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In spermatozoa, Stat1 is activated during capacitation and the acrosomal reaction

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Abstract

A role for sperm-specific proteins during the early embryonic development has been suggested by a number of recent studies. However, little is known about the participation of transcription factors in that stage. Here, we show that the signal transducer and activator of transcription 1 (Stat1), but not Stat4, was phosphorylated in response to capacitation and the acrosomal reaction (AR). Moreover, Stat1 phosphorylation correlated with changes in its localization: during capacitation, Stat1 moved from the cytoplasm to the theca/flagellum fraction. During AR, Stat1 phosphorylation increased again. In addition, blocking protein kinase A (PKA) and PKC during capacitation abolished both phosphorylation and migration of Stat1. Our results show tight spatio–temporal rearrangements of Stat1, suggesting that after fertilization Stat1 participates in the first rounds of transcription within the male pronucleus.

Reproduction (2007) **134** 425–433

Introduction

The signal transducer and activator of transcription (Stat) protein family mediates transcription of several cytokine-inducible genes and growth factors (Darnell *et al.* 1994, Leaman *et al.* 1996, Darnell 1998, Schindler 1999, Ihle 2001). In receptor-bound Stats, a conserved tyrosine residue located in the C-terminal domain is phosphorylated by the Janus kinase (Jaks). Then, the SH2 domain in each of two Stats assembles through reciprocal phosphotyrosine/SH2 interactions leading to dimerization. Once dimerized, the proteins are translocated into the nucleus, where they regulate transcription of several different genes. So far, seven Stat proteins have been identified: Stat1–6 and Stat5b (Darnell 1997, O'Shea *et al.* 2002).

Research in interferon-mediated signaling pathways led to the discovery of Stat1 and Stat2. Interferon (IFN)-γ induces Stat1 homodimerization, leading to transcription of γ activated sequence (GAS)-driven genes. Both IFN-α and Interferon (IFN)-β induce formation of either Stat1 homodimers or Stat1/Stat2 heterodimers (Kisseleva *et al.* 2002). In addition, Stat1 can be activated either by growth factor-mediated signal pathways, or by a G-protein-coupled receptor (Brivanlou & Darnell 2002).

In lymphocytes, Stat4 may be activated by IFN-α or interleukin (IL)-12 (Cho *et al.* 1996). In T cells, only IL-12 treatment activates Stat4 (Jacobson *et al.* 1996). In the immune system, Stat4 is also activated by IL-23 (O'Shea *et al.* 2002).

Even though the spermatozoon is considered as a transcriptionally silent cell, it does contain some transcription factors (Herrada & Wolgemuth 1997, D'Cruz *et al.* 2001, Pittoggi *et al.* 2001). In spermatozoa, the Jak–Stat members detected to date are tyrosine kinase 2 (Tyk2), Stat4 and Stat1 (Herrada & Wolgemuth 1997, D'Cruz *et al.* 2001). Stat4 was identified in the perinuclear theca (PT) of mouse sperm (Herrada & Wolgemuth 1997).

Freshly ejaculated spermatozoa are unable to fertilize the egg. These cells need to undergo two physiological processes in the female reproductive tract in order to acquire the capacity to fertilize. The first process is known as capacitation. During capacitation, membrane fluidity increases, cholesterol is lost, and intracellular calcium and cAMP concentrations increase; protein phosphorylation occurs and the swimming sperm pattern is modified (Breitbart & Naor 1999). The second process occurs after gamete capacitation and is known as the acrosome reaction (AR). In AR, the outer acrosomal membrane fuses with the plasma membrane (Yanagimachi 1995). After capacitation and AR, spermatozoa become able to fertilize the egg.

It has become clear that in addition to DNA, sperm cells contribute to the egg with RNA and proteins. These molecules are very likely crucial in the early stages of embryonic development. Following this order of ideas, we decided to test guinea pig spermatozoa for the

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ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-06-0264
Online version via www.reproduction-online.org

presence of transcription factors and their possible activation during the processes of sperm capacitation and AR. In guinea pig spermatozoa, we determined the presence and location of Stat1 and Stat4, as well as their possible activation during capacitation and AR. Here, we show that Stat1 was phosphorylated both during capacitation and AR. Additionally, during capacitation, the distribution of Stat1 was modified: it increased in the theca/flagellum fraction, while it decreased in the cytoplasm. In addition, in acrosome-reacted sperm, Stat1 was reorganized in the nucleus. In contrast to Stat1, Stat4 was not phosphorylated, neither at capacitation nor at AR. Furthermore, we did not detect any change in its localization pattern.

Materials and Methods

Antibodies and reagents

Pyruvic acid, lactic acid, DL-dithiothreitol (DTT), sucrose, Triton X-100, aprotinin, phenylmethylsulfonyl fluoride (PMSF), Tris, sodium orthovanadate, SDS, cetyltrimethylammonium bromide (CTAB), bisindolylmaleimide I (Bis I), genistein and EGTA, *N*-[2-(*p*-bromocynamilamino)etil-]-5-isoquinesulfonamide (H-89), and genistin were purchased from Sigma. Protein A-agarose and the protease inhibitor cocktail Complete were purchased from Roche. Nitrocellulose membranes, acrylamide, and *N,N′*-methylene-*bis*-acrylamide were purchased from Bio-Rad. The antibodies used were: rabbit polyclonal anti-Stat1 (sc-592), rabbit polyclonal anti-Stat4 (sc-486), and rabbit polyclonal anti-*p-Tyr* (sc-508), all of them from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-labeled anti-rabbit and anti-mouse, tetramethyl-rhodamine isothiocyanate (TRITC)-labeled anti-rabbit were from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). The ECL was obtained from Amersham.

