC (D) antibodies. Arrows with different shapes indicate the tyrosine phosphorylated spots in (A) and the respective proteins in the other Western blots. Results from a representative experiment are shown.

the shorter (~ 40 kDa) and longer (~ 50 kDa) sperm aldolase isoforms. Similarly, the anti-VDAC antibody recognized two proteins of similar molecular weights, implying that more than one VDAC is present in sperm as suggested previously (Hinsch *et al.*, 2001; Hinsch *et al.*, 2004); these molecular weights corresponded to the excised spots from the 2D gels. To further confirm this finding, aliquots of capacitated sperm protein extracts were analyzed by 2D PAGE Western blots using anti- PY, anti-aldolase, anti-PDH β and anti-VDAC antibodies. These Western blots show that in addition to the different molecular weight, both aldolase isoforms are separated as a trail of proteins depicting a series of isoelectric points. Similarly, PDH β and the two VDAC spots were confirmed (Fig. 6 C, D). These Western blots show that, in addition to the different molecular weight, both aldolase isoforms are separated as a trail of proteins depicting a series of isoelectric points. In each case, the arrows indicate the sequenced spots as shown in Fig. 6B. Similarly, PDH β and the two VDAC spots were confirmed (Fig. 6C, D).

The localization of these proteins was analyzed by immunofluorescence. As expected, VDAC and PDH β were found to be present in the midpiece where the mitochondria reside, while aldolase was found in the principal piece, the sperm cell compartment in which glycolysis has been reported. However, these three tyrosine phosphorylation substrates also localized to unexpected regions in the sperm. For example, both aldolase and VDAC were also found in the anterior head; this localization is atypical for these proteins and could be related with specific functions in the acrosomel compartments. Interestingly, both proteins are lost from the head after the acrosome reaction (Fig. 7 and data not shown).

To further analyze the sperm distribution of these proteins, the sperm were extracted in the presence of 1% Triton X-100 for 30 min at 4°C and the solubility of aldolase, PDH β and VDAC analyzed by Western blot. Interestingly, these three proteins distribute in both Triton X-100 soluble and insoluble fractions, and their distribution does

not appear to change during capacitation (Fig. 8A). In addition, anti-aldolase antibodies were used to test whether the high molecular weight aldolase isoform is also found in other tissues. Multi-tissue Western blots indicate that the high MW aldolase is only found in sperm and testis extracts (Fig. 8B), confirming previous observations (Vemuganti *et al.*, 2007).



Fig. 7. Immunolocalization of phosphorylated protein targets in mouse sperm. Mouse sperm were air-dried, fixed, permeabilized and probed with antibodies for anti-aldolase (A), anti-pyruvate dehydrogenase E1 β chain (B) and anti-VDAC (C). PNA (peanut agglutinin) stains the acrosome and mito-tracker is used as a mitochondrial marker (D-F). DIC images are shown in (G-I). As negative controls, normal IgG from goat (aldolase), mouse (dehydrogenase E1 β chain) and rabbit (VDAC) were run in parallel. Images shown are representative of at least three repetitions with similar results.



Fig. 8. Solubility of tyrosine phosphorylation targets in Triton X 100 and Western blot analysis of mouse tissues using α -aldolase antibodies. (A) Cauda mouse sperm were incubated in conditions that support (Cap) or not (Non) capacitation for 90 min. After this incubation the sperm were centrifuged and resuspended in buffer containing 1 % Triton X 100. After 30 min. the sperm were centrifuged and the pellet (Pt) and supernatant (Sn) analyzed by Western blots using anti-aldolase, anti- PDH β and anti-VDAC antibodies as described. The experiment shown is representative of three similar ones. (B) Immunoblot showing the presence of higher molecular weight aldolase in sperm and testis protein extracts. Ten micrograms of RIPA extracted tissues of CD1 male or female mouse were separated in 10 % SDS-PAGE and immunoblotted with anti-aldolase antibody. A representative experiment of three repetitions is shown. Abbreviations: Sp(sperm); T (testis); M (muscle); H (heart); O (ovary); Li (liver); K (kidney); Lu (lung); Hy (hypothalamus); Cx (cortex); Ce (cerebellum); P (pituitary).

