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Targeting of a Germ Cell-specific Type 1 Hexokinase Lacking a Porin-binding Domain to the Mitochondria as Well as to the Head and Fibrous Sheath of Murine Spermatozoa

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Multiple isoforms of type 1 hexokinase (HK1) are transcribed during spermatogenesis in the mouse, including at least three that are presumably germ cell specific: HK1-sa, HK1-sb, and HK1-sc. Each of these predicted proteins contains a common, germ cell-specific sequence that replaces the porin-binding domain found in somatic HK1. Although HK1 protein is present in mature sperm and is tyrosine phosphorylated, it is not known whether the various potential isoforms are differentially translated and localized within the developing germ cells and mature sperm. Using antipeptide antisera against unique regions of HK1-sa and HK1-sb, it was demonstrated that these isoforms were not found in pachytene spermatocytes, round spermatids, condensing spermatids, or sperm, suggesting that HK1-sa and HK1-sb are not translated during spermatogenesis. Immunoreactivity was detected in protein from round spermatids, condensing spermatids, and mature sperm using an antipeptide antiserum against the common, germ cell-specific region, suggesting that HK1-sc was the only germ cell-specific isoform present in these cells. Two-dimensional SDS-PAGE suggested that all of the sperm HK1-sc was tyrosine phosphorylated, and that the somatic HK1 isoform was not present. Immunoelectron microscopy revealed that HK1-sc was associated with the mitochondria and with the fibrous sheath of the flagellum and was found in discrete clusters in the region of the membranes of the sperm head. The unusual distribution of HK1-sc in sperm suggests novel functions, such as extramitochondrial energy production, and also demonstrates that a hexokinase without a classical porin-binding domain can localize to mitochondria.

INTRODUCTION

Somatic type 1 hexokinase (HK1) catalyzes the conversion of glucose to glucose-6-phosphate in the initial step of glycolysis. It exists either as a cytosolic protein or as a protein associated with the outer mitochondrial membrane via an interaction with porin, a voltage-dependent anion channel (Felgner and Wilson, 1977; Polakis and Wilson, 1985; Smith and Wilson, 1991). The association of HK1 with porin is mediated through a highly conserved porin-binding domain (PBD) in the amino terminus of the enzyme (Smith and Wilson, 1991; Gelb *et al.*, 1992). However, recent findings suggest that somatic hexokinases may have additional locations and different functions within cells. For example, hexokinase translocates to the plasma membrane and/or cell cortex of rat macrophages activated by phorbol 12-myristate 13-acetate (Pedley *et al.*, 1993). In addition, somatic hexokinases may possess protein kinase activities. Rat brain HK1

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displays protein kinase activity toward exogenous histone 2A in the absence of glucose (Adams *et al*., 1991) and also can autophosphorylate on serine, threonine, or tyrosine residues (Arora and Pedersen, 1993; Adams *et al*., 1994).

Our recent work regarding HK1 in spermatozoa has arisen not from studies on metabolism, but rather from investigations of the initial interaction between the mouse sperm and egg. This interaction involves the binding of the plasma membrane overlying the acrosome of acrosome-intact sperm to ZP3, a ligand on the egg's extracellular matrix, the zona pellucida (ZP) (Bleil and Wassarman, 1980, 1983). A phosphotyrosine-containing plasma membrane protein, initially identified as "p95," has been hypothesized to be a sperm surface receptor for ZP3 (Leyton and Saling, 1989; Leyton *et al*., 1992). This protein was subsequently purified and identified as an HK1 (Kalab *et al*., 1994).

Mouse male germ cells possess at least four potential HK1 isoforms. Clones from a mouse testis cDNA library reveal three germ cell-specific mRNAs, HK1-sa, HK1-sb, and HK1-sc (Mori *et al*., 1993). These three mRNAs share a common sequence in their N-terminal region, which replaces the PBD found in the somatic isoform (Mori *et al*., 1993). The lack of a PBD suggests that these isoforms, if translated, may have alternative localizations within spermatogenic cells and sperm. The somatic HK1 isoform may also be present, based on results from reverse transcription-polymerase chain reaction (PCR) using RNA from male germ cells (Visconti *et al*., 1996). Somatic HK1 and HK1-sa are transcribed in meiotic and postmeiotic cells, whereas HK1-sb and HK1-sc are transcribed postmeiotically (Mori *et al*., 1993; Visconti *et al*., 1996). In the human, three germ cell-specific mRNAs for isoforms of HK1 that do not contain a PBD have been found (Mori *et al*., 1996). The sequences of these mRNAs are similar but not identical to those found in the mouse. In contrast to mouse sperm, human sperm-HK1 is not tyrosine phosphorylated (Carrera *et al*., 1996; Naz *et al*., 1996).