Capacitation and acrosome reaction induction in spermatozoa

Ductus deferens and cauda epididymal guinea pig spermatozoa were obtained and washed in 154 mM NaCl, as previously described (Trejo & Mujica 1990). To start capacitation, 35×10^6 sperm cells/ml were incubated at 37 °C in minimal culture medium containing lactate and pyruvate (MCM-PL) without glucose (Rogers & Yanagimachi 1975). Incubation time varied for each experimental condition (see Results). The acrosome reaction was induced at 90 min of incubation in capacitating medium by adding the calcium ionophore A23187 (1.3 μM) and further incubating for 3 min. The sample was centrifuged at 5000 ***g***. Pelleted cells were processed for Western blotting.

Kinase inhibitor treatment

Spermatozoa at 35×10^6 cells/ml were preincubated in 154 mM NaCl with PKA inhibitor EGTA (1 mM), or H89 (30 μM), the PKC inhibitor Bis I (1 μM), or the tyrosine kinase inhibitor genistein (100 μM) or its inactive homolog genistin (100 μM) for 30 min. Then, they were incubated in MCM-PL capacitating medium in the presence or absence of the corresponding inhibitor at the same concentrations.

Sperm nuclei isolation

For nuclei isolation, a previously described technique was followed (Ocampo *et al.* 2005) with slight modifications. NaCl-washed spermatozoa were resuspended in buffer A (50 mM Tris–HCl (pH 7.4) plus a commercial mixture of protease inhibitors, Complete, according to the manufacturer's instructions), and treated with 25 mM DTT for 20 min with constant agitation at 4 °C. Afterwards, 1.2% of CTAB was added and the sample was centrifuged for 3 min at 5000 ***g*** at 4 °C. The pellet (nuclei) was washed thrice by suspension/centrifugation with 1 M sucrose, treated with 5 U heparin for 35×10^6 cells for 15 s, fixed with 1.5% formaldehyde in PBS, and handled for immunofluorescence.

Immunofluorescence

Cells or nuclei preparations were fixed with 1.5% formaldehyde on PBS (Moreno-Fierros *et al.* 1992). Smears on glass slides were prepared. Then, cells were permeabilized with acetone at −20 °C for 7 min and washed thrice in PBS. Cells were incubated overnight at 4 °C either with anti-Stat1 or with anti-Stat4 antibodies in 1% BSA in PBS. The binding of the primary antibodies was revealed using appropriate rhodamine-labeled secondary antibodies. The cells were observed in a Zeiss photomicroscope equipped with phase contrast and epifluorescence optics or using a Leica TCS SP2 confocal microscope with Krypton/Argon laser.

SDS-PAGE and Western blotting

Washed spermatozoa (35×10^6 cell) were solubilized in 1 ml lysis buffer (LB: 50 mM Tris–HCl (pH 7.4), 1 mM EGTA, 1 mM PMSF, Complete, 1 μg/ml aprotinin, 10 mM sodium orthovanadate, 25 mM sodium fluoride, and 1% Triton X-100), and then centrifuged for 3 min at 5000 ***g*** at 4 °C. The supernatants (Triton X-100 extracts) were collected. The pellets were washed thrice with LB without Triton X-100 to remove any contaminant and treated with 25 mM DTT for 20 min at 4 °C; then 1.2% CTAB (Hernández-Montes *et al.* 1973) was added and the sample centrifuged for 3 min at 5000 ***g*** at 4 °C. The supernatants (containing solubilized theca and flagellum

proteins) were collected. The pellets (nuclei) were washed thrice with LB and resuspended in LB/0.5% SDS without Triton X-100. The protein concentration of all extracts was determined (Bradford 1976). Protein extracts were denatured with Laemmli's sample buffer (Laemmli 1970) and resolved through 10% polyacrylamide-SDS gels and transferred to nitrocellulose membranes (Towbin *et al.* 1979). Membranes were incubated in 5% dried skimmed milk in TBS/T (TBS–T: 150 mM NaCl, 100 mM Tris–HCl (pH 7.6) plus 0.1% Tween 20) for 1 h to block non-specific binding sites. Then they were incubated overnight at 4 °C with the appropriate primary antibody diluted in blocking solution and washed. Incubation with the HRP-labeled secondary antibody was carried out for 1 h at 37 °C. HRP was developed by chemiluminescence kit, ECL.

Immunoprecipitation

Ten microliters of protein A-agarose were incubated with 0.4 µg of the appropriate antibody for 5 min. Then 1 mg sperm protein from the selected extract to be assayed was added. The mixture was kept under constant agitation overnight at 4 °C. Immune complexes were recovered by centrifugation (5000 ***g*** for 5 min). Then the samples were washed thrice with NET buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 20 mM sodium orthovanadate, 20 mM sodium molybdate, 50 mM sodium fluoride, and 1% Triton X-100 (pH 7.5)). The pellet was boiled for 5 min in 30 µl Laemmli's sample buffer. Equal amounts of solubilized material were analyzed through 10% polyacrylamide-SDS gels, and the proteins revealed by Western blotting.