Aldolase activity does not change during capacitation

Although the capacitation-associated increase in protein tyrosine phosphorylation was described more than 10 years ago, still it is not clear how these posttranslational changes affect sperm function. Because glycolysis is the main source of energy in mouse sperm, the finding that aldolase is tyrosine phosphorylated during capacitation opens the possibility that tyrosine phosphorylation is involved in the regulation of aldolase activity and glycolysis during this process. To analyze whether this was the case, total sperm aldolase activity was measured, as well as the activity associated to soluble and particulate sperm compartments. To measure aldolase activity, a modified indirect spectrofluorometric method was used taking into consideration the different emission of NADH and NAD when excited at 367 nm (Fig. 9A). At low concentrations of NADH, the fluorescence using 367/460 nm excitation/emission pair was linear (Fig. 9B). Because aldolase activity is coupled to the transformation of NADH to NAD when α -glycerophosphate dehydrogenase, triose phosphate isomerase (αGDH/TPI) and FBP are present, the decrease in NADH was used to measure aldolase activity. Total sperm aldolase activity was linear in the conditions used in this assay (Fig. 9C); when the cytosolic and particulate fractions were assayed, similar linearity was found (data not shown). As explained in methods, the kinetics were evaluated in real time and the activity was calculated from the slope of NADH consumption (Fig. 9D). As shown (Fig. 9 E,F), both kinetic parameters (Km and Vmax) indicative of aldolase activity in sperm incubated in media that support (Cap) or not (NCap) capacitation did not change. Similar results were obtained when the particulate and the cytosolic fractions were assayed independently (data not shown).



Fig. 9. Aldolase activity does not change during capacitation. (A) Emission spectrum of NADH and NAD. The spectra for 1mM β-NADH and 1 mM β-NAD were obtained with an excitation wavelength of 367nm. The emission peak was observed at 460 nm; using these parameters NAD had very low fluorescent emission. (B) Fluorescence is linear with NADH when low concentrations are used. NADH was dissolved in Tris/HCl buffer at different concentrations and the fluorescence was determined as described using a 367/460 nm excitation/ emission pair. A NADH concentration of 130 uM was used in the aldolase enzymatic assays. (C) Aldolase activity is linear with the sperm concentration. Sperm were resuspended in Triton buffer and serial dilutions were made and assayed for aldolase activity as described. (D) NADH consumption vs. time after addition of sperm as a source of aldolase. The substrates for the coupled enzymatic reactions: Fructose 1,6-Di phosphate, G 3-P,á-GDH, TPI and NADH in Tris- HCl buffer were placed in the same cuvette and continuous NADH fluorescence was registered before and after addition of sperm pellets. The slope of each curve was used to determine the Vo. (E) Aldolase activity in non capacitated and capacitated sperm as a function of substrate (Fructose 1,6biphosphate) concentration. Sperm were incubated in either non capacitating or capacitating conditions and aldolase activity was assayed at different Fructose 1,6biphosphate concentrations. Each measurement was repeated three times in three different experiments, the values represent the mean \pm SEM. (F) Kinetic parameters for aldolase from non capacitated and capacitated sperm. The curves of Vo vs. substrate concentration shown in (E) were adjusted to the Michaelis-Menten equation: Y=Vmax*X/(Km+X), where X is the substrate concentration (mM) and Y is the enzyme velocity (mM/sec).

Discussion

Protein phosphorylation plays a role in the regulation of intracellular processes such as transduction of extracellular signals, intracellular transport, and cell cycle progression. In sperm, capacitation is associated with a phosphorylation cascade which involves a PKAregulated increase in protein tyrosine phosphorylation (for review see Salicioni et al., 2007)). Although the correlation between sperm capacitation and the increase in tyrosine phosphorylation has been reproduced in several mammalian species (Visconti et al., 1995a; Visconti et al., 1995b; Leclerc et al., 1996; Galantino-Homer et al., 1997; Kalab et al., 1998), the identity and functions of kinases and their targets in sperm are not well defined. An initial approach to investigate the role of phosphorylation in capacitation is to identify proteins phosphorylated during this process and to characterize the kinases involved in their phosphorylation. In this respect, the use of 2D PAGE followed by MS/MS provides a comprehensive approach to the analysis of proteins involved in cell signaling (Blomberg, 1997; Alms et al., 1999; Soskic et al., 1999; Lewis et al., 2000). Specifically, changes in tyrosine phosphorylation can be monitored using 2-D gel electrophoresis (O'Farrell, 1975, Gorg *et al.*, 1988) followed by Western blot analysis with α -PY antibodies. Proteins that undergo changes in tyrosine phosphorylation during capacitation can then be isolated from a duplicated gel stained with silver and sequenced by MS/MS. This strategy has been used before for the identification of tyrosine phosphorylated proteins in sperm from human (Naaby-Hansen et al., 1997; Ficarro et al., 2003), hamster (Kumar et al., 2006) and boar sperm (Bailey et al., 2005). In this work, a similar approach was used to start the characterization of tyrosine phosphorylated substrates in mouse sperm. Capacitation prepares the sperm to undergo the acrosome

