

Changes in Sperm Glycogen Synthase Kinase-3 Serine Phosphorylation and Activity Accompany Motility Initiation and Stimulation

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Abstract: Sperm motility is regulated by protein phosphorylation. We have shown that the signaling kinase, glycogen synthase kinase-3 α (GSK-3 α), is present in spermatozoa. In somatic cells, GSK-3 is regulated by serine and tyrosine phosphorylation. In this report, we document that both GSK-3 α and GSK-3 β isoforms are present in spermatozoa, with GSK-3 α being the predominant isoform. The relationship between GSK-3 serine phosphorylation and motility was investigated. Serine phosphorylation of GSK-3 increases significantly in spermatozoa during their passage through the epididymis. Initiation and stimulation of motility *in vitro* by isobutyl-methyl-xanthine, 2-chloro-2'-deoxy-adenosine, and calyculin A lead to a dramatic increase in GSK-3 serine phosphorylation. The concentration-dependent induction of motility by calyculin A is closely associated with GSK-3 serine phosphorylation. Immunoprecipitation of GSK-3 α and GSK-3 β shows that both of the GSK-3 isoforms are more active

in caput than in caudal spermatozoa. Calyculin A treatment decreased the activity of both isoforms. Column chromatography was used to purify inactive GSK-3 α from the caudal sperm extracts. This GSK-3 α species was phosphorylated at amino acid residues serine 21 and tyrosine 214. Inactive GSK-3 α is present in caudal but not in caput epididymal spermatozoa. The enzymes protein kinase B (PKB; also known as cAkt) and phosphoinositide 3-kinase (PI3-kinase), the upstream signaling proteins involved in GSK-3 phosphorylation, are both present in spermatozoa. Fluorescence immunocytochemistry showed that GSK-3 is present in the head and tail regions of sperm. Our work suggests a novel role for the signaling system involving GSK-3 in the regulation of sperm motility.

Key words: Epididymis, protein kinase B, phosphoinositide 3-kinase.

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The long-term goal of our laboratory is to understand the biochemical mechanisms underlying the regulation of sperm motility. Motility is essential for spermatozoa to reach and penetrate the ovum. Biochemical pathways regulating motility are still unknown. Phosphorylation is a universal mechanism by which protein activity is regulated in cells. Protein phosphorylation is involved in the initiation and regulation of sperm motility. In a recent study, we showed that the tyrosine phosphorylation of a 55-kd protein varied in direct proportion to motility. The tyrosine phosphorylation of the 55-kd protein measured by immunoreactivity to phosphotyrosine antibody 4G10 was low in immotile compared to motile epididymal bovine spermatozoa. Inhibition or stimulation of motility resulted in a corresponding decrease or increase in the tyrosine phosphorylation of the protein (Vijayaraghavan et al, 1997). Further studies showed that this motility-associated, tyrosine-phosphorylated protein was glycogen synthase kinase-3 α (GSK-3 α) (Vijayaraghavan et al, 2000).

GSK-3 is a signaling enzyme involved in the biochemical pathways mediating insulin and growth hormone action (Hughes et al, 1993). GSK-3 has also been implicated in cell survival and apoptosis (Wang et al, 1994b; Welsh et al, 1994). Two isoforms of GSK-3, α and β , encoded by 2 independent genes, are present in mammalian cells (Woodgett, 1990). GSK-3 is regulated through phosphorylation at its tyrosine 214 amino acid residue and also at its serine 21 (serine 9 in GSK-3 β) amino acid residue (Hughes et al, 1993). In somatic cells, phosphoinositide 3-kinase (PI3-kinase) and cAkt are the upstream regulators of GSK-3 (Hemmings, 1997). In somatic cells, constitutively active GSK-3 is tyrosine phosphorylated. Most of the studies of somatic cells that are focused on GSK-3 β show that the enzyme becomes serine phosphorylated and partially inactivated in response to external signals (Woodgett, 2001).

In this study, we show that both isoforms of GSK-3 are present in spermatozoa, with GSK-3 α being the predominant isoform. Our results also show that sperm GSK-3 α and GSK-3 β are serine phosphorylated in direct proportion to motility. The serine phosphorylation of GSK-3, measured by immunoreactivity to an antibody specific for phosphorylated GSK-3, is low in immotile compared to motile bovine epididymal spermatozoa. This relationship between sperm motility and GSK-3 serine phos-

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phorylation could also be demonstrated *in vitro* using motility inhibitors and stimulators. Immotile caput spermatozoa, when exposed to a motility stimulator such as calyculin A, become active, with a concomitant increase in the serine phosphorylation of GSK-3. Increased serine phosphorylation of GSK-3 resulted in lower catalytic activity of the enzyme. We also used column chromatography techniques to confirm our observation that the higher-molecular-weight species of GSK-3 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is the tyrosine- and serine-phosphorylated GSK-3 α isoform. This form of GSK-3, phosphorylated at 2 residues, is present in motile caudal but not immotile caput epididymal spermatozoa. The upstream signaling enzymes, cAkt and PI3-kinase, involved in the regulation of GSK-3 serine phosphorylation are present in spermatozoa. This report suggests a novel role in sperm motility for a signaling system known to be involved in the regulation of growth and survival of somatic cells.

Materials and Methods

Sperm Preparation

Testes from mature bulls with intact tunica were obtained from a local slaughterhouse, and sperm were isolated from the caput or caudal epididymis and washed as previously described (Vijayaraghavan et al, 1996, 2000) in a sperm diluent buffer (10 mmol of Tris-HCl per liter [pH 7.2] containing 10 mmol of KCl per liter, 120 mmol of NaCl per liter, and 5 mmol of MgSO₄ per liter) supplemented with 10 mmol of glucose per liter.

Sperm Extracts

After incubation, the sperm suspensions, with treatments such as isobutyl-methyl-xanthine (IBMX; Sigma Chemical Co, St Louis, Mo), 2-chloro-2'-deoxy-adenosine (CdA) (Sigma), calyculin A (Upstate Biotechnologies, Lake Placid, NY), and sHT31 (Promega, Madison, Wis), were pelleted by centrifugation at 600 \times g for 5 minutes at 4°C. Sperm pellets were suspended in homogenization buffer (10 mmol of Tris per liter [pH 7.2] containing 1 mmol of EDTA per liter and 1 mmol of EGTA [ethyleneglycoltetraacetic acid] per liter) supplemented with proteolytic inhibitors (10 mmol of benzamidine per liter, 0.1 mmol of N-tosyl-L-lysyl chloromethyl ketone [TPCK] per liter, 0.1% β -mercaptoethanol, and 1 mmol of phenylmethylsulfonyl fluoride [PMSF] per liter), phosphatase inhibitors (1 mmol of sodium vanadate per liter and 1 nmol of calyculin A per liter), and 1% Triton X-100 (Vijayaraghavan et al, 1996). The sperm suspensions were then centrifuged at 16000 \times g for 15 minutes at 4°C. The supernatants thus obtained were stored at -20°C until further use for biochemical studies.

SDS-PAGE and Western Blot Analysis

Sperm extracts (50 μ g) were separated through 12% polyacrylamide slab gels (Laemli, 1970). After gel electrophoresis, proteins were electrophoretically transferred to a polyvinylidene

fluoride membrane (Immobilon-P; Millipore Corp, Bedford, Mass). Nonspecific protein binding sites on the membrane were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 25 mmol of Tris-HCl per liter, pH 7.4, and 150 mmol of NaCl per liter). The blots were washed twice for 15 minutes with TBS containing 0.1% Tween 20 (TTBS) and then incubated overnight with anti-phosphoserine GSK-3 antibody (Upstate Biotechnologies, Waltham, Mass, or Cell Signaling Technology, Beverly, Mass) or anti-phosphotyrosine GSK-3 antibody (Biosource, Camarillo, Calif) at a 1:1000 dilution in 5% milk in TTBS. After washing, the blots were incubated with rabbit anti-sheep (for anti-phosphoserine GSK-3 antibody; Upstate Biotechnologies) or with anti-rabbit (for phosphotyrosine) secondary antibody. The blots were then washed twice (15 minutes each) and 4 times (5 minutes each) before development with an enhanced chemiluminescence system (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. Parallel blots were developed with anti-GSK-3 α primary antibody (Zymed, San Francisco, Calif) to ensure equal protein loading. Similar protocols were used for the detection of PI3-kinase (Upstate Biotechnologies) and cAkt (New England Biolabs, Beverly, Mass) on Western blots.