Initial characterization of mouse sperm HK1 (ms-HK1) protein demonstrated that at least one isoform is translated (Kalab *et al*., 1994; Olds-Clarke *et al*., 1996; Visconti *et al*., 1996). A population of ms-HK1 differs from the classical somatic isoform both in its biochemistry and subcellular localization. Biochemically, ms-HK1 behaves as an integral membrane protein (Olds-Clarke *et al*., 1996; Visconti *et al*., 1996). Furthermore, the tyrosine phosphorylation of ms-HK1 is developmentally regulated, first appearing in condensing spermatids and then reaching maximal levels in mature sperm (Visconti *et al*., 1996). Regarding localization, immunofluorescence reveals that some ms-HK1 can be found associated with the membranes of the sperm head, as well as in the midpiece (the sole location of mitochondria in these highly differentiated cells) and throughout the tail (Olds-Clarke *et al*., 1996; Visconti *et al*., 1996).

The developmental regulation of ms-HK1 phosphorylation, its unusual biochemistry and localization when compared with HK1 in somatic cells, and the possibility of germ cell-specific isoforms lacking a PBD all suggest an important role(s) for ms-HK1 in sperm function and/or directly in fertilization. Based on the absolute requirement for a PBD in somatic HK1 to target to mitochondria (Smith and Wilson, 1991; Gelb *et al*., 1992), it might be predicted that somatic HK1 would be found in the midpiece, whereas germ cell-specific isoforms would be found in other regions of the cell. Although the existence of multiple germ cell-specific cDNA clones and the finding of somatic HK1 mRNA in germ cells suggest that ms-HK1 may represent multiple isoforms, it should be noted that in germ cells, not all mRNA transcripts are translated into proteins (e.g., Capel *et al*., 1993). The identity, localization, and biochemistry of the possible HK1 isoforms must be elucidated to determine whether ms-HK1 might also have different functions. The objectives of this study, therefore, were 1) to determine which of the potential germ cell-specific isoforms of HK1 are present at the protein level throughout germ cell development and in mature sperm; 2) to test the hypothesis that different isoforms of HK1 have alternative localizations; and 3) to investigate the biochemical characteristics of the isoform(s) found in mature sperm.

MATERIALS AND METHODS

Immunochemicals

Polyclonal antiserum against rat brain type I hexokinase (α-HK1) was generously provided by Dr. John Wilson (Michigan State University). Anti-phosphotyrosine antibodies (α-PY, clone 4G10) were purchased from Upstate Biotechnology (Lake Placid, NY).

Generation of HK1 Isoform-specific Antisera

Polyclonal antisera against unique peptide regions encoded by the germ cell-specific transcripts were prepared by Quality Controlled Biochemicals (Hopkinton, MA). In each case, a carboxy-terminal cysteine residue was either utilized or added to permit the conjugation of the peptide to a keyhole limpet hemocyanin carrier protein. A schematic representation of the isoforms and location of the epitopes are shown in Figure 1. Three antisera were generated:

1. AntiHK1-sa (α-sa) was generated against NH_2-SENPASEDRRPLEKQC-COOH, a peptide downstream from both of the potential initiation methionines in the unique amino-terminal region of HK1-sa, but upstream from the initiation methionine in the common germ cell-specific region isoforms. This antiserum was predicted to recognize only HK1-sa.
2. AntiHK1-sb (α-sb) was generated against NH_2-ELSPDRRWYQAYMRC-COOH, a peptide contained in the HK1-sb-specific, internal domain. This antiserum was predicted to recognize only HK1-sb.
3. An antiserum recognizing the common germ cell-specific sequence (α-gcs) was generated against NH_2-MGQN[C-Acm]QRGQ-