reaction and also is associated with changes in sperm motility (e.g. hyperactivation) in a number of species (Visconti and Kopf, 1998). Therefore, it can be hypothesized that components of the sperm exocytotic and motility machinery could be found among the proteins that undergo tyrosine phosphorylation during capacitation. In particular, motility changes require elevated levels of ATP to support coordinated movements of the flagellum. The sources of ATP in the sperm are known to be compartmentalized; while oxidative phosphorylation occurs in the mid piece of the flagellum where the mitochondria are confined, glycolysis appears to be restricted to the principal piece. Although oxidative phosphorylation is more efficient than glycolysis for ATP production, recent work by Miki et al. (Miki et al., 2004) demonstrated that the absence of the spermspecific glyceraldehyde 3-phosphate dehydrogenase -S (GAPDS) in GAPDS null mice results in a decrease of the ATP levels to almost negligible levels in their sperm. These findings suggest that at least in the mouse, the main source of ATP is through the glycolytic pathway. Because of this relevant role of glycolysis, the identification of two testis-specific members of the aldolase family as tyrosine phosphorylated substrates is noteworthy. Fructose-1,6- biphosphate aldolase (commonly referred to as aldolase) catalyzes the fourth step of the glycolysis. The mechanism involves a retro-aldol condensation reaction that cleaves the 6 carbon biphosphorylated fructose into two 3carbon products: D-Glyceraldehyde 3- phosphate and dihydroxyacetone phosphate. Glycolysis has been highly conserved throughout evolution; however, in sperm, many critical glycolytic enzymes present testis-specific isoforms. These isoforms are either differentially spliced variants of somatic enzymes or they derived from independent genes. Fructose-1, 6-biphosphate aldolase is an interesting example, this gene family

contains three somatic members (AldoA, AldoB and AldoC), two testis-specific intronless genes likely evolved by retrotransposition (Aldoart1 in chromosome 4 and Aldoart2 in chromosome 12) (Vemuganti et al., 2007) and two testis-specific splicing variants of AldoA derived from the use of an extra exon in the amino-terminus region of the transcript (AldoA v1 and AldoA v2) (Vemuganti et al., 2007). In our study, sequencing of two tyrosine phosphorylated spots revealed peptides matching aldoart1 and aldoart2. Moreover, results from 2D analysis suggest that all aldolase members in sperm are tyrosine phosphorylated. This result suggests that aldolase and therefore glycolysis might be regulated by tyrosine phosphorylation in sperm. To test this hypothesis, mouse sperm were incubated in conditions that either support or not capacitation, and aldolase activity was assayed in total extracts as well as in detergent soluble and particulate fractions. In all conditions, the kinetics parameters (Km and Vmax) of aldolase were invariant. The lack of change in aldolase activity suggests that tyrosine phosphorylation does not play a direct role in activation of this enzyme. However, at present we cannot discard that the efficiency of the aldolase access to its substrates in vivo is not modified. Interestingly, four tyrosine phosphorylation sites have been found in somatic aldolases (www.phosphosite.org); three of these sites are conserved in the mouse aldoart1 and four of these sites are present in aldoart2. At present, the role of these phosphorylations is not known. The source of energy for the acrosome reaction as well as other molecular processes occurring in the sperm head is not clear. The ATP needed could be produced in the mitochondria by oxidative phosphorylation and transported to the head; or alternatively, the head could have the enzymes required for glycolytic ATP production. In addition to the expected localization of aldolase in the principal piece (Krisfalusi *et al.*,

