Tyrosine phosphorylation of HSP-90 during mammalian sperm capacitation

Heath W. Ecroyd
University of Wollongong, heathe@uow.edu.au

Russell C. Jones
University of Newcastle, russell.jones@newcastle.edu.au

Robert J. Aitken
University of Newcastle

Tyrosine phosphorylation of HSP-90 during mammalian sperm capacitation

Abstract

The process of sperm capacitation is correlated with activation of a signal transduction pathway leading to protein tyrosine phosphorylation. Whereas phosphotyrosine expression is an essential prerequisite for fertilization, the proteins that are phosphorylated during capacitation have not yet been identified. In the present study, we observed that a major target of this signaling pathway is the molecular chaperone protein, heat shock protein (HSP)-86, a member of the HSP-90 family of HSPs. We used cross-immunoprecipitation experiments to confirm the tyrosine phosphorylation of HSP-86, a process that is not inhibited by the ansamycin antibiotic, geldanamycin. The general significance of these findings was confirmed by studies in which HSP-90 was also found to be tyrosine phosphorylated in human and rat spermatozoa when incubated under conditions that support capacitation. To our knowledge, these results represent the first report of a protein that undergoes tyrosine phosphorylation during mouse sperm capacitation and the first study implicating molecular chaperones in the processes by which mammalian spermatozoa gain the ability to fertilize the oocyte.

Keywords

90, hsp, phosphorylation, tyrosine, mammalian, during, sperm, capacitation, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Tyrosine Phosphorylation of HSP-90 During Mammalian Sperm Capacitation[1]

Heath Ecroyd,[3] Russell C. Jones,[3] and R. John Aitken[2,4]

*Reproductive Science Group,[3] School of Environmental and Life Sciences, and ARC Centre of Excellence
for Biotechnology and Development,[4] University of Newcastle, Callaghan, New South Wales 2308, Australia*

ABSTRACT

The process of sperm capacitation is correlated with activation of a signal transduction pathway leading to protein tyrosine phosphorylation. Whereas phosphotyrosine expression is an essential prerequisite for fertilization, the proteins that are phosphorylated during capacitation have not yet been identified. In the present study, we observed that a major target of this signaling pathway is the molecular chaperone protein, heat shock protein (HSP)-86, a member of the HSP-90 family of HSPs. We used cross-immunoprecipitation experiments to confirm the tyrosine phosphorylation of HSP-86, a process that is not inhibited by the ansamycin antibiotic, geldanamycin. The general significance of these findings was confirmed by studies in which HSP-90 was also found to be tyrosine phosphorylated in human and rat spermatozoa when incubated under conditions that support capacitation. To our knowledge, these results represent the first report of a protein that undergoes tyrosine phosphorylation during mouse sperm capacitation and the first study implicating molecular chaperones in the processes by which mammalian spermatozoa gain the ability to fertilize the oocyte.

gamete biology, signal transduction, sperm capacitation

INTRODUCTION

Ejaculated mammalian spermatozoa are incapable of fertilizing the oocyte. They gain this ability through a series of time-dependent processes collectively referred to as capacitation [1, 2]. This maturational process is correlated with activation of a unique signal transduction cascade leading to an increase in protein tyrosine phosphorylation in all mammalian species studied to date [3] (for review, see [4]). During capacitation, phosphotyrosine expression becomes largely localized to the flagellum [5–8], suggesting that this process may be a necessary prerequisite for the initiation of hyperactivated movement, a key attribute of the capacitated state [9, 10]. Furthermore, the activation of this signaling pathway has been proposed as a necessary prerequisite for sperm binding to the zona pellucida [5, 11]. Despite the potential biological importance of this tyrosine phosphorylation event, the identity of the proteins induced to undergo tyrosine phosphorylation during capacitation has yet to be fully resolved.

At present, the best-characterized targets for tyrosine phosphorylation of human [6, 12] and hamster [13] sper-

matozoa are the A kinase-anchoring proteins, AKAP 3 and AKAP 4, which localize to the fibrous sheath. These proteins act to tether protein kinase A (PKA), through its regulatory subunit (RII), to discrete subcellular locations. By contrast, in the mouse, the AKAPs located on the sperm flagellum are phosphorylated at serine/threonine, not tyrosine, residues [14]. A recent analysis of human spermatozoa by Naaby-Hansen et al. [15] identified a third protein that becomes tyrosine phosphorylated during capacitation. This unique calcium-binding protein (CABYR) localizes to the sperm fibrous sheath and is proposed to play a role in hyperactivation. A constitutively tyrosine phosphorylated hexokinase (p95/p116) has been shown to be present in mouse spermatozoa [16, 17], but no previous reports have cited proteins that become tyrosine phosphorylated during capacitation in this species. This is significant, because knowledge of the proteins activated by this signal transduction cascade may provide insight regarding the role of tyrosine phosphorylation in regulating sperm fertilizing ability.

Therefore, in the present study, we set out to identify and characterize proteins that become tyrosine phosphorylated during capacitation of mouse spermatozoa. Here, we demonstrate, to our knowledge for the first time, that heat shock protein (HSP)-90α, a highly conserved molecular chaperone protein, becomes tyrosine phosphorylated during capacitation in this species. Because tyrosine phosphorylation of HSP-90 was also found to occur in human and rat spermatozoa when incubated under conditions that support capacitation, these findings appear to be of general significance in defining the mechanism by which capacitation confers functionality on mammalian spermatozoa.

MATERIALS AND METHODS

Reagents

The following reagents were used: BSA (Research Organics, Cleveland, OH); Hepes, penicillin, and streptomycin (Gibco, Paisley, U.K.); Protein A/G Plus sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA); goat serum (Hunter Antisera, Jesmond, NSW, Australia); ampholines (pH 3–10), immobilized pH gradient (IPG) strips, ammonium persulfate, 2-mercaptoethanol, and N,N,N_1-tetra-methylethylenediamine (Temed; BioRad, Herts, U.K.); mowoil and geldanamycin (Calbiochem, San Diego, CA); and other reagents (Sigma, St. Louis, MO). The following antibodies were used: clone 4G10 antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), anti-HSP-90 monoclonal antibody (BD Transduction Laboratories, Lexington, KY), anti-HSP-86 and anti-HSP-84 monoclonal antibodies (Affinity Bioreagents, Golden, CO), anti-HSP-60 monoclonal antibody (Calbiochem, San Diego, CA), and goat anti-mouse and goat anti-rabbit antibodies (Santa Cruz Biotechnology).

