Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa

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BACKGROUND: Protein tyrosine phosphorylation is one of the main processes associated with sperm activation. Although this process and its targets have been well characterized, only few tyrosine kinases have been identified so far and their roles in spermatozoa are still largely unknown. In this study, we report the presence and localization of Src kinase in ejaculated human spermatozoa and investigate its role in regulating the processes underlying sperm activation. METHODS AND RESULTS: Specific anti-Src antibodies, against different epitopes of the protein, identified a single band of ~70 kDa relating to a protein which is mainly localized in the post-acrosomal region of the head, neck and midpiece. By immunoprecipitation and immunofluorescence techniques performed with antibodies against Src phosphorylated at Tyr416, which identifies the active kinase, we showed an increased phosphorylation during sperm capacitation. Blocking Src activity with SU6656 resulted in a significant reduction in the protein tyrosine phosphorylation. Moreover, this inhibitor also blocked the progesterone-induced acrosome reaction and interfered with the calcium response to progesterone evaluated in fura-2-loaded spermatozoa. No effect on sperm motility and hyperactivation resulted from incubation with SU6656. CONCLUSIONS: We identified a novel Src isoform in human spermatozoa, which appears to be involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and acrosome reaction.

Keywords: tyrosine kinase; sperm; capacitation; acrosome reaction; Src

Introduction

In order to reach the site of fertilization and to be able to fertilize, ejaculated human spermatozoa need to undergo a series of activating events induced during the sperm transit in the female genital tract, culminating in the process of capacitation, a complex biochemical remodeling of the sperm surface which enables spermatozoa to interact with the zona pellucida glycoproteins surrounding the oocyte (Yanagimachi, 1994). Sperm capacitation is associated with the acquisition of hyperactivation, a peculiar type of motility generating the propulsive forces necessary to penetrate the cumulus oophorus and the zona pellucida vestments of the oocyte (Luconi et al., 2006). The release of the lytic enzymes from the acrosomal vesicle in the sperm head through the acrosome reaction induced by progesterone or by sperm binding to the zona pellucida finally enables the sperm to cross the zona pellucida and interact with the oocyte. All these events can also be induced in vitro by incubating spermatozoa in media mimicking the tubal and follicular fluids and are strictly regulated by intracellular signals consisting of post-translational modifications of key proteins. Indeed, modulation of sperm functions cannot rely on activation or repression of specific master genes, since mature spermatozoa are transcriptionally and translationally silent, due to the high condensation of sperm chromatin and total loss of the translational machinery occurring during spermiogenesis (Yanagimachi, 1994). Phosphorylation and de-phosphorylation of sperm proteins, particularly on tyrosine residues, play a pivotal role among the post-translational regulatory processes (Visconti et al., 2002). Although a great effort has been spent in identifying the protein targets of such modifications (Baker et al., 2007), only a few kinases and phosphatases have been identified and characterized so far. Recently, the tyrosine kinase Src has been proposed as mediating PKA induction of sperm capacitation in the mouse (Baker et al., 2006) and in humans (Lawson et al., 2008; Mitchell et al., 2008).

The structure and the functional role of human Src in somatic cells have been widely described. Structurally, Src is characterized by several domains in addition to the kinase one (SH1): an SH2 domain, an SH3 domain, an N-terminal
region which can be palmitoylated or myristoylated and a flexible tail which contains tyrosine residues important for Src modulation. Among them, two tyrosine residues are pivotal for Src regulation. In basal conditions, 90–95% of Src is phosphorylated at Tyr527 (Zheng et al., 2000), and an intramolecular interaction with the SH2 domain stabilizes a silent conformation of the enzyme (Roskoski, 2005). Upon stimulation, Src undergoes an intermolecular auto-phosphorylation process at Tyr416; this residue is localized inside the activation loop, and its phosphorylation allows the opening of the catalytic site inducing the kinase activity (Roskoski, 2004).

In somatic cells, Src is implicated in several functions such as cross-talk and cell–cell interactions, migration, the cell cycle and apoptosis. In particular, an interaction between Src and the focal adhesion kinase (FAK) during the organization of the focal adhesion complexes has been shown (Ruoslahti, 1999). Src is also involved in the signaling cascade downstream of several receptors with tyrosine kinase activity, such as the epidermal growth factor receptor (EGF-R). Over-expression of Src results in increased cell responses to EGF, from DNA synthesis and progression through the cell cycle to protein phosphorylation (Wilson et al., 1989; Maa et al., 1995), suggesting the existence of a complex regulatory loop between Src kinase activity and the signal transduction mediated by these receptors. Moreover, Src is involved in the regulation of mitogen-activated protein kinase and phosphatidylinositol-3-kinase (PI3K) activation, whose downstream-signaling pathways regulate the delicate balance between survival and apoptosis, finally controlling cell death (Yao and Cooper, 1995; Kauffmann-Zeh et al., 1997).