2006; Vemuganti *et al.*, 2007), anti-aldolase antibodies also stained the acrosomal region of mouse sperm suggesting the presence of at least one of the aldolase isoforms in this region. This is consistent with the finding of another glycolytic enzyme, hexokinase type I, in the sperm head (Visconti et al., 1996; Travis et al., 1998). Although more research is needed, localization of glycolytic enzymes in the sperm head opens the possibility that in addition to the established role in the principal piece, glycolysis could play a role in the sperm head. In addition to the two testis-specific aldolases, eight other proteins were identified as tyrosine phosphorylation targets; from these ones, PDHB, VDAC2, glutathione S-transferase were also mapped as capacitation-associated tyrosine phosphorylation targets in human sperm (Ficarro et al., 2003), PDHE1a chain in hamster sperm (Kumar et al., 2006) and the acrosin binding protein sp32 in boar sperm (Bailey et *al.*, 2005). In the case of VDAC and PDHE1 β chain, commercial antibodies were used to confirm tyrosine phosphorylation in the assigned proteins and to analyze their localization in sperm by immunofluorescence. In somatic cells, the voltage dependent anion channels (VDACs) were first identified in the mitochondrial outer membrane; however, VDACs have also been localized to the plasma membrane in several cell types (De Pinto et al., 2003; Wang et al., 2007). In the mitochondria, VDAC is known to function as the gate for metabolite transport from the cytosol; although it displays a rather nonspecific single channel activity, usually anionic species as phosphate, chloride, organic anions, and adenine nucleotides are transported into the mitochondria through VDAC. Recent work by Hinsch et al. (Hinsch et al., 2001; Hinsch et al., 2004) demonstrated that VDAC2 and VDAC3 are present in bovine sperm; in particular VDAC2 was shown to localize to the anterior head. Although the anti-VDAC antibodies

used in the present investigation were not able to distinguish between the three VDAC isoforms, our results also indicate that in addition to the mitochondrial localization, VDAC is also localized to the acrosomal region in mouse sperm. At present, we can only speculate that tyrosine phosphorylation of VDACs may either affect the activity of these channels or their interaction with other sperm proteins. Mammalian PDH is a tetramer consisting of two alpha and two beta subunits. Contrary to the PDH α that is encoded by two genes, one somatic (PDHA1) and one testis-specific (PDHA2), PDHβ, encoded by PDHB is common to both somatic and testicular cells (Korotchkina et al., 2006). Similar to previous findings in human sperm (Ficarro et al., 2003), PDH1beta is tyrosine phosphorylated during mouse sperm capacitation. Immunofluorescence localization of this enzyme shows the expected mid piece staining consistent with mitochondrial localization; however, this enzyme was also found in the principal piece and in the acrosomal region. These results are consistent with the finding of Fujinoki et al. (Fujinoki *et al.*, 2004) which identified PDH E1 β as part of the fibrous sheath in hamster sperm. Also in hamster sperm, functional studies revealed tyrosine phosphorylation of the testis specific PDHa subunit of this enzymatic complex. The role of the tyrosine phosphorylation in this case appears to be coupled to an increase in the PDH activity (Kumar *et al.*, 2006). In summary, although work emanating from multiple laboratories is leading to a better understanding of capacitation, most of the proteins involved in this process remain to be characterized. In addition, it is important to highlight that although the increase in tyrosine phosphorylation has been correlated to sperm capacitation; it is still unclear how the changes in tyrosine phosphorylation are coupled to physiological events that occur during capacitation. The molecular identification of tyrosine kinases,

phosphotyrosine phosphatases, tyrosine phosphorylation substrates, and Ca2+ channels present in mammalian sperm will certainly expand our understanding of the molecular basis of capacitation.

CHAPTER IV

SRC FAMILY INVOLVEMENT IN MOUSE SPERM CAPACITATION Introduction

Almost 60 years ago, Austin (Austin 1952) and Chang (Chang 1951) described independently the requirement of the sperm in the female tract to be able to fertilize. Correlating to the capacitated state, the increase of tyrosine phosphorylation of a subset of proteins has been reported in many mammalian species (Visconti et al. 1995a; Leclerc et al. 1996; Galantino-Homer et al. 1997; Kulanand and Shivaji 2001). Likewise, the dependency of an increase of cAMP for the activation of a tyrosine kinase and capacitation is supported by a bevy of literature (Visconti et al. 1995b; Leclerc et al. 1996; Galantino-Homer et al. 1997; Aitken et al. 1998; Harrison and Miller 2000). The best characterized effector of cAMP in sperm cells is PKA. The identity of tyrosine kinase(s) that mediates the signaling, however, is still elusive.