Statistical analysis

Data shown are the means ± S.D. of at least three experiments. Statistically significant differences among conditions were analyzed. In Figs 3, 4A and C and 5, we used a one-way ANOVA test with Tukey's *post hoc* multiple comparison test. In Figs 4B and 6, we used Student's *t*-test.

Results

Identification and localization of Stat1 and Stat4 in guinea pig spermatozoa

We decided to evaluate whether the transcription factors Stat1 and Stat4 were present in guinea pig spermatozoa. Immunoblot analysis of Triton X-100 sperm extracts revealed the presence of both Stat1 and Stat4 (Fig. 1A). The immunopositive band revealed in guinea pig sperm (S) comigrated with the ones from mouse testis (T), brain (B), and HeLa cells (H), which are well known for their high levels of Stat1 expression. A similar situation was observed in the immunoblot for Stat4, where the immunopositive band of guinea pig sperm (S) had the same electrophoretic mobility as the positive protein band from the mouse testis whole extract (T). Two negative controls were performed in guinea pig Triton X-100 sperm extracts: one without the first antibody and the other with preimmune serum in place of the first

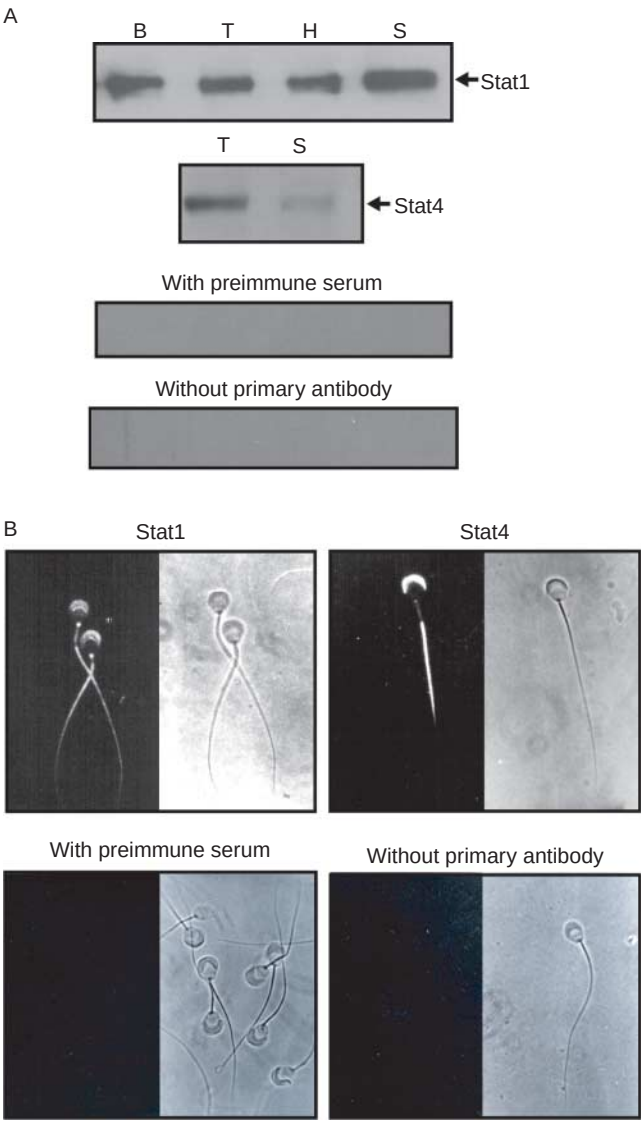


Figure 1 Stat1 and Stat4 identification in guinea pig spermatozoa. Non-capacitated ductus deferens and cauda epididymal spermatozoa were resuspended in lysis buffer. Triton X-100 extracts and controls were electrophoresed in 10% SDS-PAGE. Positive expression of Stat1 and Stat4 is shown in A. Sperm protein (S) and positive controls: mouse brain (B), mouse testis (T), and HeLa cells (H). To test the specificity of the signal, samples as in the first panel were tested in western blot by avoiding the primary antibody or using a preimmune serum. B shows the immunolocalization of Stat1 and Stat4 in fixed and permeabilized guinea pig sperm. Two negative controls were performed: spermatozoa were treated without the first antibody or stained with preimmune serum instead of the primary antibody. Each IIF is accompanied by its phase contrast image.

antibody; negative results were obtained in both controls (Fig. 1A, lower panels).

In parallel, the cellular localization of both proteins was assessed by indirect immunofluorescence. As it can be noted (Fig 1B), Stat1 was detected in the acrosomal and neck regions and also along the flagellum, although less fluorescence was observed in the middle piece. Stat4 was localized in the acrosomal region, along the flagellum with less fluorescence in the middle piece. In addition, negative controls were performed: 1) without the first antibody; and 2) with preimmune serum in place of the first antibody, both with negative results (Fig. 1B).

Stat4 has been detected in the PT of mouse sperm; but localization of Stat1 was not assessed (Herrada & Wolgemuth 1997). As mentioned previously, we localized both Stat1 and Stat4 in the Triton X-100 guinea pig sperm extracts (Fig. 1A). In acrosome–plasma membrane–free sperm, Stat1 was located mainly in the flagellum and a light fluorescence was also observed in the middle piece; on the other hand, Stat4 was observed in the apical zone of the theca and along the flagellum (Fig. 2A).