Even though the role of Src in somatic cells is well understood, the presence and the possible role of this kinase in the male gamete have been addressed only recently (Baker et al., 2006; Lawson et al., 2008; Mitchell et al., 2008). The same pp60 Src has been found to be involved in the PKA stimulatory effect on capacitation and tyrosine phosphorylation in both mouse (Baker et al., 2006) and human spermatozoa (Lawson et al., 2008; Mitchell et al., 2008). However, PKA stimulation of sperm motility appears to be mediated by Src only in mouse spermatozoa (Baker et al., 2006; Mitchell et al., 2008). Thus, the present study aims at clarifying the role of Src in ejaculated human spermatozoa, evaluating its functional activity in the main processes of sperm activation, such as protein phosphorylation, capacitation and motility, and also characterizing for the first time the possible involvement of Src in regulating intracellular calcium fluxes and acrosome reaction.

Materials and Methods

Antibodies and chemicals

Human serum albumin (HSA), human tubal fluid (HTF) and all the other reagents for human sperm preparation were from Irvine (Santa Ana, CA, USA). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and for bidimensional electrophoresis (2DE) were from GE Healthcare (Cologno Monzese, Italy). The conjugated secondary antibodies, protein A- and G-Sepharose, EGF, pentoxifylline (PF) and other not specified reagents were from Sigma Chemical Co. (St Louis, MO, USA). Peroxidase-conjugated PY20 (PY20-HRP) antibody, Src inhibitor SU6656, dibutyryl cyclic adenosine monophosphate (dbcAMP), fura-2/AM and PY20 mouse antibody against tyrosine-phosphorylated proteins were obtained from Calbiochem (La Jolla, CA, USA). Anti-AKAP3 was kindly provided by Professor Daniel Carr (Veteran Affairs Medical Center, Oregon Health Science University, Portland, OR, USA). Anti-PKA catalytic subunit antibody was from Upstate Biotechnology (Charlotteville, VA, USA); anti-phospho Tyr416 (pY416) and anti-phospho Tyr527 (pY527) Src antibodies, as well as the polyclonal antibody directed against the phosphorylated RXS/T motif specific for PKA substrates were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit anti-Src antibodies (C and N terminal) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse anti-Src antibodies were from Calbiochem and from Upstate Biotechnology (Lake Placid, NY, USA).

Preparation of spermatozoa

Human semen was collected in our laboratory according to the WHO-recommended procedure (WHO, 1999) by masturbation from men undergoing semen analysis for couple infertility due to a female factor. All the experiments were performed using samples from men meeting the WHO criteria for normozoospermia (WHO, 1999). Samples with leukocytes and/or immature germ cell concentration >10^6/ml were not included in the study. Sperm samples were processed by swim up or minipercoll techniques as described previously (Luconi et al., 2004) in non-capacitating (NC) conditions (HTF–Hepe without HSA) and then allowed to capacitate in 25 mM bicarbonate and 10% HSA-containing HTF medium or dbcAMP+PF.

Evaluation of sperm motility and viability

Motility was evaluated by computer-assisted semen analysis (CASA, Hamilton Thorn Research, Beverly, MA, USA) as described previously (Luconi et al., 2001).

For each sample, the following parameters were evaluated: VAP (average path velocity), VCL (curvilinear velocity), VSL (straight-line velocity), the percentage of motile spermatozoa and the percentage of hyperactivated spermatozoa (sort fraction, SF). All measurements were performed at 37°C. A minimum of 100 cells and 5 fields were analyzed for each aliquot. The threshold values for hyperactivated cells identification were manually set (VCL > 150 μm/s, amplitude of lateral head displacement (ALH) > 7 μm, linearity (LIN) < 50%, Mortimer et al., 1998).

Sperm viability was evaluated under a phase-contrast light microscopy using the eosin technique or the hypoosmotic swelling test according to the WHO manual (WHO, 1999).

SDS–PAGE and western blot analysis

After the different treatments, sperm samples were processed for SDS–PAGE as described previously (Luconi et al., 2004). Briefly, after protein measurement (Coomassie Kit, Bio-Rad Labs, Hercules, CA, USA), the sperm extracts, containing ~15 μg of proteins, were diluted in Laemmli’s reducing sample buffer [62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 2.5% pyridine and 200 mM dithiothreitol (DTT)], boiled and loaded onto 8 or 10% SDS–PAGE. Proteins were transferred to nitrocellulose (Sigma Chemical Co.) and processed for western blot with the primary antibodies followed by the peroxidase-conjugated secondary antibodies. The antibody-reacted proteins were revealed by the enhanced chemiluminescence system (BM, Roche, Milan, Italy). Image acquisition and apparent molecular weight evaluation were performed with Quantity One software on a ChemiDoc XRS instrument (BIO-RAD Labs). All western blots were repeated on at least three independent experiments. Membranes were stripped (Pierce, Rockford, IL, USA) and re-probed with specific

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primary antibodies, or parallel gels were stained with Coomassie blue brilliant to confirm equal protein loading.