Since the tyrosine phosphorylated proteins are present in the soluble fraction, we aim our efforts towards cytoplasmic kinases. Of such proteins, Src family had been recently proposed as mediators of the signaling pathway that leads to sperm capacitation (Baker et al. 2006). However, it should be kept in mind that at this point it is not possible to discard a role for receptor tyrosine kinases in sperm capacitation,

Src Family Kinases

The cytoplasmic tyrosine kinase family Src is involved in a wide variety of cellular responses, including cell proliferation, migration, differentiation and survival (Thomas and Brugge 1997). The first member of the family to be identified was the

tumor causative agent, isolated by Peyton Rous in 1911, demonstrated to be of viral nature in 1970 (Martin 1970) and described as a kinase in 1978 (Collett and Erikson 1978). Its cellular counterpart, c-Src protein, was the first eukaryotic tyrosine kinase to be discovered (Schartl and Barnekow 1982). The mammalian Src family is constituted by Src, Hck, Lck, Fyn, Lyn, Lck, Blk, Fgr and Yes (Ingley 2008). Src, Fyn and Yes are expressed in most tissues. However, some are highly expressed or display alternative spliced variants in certain cell types. Blk, Fgr, Hck. Lck and Lyn are primarily found in hematopoietic cells (Thomas and Brugge 1997). All members have a similar arrangement: an N-terminal highly variable domain that bears an ancillary myristoylation or palmitoylation site, followed by the SH3 proline enriched and SH2 domains, and the enzymatic activity domain SH1.

Interestingly, fibroblast cells have been used to show that Src is activated by PKA through phosphorylation on serine 17 within its amino terminus, both *in vitro* and *in vivo* (Stork and Schmitt, 2002). This phosphorylation of Src in serine 17 affects its conformation, rendering the autophosphorylation site available and triggering the activation of the kinase. The promotion of sperm phosphotyrosine activity during capacitation arises from a crosstalk between PKA activity and TKs. The tyrosine kinase(s) that is activated downstream of PKA upon capacitation still remains an open question. Although not in a conclusive manner, Src tyrosine kinase have been recently proposed to act as a key player of the cross talk between PKA activity and tyrosine phosphorylation of proteins, both in human (Lawson et al. 2008; Mitchell et al. 2008; Varano et al. 2008) and mouse sperm capacitation .

A decrease in protein tyrosine phosphorylation has been observed when spermatozoa were incubated with Src tyrosine kinase inhibitors, such as herbimicyn A, erbstatin, SU6656, PP1, or PP2 (Baker 2006). These inhibitors are expected to be specifically directed to Src-related tyrosine kinases, suggesting the involvement of this family of tyrosine kinases in the capacitation-mediated increase of sperm protein phosphotyrosine content.

Our objective is to elucidate the involvement of Src or Src family members in the tyrosine phosphorylation in mouse sperm capacitation.

Results

Src kinase family tyrosine inhibitors reduce the capacitation dependent tyrosine phosphorylation

To evaluate the possible involvement of Src family of tyrosine kinases in the increase of tyrosine phosphorylation we used a pharmacological approach. Mouse sperm were incubated in capacitation conducive conditions (+ BSA, + NaHCO3) and different concentrations of SU6656 and SKI606, two known specific inhibitors for Src family kinases (Coluccia et al. 2006). The phosphorylation of tyrosine residues was evaluated by Western blot using an anti-phosphotyrosine antibody. As seen (Fig 10) both inhibitors abrogated the capacitation dependent tyrosine phosphorylation in a concentration dependent manner.

Presence of Src family kinases in mouse sperm

The inhibitors SU6656 and SKI606 are specific for not only Src kinase but all the members of its family. Since mammalian cells present eight known members in the Src kinase family (Src, Hck, Lck, Fyn, Lyn, Lck, Blk, Fgr and Yes) (Ingley 2008), we



Fig. 10 Concentration dependent effects of Src family inhibitors on the appearance of tyrosine phosphorylated proteins in capacitated mouse sperm. Mouse sperm was incubated under conditions that support capacitation (+BSA, +NaHCO₃) or not (-BSA, -NaHCO₃). Incubation reaction was carried away in the presence or absence of increasing concentrations of SU6656 or SKI606. Total mouse sperm extracts (~ 2 x 10⁶ cells) were lysed in sample buffer and separated by 10 % SDS-PAGE and transferred to Inmobilon P. Western blots were performed using antiphosphotyrosine (α -pY) antibody. This experiment was performed at least three times with similar results. Shown is a representative experiment.

decided to determine which member(s) were possibly being affected by the inhibitor SU6656 and SKI606. In this regard, we assayed for the presence of the members of the Src kinase family in mouse sperm extracts by means of Western blots using specific antibodies (described in Materials and Methods) against their unique N-terminal domain (Fig.11). Mouse sperm extracts were prepared using either sample buffer with 2% SDS or RIPA buffer. On both samples the antibody specific for Src detected a band at the expected molecular weight (60kDa), similar to the positive control of mouse macrophages. This antibody also recognized a lower molecular weight band, at approximately 50 kDa. Alongside their respective positive controls, samples of mouse sperm extract were assayed for the presence of the other members of the Src family. Using this approach we were not able to find the presence of any of the other Src family



members. These results suggest that Src is the kinase that is being inhibited by the inhibitor SU6656 and SKI606.