Sperm Preparation

Caudal epididymal spermatozoa were collected from Swiss mice (age, 8–14 wk) and Wistar rats (age, 12–16 wk), bred in the University's animal facility, by backflushing the distal cauda epididymides with water-saturated paraffin oil. The samples were stored under oil at 37°C until use. Spermatozoa were activated by adding 200 µl of Biggers, Whitten, and Whittingham (BWW) medium [18] and then left to disperse in the droplet for
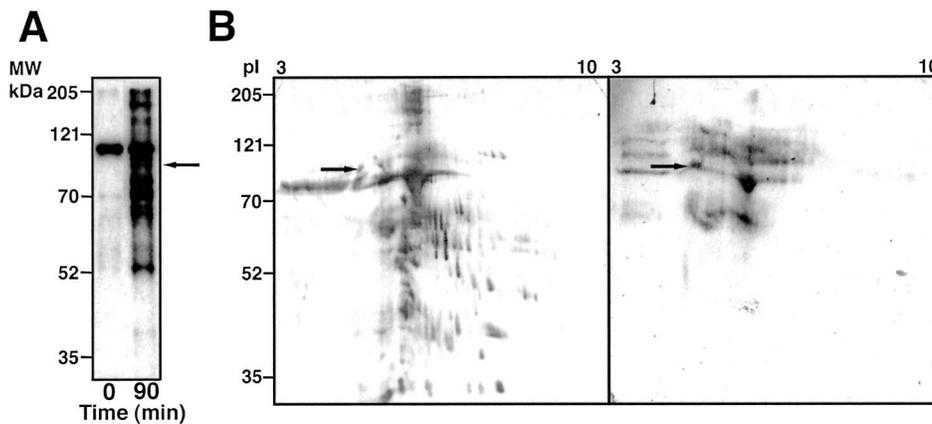
FIG. 1. The increase in protein tyrosine phosphorylation of mouse spermatozoa during incubation in medium that supports capacitation. **A**) Caudal spermatozoa were incubated in BWW for 90 min, then proteins were extracted and phosphotyrosine residues detected by immunoblot analysis. The figure shown is representative of five independent experiments. **B**) 2D separation of sperm proteins following incubation for 90 min in BWW. The silver stain (left) and parallel (right) immunoblots to detect tyrosine phosphorylated proteins are representative of four independent experiments. Note the arrows indicating a 90-kDa protein (pI 5.1) that is clearly resolvable by 2D electrophoresis and becomes tyrosine phosphorylated following incubation in BWW for 90 min. MW, Molecular weight.

10 min at 37°C, after which the concentration was determined using a Neubauer hemocytometer (Brandt, Wertheim, Germany). Spermatozoa were then diluted into treatments at a final concentration of 1×10^6 sperm/ml and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air.

Human semen samples were obtained after 2 or 3 days of abstinence by masturbation into sterile containers and then left to liquefy for at least 30 min. The spermatozoa were isolated by centrifugation through a two-step, discontinuous Percoll gradient (50% and 100% isotonic Percoll; 650 × g, 20 min, room temperature), and purified populations of highly motile spermatozoa were recovered from the base of the 100% fraction. The spermatozoa were washed with 5 ml of BWW, centrifuged (500 × g, 5 min), resuspended at a final concentration of 10×10^6 sperm/ml, and incubated at 37°C under an atmosphere of 5% CO_2 and 95% air.

Extraction of Sperm Proteins for One- and Two-Dimensional Gel Electrophoresis

Following incubation, spermatozoa were centrifuged (500 × g, 3 min) and washed in 1 ml of PBS (pH 7.4). Sperm proteins were extracted with extraction buffer (pH 6.8, comprised of 375 mM Tris-HCL and 10% [w/v] sucrose) containing 1 mM sodium orthovanadate and 2% (w/v) SDS for one-dimensional (1D) separation and containing 0.25% (w/v) SDS for two-dimensional (2D) separation of proteins. The samples were heated to 100°C (5 min) and centrifuged (20 000 × g, 15 min, 4°C), and the supernatant was boiled in SDS-sample buffer (375 mM Tris-HCL, 2% [w/v] SDS, and 10% [w/v] sucrose, pH 6.8) [19] containing 2% (v/v) 2-mercaptoethanol. The protein concentration for each sample was determined on microtiter plates using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL) according to the manufacturer's instructions. For 1D gel electrophoresis, 4 μg of protein were loaded per lane. For 2D electrophoresis, extracted sperm proteins were diluted directly into rehydration buffer such that final concentrations were 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropy)dimethylammonio]-1-propanesulfonate, 1% (w/v) dithiothreitol (DTT), and 2% (v/v) ampholines (pH 3–10). Proteins (100 μg) were loaded passively onto IPG strips (pH 3–10) overnight at room temperature, and isoelectric focusing was performed using a Multiphor II apparatus (BioRad). After focusing, strips were equilibrated first in equilibration buffer (25 mM Tris, 192 mM glycine, and 0.1% [w/v] SDS) containing 1% (w/v) DTT (15 min, room temperature) and then in equilibration buffer in which 2.5% (w/v) iodoacetamide replaced the DTT (15 min, room temperature). Next, SDS-PAGE was conducted on 7.5–10% acrylamide gels, after which either silver staining [20] or electrotransfer to nitrocellulose hybond super-C membrane [21] was performed.

Immunoblotting

Membranes were blocked with 3% (w/v) BSA in Tris-buffered saline (TBS; 20 mM Tris-HCl and 150 mM NaCl, pH 7.6) for 1 h at room temperature, then incubated in TBS containing 1% (w/v) BSA, 0.1% (v/v) Tween-20, and primary antibody for 2 h at room temperature. The primary antibody concentrations used were 1:4000 for antiphosphotyrosine

antibody (clone 4G10) and 1:1000 for the monoclonal anti-HSP-90, anti-HSP-86, and anti-HSP-84 antibodies. The membranes were washed four times for 5 min each wash with TBS containing 0.01% (v/v) Tween-20 and incubated in TBS containing 1% (w/v) BSA, 0.1% (v/v) Tween-20, and the appropriate secondary antibody conjugated to peroxidase for 1 h at room temperature. The secondary antibodies used were goat anti-mouse immunoglobulin (Ig) G (1:3000) and goat anti-rabbit IgG (1:1000). The membranes were washed as described above, and the labeled proteins were detected using an enhanced chemiluminescence kit (Amersham International, Buckinghamshire, U.K.) according to the manufacturer's instructions. To ensure antibody specificity of the antiphosphotyrosine antibody, separate membranes were incubated with clone 4G10 after it had been absorbed with 20 mM O-phospho-L-tyrosine (1 h, room temperature, constant rotation) before use in immunoblotting. The specificity of the HSP antibodies was ensured by incorporating a positive cell lysate control (HeLa cell lysate) provided by the manufacturer and mouse liver cell lysate (proteins were extracted in the same manner as described for sperm proteins) into some gels. Immunoblots shown in *Results* are representative of three or more independent replicates of the experiments using material from different animals.