GSK-3 Kinase Assay

GSK-3 activity was measured by counting the amount of ³²P transferred from [³²P]- γ -adenosine triphosphate ([³²P]- γ -ATP) to phospho-cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB; Lerner Research Institute, Cleveland, Ohio). Briefly, sperm extracts were added to phospho-CREB (1 mg/mL) along with the ATP cocktail mix (200 mmol of HEPES per liter, pH 7.5, 50 mmol of MgCl₂ per liter, 8 mmol of dithiothreitol per liter, 1 mmol of β -glycerol phosphate per liter, 400 mmol of ATP per liter, and 0.4 μ Ci of [γ -³²P] ATP per liter [10 mCi/mL; 3000 Ci/mmol]). The mixture was incubated at 30°C for 5 minutes. GSK-3 activity was also measured in the presence of 67 mmol of LiCl per liter. At the end of the incubation, a 12- μ L aliquot of the reaction mixture was applied to a phosphocellulose cation exchanger (P81; Whatman Inc, Clifton, NJ) paper cut into 1.5- \times 1.5-cm squares and washed with 0.5% (vol/vol) phosphoric acid. After 3 washes (10 minutes each) in phosphoric acid, the squares were placed into scintillation vials with 2 mL of distilled water and counted in a scintillation counter. The LiCl-sensitive protein kinase activity was assumed to have originated from GSK-3 (Ryves et al, 1998). Assays were conducted in duplicate, and means of 2 or more separate experiments are shown.

Purification of GSK-3

Caudal sperm extracts (50 mL prepared from 5 \times 10¹⁰ spermatozoa in homogenizing buffer) were passed through a diethylaminoethyl (DEAE)-cellulose (0.5 \times 13 cm) column preequilibrated with homogenizing buffer with additional 0.05 M KCl (buffer A). The column was washed with 20 mL of buffer A, and the 70-mL flow through containing all of the GSK-3 in the original extract was concentrated and applied to a Mono S column (1 mL, prepacked, high-resolution FPLC; Amersham Pharmacia). The column was washed with 5 mL of buffer A, which was followed by a linear gradient of 0.05–0.65 M KCl in homoge-

nizing buffer (buffer B). Immunoreactive fractions containing GSK-3 were pooled and concentrated to 0.5 mL. The same protocol was used for the purification of GSK-3 from caput spermatozoa.

Immunoprecipitation

Immunoprecipitation of GSK-3 α from sperm extracts was performed following the protocol of Fang et al (2000) with modifications. Sperm extracts (from 1×10^8 sperm in 100 μ L) were diluted 1:1 in immunoprecipitation buffer A (50 mmol of Tris per liter, pH 7.2, 1 mmol of EGTA per liter, 1 mmol of EDTA per liter, 150 mmol of NaCl per liter, 10% glycerol [vol/vol], 1% Triton X-100 [vol/vol], 10 mmol of benzamidine per liter, 0.1 mmol of TPCK per liter, 0.1% β -mercaptoethanol [vol/vol], 1 mmol of PMSF per liter, 1 mmol of sodium vanadate per liter, and 1 nmol of calyculin A per liter) and incubated for 2 hours at 4°C by rocking with 5 μ g of rabbit GSK-3 α (Zymed). Protein G-sepharose beads (40 μ L) were washed 2 times with 500 μ L of distilled water and once with buffer A. Washed beads were then incubated for approximately 1 hour with 100 μ L of buffer B (buffer A with 10% bovine serum albumin [BSA]). The beads, complexed with the GSK-3 α antibody, were pelleted and washed 3 times with 250 μ L of buffer A and then mixed with the sperm extracts, which had been pretreated with DEAE-cellulose. After further incubation for 30 minutes, this mixture was centrifuged to collect the supernatant. The pellet was washed 2 times in buffer A and resuspended in a final volume of 100 μ L of buffer A. The crude extracts, the supernatants, and the immunoprecipitated pellets were then used for the GSK-3 assay.

Immunocytochemistry

Spermatozoa were isolated as described above, washed twice, and resuspended in phosphate-buffered saline (PBS). Approximately 1×10^8 sperm were added to 5 mL of 4% formaldehyde in PBS (pH 7.0) and left on ice for 30 minutes, which was followed by a 30-minute incubation with 5 mL of a PBS/4% formaldehyde mixture containing 0.2% Triton X-100. Fifty microliters of the mixture was then layered onto a polylysine-coated coverslip and allowed to air dry. The coverslips were washed in TTBS 3 times for 10 minutes each. To block nonspecific binding, 200 μ L of BSA/TTBS solution was layered onto the coverslips and further incubated in a humidified chamber at room temperature for 3 hours. The blocking solution was poured off, and the primary antibody, anti-GSK-3 α (Zymed), diluted 1:500 in the BSA/TTBS/goat serum solution, was added to the coverslips. The coverslips were then left to incubate overnight in a humidified chamber at 4°C. After three 10-minute washes in TTBS, goat anti-rabbit conjugated to indocarbocyanine (CY3) secondary antibody (Jackson Laboratories, West Grove, Pa) was layered onto the coverslips at a 1:500 dilution in BSA/TTBS/goat serum solution. The secondary antibody was incubated in a humidified chamber at room temperature for 2 hours and covered with aluminum foil to shield it from light. After another 5 washes in TTBS for 10 minutes each (shielded from light), the coverslips were air dried, mounted to slides using mounting solution, and pressed between tissue paper to remove excess fluid. The edges were sealed using clear nail polish to prevent the

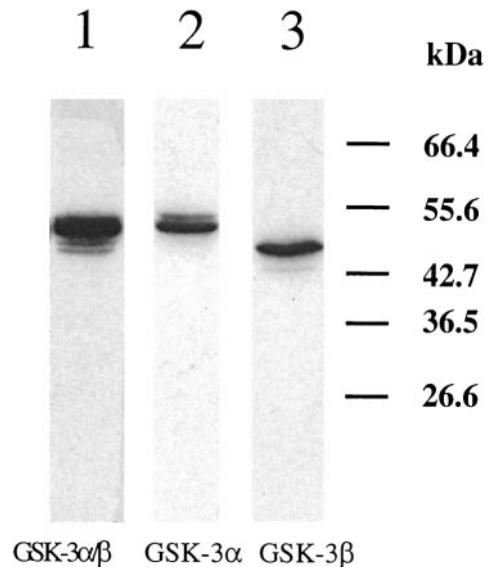


Figure 1. Western blot analysis of glycogen synthase kinase-3 (GSK-3) in bovine epididymal spermatozoa. Lane 1 shows caudal 16K supernatant immunoreactivity to an antibody against the α and β forms of GSK-3. Lane 2 uses an affinity-purified antibody against GSK-3 α , and lane 3 shows the immunoreactivity against GSK-3 β .

coverslip from drying. Finally, the coverslips were viewed using a fluorescence microscope.

Results

Two Isoforms of GSK-3 Are Present in Bovine Spermatozoa

We have previously shown that spermatozoa contain GSK-3 α (Vijayaraghavan et al, 2000). However, it is not known whether the GSK-3 β isoform is also present in spermatozoa and what the relative levels of α and β GSK-3 isoforms are. Western blot shown in Figure 1 (lane 1) was developed with an antibody raised against the catalytic domain (KQLLHGEPNVS YICSRYY) of *Drosophila* GSK-3. The catalytic domain is virtually identical in GSK-3 from diverse organisms and in the GSK-3 α and GSK-3 β isoforms of the enzyme. The antibody reacts with both GSK-3 α and GSK-3 β . The antibody reacts with 3 protein bands in Western blot analysis of caudal sperm extracts (Figure 1, lane 1). The most prominent band at 51 kd corresponds to GSK-3 α . The band of lower intensity is a doublet. The lowermost band (47 kd) of this doublet corresponds to GSK-3 β , and the identity of the middle band is not known. This assignment is based on a comparison of lane 1 with lanes 2 and 3. Figure 1, lane 2, was developed using an affinity-purified antibody raised against the unique carboxy terminus of GSK-3 α . This GSK-3 α -specific antibody shows 1 immunoreactive protein at 51 kd. Development of the blot (lane 3) with a