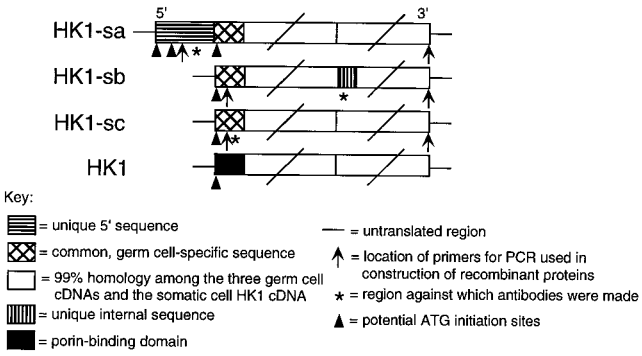

Figure 1. Schematic representation of four potential HK1 isoforms found in mouse male germ cells. Based on clones from a mouse testis cDNA library (Mori *et al*., 1993), HK1-sa is predicted to have a unique amino-terminal sequence, HK1-sb is predicted to have a unique internal domain, and HK1-sc is predicted to have no domains not found in the other two germ cell-specific messages. All three germ cell-specific isoforms lack the classical PBD found in the somatic HK1 isoform and have in its place the common, germ cell-specific sequence.

AVDVECUEC-COOH, a peptide downstream from the only start site in the common, germ cell-specific region. Given the fact that the entire sequence of HK1-sc is contained within the HK1-sa and HK1-sb isoforms (see Figure 1), it was not possible to generate an antiserum that would recognize HK1-sc specifically. This point is critical to the experimental method employed in this investigation. The α-gcs antiserum was generated to cross-react with all three potential germ cell-specific isoforms, but not with the somatic HK1 isoform.

Purified immunoglobulin G (IgG) fractions of these polyclonal antisera, as well as their preimmune sera, were prepared by running the sera over Protein A-Sepharose (CL-4B, Sigma, St. Louis, MO) columns, washing with Tris-buffered solutions, and eluting with 100 mM glycine at pH 3.0 (adapted from Ey *et al*., 1978).

Generation of Recombinant HK1 Proteins

Full-length recombinant proteins representing the three germ cell-specific isoforms were expressed in the Novagen pET 25b+ system. Clones of the cDNAs encoding the potential HK1 isoforms were provided by E.M. Eddy (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The oligonucleotides used as primers for PCR were based on sequences from Mori *et al*. (1993) and can be found in Table 1.

Briefly, PCR was performed with the use of the cDNA clones and the oligonucleotide primers (Table 1), using *Taq*+ DNA polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA). The PCR conditions were as follows: one cycle of 5 min at 93°C, 5 min at 48°C, 2 min at 72°C; 30 cycles of 1 min at 93°C, 1 min at 48°C, 3 min at 72°C; and final extension for 10 min at 72°C. DNA products were analyzed with ethidium bromide after electrophoresis on 1% agarose gels. The PCR products and pET plasmid were double digested using *Eco*RI and *Hin*dIII. The plasmid was dephosphorylated, and the PCR products were ligated into the plasmid. After the plasmids were transformed into competent cells, plasmid DNA was purified and analyzed to verify the presence of an insert. DNAs from plasmids containing inserts were sequenced using an Applied Biosystems (Foster City, CA) model 373A automated sequencer, with the DyeDeoxy Terminator Cycle Sequencing Kit, to ensure that the coding regions were in frame. Plasmids with correct inserts were transformed into a BL21-DE3 expression cell system.

The transformed BL21-DE3 cells were grown in Luria-Bertani medium in the presence of 50 μg/ml carbenicillin. Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested by centrifugation, resuspended in a buffer containing a protease inhibitor cocktail (Complete, Boehringer Mannheim, Mannheim, Germany), and treated with several rounds of sonication on ice and centrifugation to isolate inclusion bodies containing the recombinant proteins. The pellets were resuspended and washed in the same buffer with 1% Triton X-100, yielding final pellets of partially purified inclusion bodies, which were then solubilized overnight in buffer containing 6 M guanidine-HCl. This solution was centrifuged, and the supernatant was filtered and passed over a nickel-charged His-bind column (Novagen, Madison, WI). Recombinant proteins were eluted with a 1 M imidazole buffer and then dialyzed against solutions containing successively lower concentrations of guanidine-HCl to allow for a gradual refolding. The final volume was divided and lyophilized before storage at −80°C.