Immunoprecipitation

Sperm proteins were extracted with 0.25% (w/v) SDS, 1 mM sodium orthovanadate, in 10 mM Tris-HCl (pH 7.4), boiled (100°C, 5 min), and centrifuged (20 000 × g, 15 min, 4°C), and the supernatant was precleared with Protein A/G Plus agarose beads (50 μl; 1 h, 4°C, constant rotation). Protein concentrations following this preclear step were then determined on microtiter plates using a BCA kit and 100 μg of the lysate used in the immunoprecipitation procedure. The anti-HSP-90 antibody (2 μg), anti-HSP-60 antibody (2 μg), or antiphosphotyrosine antibody (2 μg) in immunoprecipitation buffer (1% [v/v] Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 1 mM sodium orthovanadate, and 1 mM EDTA, pH 7.4) was added to the precleared lysates and incubated for 1 h (4°C, constant rotation). Protein A/G Plus agarose beads (50 μl) were added and incubated overnight (4°C, constant rotation). Immunoprecipitants were washed three times in 1 ml of ice-cold immunoprecipitation buffer, eluted from the agarose beads by boiling in SDS-sample buffer for 5 min, and separated by SDS-PAGE. Proteins were then transferred to membranes and immunoblotted as described above. After probing with antiphosphotyrosine antibody, membranes were stripped (62.5 mM Tris, 2% [w/v] SDS, and 5% [v/v] 2-mercaptoethanol, pH 6.8; 1 h, 60°C), washed three times with TBS, and then reprobed with HSP-90 monoclonal antibody (as described above) to ensure equal loading.

RESULTS

Protein Tyrosine Phosphorylation During Mouse Sperm Capacitation

Figure 1A shows that one protein from mouse spermatozoa (116 kDa) was constitutively tyrosine phosphorylat-

```
  1   mpeetqtqdqpmeeeevetfafqaeiaqlmsliintfysnkeiflrelisnssdaldkirye
 63   sltdpskldsgkelhinlipskqdrtltivdtgigmtkadlinnlgtiaksgtkafmealqa
125   gadismigqfgvgfysaylvaekvtvitkhnddeqyawessaggsftvrtdtgepmgr
183   gtkvilhlkedqteyleerrikeivkkhsqfigypitlfvekerdkevsddeaeekeekee
244   ekekeekesddkpeiedvgsdeeeeekkdgdkkkkkkikekyidqeelnktkpiwtrn
302   pdditneeygefykslntndweehlavkhfsvegqlefrallfvprrapfdlfenrkkknni
363   klyvrrvfimdnceelipeylnfirgvvdsedlplnisremlqqskilkvirknlvkkclel
425   ftelaedkenykkfyeqfskniklgihedsqnrkklsellryytsasgdemvslkdyctr
485   mkenqkhiyfitgetkdqvansafverlrkhgleviymiepideycvqqlkefegktlvs
545   vtkeglelpedeeekkkqeekktkfenlckimkdilekkvekvvvsnrlvtspccivtst
605   ygwtanmerimkaqalrdnstmgymaakkhleinpdhsiietlrqkaeadkndksvkd
663   lvillyetallssgfsledpqthanriyrmiklglgideddptvddtsaavteempplegdd
725   dtsrmeevd
```

FIG. 2.   Mouse amino acid sequence of HSP-86. The peptide sequences obtained by mass spectrometry from the 2D protein spot are underlined. Potential tyrosine phosphorylation sites, as predicted by PROSCAN, are shown in bold, and the position of a potential Src-kinase phosphorylation site predicted by Scansite is shown in bold italics.
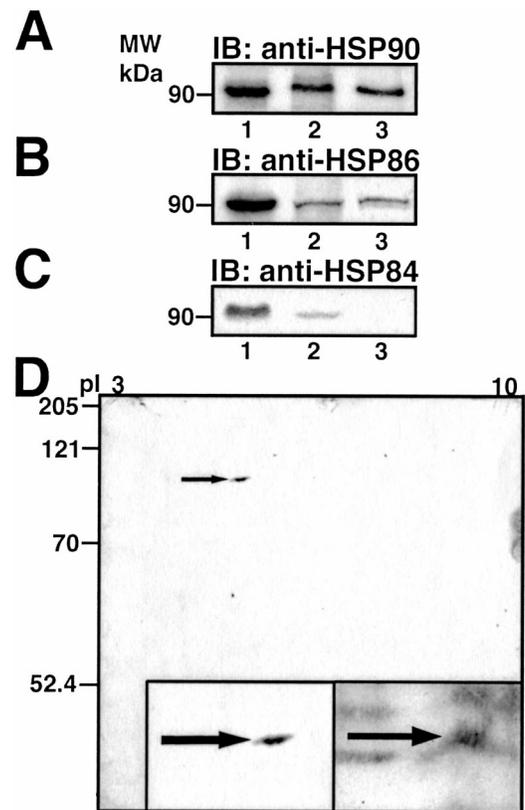

FIG. 3.   HSP-86 is present in mouse spermatozoa. Cell lysates from HeLa cells (lane 1), mouse liver (lane 2), and mouse caudal spermatozoa probed with monoclonal anti-HSP-90 (**A**), anti-HSP-86 (**B**), and anti-HSP-84 (**C**) are shown. The results are representative of four independent experiments. Also shown is a 2D immunoblot of mouse caudal spermatozoa with monoclonal anti-HSP-90 (**D**), which is representative of three independent experiments. Note the arrows indicating a specific protein spot of 90 kDa and pI 5.1. Finally, an enlargement of the protein spot on the membrane detected by the anti-HSP-90 antibody is shown (**inset**, right), as is an enlargement of the same region of a 2D immunoblot probed with the antiphosphotyrosine antibody (**inset**, left). MW, molecular weight, IB, immunoblot.