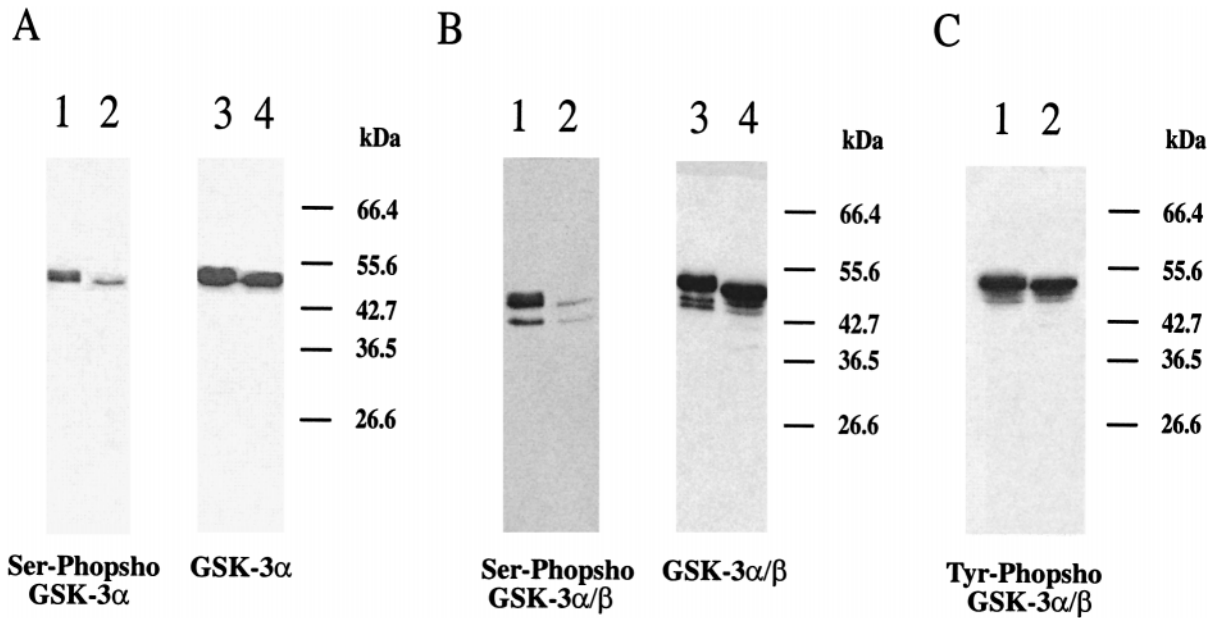


Figure 2. **(Panel A)** Western blot analysis of serine-phosphorylated glycogen synthase kinase-3 α (GSK-3 α) in caudal and caput spermatozoa (lanes 1 and 2). In a duplicate blot, GSK-3 α is shown in caudal and caput spermatozoa (lanes 3 and 4). **(Panel B)** Western blot analysis of serine-phosphorylated GSK-3 α and GSK-3 β in caudal and caput spermatozoa (lanes 1 and 2). A duplicate blot in which the antibody detects both GSK-3 α and GSK-3 β shows equal amounts of protein (lanes 3 and 4). **(Panel C)** Western blot analysis of tyrosine-phosphorylated GSK-3 α and GSK-3 β in caudal and caput spermatozoa (lanes 1 and 2).

mouse monoclonal antibody specific for GSK-3 β showed 1 band at 47 kd.

Higher Levels of Serine-Phosphorylated GSK-3 in Caput Than in Caudal Epididymal Spermatozoa

In a previous study, we showed that the tyrosine phosphorylation of GSK-3 increased in direct proportion to sperm motility (Vijayaraghavan et al, 2000). In somatic cells, GSK-3 is also serine phosphorylated (Wang et al, 1994a). The purpose of the following experiments was to examine whether sperm GSK-3 was serine phosphorylated and whether this phosphorylation was related to motility. We used an antibody specific to serine-phosphorylated GSK-3 α . Western blot analysis shows that GSK-3 α is serine phosphorylated in bovine epididymal spermatozoa and that its phosphorylation is significantly higher in motile caudal than in immotile caput spermatozoa (Figure 2, panel A, lane 1, compared to lane 2). The difference in intensities of immunoreactive serine phosphorylation of GSK-3 is not due to higher amounts of GSK-3 in caudal sperm extracts. A duplicate Western blot developed with an antibody against GSK-3 α shows roughly equal amounts of GSK-3 in caput and caudal epididymal sperm extracts (Figure 2, panel A, lanes 3 and 4). Note that GSK-3 α in caudal sperm (Figure 2A, lanes 1 and 3) has an apparently higher molecular weight than its counterpart in caput sperm (Figure 2B, lanes 2 and 4). We also used an antibody for serine-phosphorylated GSK-3 that reacts against serine-phosphorylated GSK-3 α and GSK-

3 β . This antibody shows 2 bands corresponding to the 2 isoforms. Figure 2 (panel B) also shows that both GSK-3 α and GSK-3 β in caudal spermatozoa are phosphorylated to a greater extent than in caput epididymal spermatozoa (Figure 2, panel B, lane 1, compared to lane 2). Figure 2, panel B, lanes 3 and 4, shows equal amounts of GSK-3 α and GSK-3 β in the extracts. Once again, note that GSK-3 α is at a slightly higher molecular weight in caudal than in caput sperm (Figure 2B, lanes 1 and 3 compared to lanes 2 and 4). Western blot probed with anti-phosphotyrosine GSK-3 antibody showed that GSK-3 α in both caudal and caput sperm is tyrosine phosphorylated (Figure 2, panel C).

Serine Phosphorylation Varies in Direct Proportion to Motility

Note that in somatic cells, GSK-3 is serine phosphorylated in response to external signals. It was therefore surprising that GSK-3 in spermatozoa was serine phosphorylated in the apparent absence of any external signaling molecules. Since GSK-3 was serine phosphorylated to a greater extent in motile caudal spermatozoa, we next examined whether GSK-3 serine phosphorylation could be altered in vitro when the motility status of spermatozoa was altered by pharmacological means. The relationship between GSK-3 α serine phosphorylation and sperm motility was investigated using 2 motility stimulators, IBMX and CdA, and a motility inhibitor, sHT31 (Vijayaraghavan et al, 1996). Immotile caput spermatozoa were treated