Preparation of Sperm

Cauda epididymal sperm were collected from retired breeder CD1 mice (Charles River, Wilmington, MA) in a medium containing 20 mM Tris-HCl, 130 mM NaCl, 1 mM EDTA, pH 7.5 (TN/EDTA). The sperm were washed in this medium, centrifuged, resuspended in 20 mM Tris-HCl and 1 mM EDTA (TE), and homogenized 10 times on ice in a Teflon/glass homogenizer. Samples were then split, with one fraction being boiled directly in sample buffer (Laemmli, 1970). This fraction was centrifuged and the supernatant designated as "whole sperm extract." After homogenization, aliquots of the sample were spun at 10,000 × *g* for 10 min at 4°C. The resultant pellet was boiled in sample buffer and centrifuged, and the supernatant was designated "P_{10}." The supernatant of the 10,000 × *g* spin was centrifuged for 1 h at 100,000 × *g* at 4°C with a Beckman (Fullerton,

Table 1. Oligonucleotide primers used for PCR in the generation of full-length recombinant proteins

	Sense oligonucleotides[a]		Antisense oligonucleotides[b]	
	Nucleotides[c]	Nucleotide sequence	Nucleotides[c]	Nucleotide sequence
HK1-sa	36-55	GCATGGTCCAGGACAGGCTG	2916-2898	CGTAGGGTCTCCTCTGAGC
HK1-sb	6-25	ACAGAACTGCCAGCGAGGAC	2829-2811	CGTAGGGTCTCCTCTGAGC
HK1-sc	6-25	ACAGAACTGCCAGCGAGGAC	2760-2742	CGTAGGGTCTCCTCTGAGC

[a] Sense oligonucleotides contained an *Eco*RI site at their 5′ end.

[b] Antisense oligonucleotides contained a *Hin*dIII site at their 5′ end.

[c] Nucleotides are numbered from the first in-frame ATG.

CA) Airfuge ultracentrifuge. Both the supernatant and pellet fractions were boiled in sample buffer and centrifuged, and the resultant supernatants were designated "S_{100}" and "P_{100}," respectively. The P_{10} fraction corresponded to proteins found in the sperm cytoskeleton, nuclei, and mitochondria as well as intact sperm that survived homogenization. The S_{100} fraction contained soluble proteins, and the P_{100} contained membrane proteins (Visconti *et al.*, 1996).

For all cell or tissue preparations, one of two methods was utilized to assay protein concentration depending on the presence or absence of reducing agents. These were the MicroBCA kit (Pierce, Rockford, IL) and the amido black assay (Schaffner and Weissmann, 1973).

Preparation of Germ Cell and Tissue Extracts

Testes were collected from retired breeder CD1 mice, decapsulated, and subjected to sequential enzymatic digestion with collagenase and trypsin/DNase 1 (Romrell *et al.*, 1976; Bellvé *et al.*, 1977) to yield a population of mixed germ cells. Separation of the mixed germ cells into purified populations of pachytene spermatocytes, round spermatids, and condensing spermatids was accomplished by sedimentation velocity at unit gravity in a 2–4% BSA gradient in an enriched Krebs-bicarbonate medium (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). Both the pachytene spermatocyte and round spermatid populations were approximately 85% pure, while the condensing spermatid population was approximately 40–50% pure, being contaminated with anuclear residual bodies and some round spermatids.

Various tissues were obtained from retired breeder CD1 mice, placed in 3 ml TN/EDTA medium, and minced using small dissection scissors. The tissues were washed free of excess blood, and visible blood clots were removed. Tissues were homogenized in Teflon/glass homogenizers 10–20 times on ice. SDS-sample buffer was added to the samples, which were then sonicated on ice, boiled, and centrifuged. The supernatants were aliquoted and stored at −80°C.