Fig.11. Src kinase is present in mouse sperm extract. Total mouse sperm extracts (~ 2×10^6 cells) were lysed in sample buffer or extracted in RIPA buffer, separated by 10 % SDS-PAGE and transferred to Inmobilon P. Western blots were performed using anti-Src, anti-fyn, anti- lck, anti- lyn, anti- blk, anti- yes, anti- hck and anti-fgr antibodies. Experiments shown are representative of experiments performed at least three times with similar results. In the rightmost lane 20 µg of the respective positive control was run in parallel. A representative of three repetitions is shown. Abbreviations: MACS (Mouse macrophages); NAM (namalwa cells); MDCK (Madin-Darby canine kidney); spleen (mouse spleen extract). Membranes were reblotted with anti-actin as a loading control.

Src activity in mouse sperm

Since we were able to observe a inhibitory effect on the tyrosine phosphorylation normally correlated with the capacitated sperm state when sperm were incubated in the presence of Src inhibitors, we decided to analyze whether there is a change in Src activity levels during capacitation. We measured the kinase activity using the well characterized Src peptidic substrate cdc2 (KVEKIGEGTYGVVYK).



Fig.12. Src kinase activity in non capacitated and capacitated mouse sperm. Mouse cauda sperm was incubated in conditions that elicit (+BSA, +NaHCO₃), or not capacitation and extracted as described in "Material and Methods" A) Mouse sperm extracts were incubated with anti-Src (GD11) antibody for 2 hr. Protein G Sepharose was added for another h. Sepharose beads were then washed three times with the same buffer and proteins bound were eluted from the beads by boiling. Immunoprecipitated proteins were then separated by 10%gel, transferred and then probed with anti- Src antibody (36D10). Anti-tubulin antibody was used as a control. As a second control sperm extracts were incubated with beads without antibody. B) Posterior to incubation with anti-Src (GD11) antibody, proteins were immunoprecipitated and subjected to a kinase reaction using cdc2 as a substrate. The incorporation of ^{32}P (nmol) per minute per 5 million sperm cells was assayed by scintillation counting. C) Sample as in B was separated by 8% gel, transferred and 32 P was visualized by autoradiography. Same membrane was probed with anti-Src antibody. Results illustrate one of three replicated performed experiments.

Sperm were incubated in conditions that support (+ BSA, + NaHCO3) or do not (- BSA, - NaHCO3) support capacitation for 60 min. Src was then isolated from sperm extracts by immunoprecipitation using a monoclonal antibody against Src (GD11). The immuno precipitate was incubated in a kinase buffer containing ³²ATP and the specific substrate (cdc2). As shown in Fig. 12, Src activity was significantly higher in capacitated sperm than in the non capacitated sperm. In addition to the effect of Src inhibitors on the phosphorylation state of sperm proteins, this observation points toward an active role of Src during sperm capacitation

Specificity of Src inhibitors

In order to discard possible nonspecific effects of SU6656 and SK1606 on other kinases than Src kinase, we analyzed the effect of these inhibitors on PKA activity. PKA is a known player of capacitating pathway. Moreover, it has been recently suggested that in mouse sperm, Src lies downstream of PKA in the signaling pathway that leads to the phosphorylation of sperm proteins (Baker et al. 2006). With this hypothesis in mind, SU6656 and SK1606 should have no effect on PKA activity. In other to analyze PKA activity *in vivo*, we used a specific monoclonal antibody against phosphor-PKA substrates (RRXpS/pT). Sperm protein extracts were prepared following incubating conditions that support capacitation; western blots were performed to assay PKA activity (Morgan et al. 2008). To our surprise, in the presence of the same concentration that abrogates the capacitation-dependent tyrosine phosphorylation, the inhibitors strongly abolishes the phosphorylation of serine/threonine residues of PKA substrates (Fig. 13). This striking result suggests that tyrosine phosphorylation is being inhibited due to

nonspecific inhibition of PKA. The same effect was observed when specific PKA inhibitors were used (not shown).