Bidimensional electrophoresis
Following capacitation, spermatozoa were washed in PBS and the pellet was extracted in RIPA buffer for protein measurement. Aliquots of 100 μg of lysate were further extracted in 2D IPG rehydration buffer consisting of 8 M urea, 2% (w/v) 3-[[(cholamidopropyl)dimethylammonio]-1-propanesulfonate) CHAPS, 0.5% (v/v) IPG buffer, 18.6 mM DTT. Following centrifugation at 10,000 rpm for 5 min, the supernatant was collected and applied on a 13 cm Immobiline Dry Strip pH 3–10 NL (GE Healthcare). The IPG strips were then covered in mineral oil and left to rehydrate overnight at room temperature under 10 V. Isoelectric focusing was performed with IPGphor (GE Healthcare) using the following program: 500 V (step and hold) 2 h, 1000 V (step and hold) 1 h, 8000 V (gradient) 3 h and 8000 V (step and hold) 4 h. Following focusing, the IPG strips were immediately incubated in equilibration buffer [30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea, 50 mM Tris, pH 8.8, and trace amounts of bromophenol blue] supplemented with 0.5% (w/v) DTT for 10 min at room temperature, followed by fresh equilibration buffer supplemented with 2.5% (w/v) iodoacetamide for a further 10 min at room temperature. The strips were then washed in SDS–running buffer and placed on top of 10% polyacrylamide gels and held in place using 0.5% (w/v) agarose prepared in SDS–running buffer. Proteins were then resolved by electrophoresis, and the separated proteins were either subjected to silver staining (Silver Staining Plus, Biorad) according to the manufacturer’s instruction or transferred onto nitrocellulose membranes and immunoblotted as described previously.

Immunoprecipitation analysis
After protein measurement, aliquots of cell lysates containing equal amount of proteins (150 μg for spermatozoa or 800 μg for PC3 cells) were incubated for 1 h with 30 μl of protein G- or A-Sepharose for preclearing. Precleared lysates were then incubated for 1 h using 1 μg of the appropriate primary antibodies followed by overnight incubation at 4°C with 50 μl of protein G- or A-Sepharose. The immunobeads were washed and subjected to reducing SDS–PAGE followed by western blot analysis using different antibodies to reveal Src or co-immunoprecipitated proteins.

Sperm samples immunodepleted of AKAP3 were obtained by collecting supernatants after AKAP3 immunoprecipitation with an excess of anti-AKAP3 antibody (5 μg). Since AKAP3 is detergent-resistant because of its strong association with the fibrous sheath compartment, we used an SDS extraction method to lyse spermatozoa before performing immunoprecipitation (Luconi et al., 2004). Extracted supernatants were then subjected to AKAP3 immunoprecipitation (as described previously), and the AKAP3-depleted supernatants were collected and subjected to SDS–PAGE.

Immunocytochemistry
NC and capacitated spermatozoa (2 × 10⁵ cells) were layered on slides, fixed in 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. After washing, cells were blocked with 3% BSA–PBS, pH 7.4, at 37°C for 1 h and then incubated overnight with anti-Src, anti-phospho Tyr416 Src or PY20 primary antibodies (1:1000 dilution). After three washes in PBS, cells were incubated for 1 h with Texas Red anti-rabbit IgG (1:50 dilution) or FITC anti-mouse IgG (1:100 dilution). Washed slides were air-dried and mounted on 10% glycerol–PBS. Staining avoiding primary antibody was used as a negative control.

Measurement of [Ca²⁺],
[Ca²⁺], was measured in fura-2-loaded spermatozoa in HTF/HSA medium (with or without inhibitors) by a fluorimetric method (Gryniwicz et al., 1985). Treated spermatozoa were loaded with 2 μM fura-2/AM for 45 min at 37°C, washed and resuspended in FM buffer (125 mM NaCl, 10 mM KCl, 2.5 mM CaCl₂, 0.25 mM MgCl₂, 19 mM Na lactate, 2.5 mM Na piruvate, 20 mM HEPES/Na, 0.3% BSA, pH 7.5) for 30 min at 37°C. Fluorescence was measured in a double-wavelength spectrophotometer (LS50B, Perkin Elmer) set at 340/380 nm excitation, with emission at 510 nm. Spermatozoa were stimulated with 10 μM progesterone.

Evaluation of acrosome reaction
Sperm acrosome reaction was evaluated through the use of the fluorescent probe FITC-labeled Arachis hypogaea (peanut) lectin as described previously (Krausz et al., 1994). Briefly, spermatozoa (10⁷/ml) stimulated for 1 h with progesterone (10 μM), were first re-suspended in 0.5 ml of hypoosmotic swelling medium for 1 h at 37°C and then in 50 μl of ice-cold methanol. The sperm suspension was layered on a slide, air-dried at room temperature and stored at −20°C. Fluorescence was observed under a fluorescent microscope (Leitz, type 307–148002; Wetzlar, Germany), and the acrosome reaction was evaluated on a total of 100 spermatozoa per slide. Only curly-tailed spermatozoa were considered viable and thus scored. The difference between the percentage of the acrosome reaction in progesterone-induced and spontaneous samples is considered as the percentage of spermatozoa which are responsive to the progesterone stimulation.

Statistical analysis
Data were analyzed with Microcal Origin software, version 6.1 (MicroCal Software Inc., Northampton, MA, USA). One-way analysis of variance (ANOVA) was applied for multiple comparisons, whereas Student’s t-test was used for comparisons of two classes of data. A P-value < 0.05 was considered statistically significant. Results are expressed as mean ± SE unless otherwise stated.