Protein Extraction and Phase Separation Assays

Sperm were collected and fractionated as above without boiling in sample buffer. The P_{100} pellet was assayed for protein concentration, and five equal aliquots of P_{100} proteins (20 μg of protein each) were prepared. Individual aliquots were extracted under the following conditions: (1) TE buffer, 2) 0.5 M NaCl, 3) 100 mM Na_2CO_3 in TE buffer, pH 11, 4) 2 mM glucose-6-PO_4 in TE buffer, and 5) 1% Triton X-100 in TE buffer. Aliquot 4 was incubated at 30°C for 30 min and then placed on ice. All other aliquots were incubated at room temperature for 1 h. The aliquots then were centrifuged at 100,000 × *g* for 1.5 h, and the top half of each of the supernatants and the pellets were collected, boiled in sample buffer, and analyzed by SDS-PAGE. Additionally, a sperm membrane fraction (25 μg protein) was prepared as described above and was subjected to Triton X-114 phase separation using the method of Bordier (1981).

Electrophoresis and Immunoblotting

Proteins were separated under reducing conditions by SDS-PAGE with the use of 10% polyacrylamide gels (Laemmli, 1970). Detection of proteins was achieved by immunoblotting after transfer to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for at least 1 h in a Tris-buffered saline solution [25 mM Tris, 150 mM NaCl (TBS)] containing 0.1% Tween 20 (TTBS) and approximately 5% cold-water teleostean gelatin (Sigma) and probed with the appropriate primary antibody for 1 h. Blots were then washed briefly in TTBS, before being incubated with the appropriate peroxidase-conjugated secondary antibody for 35 min (1:5000 dilution). Blots were washed in TTBS for at least 2 h before visualization of immune complexes by chemiluminescence (Renaissance, DuPont NEN, Boston, MA). When appropriate, preabsorption of the germ cell-specific HK1 antisera with the peptides used to generate the antisera was utilized as a control. Preabsorption was carried out overnight on a rocker at 4°C using 0.1 mg peptide/100 μl of purified IgG.

Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975) with the following modifications. The first-dimension gel (9.5 M urea, 4.1% acrylamide, 2% NP-40) contained the following ratios of ampholytes to generate a reproducible gradient with good resolution between pH 5 and pH 7.5 (3.65%, pH 3–10; 1.17%, pH 5–7; 1.17%, pH 8–10) (Bio-Rad, Hercules, CA). Mouse sperm were homogenized, sonicated on ice, and treated with 5 μg of DNase I for 10–20 min before being extracted in sample buffer without reducing agents as above. Samples were prepared for loading onto the first dimension by mixing sperm extract proteins with a buffer containing 9.9 M urea, 4.0% NP-40, 2.2% ampholytes (80% of pH 3–10, and 20% of pH 5–7), and 100 mM dithiothreitol (DTT). Care was taken to ensure that the final urea concentration of this mixture was greater than 8.0 M. Ten micrograms of sperm protein in 50 μl of buffer were applied to each first-dimension gel. In some cases, sperm proteins were spiked with 0.2 μg of purified rat brain hexokinase (the generous gift of John Wilson, Michigan State University) to demonstrate that the germ cell-specific HK1 could be resolved from the somatic isoform. Isoelectric focusing was performed at 400 V for 18 h. First-dimension gels were removed, equilibrated briefly in a buffer containing 3.0% SDS and 50 mM DTT, and then separated in the second dimension by SDS-PAGE in 8% polyacrylamide gels.

Immunoelectron Microscopy

Mouse sperm were obtained from the caudae epididymides of retired breeder CD1 mice, and washed twice by centrifugation (5 min at 300 × *g*) and resuspension in PBS. Sperm were fixed in 0.2 M cacodylate buffer (pH 7.4) containing 2% paraformaldehyde for 1 h at 4°C, dehydrated in an increasing series of ethanol treatments, and embedded in LR Gold resin (Polysciences, Fort Washington, PA). Grids were incubated in a Tris-buffered saline blocking solution containing 10% normal goat serum for 30 min at room temperature. They were incubated subsequently in blocking buffer containing a 1:200 dilution of either purified α-gcs IgG or purified α-gcs IgG preabsorbed with peptide as described above. After three washes in TBS, grids were incubated in blocking buffer containing 18 nm colloidal gold conjugated to a goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:50. After being washed three times in TBS, all grids were fixed and counterstained with 1% osmium tetroxide followed by aqueous uranyl acetate and lead citrate. Specimens were observed and photographed using a Philips (Eindhoven, the Netherlands) 201 transmission electron microscope.