The PT is a unique cytoskeletal mammalian sperm structure, which surrounds the nucleus and does not allow antibodies to enter the nucleus (Zepeda-Bastida, unpublished data). In addition, the sperm nucleus is very compact. Therefore, in order to detect Stat proteins within the nuclear compartment, we treated DTT–CTAB nuclei preparations (nuclei without nuclear membrane and PT) with heparin to promote slight nucleus decondensation before antibody staining. With this procedure, both Stat1 and Stat4 were detected inside the nucleus (Fig. 2B). Both Stat1 and Stat4 located mainly in the apical zone of the nucleus and a postacrosomal cone was also detected. No fluorescence was observed at the equatorial region. For negative controls, DTT–CTAB nuclei were treated with the second antibody, but without the first one or, alternatively, with preimmune serum instead of the first antibody. Both controls gave negative results. Finally, the immunoblot analysis of protein extracts from Triton X-100 (Cyt), theca/flagellum (T/F), and nucleus (Nuc) revealed the presence of both Stat1 and Stat4 proteins in all subcellular fractions assayed (Fig. 2C). In addition, no contamination of the soluble fraction to the theca/flagellum and nucleus was found using immunodetection of the focal adhesion kinase as a marker (data not shown).

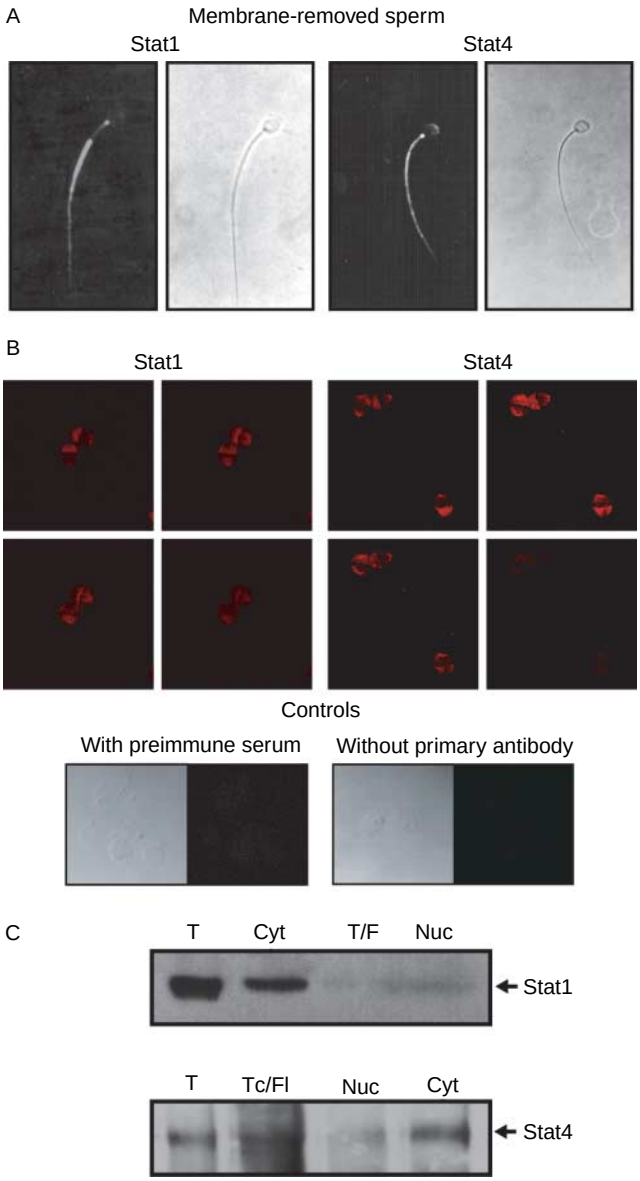


Figure 2 Determination of Stat1 and Stat4 in subcellular regions of guinea pig sperm. Non-capacitated sperm were treated with lysis buffer in order to solubilize the plasma membrane and the acrosomal membrane. Membrane-free spermatozoa were fixed and handled for immunofluorescence (A). Spermatic nuclei (DTT–CTAB) were fixed and stained with anti-Stat1 and anti-Stat4 antibodies; B shows different focal planes of the same preparation. Negative controls were as in Fig. 1 and each IIF is accompanied by its phase contrast image in A and B. Proteins from subcellular fractions: cytosol/membranes (Cyt), theca/flagellum (T/F), nuclei (Nuc), and mouse testis (T) were subjected to SDS-PAGE and Western blotting (C).

Capacitation-induced phosphorylation of Stat1

Phosphorylation at a tyrosine residue promotes dimerization of Stat proteins (Heim *et al.* 1995, Gupta *et al.* 1996, Ihle 1996), as well as their nuclear translocation (Shuai *et al.* 1994), and DNA-binding activity (Mowen & David 1998). Sperm capacitation has been shown to be highly correlated with protein phosphorylation at tyrosine residues (Visconti *et al.* 1995*a*, 1995*b*). In order to know whether the capacitation process induces changes in the pattern of Stat protein phosphorylation, Triton X-100 extracts from cells incubated in the capacitating medium, MCM-PL, were immunoprecipitated with anti-phosphotyrosine antibodies and immunoblotted with either anti-Stat1 or anti-Stat4. A significant increase in phosphorylation of Stat1 was detected after 30 min of capacitation; however, after

60 min, phosphorylated Stat1 decreased to a basal level, as seen in non-capacitated sperm. The reciprocal experiment, immunoprecipitation with anti-Stat1 antibody and Western blotting with anti-phosphotyrosine, produced the same result (Fig. 3A). In contrast, phosphorylation of Stat4 was not observed regardless of whether spermatozoa were capacitated or not (Fig. 3B).