ed. This protein corresponds to a previously identified hexokinase [17]; therefore, it was used in the antiphosphotyrosine immunoblots to confirm equal loading of protein samples extracted from mouse spermatozoa. Following incubation for 90 min in medium that supports capacitation, a dramatic increase was observed in tyrosine phosphorylation of a broad range of proteins (55–220 kDa). The 2D immunoblot analysis showed that multiple proteins are tyrosine phosphorylated after 90-min incubation in medium that supports capacitation (Fig. 1B). These are predominantly acidic (pI < 7), and some have the same molecular weight but different pIs, demonstrating that multiple tyrosine phosphorylated proteins may constitute a single positive band resolved by 1D analysis. Of particular interest was a protein found to be tyrosine phosphorylated during incubation under capacitating conditions that was clearly resolved by 2D gel electrophoresis (Fig. 1, arrows). This protein has an approximate molecular mass of 90 kDa and a pI of 5.1. It was excised from the gel, trypsin digested, and subjected to tandem mass spectrometry (MS/MS) protein sequencing, and the resultant peptides were used to search protein databases against known protein sequences. FASTA [22] searches were carried out (http://fasta.bioch.virginia.edu/), and the BLAST server was accessed (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_blast.html). Potential tyrosine phosphorylation sites were determined using PROSCAN (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) with the similarity level set at 100%. Peptide sequences obtained from MS/MS spectra all significantly aligned with the 90-kDa molecular chaperone proteins (HSP-86 and HSP-84) that have a predicted pI of 5.1–5.2. Figure 2 shows the mouse amino acid sequence of HSP-86, and the peptides obtained via MS/MS sequencing are underlined. Potential tyrosine phosphorylation sites that were predicted by PROSCAN are shown in bold, and the position of a potential Src-kinase phosphorylation site, predicted by Scansite, is shown in bold italics.

HSP-86 Is Present in Mouse Spermatozoa and Is Tyrosine Phosphorylated During Capacitation

To confirm the presence of HSP-90 in mouse spermatozoa, protein extracts were probed with a series of anti-HSP-90 antibodies. Figure 3A shows that a monoclonal antibody against HSP-90 recognizes a 90-kDa protein in lysates from HeLa cells (provided as a positive control for the antibody; lane 1), mouse liver (lane 2), and mouse spermatozoa (lane 3). Because the HSP-90 family is made of two closely related isoforms in the mouse, HSP-86 and HSP-84, antibodies specific to these isoforms were used to probe cell lysates to distinguish the isoform(s) present in mouse spermatozoa. Both HSP-86 and HSP-84 were found to be present in HeLa cells (Fig. 3, B and C, lane 1) and mouse liver (Fig. 3, B and C, lane 2), but only the 86-kDa isoform (HSP90α) was found to be present in mouse spermatozoa (Fig. 3, B and C, lane 3). Figure 3D shows that immunoblotting of mouse sperm extracts, separated by 2D gel electrophoresis, with the monoclonal antibody to HSP-90 resulted in a single protein spot being recognized. This protein has a molecular mass of 90 kDa and a pI of 5.1, and this spot on the 2D-immunoblot corresponds with the aforementioned protein that is tyrosine phosphorylated during capacitation (Fig. 3D, inset).

To confirm that HSP-86 is tyrosine phosphorylated during mouse sperm capacitation, cross-immunoprecipitation experiments were conducted using the monoclonal HSP-90 and antiphosphotyrosine (clone 4G10) antibodies. Figure 4A shows that when monoclonal HSP-90 antibody alone is separated by SDS-PAGE and the gel silver stained to visualize the protein bands, no 90-kDa protein is evident (lane 1). However, immunoprecipitation of mouse sperm extracts with this antibody resulted in the appearance of a 90-kDa protein band in the gel (lane 2). The specificity of this interaction was confirmed by showing that another antibody (monoclo-
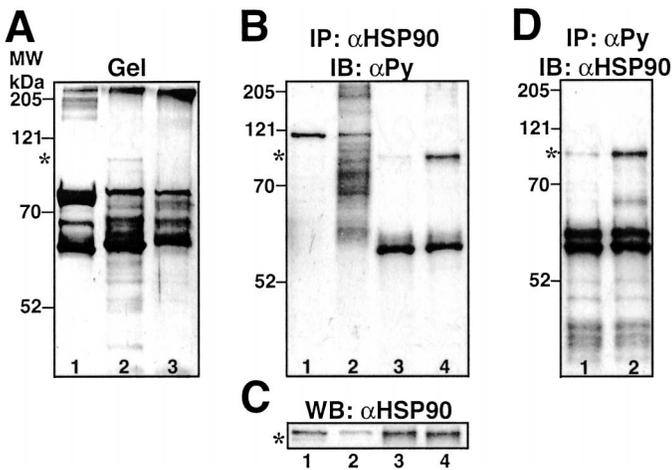
FIG. 4. HSP-90 is tyrosine phosphorylated during mouse sperm capacitation. **A**) Silver-stain gel of proteins following immunoprecipitation with anti-HSP-90 and a "control" antibody. Anti-HSP-90 antibody (2 μg) was run into the gel alone (lane 1). Total protein extracts were immunoprecipitated with anti-HSP-90 (lane 2) or with a control antibody, HSP-60 (lane 3). **B**) Immunoblot (IB) of total protein extracts of mouse caudal spermatozoa at 0 min (lane 1) and 90 min (lane 2) and then immunoprecipitated (IP) with anti-HSP-90 (αHSP90) (lanes 3 [0 min] and 4 [90 min]) and probed with antiphosphotyrosine (αPy). **C**) Reprobing of total and immunoprecipitated proteins with anti-HSP-90 following stripping of the membrane with stripping buffer; WB, western blot. **D**) Immunoblot of proteins from mouse caudal spermatozoa immunoprecipitated with antiphosphotyrosine antibody following 0 min (lane 1) and 90 min (lane 2) incubation in BWW. Asterisks indicate the position of HSP-90. The results shown are representative of five independent experiments.

nal anti-HSP-60) used in this procedure did not immunoprecipitate the 90-kDa protein (lane 3). Furthermore, neither the anti-HSP-90 nor the antiphosphotyrosine antibody detected a 90-kDa protein by immunoblot when either no antibody or the anti-HSP-60 antibody was used to immunoprecipitate proteins (data not shown). Figure 4B confirms that an increase in protein tyrosine phosphorylation occurs in mouse spermatozoa incubated under capacitating conditions for 90 min (lane 1, 0 min; lane 2, 90 min). During this time, a dramatic increase also occurs in tyrosine phosphorylation of HSP-90 (lane 3, 0 min; lane 4, 90 min). The increased signal was caused by an increase in tyrosine phosphorylation of the protein, because both immunoprecipitation lanes contained the same amount of HSP-90 protein (Fig. 4C, lanes 3 and 4). This result was confirmed by cross-immunoprecipitation with the antiphosphotyrosine antibody, which showed an increase in the amount of HSP-90 that was immunoprecipitated following 90-min incubation (Fig. 4D, lanes 1 [0 min] and 2 [90 min]).

