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

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Tyrosine Phosphorylation of HSP-90 During Mammalian Sperm Capacitation

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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.

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] spermatozoa 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)-90, 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); Heps, penicillin, and streptomycin (Gibco, Paisley, U.K.); Protein A/G Plus sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA); goat serum (Hunst Antisera, Jesmond, NSW, Australia); amphotelines (pH 3–10), immobilized pH gradient (IPG) strips, ammonium persulfate, 2-mercaptoethanol, and N,N,N’,N’-tetra-methylethylenediamine (Temed; BioRad, Herts, U.K.); mowiol 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 epidydimal 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

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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, 30 min). The spermatozoa were washed with 5 ml of BWW, centrifuged (500 g, 3 min), and the supernatant was 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% CO₂ 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-cholamidopropyl)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% (v/v) iodoacetamide replaced the DTT (15 min, room temperature). Next, SDS-PAGE was conducted on 7.5–10% acrylamide gels. After loading, strips were transferred to membranes and immunoblotted as described above. To ensure equal loading, 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 anti-phosphotyrosine antibody (2 µg) in immunoprecipitation buffer (1% [w/v] Trition 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 anti-phosphotyrosine 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 phosphorylated.
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 pls, 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 this spot on the 2D-immunoblot corresponds with the protein having a molecular mass of 90 kDa and a pI of 5.1, which is representative of three independent experiments. Note the arrows indicating a specific protein spot of 90 kDa and pl 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.

**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, A 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 pl 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.

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-
ruptured 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 phosphorylated 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-90 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 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 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.

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