It has been reported that tyrosine phosphorylation of the cytosolic Stats induces them to dimerize and translocate into the nucleus (Heim *et al.* 1995, Gupta *et al.* 1996, Ihle 1996). Therefore, we tested whether tyrosine phosphorylation decreases the cytosolic levels of Stat1. In comparison with non-capacitated spermatozoa at 30, 60, and 90 min of capacitation, we observed a significant decrease in cytosolic Stat1 protein (Fig. 4A); it is noteworthy that after 30 min of capacitation, there are no significant differences in Stat1 diminution ($P > 0.05$). In addition, when Stat1 was measured in the theca/flagella fraction, the protein was increased in capacitated sperm (Fig. 4B). When the cells were treated with genistein, a broad tyrosine kinase inhibitor, it prevented the decrease of cytosolic Stat1 protein. As a control, genistin, an inactive molecule structurally related to genistein, was tested. Genistin did not cancel Stat1 translocation (Fig. 4C). The result indicated the specificity of a tyrosine kinase inhibitor. Tubulin, which is observed in the sperm head (Dvorakova *et al.* 2005), was used as loading control. Gel densitometry was performed in each case and it is shown below each panel.

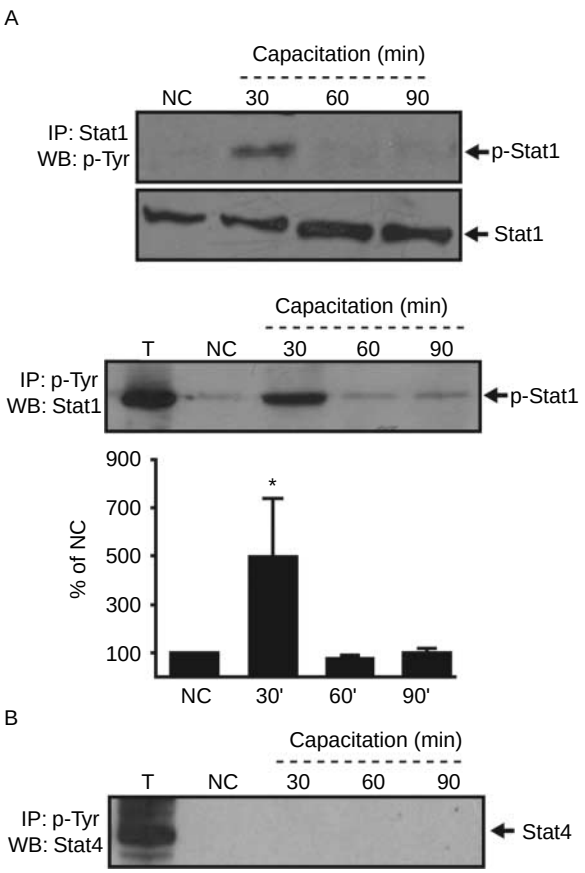


Figure 3 Capacitation induces phosphorylation of Stat1. Phosphorylation of Stat1 and Stat4 was tested in sperm incubated in MCM-PL capacitating medium for the indicated time. Triton X-100 extracts were immunoprecipitated with anti-phosphotyrosine antibody (p-Tyr) or anti-Stat1 antibody. Coprecipitated proteins were washed and subjected to western blotting using anti-Stat1 or anti-p-Tyr respectively (A). B shows immunoprecipitation from Triton X-100 extracts with anti-pTyr antibody and western blotting with anti-Stat4 antibody. Quantification of Stat1 results by densitometry is shown below in A. Results are means ± S.D. of triplicate experiments. *$P < 0.05$ versus non-capacitated group (ANOVA).