HSP-90 Is Tyrosine Phosphorylated in Human and Rat Spermatozoa

To test whether HSP-90 is also tyrosine phosphorylated during capacitation of spermatozoa from other species, immunoprecipitation with the anti-HSP-90 antibody was performed on human and rat sperm extracts. In contrast to the mouse, human spermatozoa were found to display widespread protein tyrosine phosphorylation before incubation (Fig. 5A, lane 1 [0 h]). However, in agreement with findings in the mouse, human spermatozoa also showed an increase in tyrosine phosphorylation when incubated in medium that supports capacitation (Fig. 5A, lane 2 [3 h]). During this time, a corresponding increase was observed in tyrosine phosphorylation of HSP-90 in these cells (Fig. 5A, lanes 3
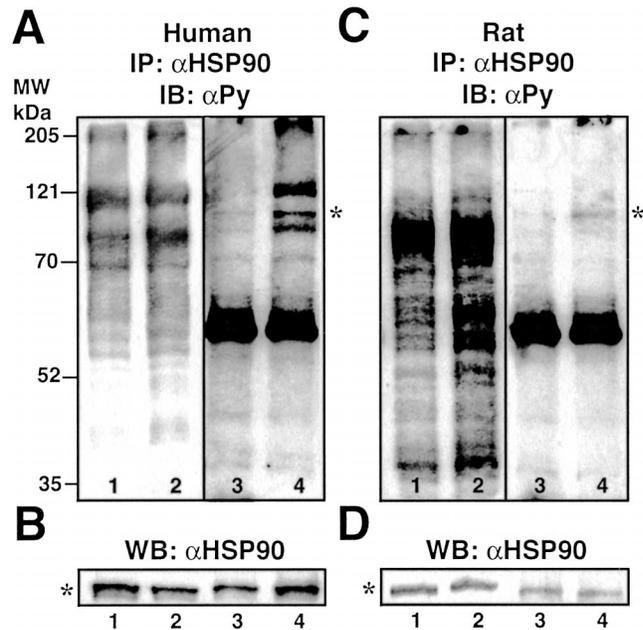

FIG. 5. HSP-90 is tyrosine phosphorylated in human and rat spermatozoa. **A**) Immunoblot (IB) of total protein extracts of human spermatozoa after 0 h (lane 1) and 3 h (lane 2) of incubation and then immunoprecipitated (IP) with anti-HSP-90 (αHSP90) (lanes 3 [0 h] and 4 [3 h]) and probed with antiphosphotyrosine (αPy). The figures for total and immunoprecipitated proteins were obtained by exposing film for 1 and 5 min, respectively. **B**) Reprobing of total and immunoprecipitated proteins with anti-HSP-90 following stripping of the membrane with stripping buffer; WB, western blot. **C**) Immunoblot of total protein extracts of rat spermatozoa following incubation for 0 h (lane 1) and 3 h (lane 2) and then immunoprecipitated with anti-HSP-90 (lanes 3 [0 h] and 4 [3 h]) and probed with antiphosphotyrosine. The figures for total and immunoprecipitated proteins were obtained by exposing the film for 1 and 5 min, respectively. **D**) Reprobing of total and immunoprecipitated proteins with anti-HSP-90 following stripping of the membrane with stripping buffer. Asterisks indicate the position of HSP-90. The results shown are representative of three independent experiments.

[0 h] and 4 [3 h]), and this was not caused by an increase in the amount of HSP-90 that could be immunoprecipitated (Fig. 5B, lanes 3 and 4). Rat spermatozoa were also found to have widespread tyrosine phosphorylation before incubation (Fig. 5C, lane 1 [0 h]) that increased during a 3-h incubation in medium that supports capacitation (Fig. 5C, lane 2 [3 h]). During this time, an increase was observed in tyrosine phosphorylation of HSP-90 (Fig. 5C, lanes 3 [0 h] and 4 [3 h]), and this was not caused by an increase in the amount of HSP-90 that was able to be immunoprecipitated (Fig. 5D, lanes 3 and 4).

Geldanamycin, an Inhibitor of HSP-90, Does Not Affect Its Tyrosine Phosphorylation

Geldanamycin, an ansamycin antibiotic, is able to bind with high affinity to the ATP-binding site of HSP-90 and, thus, disrupt its ATP-dependent chaperoning function. Therefore, we tested whether the signaling pathway leading to tyrosine phosphorylation required the ATP-dependent chaperoning function of HSP-90. Figure 6A shows that geldanamycin had no effect on protein tyrosine phosphorylation of mouse spermatozoa incubated under capacitating conditions. Furthermore, immunoprecipitation of HSP-90 showed that its tyrosine phosphorylation is not disrupted by geldanamycin in the incubation medium (Fig. 6B). Studies were also conducted to test whether geldanamycin dis-

rupted significant parameters associated with sperm capacitation. Geldanamycin (0–20 μM) was found not to affect sperm motility, hyperactivation, their ability to acrosome react (either spontaneously or in response to 5 μM A23187 or progesterone), or the number of spermatozoa able to bind to isolated mouse zona pellucida (data not shown). At higher concentrations (50 μM), geldanamycin induced sperm agglutination (data not shown).

DISCUSSION

To our knowledge, this is the first study to identify a protein that becomes tyrosine phosphorylated during capacitation of mouse spermatozoa. Furthermore, we demonstrate that the tyrosine phosphorylation of HSP-90 during capacitation also occurs in human and rat spermatozoa when incubated under conditions that support capacitation and, as such, may represent an important fundamental requirement for mammalian capacitation in general. This is also, to our knowledge, the first report implicating a role for protein chaperones in the signaling pathway leading to sperm capacitation and, thus, represents an important contribution toward our further understanding of this process.

The evidence that HSP-90 is tyrosine phosphorylated during sperm capacitation is as follows: First, a 90 kDa protein, tyrosine phoshophorylated during sperm capacitation, was excised from a 2D gel and shown to be HSP-90 by tandem MS/MS protein sequencing. Second, 1D immunoblotting with anti-HSP-90 antibodies confirmed that the 86-kDa isoform of this protein is present in mouse spermatozoa. Third, 2D immunoblotting with anti-HSP-90 antibodies confirmed the presence of a single immunoreactive protein that exactly matched the position of a major tyrosine phosphorylated protein, which was shown to be HSP-90 by direct sequencing. Fourth, immunoprecipitation with anti-HSP-90 antibodies brought down a protein that cross-reacted with antiphosphotyrosine antibodies and, moreover, showed an increase in phosphotyrosine expression during capacitation (Fig. 4B, lanes 3 and 4). Fifth, immunoprecipitation with an antiphosphotyrosine antibody brought down a protein that cross-reacted with the anti-HSP-90 antibody. Furthermore, the amount of HSP-90 protein immunoprecipitated by this antiphosphotyrosine antibody increased during capacitation in concert with the cellular increase in tyrosine phosphorylation.