Results
A Src isoform is present in human spermatozoa
To evaluate the presence of Src in human spermatozoa, we performed western blot analysis of sperm lysates using both a polyclonal (Santa Cruz; Fig. 1A, upper panel) and a monoclonal (Upstate Biotechnology; Fig. 1A, lower panel) anti-Src antibody that revealed a positive band migrating at a higher molecular weight than the expected 60 kDa, obtained in prostatic cell line PC3 used as positive control. No difference in the apparent molecular weight of the band was detected at different times of capacitation (1 and 3 h; Fig.1A, upper panel). The higher molecular weight of sperm Src isoform was confirmed by Src immunoprecipitation from both sperm and PC3 total lysates (Fig.1B). The specificity of the antibody was demonstrated by immunoprecipitating sperm lysates with the anti-isotypic IgG (Fig.1C). Moreover, western blot analysis with anti-Src antibody (Fig. 1D, b) of total sperm lysates separated by 2DE (Fig. 1D, a) revealed a train of spots corresponding to Src isoforms migrating at 68.9 ± 0.3 kDa, a higher molecular weight than the somatic Src isoform. The different isoelectric points of the four spots may be due to different grades of phosphorylation.
Src in human spermatozoa

The calculated average molecular weight of sperm Src as evaluated from different western blots obtained by both monodimensional electrophoresis and 2DE of sperm lysates was 69.4 ± 3.7 kDa (n = 20).

Furthermore, Src is characterized by a clearly defined localization in human spermatozoa (Fig. 2). The immunocytochemical analysis of fixed and permeabilized spermatozoa revealed positivity for Src in ~85% of the spermatozoa in the sample (Fig. 2B, ×25 magnification). At a higher magnification (×100, Fig. 2C–F), Src positivity (panel F) can be appreciated in the post-acrosomal region of the sperm head (equatorial segment and neck) and in the midpiece, although the principal piece appeared to be negative. A weak positivity in the acrosomal region was detected in ~10% of Src-positive spermatozoa.

**Src is activated during capacitation**

In order to study the role of Src during the main processes of sperm activation, we first evaluated Src activity during *in vitro* sperm capacitation by using two specific antibodies directed against the phosphorylated tyrosines (Tyr) in 416 or 527, identifying, respectively, the active or inactive form of the kinase (Roskoski, 2004). In somatic cells, these two Tyr are the main regulatory phosphorylation sites of Src. Phosphorylation in Tyr527 stabilizes a restrained form of the enzyme, whereas, upon de-phosphorylation of this residue, the SH2 and SH3 domains become accessible to phosphorylation/activation, and the Tyr416 can undergo an intermolecular auto-phosphorylation, which promotes the kinase activity (Roskoski, 2004).

During the time-dependent process of capacitation, there is a significant increase in phosphorylation in Tyr416, which is accompanied by a slight decline in phosphorylation in Tyr527 between 1 and 3 h capacitation (intensity of Tyr527 band, arbitrary units, NC: 40.0 ± 0.5; C1h: 46.5 ± 8.5; C2h: 27.0 ± 1; C3h: 15.5 ± 4.5; n = 2), resulting in Src activation (Fig. 3A). The increased auto-phosphorylation occurring during capacitation was further confirmed by western blot analysis with the anti-pY416 antibody of Src immunocomplex obtained with an anti-Src antibody from sperm lysates (Fig. 3B). To demonstrate that the increased phosphorylation in Tyr416 was mediated by Src itself, we allowed spermatozoa to capacitate in the presence or absence of SU6656, a specific inhibitor of the enzyme (Blake et al., 2000). Addition of SU6656 during capacitation resulted in a block of phosphorylation in Tyr416, confirming that the capacitation process was accompanied by Src self-activation (Fig. 3C). The inhibitory effect of SU6656 on Src phosphorylation in Tyr416 was also observed when capacitation was induced in the presence of 5 mM dbcAMP + 1 mM PF, a treatment which is supposed...
PKA catalytic subunit (PKA) is shown in the lower blot. Lane protein normalization for PKA catalytic subunit (PKA) is shown in the lower blot. (A) Western blot analysis of NC spermatozoa or spermatozoa capacitated for 1 (C1h), 2 (C2h) or 3 h (C3h) revealed with the anti-pY416 (upper blot) and anti-pY527 (middle blot) Src antibodies, identifying, respectively, the Src-active and -inactive status. Src tyrosine phosphorylation in the Y416 position increases from NC to C3h, and conversely, Src tyrosine phosphorylation in Y527 decreases during capacitation. Lane protein normalization for PKA catalytic subunit (PKA) is shown in the lower blot. (B) The same samples as in panel A were immunoprecipitated with a mouse anti-Src antibody (IP: Src Ab) and revealed with anti-pY416 Src or anti-Src antibodies. The immunoprecipitation confirms the increased phosphorylation in Y416 during capacitation. A sperm lysate immunoprecipitated with an anti-iso IgG (IP: IgG) is shown on the left as a negative control of the immunoprecipitation technique. (C) Spermatozoa capacitated for 1 h in the absence or in the presence of the Src inhibitor SU6656 (SU 280 nM) were immunoprecipitated with a mouse anti-Src antibody (IP: Src Ab) and revealed by western blot analysis using the anti-pY416 antibody. SU6656 blocks Src phosphorylation in Y416, confirming that the capacitation process is accompanied by Src self-activation. (D) Western blot analysis of NC spermatozoa or spermatozoa capacitated for 3 h in 10% HSA–HTF medium supplemented (dbcAMP + PF) or not (C3h) with 5 mM dbcAMP + 1 mM PF in the presence or absence of 280 nM SU6656 (SU) reveals a similar increase in Src phosphorylation in Tyr416 and a similar inhibition by SU, independent of the presence of dbcAMP + PF (upper blot). Lane protein normalization for PKA catalytic subunit (PKA) is shown in the lower blot.