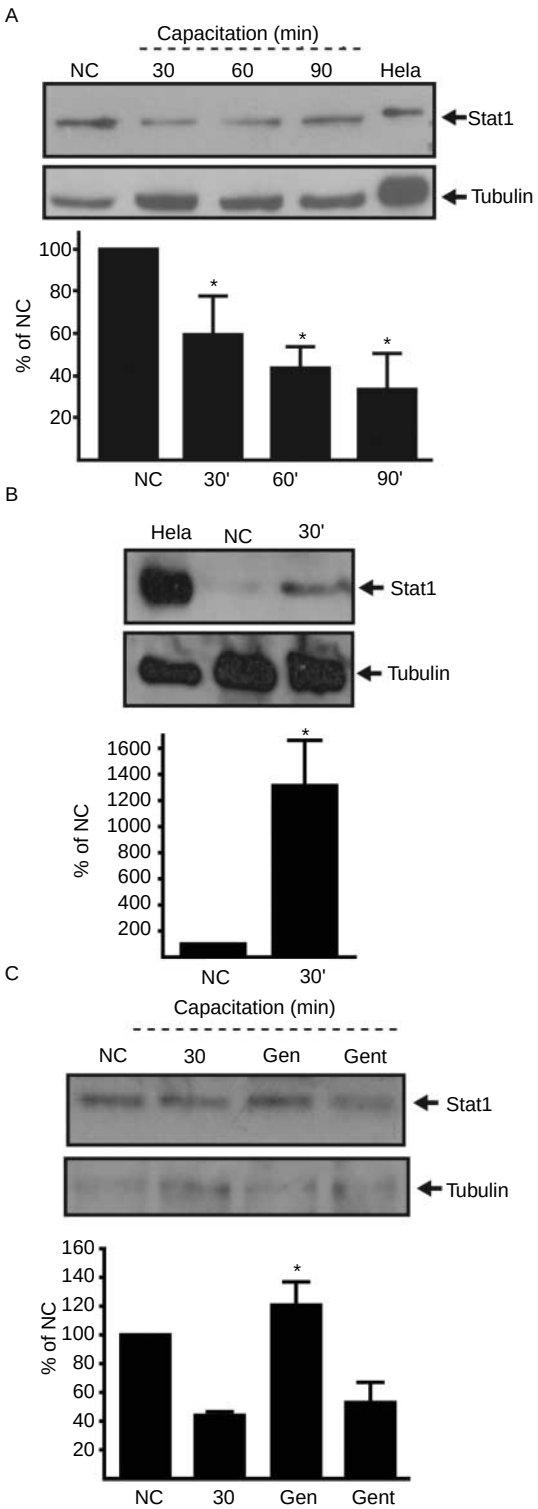
Effect of PKA, PKC, and calcium on cytosolic Stat1

Capacitated sperm exhibited a significant decrease in cytosolic Stat1 levels (Fig. 4). Additionally, inhibition of tyrosine kinase with genistein prevented the decrease in cytosolic Stat1 normally seen during capacitation (Fig. 4C). The activation of PKA plays a major role in capacitation. This is true also for the calcium-mediated activation of PKC. Thus, both protein kinases should regulate protein phosphorylation to promote activation. In addition, it has been demonstrated that PKA and PKC are involved in Stat1 activation (Lee & Rikihisa 1998,Sakhalkar *et al.* 2005, Salonen *et al.* 2006). Therefore, we decided to determine whether treatment of sperm with inhibitors of both kinases and/or chelation of external calcium modified Stat1 translocation. The results shown in Fig. 5 indicate that by preventing PKA or PKC activation or by chelating calcium with EGTA, Stat1 translocation was blocked. The cytosolic Stat1 concentration in the cells treated with inhibitors was not significantly different from that found in non-capacitated sperm.

Phosphorylation of Stat1 during acrosome reaction

Considering that phosphorylation of cytosolic Stat1 decreased some time after capacitation, and also that Stat4 was never phosphorylated during the capacitation process, we decided to determine whether further changes in phosphorylation occur during the acrosome reaction (AR). To do this, synchronous AR was induced with A23187 at 90 min capacitation, when Stat1 phosphorylation was decreased to basal level (Fig. 3A). Then Triton X-100 extracts were obtained and immunoprecipitated with anti-phosphotyrosine. Increased phosphorylation was observed in the western blot of immunoprecipitated proteins revealed with anti-Stat1 (Fig. 6A). However, as in capacitation, Stat4 was not phosphorylated in the acrosome-reacted spermatozoa (Fig. 6B). In order to determine whether AR induces changes in the location pattern of the Stat1 in the nuclear compartment, nuclei obtained from non-capacitated and AR sperm were stained with anti-Stat1 antibodies.

Stat1 was detected in the two nuclear hemispheres in both samples. In acrosome-reacted spermatozoa, Stat1 was detected in the equatorial region, forming a belt in the middle of the nucleus, which was not present in non-capacitated sperm (Fig. 6C).



Discussion

In order for spermatozoa to acquire the ability to fertilize, capacitation and AR must occur as these cells travel through the female reproductive tract. The spermatozoon is a highly differentiated and transcriptionally silent cell; nevertheless, it does contain some transcription factors (Herrada & Wolgemuth 1997, D'Cruz *et al.* 2001, Pittoggi *et al.* 2001). Among them, Stat1 was reported in human spermatozoa, whereas Stat4 was reported in both human and mouse spermatozoa; mouse Stat4 was located in the sperm PT (Herrada & Wolgemuth 1997, D'Cruz *et al.* 2001).

Recent reports suggest a new view, in which sperm deliver not only the male genome to the egg, but also several crucial molecules needed for fertilization as well as development of the embryo (Ostermeier *et al.* 2002, 2004, 2005, Saunders *et al.* 2002, Piston *et al.* 2004, Ainsworth 2005). Here, we report the presence of both Stat1 and Stat4 transcription factors and their location in guinea pig sperm subcellular fractions. Additionally, we show that the Stat1-phosphorylated state varies during capacitation (Fig. 3A) and AR (Fig. 6A). Interestingly, Stat1 phosphorylation during capacitation occurred together with a decrease in the cytosolic level of Stat1, which paralleled an increase in the concentration of Stat1 in the theca/flagellum fraction (Fig. 4A and B).