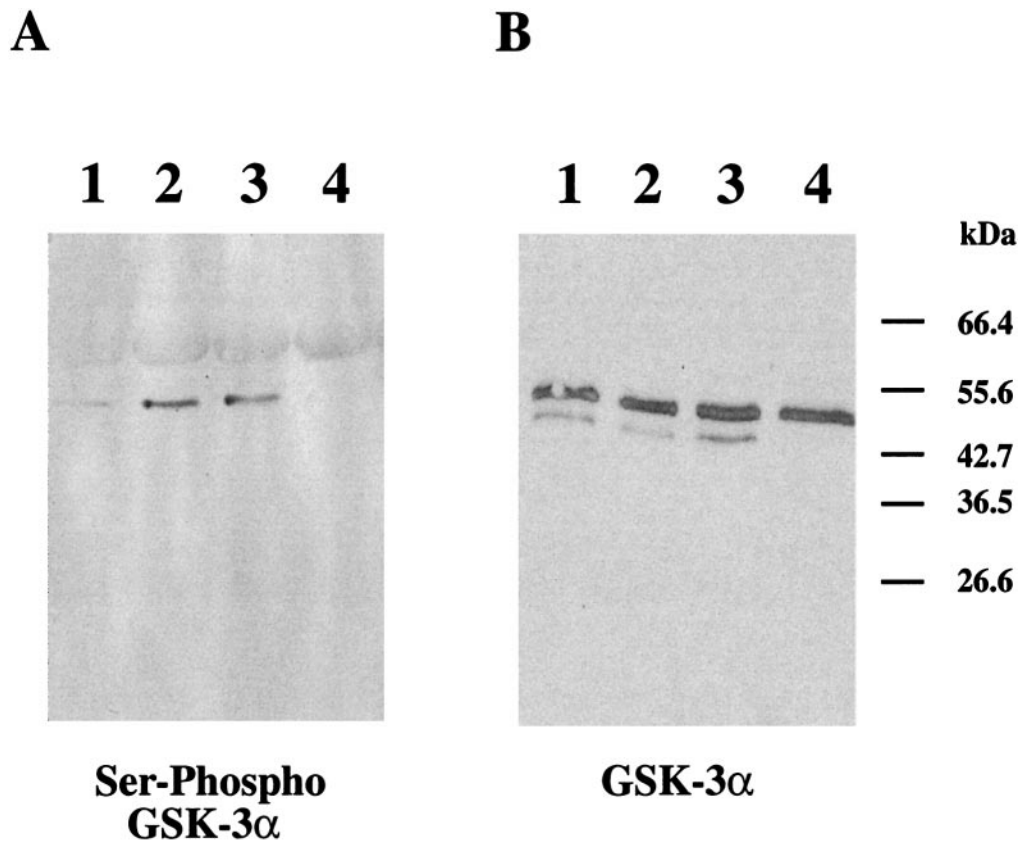


Figure 3. **(Panel A)** Western blot analysis of serine-phosphorylated glycogen synthase kinase-3 (GSK-3) in which caput spermatozoa are exposed to motility stimulators and a motility inhibitor. Caput spermatozoa were exposed to 2 motility stimulators, isobutyl-methyl-xanthine (lane 2) and 2-chloro-2'-deoxy-adenosine (lane 3), and to 1 motility inhibitor, sHT31 (lane 4). The 16K supernatants were then subjected to Western blot analysis using an antibody against serine-phosphorylated GSK-3. **(Panel B)** A duplicate Western blot that was run using an affinity-purified antibody against GSK-3 α shows equal pools of GSK-3 α in all lanes.

with 0.5 mmol of IBMX per liter, 10 μ mol of CdA per liter, or 10 μ mol of sHT31 per liter. Extracts from control and treated sperm were subjected to Western blot analysis with antibodies against serine-phosphorylated GSK-3. Data in Figure 3, panel A, show that the motility stimu-

lators caused an increase in GSK-3 serine phosphorylation (lanes 2 and 3), whereas treatment with the motility inhibitor sHT31 (lane 4) caused a virtual elimination of serine phosphorylation when compared to control sperm (lane 1). Duplicate blots probed with GSK-3 α antibody show that equal amounts of protein are present in extracts from control and treated spermatozoa (Figure 3, panel B).

Table 1. Summary of GSK-3 purification from caudal sperm extracts*†

Purification Step	Protein	Specific Activity (U/mg protein)
Caudal sperm extract	80.5 mg	9.18
DEAE-cellulose flow through	26.4 mg	14.4
Mono S flow through of DEAE-cellulose flow through	210 μ g	7.82
Mono S gradient of DEAE-cellulose flow through (0.125–0.165 M KCl)	104 μ g	39.9

* Table 1 summarizes the purification of GSK-3 from 50 mL of caudal sperm extracts as outlined in "Materials and Methods." One unit (U) of GSK-3 activity is defined as the millimoles of P^{32} incorporated onto phospho-CREB/min.

† DEAE indicates diethylaminoethyl; GSK-3, glycogen synthase kinase-3; and CREB, cyclicadenosine monophosphate-responsive element binding protein.

Purification of GSK-3 From Caudal and Caput Sperm Extracts

Next, we wanted to further confirm the identity and serine phosphorylation status of the GSK-3 species present in sperm extracts using column chromatography. We used DEAE-cellulose and Mono S columns for partial purification of GSK-3 from caudal and caput sperm extracts. Column fractions were analyzed for GSK-3 immunoreactivity and catalytic activity. Specific activity of GSK-3 in the pooled caudal sperm extracts was 9.18 U of protein per milligram. A summary of the purification steps is provided in Table 1. The extracts were first passed through a DEAE-cellulose column. All of the GSK-3 in the extracts was present in the flow-through fraction (specific

Table 2. Summary of GSK-3 purification from caput sperm extracts*†

Purification Step	Protein	Specific Activity (U/mg protein)
Caput sperm extract	61.3 mg	10.73
DEAE-cellulose flow through	21.7 mg	15.6
Mono S flow through of DEAE-cellulose flow through	820 μ g	...
Mono S gradient of DEAE-cellulose flow through (0.125–0.165 M KCl)	104 μ g	64.08

* Table 2 summarizes the purification of GSK-3 from 30 mL of caput sperm extracts as outlined in "Materials and Methods." One unit (U) of GSK-3 activity is defined as the millmoles of P³² incorporated onto phospho-CREB/min.

† Abbreviations are explained in the second footnote to Table 1.

activity, 14.4 U of protein per milligram). The flow-through fraction was concentrated and passed through a Mono S column. GSK-3 immunoreactivity was present in the flow-through and gradient (0.125–0.165 M KCl) fractions.

The enzyme from caput sperm extracts was purified using the same protocol. The extracts with a specific activity of 10.7 U of protein per milligram were passed through DEAE-cellulose column. As with caudal sperm extracts, the flow-through fraction contained all of the immunoreactive GSK-3. The concentrated DEAE-cellulose flow through (specific activity, 15.6 U of protein per milligram) was applied to a Mono S column, and GSK-3 (α and β) was obtained in the Mono S gradient (0.125–0.165M KCl) with a specific activity of 64.08 U of protein per milligram (Table 2).

Figure 4 shows the Western blot data of the column fractions. Figure 4, panel A, shows blots probed with GSK-3 α antibodies, while panel B shows duplicate blots probed with GSK-3 β antibody. The notable difference between caudal and caput sperm is that a portion of GSK-3 α in caudal sperm extracts is present in the Mono S flow-through fraction, whereas there is no detectable GSK-3 in the corresponding fraction in caput sperm extracts (lane 4 in Figure 4, panels A and B). The Mono S gradient fractions of caput and caudal sperm extracts contained both GSK-3 α and GSK-3 β (lane 3 in Figure 4, panels A and B).

Blots probed with serine-phosphorylated GSK-3 α/β antibody showed that both GSK-3 α and GSK-3 β were phosphorylated at their serine positions in caudal and caput sperm extracts (Figure 4, panel C). Individual lanes show DEAE-cellulose flow through (lane 1), Mono S gradient (lane 2), and Mono S flow through (lane 3). Significantly, in caudal sperm, most of the serine-phosphorylated GSK-3 α was present in the Mono S flow-through fraction as a high-molecular-weight form with a low specific activity of 7.8 U of protein per milligram. The enzyme is absent

in the Mono S flow-through fraction of caput sperm extracts (Figure 4, panel C, lane 3).

Caput and Caudal Spermatozoa Incubated With Calyculin A

We further examined the relationship between motility and GSK-3 phosphorylation using the motility stimulator calyculin A. Calyculin A is a protein phosphatase inhibitor that initiates or stimulates motility at nanomolar concentrations (Vijayaraghavan et al, 1996). Caput spermatozoa were treated with 50 nmol of calyculin A per liter and extracts of control and treated spermatozoa were analyzed for GSK-3 serine phosphorylation. Western blots in panel A were developed with antibodies specific for GSK-3 α . The data show that calyculin treatment increases GSK-3 serine phosphorylation (lane 4 compared to lane 3 in panel A). Figure 5, lanes 1 and 2, shows that extracts from control and calyculin-treated sperm contain equal amounts of GSK-3 α . Blots in Figure 5, panel B, were developed with antibodies that react against both GSK-3 α and GSK-3 β . These data show that the serine phosphorylation of both GSK-3 α and GSK-3 β is increased by calyculin treatment (lane 4 compared to lane 3 in panel B, Figure 5). Figure 5, lanes 1 and 2, shows that equal amounts of protein were loaded. Visual evaluation confirmed our earlier reports (Vijayaraghavan et al, 1996, 2000) that this concentration of calyculin A induced motility in immotile caput spermatozoa.

Next, we examined whether there was a dose-dependent increase in serine phosphorylation and an increase in the molecular weight of GSK-3 α in sperm treated with calyculin A. Caput epididymal spermatozoa were incubated with concentrations of calyculin A that varied from 0 to 50 nmol/L. Western blot analysis of extracts of control and treated sperm showed that calyculin A caused a dose-dependent increase in GSK-3 serine phosphorylation at concentrations between 10 and 50 nmol/L (Figure 6, panel A). Panel B shows that equal amounts of GSK-3 are present. Note that there is an increase in the apparent molecular weight of GSK-3 α in SDS-PAGE with increased serine phosphorylation. We have previously shown that increasing the calyculin A concentration caused a dose-dependent increase in the motility of immotile caput epididymal spermatozoa, with a maximum motility effect observed at concentrations of calyculin A that varied from 15 to 25 nmol/L (Vijayaraghavan et al, 2000).