to better stimulate this process (Fig. 3D). Immunofluorescence cytochemistry of fixed and permeabilized human NC spermatozoa (A), or spermatozoa capacitated for 1 (B) and 3 h (C), performed using the anti-pY416 antibody identifies the active form of Src (arrowheads). Corresponding phase-contrast images are shown (D–F). The fluorescence intensity corresponding to the active Src increases from NC (A) to 1 (B) up to 3 h (C) of capacitation in spermatozoa and shows a similar localization as for total Src (head and midpiece).

Figure 4:

Src is activated during capacitation: immunocytochemistry analysis. Immunofluorescence cytochemistry of fixed and permeabilized human NC spermatozoa (A), or spermatozoa capacitated for 1 (B) and 3 h (C), performed using the anti-pY416 antibody identifies the active form of Src (arrowheads). Corresponding phase-contrast images are shown (D–F). The fluorescence intensity corresponding to the active Src increases from NC (A) to 1 (B) up to 3 h (C) of capacitation in spermatozoa and shows a similar localization as for total Src (head and midpiece).

No significant effect of Src inhibition on either sperm motility or hyperactivation parameters such as VAP, VCL, VSL, motile sperm and sort fraction, was observed when SU6656 was added from the very beginning of the process (Fig. 5A–E) or once capacitation had already started (15 min before stopping) (data not shown). SU6656 was also ineffective when capacitation was achieved by 3 h sperm stimulation with 5 mM dbcAMP + 1 mM PF (Fig. 5F). These results indicate a lack of an involvement of Src in the regulation of motility in ejaculated human spermatozoa. Although they are in contrast with those obtained in epididymal mouse spermatozoa (Baker et al., 2006), they are in agreement with the results obtained by Mitchell et al. (2008) in human spermatozoa.

Since sperm capacitation is strictly associated with a rapid increase in protein tyrosine phosphorylation, we next evaluated the effect of Src inhibition on the tyrosine phosphorylation status of proteins. As can be observed in Fig. 6A, following capacitation, the increase in tyrosine phosphorylation observed in some proteins (60–110 kDa) was prevented by SU6656 at 1 h and partially inhibited at 2 h. No dose effect was present over the range of concentrations of SU6656 used (Fig. 6A). Among the protein bands which appeared to be tyrosine-phosphorylated during capacitation and whose phosphorylation was remarkably prevented by SU6656 addition, there was one of an apparent molecular weight of ~110 kDa. A protein of a similar molecular weight, the protein kinase A anchoring protein 3 (AKAP3), has been described recently as undergoing tyrosine phosphorylation during capacitation in response to bicarbonate administration as well as to PI3K inhibition (Luconi et al., 2004, 2005). Such an increase in tyrosine phosphorylation results in PKA recruitment and activation in sperm tails, finally leading to increased sperm motility. Thus,
an inhibitory effect of SU6656 on tyrosine phosphorylation of AKAP3 is not expected since this compound is ineffective on motility. To investigate whether SU6656 inhibition of tyrosine phosphorylation was on AKAP3 or on other proteins migrating at the same molecular weight, we performed immunodepletion experiments (see Material and Methods for protocol). AKAP3-immunodepleted (Fig. 6B, left panel) or total (Fig. 6B, right panel) lysates, obtained from spermatozoa capacitated in the presence or absence of SU6656, were separated on SDS–PAGE and subjected to western blot analysis for phosphotyrosine proteins. SU6656 inhibition of tyrosine phosphorylation of a 110 kDa band was still present following AKAP3 immunodepletion, suggesting that the band whose phosphorylation is controlled by Src is not AKAP3. The residual increase in tyrosine phosphorylation of the 110 kDa band, still present following 2 h capacitation in the presence of SU6656 (Fig. 6A), may involve AKAP3 and consequently support the associated sperm hyperactivation (Fig. 5, Luconi et al., 2004).

Immunocytochemistry of fixed and permeabilized spermatozoa following capacitation in the presence or absence of SU6656 (Fig. 7A–J) confirmed that capacitation induced a marked and time-dependent increase in the tyrosine phosphorylation of proteins mainly localized in sperm tails, which was inhibited by SU6656. In these capacitation conditions, there was a significant increase in the number of positive spermatozoa and in the intensity of this positivity (Fig. 7, lower table). Again, these two parameters were significantly reduced but not completely suppressed in the presence of SU6656 (Fig. 7, lower table). Although tyrosine phosphorylation was mainly localized in sperm tails, positivity was also present in the sperm neck (Fig. 7D, inset). Similar results have been obtained when capacitation was achieved in the presence of dbcAMP + PF (not shown).