As can be noted in Fig. 3, Stat1, but not Stat4, was phosphorylated in response to capacitation, suggesting that different signal pathways work in the sperm to activate two members of the same family. The increase in Stat1 phosphorylation was transient, peaking at 30 min of incubation in MCM-PL medium. Then, it decreased to basal levels at 60 and 90 min of capacitation. Intriguingly, cytosolic Stat1 was dephosphorylated, indicating the presence of phosphatases. Nevertheless, once the AR began, Stat1 became highly phosphorylated once again (Fig. 6). These results also implicate that the kinase-mediated phosphorylation of Stat1 is a tightly regulated process: the first phosphorylation would allow Stat1 to move to cellular compartments such as the theca, flagellum, and nucleus, all of which enter the egg at fertilization. The second phosphorylation would allow Stat1 to be active during the first rounds of transcription in the male pronucleus.

Figure 4 Stat1 concentration decreases in the cytosol of capacitated sperm. Triton X-100 extracts obtained from capacitated sperm for the times indicated were prepared for immunoblot analysis, 100 μg protein were loaded per lane and immunoblotted using anti-Stat1 (A). Extracts obtained from theca/flagella fraction were immunoblotted with anti-Stat1 (B). Sperm were preincubated in NaCl with 100 μM genistein or 100 μM genistin, then capacitated in MCM-PL medium plus 100 μM genistein or 100 μM genistin for 30 min. Proteins were subjected to Western blotting (C). Densitometry data of triplicate experiments were normalized against tubulin, used as loading control. Graphs show means±S.D. $*P<0.05$ versus non-capacitated group (ANOVA) is shown in A and B, whereas in C $*P<0.05$ versus capacitated group is shown.

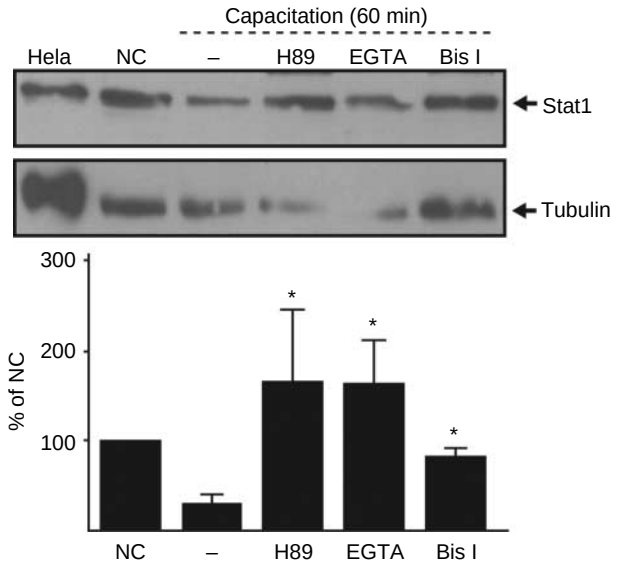


Figure 5 Effect of protein kinase inhibitors on the cytosolic Stat1 protein in capacitated sperm. Triton X-100 extracts were obtained from non-capacitated (NC) and 60-min MCM-PL capacitated sperm or this last sample plus the following kinase inhibitors: 1 mM EGTA, 30 μM H89, and 1 μM bisindolylmaleimide I (Bis I). The extracts were immunoblotted with anti-Stat1 antibody. Stat1 cytosolic levels were normalized against tubulin. Results in the graphs show means ± S.D. of triplicate experiments. $^*P<0.05$ versus capacitated group (ANOVA).

The data indicate that Stat1 phosphorylation in capacitated and acrosome-reacted spermatozoa could be significant from a functional point of view. Supporting this idea is the large decrease of Stat1 concentration in the cytosol, together with the observed increase in the theca/flagellum fraction (Fig. 4). Thus, it may be suggested that phosphorylation signals induce a change in the localization of Stat1. Furthermore, genistein, a broad tyrosine kinase inhibitor, canceled the migration of Stat1.

During capacitation, cAMP levels increase in response to production of reactive oxygen species, leading to PKA activation. Blocking this signal pathway inhibits capacitation (White & Aitken 1989, Visconti *et al.* 1997, Breitbart & Naor 1999). Moreover, in spermatozoa, activation of PKA increases the level of tyrosine-phosphorylated proteins (Thundathil *et al.* 2002). In addition, during capacitation, intracellular calcium is increased, which in turn would induce activation of PKC and regulation of tyrosine phosphorylation (Visconti *et al.* 1995*a*, 1995*b*, Luconi *et al.* 1996, Leclerc *et al.* 1998, Baker *et al.* 2004). Although PKC participation in capacitation remains controversial, there is evidence supporting the activation of PKC during capacitation (Furuya *et al.* 1993, Cohen *et al.* 2004, Breitbart *et al.* 2006, O'flaherty *et al.* 2006). In this work, inhibition of Stat1 translocation was observed in spermatozoa treated with EGTA, H-89, or Bis I. Thus, results in capacitation clearly show that for cytosolic Stat1 translocation to occur, all PKA, PKC, and calcium are needed to promote the phosphorylation of Stat1. It is well known that PKA activation increases tyrosine phosphorylation; also the phosphorylated PKA participates in Stat tyrosine phosphorylation (Deo *et al.* 2004, Sakhalkar *et al.* 2005). It is quite possible that PKA, PKC, and calcium are involved