Relationship Between GSK-3 Serine Phosphorylation and Catalytic Activity

In somatic cells, serine phosphorylation is known to decrease the catalytic activity of GSK-3. We next determined if this expected relationship between GSK-3 phosphorylation and its catalytic activity also held true in sper-

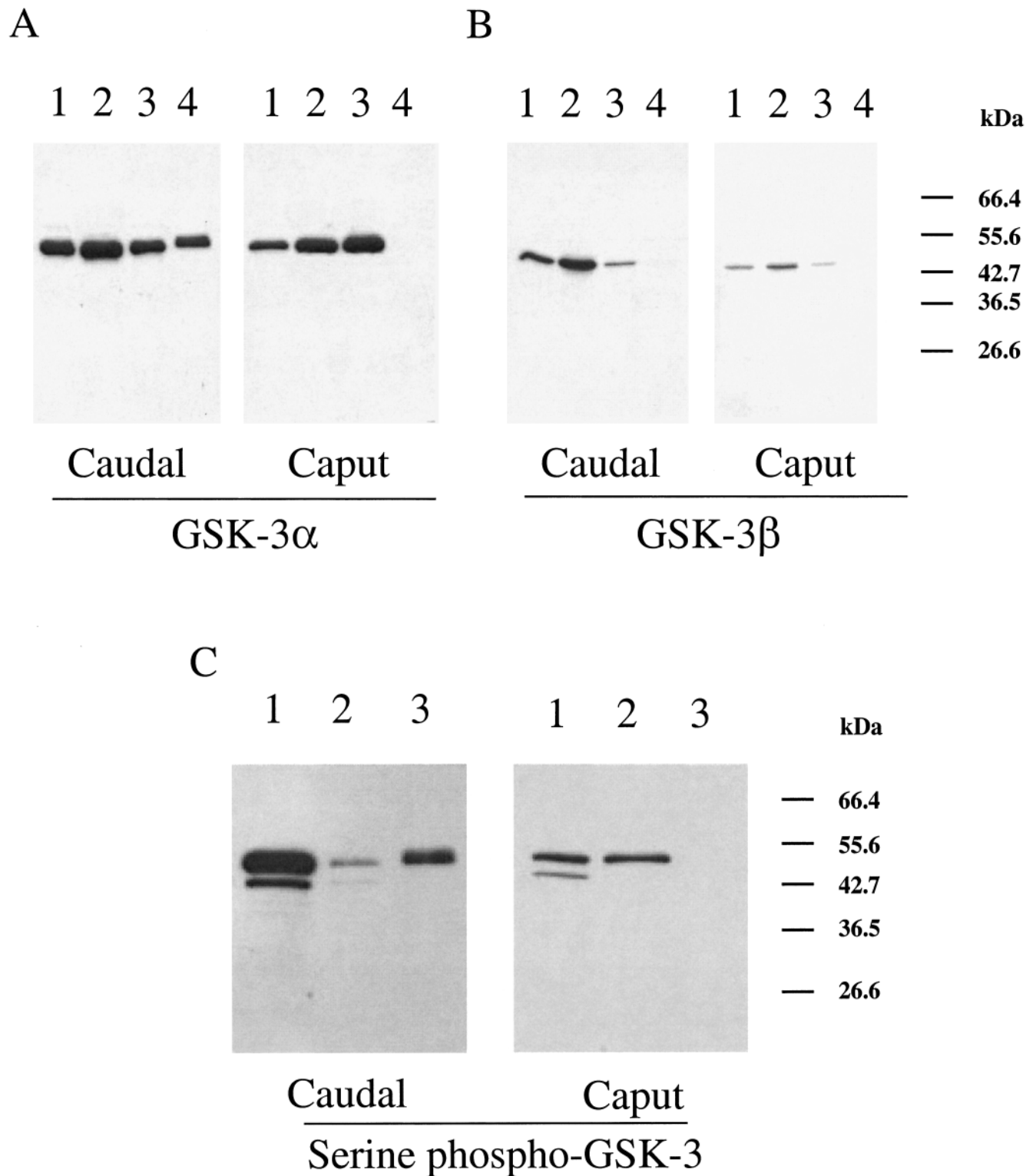


Figure 4. Purification of glycogen synthase kinase-3 (GSK-3) from caudal and caput sperm extracts. **(Panels A and B)** Western blots showing GSK-3 α and GSK-3 β , respectively, in caudal and caput sperm extracts during different stages of purification. Individual lanes show crude extract (lane 1), diethylaminoethyl (DEAE)-cellulose flow through (lane 2), Mono S gradient (lane 3), and Mono S flow through (lane 4). **(Panel C)** Western blots showing serine-phosphorylated GSK-3 α and GSK-3 β in caudal and caput sperm extracts, respectively, during different stages of purification. Individual lanes show DEAE-cellulose flow through (lane 1), Mono S gradient (lane 2), and Mono S flow through (lane 3).

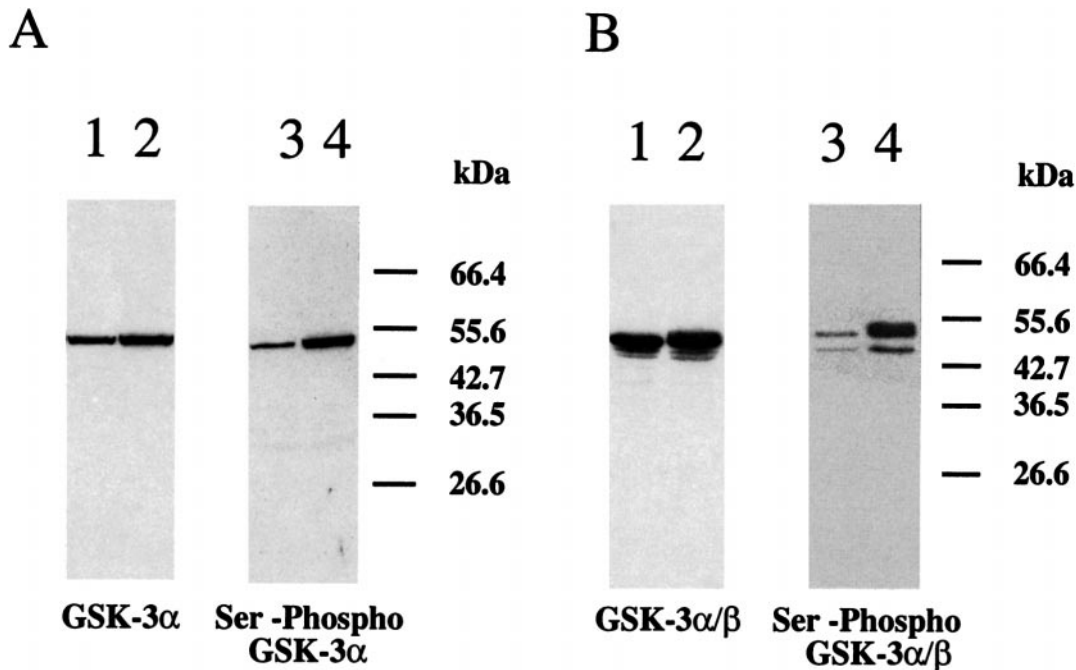


Figure 5. **(Panel A)** Western blot analysis of glycogen synthase kinase-3 α (GSK-3 α) and serine-phosphorylated GSK-3 α (lanes 3 and 4) caput sperm after treatment with 0.1% dimethylsulfoxide (DMSO) (control) and calyculin A at a concentration of 50 nmol/L. **(Panel B)** Western blot analysis showing both the isoforms of GSK-3 (lanes 1 and 2) and serine-phosphorylated GSK-3 in caput sperm after treatment with 0.1% DMSO (control) and calyculin A at a concentration of 50 nmol/L.

matozoa. We have previously shown that GSK-3 activity is high in caput compared to caudal epididymal spermatozoa. This measurement was determined by an indirect assay based on the ability of GSK-3 to activate the inactive protein phosphatase (PP1) and inhibitor I2 complex. The measurement was also complicated by the fact that sperm extracts had high endogenous protein phos-

phatase activity. A direct assay using a synthetic pre-phosphorylated amino acid sequence domain in CREB was used in this study (Wang et al, 1994b). Protein kinase activity sensitive to lithium was thought to have originated from GSK-3 (Stambolic et al, 1996). Measurement of GSK-3 in soluble sperm extracts, using this assay, showed that immotile caput spermatozoa have about 2.5-