Intracellular calcium levels and calcium influx in spermatozoa have been demonstrated to be associated with tyrosine phosphorylation; therefore, we evaluated the effect of SU6656 addition on intracellular calcium levels in capacitated spermatozoa loaded with the calcium probe fura-2 (Fig. 8A). Although SU6656 exerted no effect on the basal intracellular calcium levels, it was able to interfere with the calcium influx triggered by progesterone. Indeed, both the peak and the plateau components of the calcium wave in response to progesterone were significantly reduced by SU6656. The mean ± SE-fold increase of intracellular calcium concentration following 10 μM progesterone over basal (n = 6) was: peak phase 7.43 ± 1.87 and 4.97 ± 1.41 in the absence and in the presence of 280 nM SU6656, respectively, P < 0.05; plateau phase 2.35 ± 0.26 and 1.79 ± 0.15 in the absence and in the presence of 280 nM SU6656, respectively, P < 0.05. Since the peak phase of calcium response has been demonstrated to be responsible for the induction of acrosome reaction in response to progesterone (Harper et al., 2006), we measured acrosome reaction in response to progesterone in spermatozoa treated and untreated with SU6656. Acrosome reaction in response to progesterone was blunted when SU6656 was present from the beginning or at the end of capacitation, suggesting the involvement of Src activation in the processes of capacitation and acrosome reaction (Fig. 8B).
Src is not a direct substrate of PKA

Since our findings suggested the existence of some differences in the role of Src between mouse (Baker et al., 2006) and human spermatozoa, we sought to study whether PKA could directly interact with Src, resulting in its activation through serine phosphorylation, as has been demonstrated in the mouse (Baker et al., 2006). Immunocomplexes obtained with an anti-Src antibody from sperm lysates (Fig. 9A, right panel) or the corresponding total sperm lysates revealed that Src is not a direct substrate of PKA. In fact, a band co-migrating with Src (Fig. 9D, left panel) could not be detected on the same membrane by using the RRXS antibody (Lefievre et al., 2002) directed against PKA-phosphorylated substrates (Fig. 9D, right panel).

**Discussion**

The present study identifies a human sperm isoform of Src of ~70 kDa, localized in the post-acrosomal region of the sperm head and midpiece, and clearly demonstrates its involvement in regulating sperm capacitation and acrosome reaction, but rules out any significant role of this kinase in the control of the processes of sperm motility and hyperactivation associated with capacitation as well as any direct interaction of this kinase with PKA. Interestingly, the molecular weight of the human sperm Src isoform (68.9 kDa), detected in SDS–PAGE by antibodies directed against different Src epitopes, appears to be higher than the 60 kDa isoform found in somatic cells and in mouse spermatozoa (Baker et al., 2006), confirming previous results which demonstrated the presence of a 66 kDa Src isoform in capacitated human spermatozoa (Kumar and Meizel, 2005). Whether this weight difference may be due to different degrees of post-translational modifications, such as palmitoylation or myristoylation, already described to be essential for somatic Src recruitment to the plasma membrane, or, alternatively, to the presence of mRNA splicing variants of Src in spermatozoa, as already described in neuronal cells (Matsunaga et al., 1993), requires further investigations. From our data, we can rule out any molecular weight change detectable by western blot or immunoprecipitation techniques due to Src post-translational modifications associated with capacitation.