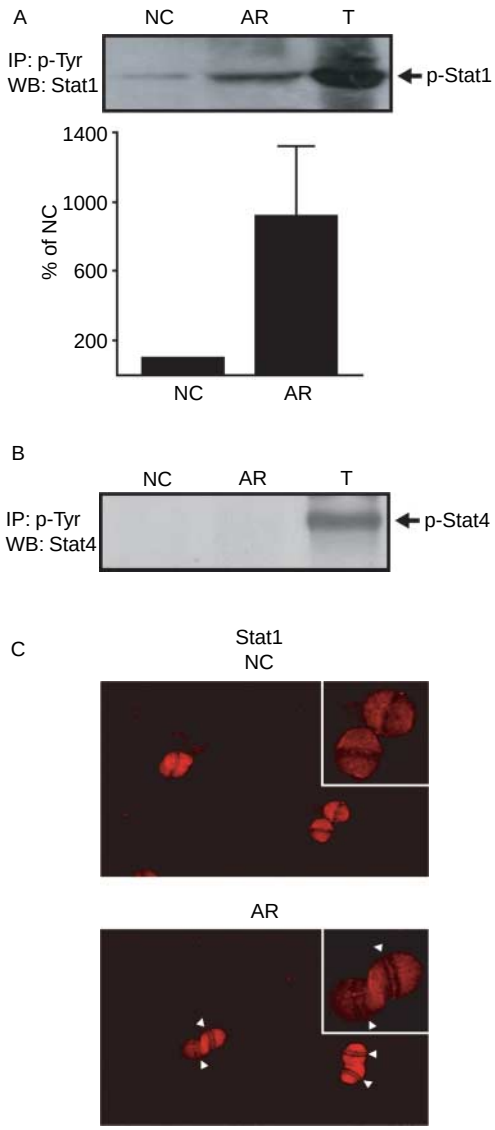


Figure 6 Acrosome reaction enhances tyrosine phosphorylation of Stat1. Phosphorylation of Stat1 and Stat4 was determined in acrosome-reacted spermatozoa. In spermatozoa capacitated in MCM-PL for 90 min, the acrosomal reaction was induced with 1.3 μM calcium ionophore A23187. Acrosome-reacted spermatozoa were collected at 5000 ***g***. Pelleted spermatozoa were added with lysis buffer. Triton X-100 extracts immunoprecipitated with anti-p-Tyr were subjected to immunoblot analysis and revealed with anti-Stat1 (A). Quantification of the results by densitometry is shown below in A. Mean ± S.D. of at least three experiments. $^*P<0.05$ versus non-capacitated group. B, Stat4 phosphorylation in Triton X-100 extracts of spermatozoa as in A. Acrosome-reacted (AR) or non-capacitated sperm (NC) extracts are shown alongside with mouse testis (T) extracts as positive control. Nuclei (DTT–CTAB) from non-capacitated and acrosome-reacted spermatozoa were fixed and stained with anti-Stat1. Arrows indicate a belt area formed by Stat1 in the middle of the nucleus in AR sperm (C).

in the same signal pathway, where either PKA is necessary to activate PKC (Sakhalkar *et al.* 2005), or calcium and cAMP work together to activate PKA. Subsequently, calcium channels would become activated with a concomitant activation of PKC through PLCγ. However, that scenario would only be possible in the late phase of capacitation (Breitbart 2002). On the other hand, it has recently been shown that PKC is active during capacitation, but activation of PKA leads to an inhibition of PKC (Cohen *et al.* 2004, Breitbart *et al.* 2006). It is possible that PKC-dependent Stat1 phosphorylation occurs before PKA blocks PKC activity. In the short term, this would result in the first calcium waves. If that were the case, it would indicate that two different signal pathways converge in a common substrate: a candidate is Stat1 by itself, and another could be a Src-family member (Chang *et al.* 2004, Deo *et al.* 2004, Salonen *et al.* 2006), like c-yes (Leclerc & Goupil 2002). In addition, another non-receptor tyrosine kinase like PYK2 might be involved (Takaoka *et al.* 1999, Chieffi *et al.* 2003).

As mentioned previously, Stat1 becomes phosphorylated a second time during AR; interestingly, during AR, intranuclear Stat1 was reorganized (Fig. 6C). A question that comes to mind is whether intranuclear Stat1 is moving toward the site where the first transcription will take place. The fact that Stat1 locates at the internuclear equatorial zone of the acrosome-reacted sperm is very suggestive.

The present study provides evidence that the transcription factor Stat1, but not Stat4, can be activated during the last two steps of sperm preparation for fertilization. In addition, we provide evidence for the mechanism by which a transcription factor can move toward spermatic structures that will enter the female oocyte during fertilization. In opposition to somatic cells, where transcription factors move directly from the cytoplasm into the nucleus, in sperm the theca does not allow a direct movement. Thus, the strategy has to be different: transcription factors should move into the theca and flagellum and wait until the fertilization process begins to reach their final destination, the pronucleus. In addition, we show two kinases involved in Stat1 activation. Current work in our lab is underway to establish the signal pathway involved in Stat1 activation.

Acknowledgements

This work was supported by Consejo Nacional de Ciencia y Tecnología (Conacyt)-Mexico (grant 41725-Q to A M). Y B and A Z were supported by a Conacyt doctoral fellowship. The technical assistance of M en C Ana Lilia Roa Espitia, Víctor Cortés, M en C Alma Olivia Monrroy Lopez and QBP, Blanca Estela Reyes Marquez is acknowledged. We wish to thank Dr J Eduardo Perez Salazar for the critical reading and comments on the manuscript. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 11 October 2006
First decision 9 November 2006
Revised manuscript received 20 June 2007
Accepted 25 June 2007