A recent study by Ficarro et al. [23] used 2D gel electrophoresis to sequence proteins from human spermatozoa that matched those found to be tyrosine phosphorylated after incubation under capacitating conditions. This "phosphoproteome" study identified HSP-90α as a target for tyrosine phosphorylation in human spermatozoa. In the present study, we demonstrate, by cross-immunoprecipitation, that HSP-90 is a target for tyrosine phosphorylation and, therefore, is activated during sperm capacitation. This is significant because of the key role of HSP-90 in signal transduction cascades in other cellular systems (see below).

Studies to date that have identified proteins phosphorylated during capacitation have been limited, especially in the mouse. Yet, this tyrosine phosphorylation event is thought to play an important role in promoting the fertilizing ability of spermatozoa, because it has been correlated with hyperactivation [8, 9] and with sperm-egg interaction [5, 11]. Significantly, a number of identified tyrosine phosphoproteins have been directly implicated in mediating these processes [11, 15, 24, 25]. The identification of HSP-90 as a target for tyrosine phosphorylation suggests that the coordinated involvement of molecular chaperones with
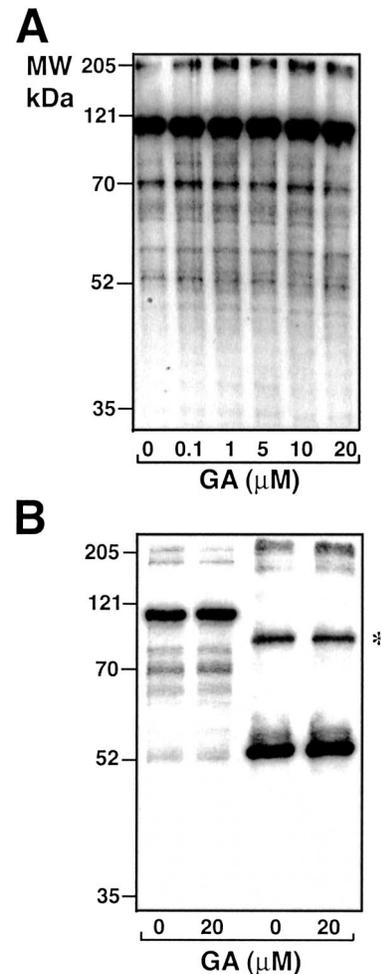
FIG. 6. The effect of geldanamycin (GA) on protein tyrosine phosphorylation. **A**) Immunoblot of mouse caudal sperm extracts following incubation in BWW for 90 min in the presence of geldanamycin (0–20 μM). **B**) Mouse caudal sperm incubated in BWW for 90 min, alone, or in the presence of 20 μM geldanamycin were immunoprecipitated with anti-HSP-90 antibody and then immunoblotted with antiphosphotyrosine antibody. Asterisks indicate the position of HSP-90. The results shown are representative of three independent experiments.

their client proteins may play key roles in these events leading to fertilization.

The HSP-90 is a highly conserved, 90-kDa protein that, apart from being induced under stress conditions, is also one of the most abundant cellular proteins, accounting for 1–2% of the total cellular protein pool in most cells (see [26, 27] and references therein). Along with its role in heat stress, HSP-90 has been shown to be a necessary component of a variety of signaling pathways, where it acts in concert with other proteins, such as Cdc37/p50 [28, 29], to ensure the correct folding and, therefore, activity of client proteins. The signaling pathway leading to tyrosine phosphorylation is mediated via cAMP and PKA [3, 4, 30], suggesting the involvement of protein serine/threonine and tyrosine phosphorylation. Thus, HSP-90 has the potential to mediate serine/threonine and/or tyrosine kinase signaling in the pathway leading to the global increase in protein tyrosine phosphorylation that occurs during this process.

Studies have shown that mouse HSP-90 exists in two forms, HSP-84 and HSP-86 (76% homologous), which are encoded by separate genes [31], but HSP-84 is not present in the male gamete [32], which was confirmed in the pre-

sent study (Fig. 3). HSP-90 has been demonstrated to play an important role during spermatogenesis [33] such that reductions in HSP-90 function correlate with male sterility in viable transheterozygotes of *Drosophila* sp. [34]. In studies of boar spermatozoa, HSP-90 has been implicated as playing a role in sperm motility, because geldanamycin was found to reduce the percentage of motile spermatozoa [35, 36]. The failure of geldanamycin to inhibit motility in the present study may reflect species differences in the role of HSP-90 in sperm motility. Experiments aimed at determining whether geldanamycin inhibits sperm functions (including motility) in other species are currently being conducted in our laboratory. Despite these studies demonstrating the importance of HSP-90 in spermatozoa, its participation in the signaling events associated with capacitation has not been previously recognized.

Both the serine/threonine phosphorylation of HSP-90 [37, 38] and its association with a large number of tyrosine kinases are well established (see [27] and references therein). However, previous to the present study, the tyrosine phosphorylation of HSP-90 had only been reported in endothelial cells following their exposure to agents aimed at increasing nitric oxide production [39, 40]. Thus, little is known about the cellular effects of HSP-90 tyrosine phosphorylation or the manner by which it is regulated. The studies in endothelial cells demonstrated that the tyrosine phosphorylation of HSP-90 was an important step in the activation of endothelial nitric oxide synthase [39, 40]. Significantly, endothelial nitric oxide synthase has been localized to the head and midpiece of human spermatozoa [41, 42], and nitric oxide is thought to play a role in events associated with capacitation [43, 44]. Thus, the tyrosine phosphorylation of HSP-90 during sperm capacitation may represent an important component of the signaling pathway leading to nitric oxide production in these cells.

Alternatively, the role of HSP-90 in tyrosine kinase function and antigen presentation in other systems suggests that it may also play a significant role in mediating the fertilizing ability of the spermatozoon through similar mechanisms. Indeed, some well-characterized binding substrates of HSP-90 include Src family tyrosine kinases and Raf serine/threonine kinase (see [27] and references therein), both of which have been implicated in mediating events associated with sperm fertilization [45, 46]. Furthermore, the association of HSP-90 with client proteins, such as extracellular signal-regulated kinases (ERKs) [47], phosphatases [48], and calmodulin [49], as well as its capacity to bind calcium [49] suggest possible pathways by which this chaperone might be involved in the regulation of sperm function given the proposed roles of these proteins in sperm motility, capacitation, and sperm-egg interaction [46, 50–53]. Tyrosine phosphorylation of HSP-90 may result in its conformational change and concomitant change in substrate specificity during capacitation, which then facilitates downstream events associated with this process. Thus, future studies aimed at determining the client proteins of the tyrosine phosphorylated HSP-90 are essential to elucidate its role in the events leading up to fertilization. Furthermore, it remains to be determined whether tyrosine phosphorylation of HSP-90 produces a shift in its subcellular localization to facilitate a change in substrate specificity. This is the focus of work currently being conducted in our laboratory.