Fig. 13. Effects of Src family inhibitors on the appearance of phosphorylation of PKA substrates in capacitated mouse sperm. Mouse sperm was incubated under conditions that support capacitation (+BSA, +NaHCO₃) or not (-BSA, -NaHCO₃). Incubation reaction was performed in the presence or absence of 50 μ M of SU6656 or SKI606. Total mouse sperm extracts (~ 2 x 10⁶ cells) were lysed in sample buffer and separated by 10 % SDS-PAGE and transferred to Inmobilon P. Western blots were performed using an antibody that recognizes phosphorylated PKA substrates. This experiment was performed thrice with similar results. Shown is a representative experiment.

Should the effect of Src inhibitors on tyrosine phosphorylation be exerted through the blockade of PKA activity, PKA should also be inhibited when an *in vitro* kinetic assay are conducted. To confirm the effect of Src inhibitors on PKA, we analyzed vitro PKA activity in the presence of SU6656 and SKI606. Sperm were lysed with 1% Triton X-100 in the presence of protease and phosphatase inhibitors Then, the reactions were conducted in the presence of cAMP (with the phosphodiesterase inhibitor IBMX), and different concentrations of SU6656 or SKI606. The well known PKA inhibitor, H-89, was used as a control. After 30 min, ATP incorporated to the PKA substrate (kemptide) was estimated using a scintillation counter. Surprisingly, no inhibition of PKA activity was found when Src inhibitors were present. In light of these results, it is understood that PKA activity is indirectly affected by Src inhibitors. It could be then hypothesized that Src could be an important player of the capacitating signaling pathway that is located upstream of PKA.



Discussion

One of the missing links in the signaling pathways that lead to sperm capacitation is the identity of the tyrosine kinase downstream of PKA. In terms of temporal regulation of capacitation events, PKA mediates both slow and fast events (Salicioni et al. 2007). The phosphorylation of tyrosine residues is a slow associated event and is mediated by a crosstalk among PKA and one or several tyrosine kinases(Visconti et al. 1995b). Recently, Baker et al (2006) proposed a model in which PKA regulates Src kinase activity both directly by phosphorylation of serine 17 and indirectly by inhibition of Src inhibitor, Csk. According to the model, these signaling events will lead to a change in the conformation of Src, triggering the autophosphorylation and rendering the enzyme active.

Src family members had been reported in sperm of several mammalian species, using an immunological approach, here we show the presence of Src kinase and the absence of the other members of the family. Likewise, we show the abrogation of the capacitation associated tyrosine phosphorylation in the presence of Src family inhibitors. Importantly, these inhibitors have been characterized as highly specific for Src and Src family members, SU6656 IC₅₀ is reported as $0.28\pm0.03 \mu$ M (Blake et al. 2000) and SKI-606 reported as 250 nM (Vultur et al. 2008). Taken together, our results suggest Src involvement in the capacitation associated tyrosine phosphorylation.

To confirm such results, we performed a Src kinase assay by immunoprecipitating Src from sperm incubated in non capacitated and capacitated conducive conditions for 60 minutes. Consistent with our results, we found a significant increase in the activity of Src in the capacitated sperm. Baker et al (2006) recently proposed a model in which Src is placed downstream of PKA. In fibroblast cells it has been shown *in vivo* and *in vitro* (Stork and Schmitt 2002) PKA phosphorylates Src in serine 17, which triggers the activation of Src.

In sperm capacitation, PKA activation occurs during the first 30 min of capacitation (Morgan et al. 2008). In order to rule out the effect of the inhibitors on PKA activity, using the same concentration of SU6656 and SKI-606 that abrogated the

44

tyrosine phosphorylation, we performed a western blot using an antibody that recognizes subtrates phosphorylated by PKA. Unexpectedly, we found that Src inhibitors were strongly abolishing the phosphorylation of PKA substrates.

To validate this data, we capacitated sperm in the presence of Triton X-100, PKA agonists (+dbcA, +IBMX) and Src inhibitor for 30 minutes, and PKA activity was assayed. The activity of PKA in these conditions was not significantly affected by the presence of Src inhibitors. The *in vitro* assay results are incongruous with the results we obtained in the PKA substrates western blot. PKA activity is regulated by spatial cellular distribution that the PKA anchoring proteins provide. Sperm cells are highly compartmelized; therefore, the lack of spatial organization may have affected the activity of PKA.

Our results suggest a crosstalk between Src and PKA during capacitation. The involvement of PKA signaling as a trigger of tyrosine kinase phosphorylation during capacitation is supported by a work from different labs in several mammalian species, and a very accepted model (Visconti and Kopf 1998) suggest a tyrosine kinase downstream of PKA. The identity and the signaling pathways that lead to the increase of tyrosine phosphorylation still remain outstanding.