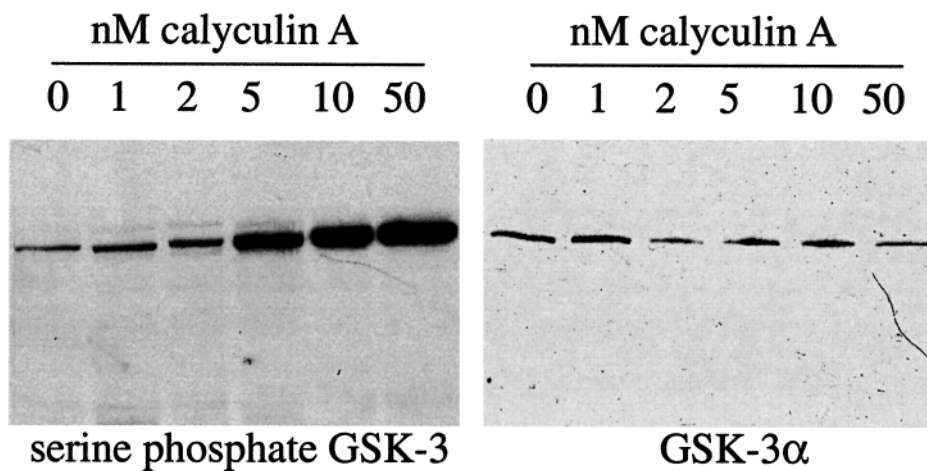


Figure 6. **(Panel A)** Western blot analysis showing a dose-dependent effect of calyculin A on the serine phosphorylation of glycogen synthase kinase-3 (GSK-3). Calyculin A was added to immotile caput spermatozoa in a concentration gradient of 0, 1, 2, 5, 10, and 50 nmol/L. An antibody binding to serine-phosphorylated GSK-3 was used to demonstrate the gradual increase in the serine phosphorylation of GSK-3 corresponding to the increasing concentrations of calyculin A. **(Panel B)** A parallel Western blot using an affinity-purified antibody against GSK-3 α shows equal pools of GSK-3 α in all of the groups.

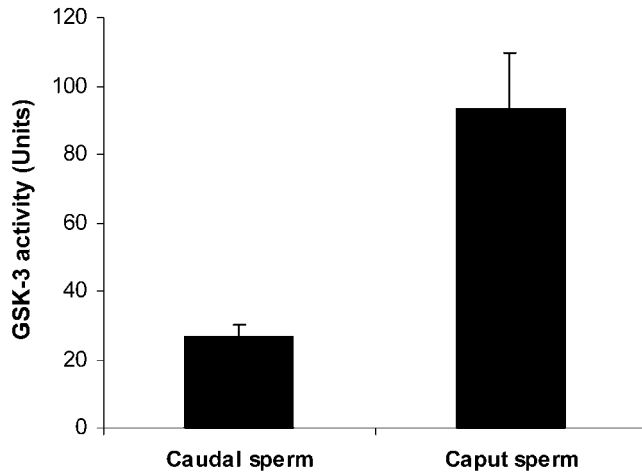


Figure 7. Activity comparison of glycogen synthase kinase-3 (GSK-3) in caudal and caput sperm extracts. Sperm number-adjusted caudal and caput sperm extracts were subjected to an activity assay for GSK-3 using phospho-CREB (cyclic adenosine monophosphate-responsive element binding protein) as the substrate. Immobile caput sperm were shown to possess higher GSK-3 activity than motile caudal sperm. (A unit is defined as the nanomoles of ^{32}P incorporated onto phospho-CREB/min/ 1×10^7 sperm).

fold higher catalytic activity than do caudal epididymal spermatozoa (Figure 7).

Next, we investigated whether increased GSK-3 serine phosphorylation due to calyculin A treatment in vitro (Figure 4) was also accompanied by a decrease in GSK-3 activity. Data in Figure 8, panel A, show that in both caput and caudal sperm extracts, calyculin A treatment resulted in a 50% reduction in GSK-3 activity compared to controls. Data in Figures 7 and 8 (panel A) are activity measurements in crude extracts. Activity measurements in crude extracts are due to both GSK-3 α and GSK-3 β and are subject to potential interference by nonspecific factors present in extracts. We therefore measured GSK-3 activity following immunoprecipitation with GSK-3 α antibodies. Immunoprecipitation using the GSK-3 α antibody completely pulled down GSK-3 α from sperm extracts, leaving behind the GSK-3 β antibody in the supernatant. This technique, confirmed by Western blotting (figures not shown), permitted measurement of the individual activities of the 2 isoforms of GSK-3 in caudal and caput sperm extracts. The activities of both GSK-3 α and

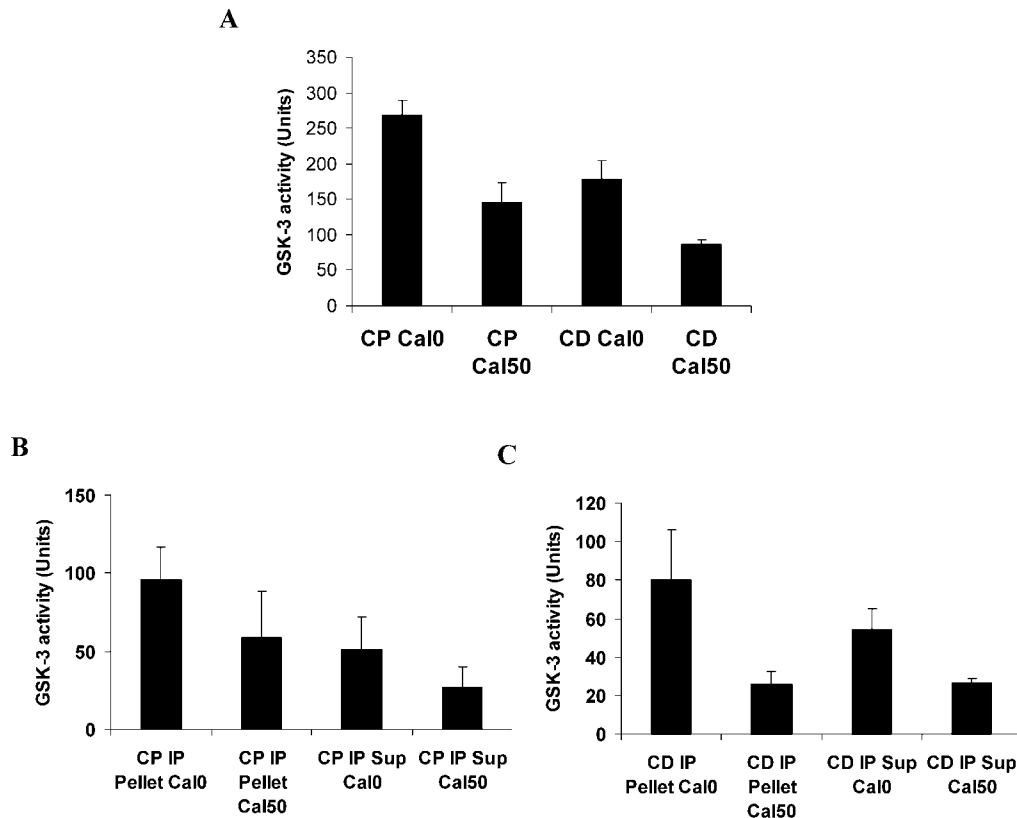


Figure 8. Effect of calyculin A on sperm glycogen synthase kinase-3 (GSK-3) activity (**Panel A**). Activity after immunoprecipitation of GSK-3 α from caput (**Panel B**) and caudal (**Panel C**) sperm extracts before and after treatment with calyculin A are also shown. Sperm number-adjusted caudal and caput sperm suspensions were incubated in the presence of calyculin A at a concentration of 50 nmol/L using 0.1% dimethylsulfoxide (DMSO) as a control and were extracted using 1% Triton X-100 containing homogenizing buffer. Extracts were subjected for immunoprecipitation of GSK-3 α , and the pellets containing GSK-3 α and the supernatants with GSK-3 β were collected. An activity assay in these showed that caput sperm have greater activity for both forms of GSK-3 and that the addition of calyculin A decreased the activity of both isoforms. (A unit is defined as the nanomoles of ^{32}P incorporated onto phospho-CREB [cyclic adenosine monophosphate-responsive element binding protein]/min/ 1×10^7 sperm).