RESULTS

Generation and Characterization of HK1 Isoform-specific Antisera

As predicted, the purified IgG fractions of the different antisera were isoform specific: α-sa recognized only HK1-sa, α-sb recognized only HK1-sb, and α-gcs recognized all three recombinant proteins (Figure 2A). Lanes containing proteins from the expression cells transformed with plasmid without insert (labeled "pET") demonstrated that the recombinant proteins, and not bacterial proteins, accounted for the immunoreactive signals. It should be noted also that differences in the molecular weights among the recombi-

nant proteins corresponded to the predicted differences based on sequence analysis (Table 2).

Preabsorption of the antibodies with the specific peptides against which the antisera were generated further demonstrated the specificity of the individual antisera. Figure 2B demonstrates a loss of the recombinant HK1-sa and HK1-sb immunoreactive signals with the respective preabsorbed IgG fractions, and Figure 3D shows a loss of the immunoreactive signal of the native protein with the preabsorbed α-gcs IgG fraction. Sensitivity of the three purified IgG fractions was established by the use of serial dilutions of the respective recombinant protein-positive controls. All three antisera were of equal sensitivity, i.e., able to detect at least 0.05 μg of full-length specific protein(s) (Figure 2C shows data for α-sb; identical results were seen with α-sa and α-gcs.).

Expression of HK1-sc in Mature Mouse Sperm

To establish which, if any, of the potential germ cell-specific HK1 isoforms were present in mature sperm, whole sperm extracts were probed with the three isoform-specific antisera. Only the α-gcs antiserum revealed an immunoreactive band (compare Figure 3C with Figure 3, A and B). To determine whether the HK1-sa and/or HK1-sb isoforms might exist in small amounts in specific sperm compartments, sperm were fractionated into organelles and cytoskeletal elements (P_{10}), soluble proteins (S_{100}), and membrane proteins (P_{100}). No evidence of the HK1-sa and HK1-sb isoforms was found in any of these fractions, even after up to 40 μg of P_{10} or P_{100} proteins per lane were loaded (Figure 3, A and B). Because soluble proteins make up a relatively small percentage of total sperm protein and are recovered at dilute concentrations, 20 μg of S_{100} were loaded per lane.

Although the purified α-gcs IgG was shown to recognize all three recombinant isoforms, which are disparate in size (Figure 2A and Table 2), this antiserum detected a single immunoreactive band in sperm. The lack of immunoreactivity with the α-sa and α-sb antisera, combined with the presence of only one band of α-gcs immunoreactivity and the fact that all three antisera were equally sensitive, suggested that HK1-sc was the only isoform present in mature sperm (Figure 3C). HK1-sc localized biochemically to the membrane fraction (P_{100}), as well as to the fraction enriched in organelle and cytoskeletal elements (P_{10}), a result that correlated with the immunolocalization of this protein (see below). Immunoreactivity in the S_{100} fraction was weak and variable and potentially represented contamination with membrane proteins. The difference in apparent molecular weight between the native protein and its counterpart recombinant protein (see Figure 3C) can be explained by the presence of the amino-terminal signal sequence and the carboxy-terminal herpes simplex virus tag and histidine repeat in the recombinant protein.