In summary, our results suggest that the only Src family present in mouse sperm is the first characterized member of the Src family members and its inhibition results in abrogation of tyrosine phosphorylation. We also present evidence that point towards a link between Src and PKA, in the regulation of the capacitation associated tyrosine phosphorylation. However, our results indicated that Src might be activated either prior or concomitant to PKA activation. Noteworthy, Src inhibitions impairs PKA activity in vivo.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Sperm capacitation correlates with an increase of tyrosine phosphorylation of a subset of proteins in several mammalian species (Visconti et al. 1995; Leclerc et al. 1996; Galantino-Homer et al. 1997; Kulanand and Shivaji 2001). The role of the phosphorylation signaling in the capacitation events is still unclear. One step towards the understanding of the possible role of the sperm capacitation associated tyrosine phosphorylation is the identification of the proteins that undergo these conserved post translational changes. By separating the phosphoproteins with two dimensional SDS-PAGE followed by MS/MS, we identified: tubulin, VDAC, PDH E1 β chain, glutathione S-transferase, NADH dehydrogenase (ubiquinone) Fe-S protein 6, acrosin binding protein precursor, proteasome subunit alpha type 6b, and cytochrome b-c1 complex as tyrosine phosphoproteins upon mouse sperm capacitation. Of such proteins, we focused on characterizing those that are involved in the glycolytic and cellular respiration pathway, due to the expected high requirement for ATP of sperm during capacitation events. We showed the presence by western blot and immunofluorescence of VDAC, Pyruvate dehydrogenase E1 β chain, found in the midpiece. Likewise, we found aldolase in the principal piece.

Two isoforms of aldolase were identified as phosphoproteins associated with capacitation: aldoart1and aldoart2. These two isoforms have been recently reported (Vemuganti et al. 2007) in mouse sperm as two testis specific intronless genes, most

likely, retrotrasposons (*Aldoart1* in chromosome 4 and *Aldoart2* in chromosome 12). Two additional isoforms of aldolase are splicing variants of the somatic AldoA. Althought we could not detect a significant difference in the activity of aldolase by fluorimetry upon capacitation, it cannot be discarded a possible role in the capacitation events.

The signaling events that bring about the tyrosine phosphorylation increase upon capacitation are far from being elucidated (Salicioni et al. 2007). Early on the capacitation events is the influx of HCO_3^- One of the likely targets of HCO_3^- is the unique soluble adenylyl cyclase that will bring about the increase of cyclic AMP (reviewed by Salicioni et al., 2007). In sperm the best characterized effector is the serine/threonine kinase PKA. The increase of PKA activity proposed to be the first step of following tyrosine kinase cascade activation. Here we show that Src is the only member of the Src family of kinases present in sperm.

We show that the use of specific inhibitors abrogates the tyrosine phosphorylation associated with capacitation. Moreover, the activity of the Src kinase during capacitation increases significantly. However, the activity of PKA, as determined using a specific antibody that recognized the serine/threonine phosphoproteins was inhibited by both Src inhibitors, SU6656 and SKI-606 at a 50 μ M concentration. The *in vitro* activity of PKA, unexpectedly, not affected in the presence of SU6656 or SKI-606. Our results suggest an involvement of a crosstalk between Src and PKA activities in capacitation associated events.

Future Directions

Future studies will focus in the characterization of the tyrosine phosphoproteins that we identified. This information is crucial to the understanding of the biological relevance of tyrosine phosphorylation in capacitation events. We speculate that the proteins involved in the production of ATP; glycolytic and electron chain transport pathways (VDAC, PDH E1 β chain, NADH dehydrogenase (ubiquinone) Fe-S protein 6 and cytochrome b-c1) role is critical for capacitation and we expect that the change of the phosphorylation status mediates the activity level. Tubulin has been shown (Ficarro et al. 2003) to be tyrosine phosphorylated upon capacitation as well, the exact role is still unclear. The function of glutathione S-transferase, acrosin binding protein precursor, proteasome subunit alpha type 6b need as well to be elucidated.

Looking upstream on the regulation of the tyrosine kinase(s) that bring about the tyrosine phosphorylation, our results suggest a crosstalk between Src tyrosine kinase and PKA. The activity of Src might be upstream of the activation of PKA. This findings report the first tyrosine kinase in sperm that regulates a key step of capacitation events. Further studies will focus on the identification of the molecules that mediate that crosstalk and its regulation.

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