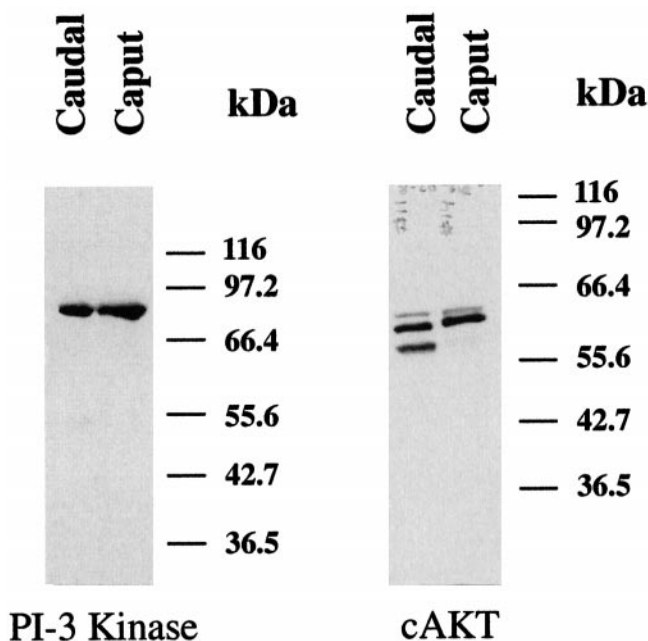


Figure 9. Western blot analysis of bovine epididymal sperm extracts demonstrating the presence of the upstream regulators of glycogen synthase kinase-3 (GSK-3). Antibodies against phosphoinositide 3-kinase and cAkt, upstream regulators of GSK-3, were used on caput and caudal 16K supernatants. Both enzymes were present in bovine spermatozoa.

GSK-3 β were higher, nearly double in caput vs caudal sperm extracts. Treatment with 50 nmol of calyculin A per liter decreased GSK-3 activity in both caput and caudal sperm (Figure 8, panel A). This decrease in activity was observed for both GSK-3 isoforms (ie, in GSK-3 α immunoprecipitates and in the supernatants of extracts following immunoprecipitation) (Figure 8, panels B and C). In caput sperm treated with 50 nmol of calyculin A per liter, GSK-3 α activity was reduced by about 39% (from 96.0 to 59.0 U) (Figure 8, panel B), while the activity for the GSK-3 β isoform was reduced by about 48% (from 51.0 to 26.0 U) (Figure 8, panel C). Activity measurements in crude extracts showed a net inhibition of 46%. In caudal sperm, with the addition of 50 nmol of calyculin A per liter, GSK-3 α activity was reduced by approximately 68% (from 80.3 to 25.7 U), while the GSK-3 β activity was reduced by about 52% (from 54.3 to 26.4 U). Inhibition in crude caudal extracts was 52% (Figure 8, panel C). Also note that GSK-3 activity in crude sperm extracts is higher in caput than in caudal sperm, which further confirms the data in Figure 7. Enzyme activity values for extracts shown in Figure 8 are higher than those in Figure 7, because the activity assay in Figure 8 was performed with the flow-through fraction of extracts passed through DEAE-cellulose mini columns. The DEAE column appears to remove unknown GSK-3 inhibitors from sperm extracts.

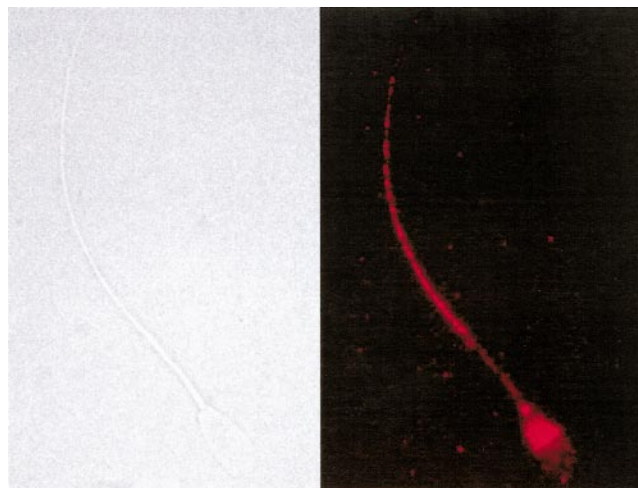


Figure 10. Immunocytochemistry of glycogen synthase kinase-3 α (GSK-3 α) shows the localization in sperm. An affinity-purified antibody against GSK-3 α was used on formalin-fixed sperm cells. Intense staining was seen in the posterior portion of the head, in the equatorial segment, and along the length of the tail.

Western Blot Analysis of PI3-Kinase and cAkt

In somatic cells, GSK-3 is phosphorylated and inactivated by protein kinase B (PKB; also known as cAkt). cAkt, in turn, is activated by PI3-kinase. We examined whether these 2 upstream GSK-3-regulating enzymes are present in spermatozoa. Antibodies against PI3-kinase and cAkt were used in Western blot analysis of bovine epididymal sperm extracts. PI3-kinase is a heterodimer composed of an 85-kd regulatory subunit and a 110-kd catalytic subunit. Antibodies against the 85-kd subunit show that it is present in spermatozoa (Figure 9, lane 1). Carboxy terminus antibodies against cAkt, which is a 62-kd protein, show immunoreactive bands in this expected range in caput and caudal spermatozoa. Note that the antibody shows 2 closely spaced bands in caudal sperm extracts (Figure 9, lane 2). It is possible that the slower migrating band is a phosphorylated form of cAkt.

GSK-3 Immunocytochemistry

Finally, we examined the localization of GSK-3 in spermatozoa. An antibody against GSK-3 α was used on caput and caudal spermatozoa. No specific differences could be seen in the localization of GSK-3 α between caput and caudal spermatozoa. Intense staining was observed in the posterior portion of the head, in the equatorial segment, and along the length of the tail (Figure 10).

Discussion

In mammals, molecular cloning showed the existence of 2 closely related isoforms termed GSK-3 α and GSK-3 β (Woodgett, 1990). The α -isoform is a 51-kd protein shar-

ing 95% identity with the 47-kd β -isoform in the kinase domain. Catalytic activity of GSK-3 is regulated by the phosphorylation of its tyrosine 214 in both and by its serine 21 and serine 9 residues in GSK-3 α and GSK-3 β , respectively (Wang et al, 1994a). The presence of GSK-3 in spermatozoa was first reported by Vijayaraghavan et al (1996). On the basis of an indirect assay, its activity was approximately threefold higher in immotile caput than in motile caudal spermatozoa, which suggests that it is involved in motility regulation (Smith et al, 1999). Recently, we reported the existence of a tyrosine-phosphorylated protein, GSK-3 α in bovine spermatozoa, and found that its tyrosine phosphorylation varied directly with motility (Vijayaraghavan et al, 2000).

In the present study, we first examined whether both isoforms of GSK-3 are present in spermatozoa. Western blots for caudal sperm extracts with isoform-specific GSK-3 antibodies (Figure 1) showed that both GSK-3 (α and β) isoforms are present in bovine spermatozoa. On the basis of the intensity of immunoreactivity, GSK-3 α appears to be the predominant isoform in spermatozoa. Next, we examined whether sperm GSK-3 is serine and tyrosine phosphorylated. Western blot analysis with an antibody specific to serine-phosphorylated GSK-3 α showed a dramatic enhancement in GSK-3 serine phosphorylation in motile caudal compared to immotile caput spermatozoa (Figure 2, panel A), while a slight increase in the phosphorylation of tyrosine 214 residue was observed when duplicate blots were used for the anti-phospho-GSK-3 antibody (Figure 2, panel C). Antibody-recognizing phosphorylated serine residue in both GSK-3 isoforms showed that the serine phosphorylation of GSK-3 α and GSK-3 β is increased with motility (Figure 2, panel B). Thus, 2 different phosphorylation site-specific antibodies confirmed that the serine phosphorylation of GSK-3 is higher in caudal than in caput spermatozoa. It should be emphasized that in somatic cells, GSK-3 is serine phosphorylated only in response to external signals. Remarkably, the serine phosphorylation of GSK-3 is observed in caudal epididymal spermatozoa even in the apparent absence of any cellular activator.