Studies have shown that HSP-90 is an ATP-dependent chaperone [54, 55], but not all of its chaperoning activity is ATP dependent [56] (see also [57, 58] and references therein). Significantly, in the present study, the specific HSP-90-inhibitor geldanamycin, which binds to the nucleotide-binding site of the protein, did not disrupt the tyrosine phosphorylation of HSP-90. Furthermore, addition of geldanamycin to mouse spermatozoa incubated under capacitating conditions did not disrupt their motility, hyperactivation, tyrosine phosphorylation, or ability to bind to zona pellucida proteins. Together, these results suggest either that the activity of tyrosine phosphorylated HSP-90 in mouse spermatozoa is not dependent on its ATPase activity or that the ATP-dependent chaperoning activity of HSP-90 plays a role in other events associated with fertilization (e.g., fusion with the oolemma). Regarding the ATP-independent chaperoning functions of HSP-90, it is interesting that the interaction of HSP-90 with protein phosphatase 5, a serine/threonine phosphatase, is not sensitive to geldanamycin [59].

In conclusion, the findings of the present study provide, to our knowledge, the first identification of a protein (HSP-90α) that becomes tyrosine phosphorylated during capacitation in mouse spermatozoa. We have also demonstrated that tyrosine phosphorylation of HSP-90 occurs in human and rat spermatozoa when incubated under conditions that support capacitation. Thus, the present results are of general significance in understanding the biochemistry of mammalian sperm capacitation.

REFERENCES

1. Austin CR. Observations on the penetration of the sperm into the mammalian egg. Aust J Sci Res 1951; 4:581–596.
2. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 1951; 168:697–698.
3. Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 1995; 121:1129–1137.
4. Visconti PE, Stewart-Savage J, Blasco A, Battaglia L, Miranda P, Kopf GS, Tezon JG. Roles of bicarbonate, cAMP, and protein tyrosine phosphorylation on capacitation and the spontaneous acrosome reaction of hamster sperm. Biol Reprod 1999; 61:76–84.
5. Urner F, Leppens-Luisier G, Sakkas D. Protein tyrosine phosphorylation in sperm during gamete interaction in the mouse: the influence of glucose. Biol Reprod 2001; 64:1350–1357.
6. Mandal A, Naaby-Hansen S, Wolkowicz MJ, Klotz K, Shetty J, Retief JD, Coonrod SA, Kinter M, Sherman N, Cesar F, Flickinger CJ, Herr JC. FSP95, a testis-specific 95-kilodalton fibrous sheath antigen that undergoes tyrosine phosphorylation in capacitated human spermatozoa. Biol Reprod 1999; 61:1184–1197.
7. Nassar A, Mahony M, Morshedi M, Lin MH, Srisombut C, Oehninger S. Modulation of sperm tail protein tyrosine phosphorylation by pentoxifylline and its correlation with hyperactivated motility. Fertil Steril 1999; 71:919–923.
8. Turner RM, Eriksson RL, Gerton GL, Moss SB. Relationship between sperm motility and the processing and tyrosine phosphorylation of two human sperm fibrous sheath proteins, pro-hAKAP82 and hAKAP82. Mol Hum Reprod 1999; 5:816–824.
9. Si Y, Okuno M. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. Biol Reprod 1999; 61:240–246.
10. Yeung CH, Weinbauer GF, Cooper TG. Responses of monkey epididymal sperm of different maturational status to second messengers mediating protein tyrosine phosphorylation, acrosome reaction, and motility. Mol Reprod Dev 1999; 54:194–202.
11. Flesch FM, Colenbrander B, van Golde LM, Gadella BM. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. Biochem Biophys Res Commun 1999; 262:787–792.
12. Carrera A, Moos J, Ning XP, Gerton GL, Tesarik J, Kopf GS, Moss SB. Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: identification of A kinase anchor proteins as major substrates for tyrosine phosphorylation. Dev Biol 1996; 180:284–296.
13. Kula Nand J, Shivaji S. Identification of the major tyrosine phosphorylated protein of capacitated hamster spermatozoa as a homo-

logue of mammalian sperm a kinase anchoring protein. Mol Reprod Dev 2002; 61:258–270.

14. Johnson LR, Foster JA, Haig-Ladewig L, VanScoy H, Rubin CS, Moss SB, Gerton GL. Assembly of AKAP82, a protein kinase A anchor protein, into the fibrous sheath of mouse sperm. Dev Biol 1997; 192:340–350.

15. Naaby-Hansen S, Mandal A, Wolkowicz MJ, Sen B, Westbrook VA, Shetty J, Coonrod SA, Klotz KL, Kim YH, Bush LA, Flickinger CJ, Herr JC. CABYR, a novel calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein involved in capacitation. Dev Biol 2002; 242:236–254.

16. Kalab P, Visconti P, Leclerc P, Kopf GS. p95, the major phosphotyrosine-containing protein in mouse spermatozoa, is a hexokinase with unique properties. J Biol Chem 1994; 269:3810–3817.

17. Visconti PE, Olds-Clarke P, Moss SB, Kalab P, Travis AJ, de las Heras M, Kopf GS. Properties and localization of a tyrosine phosphorylated form of hexokinase in mouse sperm. Mol Reprod Dev 1996; 43:82–93.

18. Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC (ed.), Methods in Mammalian Embryology. San Francisco: Freeman; 1971:86–116.

19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680–685.

20. Nesterenko MV, Tilley M, Upton SJ. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. J Biochem Biophys Methods 1994; 28:239–242.

21. Towbin H, Staehelin TH, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979; 76:4350–4354.

22. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 1988; 85:2444–2448.

23. Ficarro S, Chertihin O, Westbrook VA, White F, Jayes F, Kalab P, Marto JA, Shabanowitz J, Herr JC, Hunt DF, Visconti PE. Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation. J Biol Chem 2003; 278:11579–11589.

24. Brandt H, Hoskins DD. A cAMP-dependent phosphorylated motility protein in bovine epididymal sperm. J Biol Chem 1980; 255:982–987.