Our findings clearly demonstrate that several differences exist between mouse and human spermatozoa in both function and localization of Src. In fact, immunocytochemistry performed using anti-Src and anti-pTyr416 Src antibodies mainly localizes both resting and active Src in the post-acrosomal region and the neck of the sperm head and to a lesser extent in the midpiece and acrosome (Lawson et al., 2008), whereas the principal piece of sperm tail, which is the main site of tyrosine phosphorylation associated with sperm capacitation, appears to be negative (as also confirmed by Kumar and Meizel, 2005), in contrast with what has been found by other groups in human (Mitchell et al., 2008; Lawson et al., 2008) and mouse (Baker et al., 2006) spermatozoa. Some of these discrepancies may be explained by the different roles supported by the apparent different isoforms...
of Src in the two sperm species. In fact, although Src inhibition in the mouse is accompanied by an almost complete block of hyperactivation (Baker et al., 2006), in humans, SU6656 added during capacitation does not alter either motility or hyperactivation parameters such as VAP, VCL, VSL and in particular sort fraction, as demonstrated by our detailed CASA analysis. Conversely, inhibition of Src during capacitation of human spermatozoa results in inhibition of the acrosome reaction physiologically induced by progesterone. The different localization of the activated protein in the mouse and in humans may underlie the different role of Src: a tail localization, as found in the mouse, is associated with a regulation of hyperactivation, whereas a head localization, as found in humans (Kumar and Meizel, 2005; Lawson et al., 2008; present study), may account for Src control of capacitation and acrosome reaction. It must be mentioned, however, that any possible role of Src in mouse sperm acrosome reaction has not yet been investigated. In particular, by adding SU6656 from the beginning of capacitation and at the end of capacitation just before stimulation with the acrosome reaction inducer, we were able to discriminate the effect of Src inhibition on capacitation and acrosome reaction, respectively, since it is known that only capacitated spermatozoa can undergo progesterone-stimulated acrosome reaction. Thus, although the effects on acrosome reaction observed following addition of the Src inhibitor from the very beginning of sperm capacitation may be due to an inhibition of both acrosome reaction and capacitation, the effects observed following addition of SU6656 just before the induction of acrosome reaction, once capacitation has already begun (or even been completed), are dependent on the interference occurring with acrosome reaction only. Our data clearly demonstrate that Src exerts a role in both capacitation and the subsequent process of acrosome reaction, through affecting tyrosine phosphorylation of specific proteins and the calcium influx induced by progesterone, processes which have been described as fundamental in the induction of acrosome reaction (Baldi et al., 2002). In particular, due to the post-acrosomal localization of Src in human spermatozoa, it can be hypothesized that this enzyme is involved in the release of calcium from sperm intracellular stores (such as the redundant nuclear envelope; Ho and Suarez, 2003) which are located in the neck region. Alternatively, an effect on calcium channels involved in the progesterone effect in spermatozoa cannot be excluded. Src involvement in regulating intracellular calcium levels by different mechanisms has been clearly demonstrated in several cell types. Recently, Src inhibition has been shown to blunt progesterone-induced calcium release from intracellular stores in platelets (Blackmore, 2008). Moreover, in neurons, Src has been demonstrated to regulate, through tyrosine phosphorylation, the voltage-dependent calcium influxes which underlie neurotransmitter release (Ohnishi et al., 2001; Wang, 2003). The calcium requirement for Src activity associated with sperm membranes has been recently documented in human spermatozoa (Lawson et al., 2008), in contrast with the inhibitory action of this ion on activity of the other Src kinase, YES-1 (Leclerc and Goupil, 2002).

Figure 7: Capacitation-induced increase in sperm protein phosphorylation is mainly localized in sperm tails. Immunofluorescence cytochemistry of fixed and permeabilized human NC spermatozoa (A and B, NC), or capacitated for 1 h (C, C1h) and 3 h (D, C3h) with or without 280 nM SU6656 (E, C3h + SU), performed using the anti-phospho tyrosine PY20 antibody, reveals a capacitation-dependent increase in positivity mainly localized in sperm tails (A–D) and in the sperm neck (D, inset). Corresponding phase-contrast images are shown (F–J). A negative control avoiding the primary antibody was performed on NC spermatozoa (A). Data in lower table represent mean ± SE of the percentage of PY20-positive cells or of PY20 intensity, obtained by quantitative analysis performed under microscope on five different fields (two independent experiments) using Adobe Photoshop 9.0 software, and confirm the increased phosphorylation observed in sperm during capacitation, which is significantly reduced by sperm incubation with SU6656. One-way ANOVA was applied for multiple comparison (NC, 1 and 3 h), and Student’s t-test for paired data was used for comparison of two classes of data (presence or absence of SU). Statistical significance is indicated.

<table>
<thead>
<tr>
<th>% PY20 Positive Spermatozoa</th>
<th>NC</th>
<th>C1h</th>
<th>C3h</th>
<th>C3h+SU</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Positive Cells</td>
<td>11.9±1.8</td>
<td>31.4±3.2</td>
<td>45.1±5.0</td>
<td>21.8±1.4</td>
</tr>
<tr>
<td>P=0.001 vs NC</td>
<td></td>
<td>&lt;0.001 vs NC</td>
<td>&lt;0.001 vs NC</td>
<td>&lt;0.01 vs C3h</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>PY20 Intensity (Arbitrary Units)</th>
<th>NC</th>
<th>C1h</th>
<th>C3h</th>
<th>C3h+SU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>1.8±0.2</td>
<td>3.5±0.0</td>
<td>7.4±0.3</td>
<td>4.1±0.1</td>
</tr>
<tr>
<td>P=0.001 vs NC</td>
<td></td>
<td>&lt;0.001 vs NC</td>
<td>&lt;0.001 vs NC</td>
<td>&lt;0.001 vs C3h</td>
</tr>
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plasma membrane. In spermatozoa, Src may also represent one of the key enzymes involved in the transduction of the capacitation signals from the plasma membrane to the acrosome.

Several members of Src kinase family have recently been described in mammalian spermatozoa, although their function in spermatozoa has still to be addressed (Leclerc and Goupil, 2002; Kumar and Meizel, 2005; Lalancette et al., 2006; Bordeau and Leclerc 2008; Mitchell et al., 2008).