To further examine the relationship between sperm motility and GSK-3 serine phosphorylation, we used compounds that stimulate or inhibit motility. Western blot analysis showed that caput and caudal spermatozoa treated with sHT31, a motility inhibitor, resulted in a dramatic decrease in the serine phosphorylation of GSK-3 when compared to control spermatozoa (Figure 3). Motility stimulators such as IBMX and CdA also caused a noticeable increase in GSK-3 serine phosphorylation. We further examined the relationship between motility and GSK-3 serine phosphorylation using the motility activator calyculin A. Sperm suspensions with 1×10^8 sperm per milliliter, incubated for 15 minutes in the presence of ca-

lyculin A (with concentrations between 0 and 50 nmol/L), showed a dose-dependent increase in serine phosphorylation. Calyculin A at a concentration of 5 nmol/L increased serine phosphorylation in caput sperm, reaching a maximum at 20 nmol/L. This increase in serine phosphorylation matches the concentration-dependent motility initiation by calyculin A (Vijayaraghavan et al, 1996).

An increase in GSK-3 serine phosphorylation accompanies sperm maturation and the acquisition of motility in the epididymis. This increased serine phosphorylation also results in the expected decrease in GSK-3 catalytic activity. Western blot analysis also showed that in both caput and caudal sperm, GSK-3 α appears to be both serine and tyrosine phosphorylated. However, GSK-3 α in the caudal sperm appears to have a higher molecular weight in SDS-PAGE analysis than in caput sperm. One explanation for this observation is that GSK-3 α in caput sperm may be either serine or tyrosine phosphorylated, whereas in caudal sperm, it may be phosphorylated at both residues. To examine this possibility, we partially purified GSK-3 α from sperm extracts. In an anion exchange column (DEAE-cellulose), all of the GSK-3 in caput and caudal sperm extracts was found in the flow-through fraction. The enzyme in this flow-through fraction had a higher activity than the whole extracts, which is probably due to the removal of interfering proteins from the extracts. When the anion exchange flow-through fraction was analyzed using the Mono S column (cation exchanger), immunoreactive GSK-3 was obtained in both the flow-through and gradient fractions (Table 1) in the caudal sperm, whereas in the caput sperm, all of the GSK-3 eluted in the gradient fractions (Table 2). Western blots showed that, in caudal sperm, the higher-molecular-weight fraction of GSK-3 α was present in the Mono S flow-through fraction (Figure 4, panel A) with a low specific activity (7.8 U of protein per milligram) compared to the low-molecular-weight forms, GSK-3 α and GSK-3 β in the gradient fractions (Figure 4, panels A and B), which had a specific activity of 38.97 U of protein per milligram (Table 1). In the case of caput sperm, all the GSK-3 α and GSK-3 β present in the Mono S gradient had a specific activity of 64.08 U of protein per milligram. The 2 molecular-weight forms of GSK-3 α could be due to differences in phosphorylation: the low-molecular-weight form may be phosphorylated at one residue—either at serine 21 or tyrosine 214—while the higher-molecular-weight form may be phosphorylated at both residues. This higher-molecular-weight form of GSK-3 α is present in caudal but not in caput epididymal spermatozoa. An alternate possibility for the difference in molecular weight could be due to glycosylation.

Next, we investigated whether changes in GSK-3 activity accompany motility-associated changes in serine phosphorylation. We first compared GSK-3 catalytic ac-

tivity in extracts of cell number-adjusted caput and caudal spermatozoa. The activity of GSK-3 was significantly higher in caput than in caudal sperm. Furthermore, calyculin A, which increased the serine phosphorylation of GSK-3 α and GSK-3 β and initiated the motility in immotile caput spermatozoa, inhibited the GSK-3 activity in caput and caudal spermatozoa by approximately 50%. Measurement of GSK-3 α and GSK-3 β activity following immunoprecipitation showed that both isoforms are less active in caudal than in caput spermatozoa. In both caput and caudal spermatozoa, treatment with 50 nmol of calyculin A per liter inhibited the activities of both isoforms of GSK-3.

Immunocytochemistry using the GSK-3 α antibody showed that GSK-3 is located at the equatorial segment, on the postacrosomal region, and on the principal piece of the tail in both caudal and caput spermatozoa. Antibodies detecting both GSK-3 α and GSK-3 β and detecting GSK-3 β only also were used for immunocytochemistry (data not shown). These antibodies exhibited the same pattern of localization, suggesting that GSK-3 α and GSK-3 β reside in the same location. Their localization would be consistent with their role in sperm motility and fertilization.

Spermatozoa are terminally differentiated cells with little protein synthesis (Toshimori, 1998). Therefore, the phosphorylation and dephosphorylation of the proteins already present must be important mechanisms for regulating protein and cellular function. The increase in the phosphorylation of GSK-3 α and GSK-3 β upon treatments with motility stimulators suggests that a protein kinase responsible for its phosphorylation is present in spermatozoa.

To date, 2 enzymes are known to phosphorylate GSK-3—protein kinase A (PKA) (Fang et al, 2000) and PKB/cAkt (Hemmings, 1997; Brazil and Hemmings, 2001). cAkt is regulated by PI3-kinase. Upon cellular stimulation, PI3-kinases recruited to the cell surface generate inositol lipids such as PtdIns(3)P, PtdIns(3,4)P₂, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃ (Leevers et al, 1999; Vanhaesebroeck and Waterfield, 1999; Cantrell, 2001), which bind and activate PH-domain-containing enzymes. One of these is phosphoinositide-dependent kinase 1 (PDK1), which, when activated, phosphorylates Akt (Hemmings, 1997; Brazil and Hemmings, 2001). Serine phosphorylation and inhibition of GSK-3 is primarily mediated by Akt (Gold et al, 2000; Krasilnikov, 2000). We have shown that both cAkt and PI3-kinase are present in spermatozoa. Western blot analysis showed that an antibody against PI3-kinase could detect a protein at 85-kd, while the antibody against cAkt detected 2 protein bands at 62 kd. GSK-3 was first identified as a rate-limiting enzyme in glycogen synthesis (Embi et al, 1980). Recent studies have identified a number of new roles and substrates for

GSK-3 (Woodgett, 2001). Two major pathways involving GSK-3 are *Wnt/wingless* signaling and a PI3-kinase pathway involved in apoptosis and cell survival (Woodgett, 2001). The identification of PI3-kinase and cAkt in sperm suggests a new role for GSK-3 representing a novel role for this signaling mechanism in terminally differentiated spermatozoa.

An important question raised by our studies concerns the external signal responsible for the changes in sperm GSK-3 *in vivo*. In somatic cells, changes in GSK-3 phosphorylation are induced by external signals. It is not known what external signals, if any, are involved in epididymal sperm maturation and motility initiation. It is also intriguing that a variety of treatments *in vitro* such as IBMX, 2-chloroadenosine, and calyculin A alter GSK-3 phosphorylation. This suggests that the net phosphorylation of GSK-3 is not static but undergoes active turnover in spermatozoa suspended in simple salt buffers *in vitro*. The biological significance of this turnover is not known. It is possible that a paracrine factor, perhaps adenosine (Vijayaraghavan and Hoskins, 1986) secreted from spermatozoa, is one of the signaling factors responsible for controlling GSK-3 phosphorylation.

Another question raised by our studies concerns the physiological significance of GSK-3 phosphorylation. Since we have not yet identified the protein substrates of GSK-3, it is difficult to speculate how GSK-3 acts in spermatozoa. We emphasize that the present study showing a relationship between GSK-3 activity, serine phosphorylation, and sperm motility is correlative. One of the limitations in designing further studies that might shed light on the exact role of GSK-3 in sperm function is, as noted, the lack of knowledge of external signaling molecules that may activate spermatozoa. It is therefore not possible to experimentally manipulate the signaling pathway involving sperm GSK-3 to examine the physiological consequences by altering GSK-3 activity. Another limitation is our inability to specifically inactivate GSK-3 by pharmacological means. We are currently evaluating the use of a number of inhibitors, commercially available, claimed to be specific for GSK-3. Preliminary results suggest that the effects of these inhibitors on GSK-3 activity in sperm are variable. That GSK-3 is conserved in sperm from a variety of species (eg, avian, rodent, bovine, crustacean, echinoderm) suggests that the enzyme is involved in some essential function within spermatozoa. Studies in our laboratory are currently focused on identifying substrates for sperm GSK-3 and on examining the activity of the enzyme in sea urchin compared to mammalian spermatozoa. The studies may shed further light on the physiological role of the enzyme in spermatozoa.

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