25. Vijayaraghavan S, Trautman KD, Goueli SA, Carr DW. A tyrosine-phosphorylated 55-kilodalton motility-associated bovine sperm protein is regulated by cyclic adenosine 3′,5′-monophosphates and calcium. Biol Reprod 1997; 56:1450–1457.

26. Picard D. Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 2002; 59:1640–1648.

27. Richter K, Buchner J. Hsp90: chaperoning signal transduction. J Cell Physiol 2001; 188:281–290.

28. Brugge JS, Erikson E, Erikson RL. The specific interaction of the Rous sarcoma virus transforming protein, pp60src, with two cellular proteins. Cell 1981; 25:363–372.

29. Oppermann H, Levinson W, Bishop JM. A cellular protein that associates with the transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc Natl Acad Sci U S A 1981; 78:1067–1071.

30. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterized by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 1998; 111:645–656.

31. Moore SK, Kozak C, Robinson EA, Ullrich SJ, Appella E. Murine 86- and 84-kDa heat shock proteins, cDNA sequences, chromosome assignments, and evolutionary origins. J Biol Chem 1989; 264:5343–5351.

32. Gruppi CM, Wolgemuth DJ. HSP86 and HSP84 exhibit cellular specificity of expression and co-precipitate with an HSP70 family member in the murine testis. Dev Genet 1993; 14:119–126.

33. Gruppi CM, Zakeri ZF, Wolgemuth DJ. Stage and lineage-regulated expression of two hsp90 transcripts during mouse germ cell differentiation and embryogenesis. Mol Reprod Dev 1991; 28:209–217.

34. Yue L, Karr TL, Nathan DF, Swift H, Srinivasan S, Lindquist S. Genetic analysis of viable Hsp90 alleles reveals a critical role in *Drosophila* spermatogenesis. Genetics 1999; 151:1065–1079.

35. Huang SY, Kuo YH, Tsou HL, Lee YP, King YT, Huang HC, Yang PC, Lee WC. The decline of porcine sperm motility by geldanamycin, a specific inhibitor of heat-shock protein 90 (HSP90). Theriogenology 2000; 53:1177–1184.

36. Huang SY, Kuo YH, Lee WC, Tsou HL, Lee YP, Chang HL, Wu JJ, Yang PC. Substantial decrease of heat-shock protein 90 precedes the

decline of sperm motility during cooling of boar spermatozoa. Theriogenology 1999; 51:1007–1016.

37. Lees-Miller SP, Anderson CW. The human double-stranded DNA-activated protein kinase phosphorylates the 90-kDa heat-shock protein, hsp90 alpha at two NH2-terminal threonine residues. J Biol Chem 1989; 264:17275–17280.

38. Lees-Miller SP, Anderson CW. Two human 90-kDa heat shock proteins are phosphorylated in vivo at conserved serines that are phosphorylated in vitro by casein kinase II. J Biol Chem 1989; 264:2431–2437.

39. Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O. Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. Circ Res 2001; 89:866–873.

40. Harris MB, Ju H, Venema VJ, Blackstone M, Venema RC. Role of heat shock protein 90 in bradykinin-stimulated endothelial nitric oxide release. Gen Pharmacol 2000; 35:165–170.

41. Lewis SE, Donnelly ET, Sterling ES, Kennedy MS, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod 1996; 2:873–878.

42. O'Bryan MK, Zini A, Cheng CY, Schlegel PN. Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. Fertil Steril 1998; 70:1143–1147.

43. Herrero MB, Cebral E, Boquet M, Viggiano JM, Vitullo A, Gimeno MA. Effect of nitric oxide on mouse sperm hyperactivation. Acta Physiol Pharmacol Ther Latinoam 1994; 44:65–69.

44. Zini A, De Lamirande E, Gagnon C. Low levels of nitric oxide promote human sperm capacitation in vitro. J Androl 1995; 16:424–431.

45. Hickox DM, Gibbs G, Morrison JR, Sebire K, Edgar K, Keah HH, Alter K, Loveland KL, Hearn MT, de Kretser DM, O'Bryan MK. Identification of a novel testis-specific member of the phosphatidylethanolamine binding protein family, pebp-2. Biol Reprod 2002; 67:917–927.

46. De Lamirande E, Gagnon C. The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod 2002; 8:124–135.

47. Setalo G Jr, Singh M, Guan X, Toran-Allerand CD. Estradiol-induced phosphorylation of ERK1/2 in explants of the mouse cerebral cortex: the roles of heat shock protein 90 (Hsp90) and MEK2. J Neurobiol 2002; 50:1–12.

48. Silverstein AM, Galigniana MD, Chen MS, Owens-Grillo JK, Chinkers M, Pratt WB. Protein phosphatase 5 is a major component of glucocorticoid receptor.hsp90 complexes with properties of an FK506-binding immunophilin. J Biol Chem 1997; 272:16224–16230.

49. Minami Y, Kawasaki H, Suzuki K, Yahara I. The calmodulin-binding domain of the mouse 90-kDa heat shock protein. J Biol Chem 1993; 268:9604–9610.

50. Ahmad K, Bracho GE, Wolf DP, Tash JS. Regulation of human sperm motility and hyperactivation components by calcium, calmodulin, and protein phosphatases. Arch Androl 1995; 35:187–208.

51. Aitken RJ, Clarkson JS, Hulme MJ, Henderson CJ. Analysis of calmodulin acceptor proteins and the influence of calmodulin antagonists on human spermatozoa. Gamete Res 1988; 21:93–111.

52. Si Y, Olds-Clarke P. Evidence for the involvement of calmodulin in mouse sperm capacitation. Biol Reprod 2000; 62:1231–1239.

53. White DR, Aitken RJ. Relationship between calcium, cyclic AMP, ATP, and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res 1989; 22:163–177.

54. Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell 1997; 90:65–75.

55. Scheibel T, Neuhofen S, Weikl T, Mayr C, Reinstein J, Vogel PD, Buchner J. ATP-binding properties of human Hsp90. J Biol Chem 1997; 272:18608–18613.

56. Jakob U, Buchner J. Assisting spontaneity: the role of Hsp90 and small Hsps as molecular chaperones. Trends Biochem Sci 1994; 19:205–211.

57. Jakob U, Scheibel T, Bose S, Reinstein J, Buchner J. Assessment of the ATP binding properties of Hsp90. J Biol Chem 1996; 271:10035–10041.

58. Buchner J. Supervising the fold: functional principles of molecular chaperones. FASEB J 1996; 10:10–19.

59. Shao J, Hartson SD, Matts RL. Evidence that protein phosphatase 5 functions to negatively modulate the maturation of the Hsp90-dependent heme-regulated eIF2α kinase. Biochemistry 2002; 41:6770–6779.