A fundamental difference between the present study and the one from Baker et al. (2006) in the mouse concerns the way capacitation is achieved. Although in our study, human spermatozoa have been obtained from ejaculated samples, in the mouse, spermatozoa have been obtained from the epididymis, a condition which is not physiological and may underlie a non-complete functional maturation of spermatozoa (Yanagimachi, 1994). Furthermore, although in our study, human spermatozoa have been physiologically capacitated by up to 3 h incubation in HTF media containing bicarbonate and albumin, capacitation of mouse spermatozoa was achieved by stimulation with a stable analog of cAMP, dbcAMP, in combination with the phosphodiesterase inhibitor PF (Baker et al., 2006). The same ‘pharmacological’ capacitating condition has been used also for human spermatozoa (Mitchell et al., 2008). However, when we compared such an ‘aggressive’ condition with the more physiological capacitation achieved in HTF medium, we obtained quite similar results in terms of Src activation, protein tyrosine phosphorylation, motility and of SU6656 modulation of these processes. Other differences in the incubation conditions exist between our paper and the ones from Mitchell et al. (2008) and Lawson et al. (2008) which may account for the differences in some of the obtained results. In particular, we investigated the role of Src during in vitro capacitation obtained in the most physiological in vitro conditions (i.e. by incubating in HTF medium supplemented with albumin), whereas, on one hand, Lawson et al. (2008) measured Src activity once capacitation had occurred, and on the other hand, Mitchell et al. (2008) used harsher capacitating conditions, even avoiding the presence of calcium in order to stress protein tyrosine phosphorylation and motility processes. Interestingly, after Mitchell et al.’s paper had been published, Lawson et al. (2008) clearly demonstrated that calcium is required for Src activity.

Finally, in our study, a direct interaction between PKA and Src in human spermatozoa during physiological induction of capacitation was not demonstrated. Moreover, differently from previous studies (Lawson et al., 2008; Mitchell et al., 2008), we were unable to demonstrate any PKA co-immunoprecipitation and direct phosphorylation of Src even if capacitation was achieved by using the cAMP stable analog. In particular, although Lawson et al. succeeded in co-immunoprecipitating Src and PKA in human spermatozoa, such an interaction seems to be very weak and does not result in a strong activation of Src. In fact, PKA-mediated direct phosphorylation of Src in human spermatozoa can be achieved only following addition of an excess of exogenous PKA catalytic subunit (Lawson et al., 2008). Moreover, Src activation during physiological capacitation, evaluated as the ability of the immunoprecipitated Src to in vitro-phosphorylate the percentage of spermatozoa showing tyrosine phosphorylation, but also the intensity of this phosphorylation. Since Src is mainly localized in the sperm post-acrosomal region, neck and midpiece, whereas tyrosine phosphorylation in response to its activation mainly involves proteins in the principal piece of the tail, it could be hypothesized that Src does not directly phosphorylate the downstream protein targets but may act on intermediate tyrosine kinases which, upon activation, shuttle between post-acrosomal Src and protein targets in the flagellum. However, in some spermatozoa, tyrosine phosphorylation is not restricted to the principal piece, but also occurs in sperm neck, where Src is mainly localized, suggesting a possible direct interaction between Src and phosphorylated targets in this compartment.

In somatic cells, active Src has been described to interact with a number of receptors and second messengers in multimeric complexes associated with the plasma membrane, carrying on the transduction of the signaling cascade initiated at the

![Figure 8: Src is involved in the regulation of calcium influx in response to progesterone and in acrosome reaction.](https://example.com/figure8.png)
the generic substrate enolase, seems to be modestly regulated by PKA, since modulation of PKA activity by addition of IBMX, dbcAMP or the PKA inhibitor H89 does not result in a marked alteration of Src activity, compared with the one obtained by addition of an excess of PKA catalytic subunit (Lawson et al., 2008). Nevertheless, from our data, we cannot exclude that Src may represent a PKA-regulated intermediary kinase controlling tyrosine kinase-associated events, as suggested for mouse (Baker et al., 2006) and human (Lawson et al., 2008; Mitchell et al., 2008) spermatozoa. Baker et al. (2006) demonstrated that PKA can directly phosphorylate and activate Src in mouse spermatozoa, triggering not only capacitation as in human spermatozoa, but also hyperactivation. Direct activation through PKA-induced tyrosine phosphorylation in Ser17 has been described in somatic cells (Obara et al., 2004), although PKA-stimulated phosphorylation and inactivation of the Src inhibitor, kinase Csk remain the most common molecular mechanisms of Src activation (Roskosky, 2004). Dephosphorylation of the regulatory Y527 Src residue through PKA activation of the protein tyrosine phosphatase PTP1B (Bjorge et al., 2000), localized in the sperm equatorial segment and tail (Tomes et al., 2004), has been suggested as an alternative mechanism of an indirect activation of Src by PKA (Lawson et al., 2008). Overall, these results may underlie the existence of species-specific differences in the mechanisms driving sperm activation. Indeed, strong species-specific differences exist in the molecular mechanisms which drive the sperm–oocyte interaction, thus accounting for the species specificity of the gamete interaction. Even processes and enzymes which are similar between spermatozoa of different species may present functional differences. Tyrosine phosphorylation of proteins has been demonstrated to be essential for sperm capacitation and zona pellucida binding in mammalian spermatozoa. However, the capacitation-induced tyrosine phosphorylation of chaperones results in the expression of a zona pellucida receptor complex on sperm head in the mouse (Asquith et al., 2004) but not in humans (Mitchell et al., 2007).

In conclusion, we demonstrate the presence, localization and functional activity of a 70 kDa Src isoform in human spermatozoa, elucidating the important role exerted by this kinase in the process of capacitation and acrosome reaction, and ruling out its involvement in induction and maintenance of sperm motility and hyperactivation.

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References