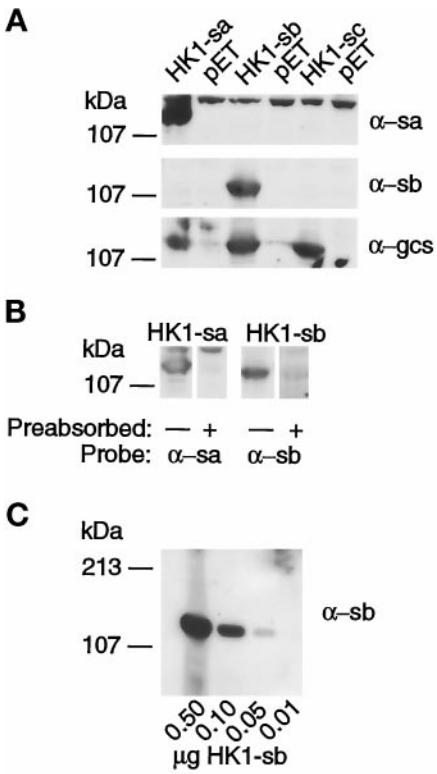

Figure 2. Specificity of the α-sa, α-sb, and α-gcs antisera. Extracts of BL21-DE3 cells expressing recombinant proteins were separated using SDS-PAGE. Lanes labeled "pET" represented extracts from these cells, which contain the plasmid without the insert. (A) α-sa and α-sb reacted specifically with recombinant HK1-sa and HK1-sb, respectively, and did not cross-react with recombinant proteins representing the other potential germ cell-specific isoforms, whereas α-gcs antiserum was shown to cross-react appropriately with all three recombinant proteins. The size differences among the recombinant proteins can be explained by differences in the predicted molecular weights of the isoforms as seen in Table 2. (B) Specificity of the recognition of HK1-sa and HK1-sb recombinant proteins by their respective antisera was demonstrated by the disappearance of immunoreactivity when the antisera were preabsorbed with the peptides against which they were made. The resultant loss of immunoreactivity of endogenous HK1-sc after preabsorption of the α-gcs antisera is shown in Figure 3D. (C) Sensitivity of the α-sb antiserum was shown by the detection of immunoreactivity with as little as 0.05 μg of full-length recombinant protein. Identical results were obtained with the α-sa and α-gcs antisera (our unpublished results).

Expression of HK1-sc Protein during Spermatogenesis

Although not found in mature sperm, HK1-sa and/or HK1-sb might be expressed transiently or synthesized and processed/degraded during germ cell development. To test these possibilities, protein extracts from purified populations of pachytene

Table 2. Comparison of predicted size, molecular weight, and isoelectric points of the four potential HK1 isoforms based on MacVector analysis[a]

	Predicted number of amino acids	Predicted molecular mass (kDa)	Predicted isoelectric point
HK1-sa[b]	973	108.138	6.55
HK1-sb[b]	943	105.282	6.62
HK1-sc[b]	921	102.472	6.59
somatic HK1[c]	918	102.266	6.47

[a] Note that the measurements given are for the predicted endogenous proteins and do not include the amino- or carboxy-terminal domains present in the recombinant proteins.

[b] Sequence taken from Mori *et al.* (1993).

[c] Sequence taken from Arora *et al*. (1990).

spermatocytes, round spermatids, and condensing spermatids were generated. These cell types were chosen because they correspond to the known expression patterns for the mRNAs of these isoforms (Mori *et al*., 1993). Again, even after loading up to 40 μg of each extract per lane, no evidence of HK1-sa or HK1-sb was found (Figure 4, A and B). However, when the α-gcs antiserum was used, one immunoreactive band was detected, first in round spermatids and then in increased amounts until a maximal and constant level was reached in mature sperm (Figure 4C). This observation is consistent with the previously established expression of HK1-sc mRNA (Visconti *et al*., 1996). Again, the lack of signal with the α-sa and α-sb antisera, combined with a single immunoreactive signal with the α-gcs antiserum, suggested that HK1-sc was the only germ cell-specific HK1 isoform present in these cells.

Lack of Expression of HK1-sc Protein in Somatic Tissues

To demonstrate that HK1-sc was germ cell specific at the protein level, extracts of various somatic tissues were probed with the α-gcs antiserum. As seen in Figure 5, no immunoreactive signal for HK1-sc was found in any of the somatic tissues tested. Probing of identical blots with α-sa and α-sb also yielded no immunoreactivity in the somatic tissue extracts, demonstrating that these isoforms were not detectable in somatic tissues (our unpublished results). Nevertheless, reprobing of these blots with antisera capable of recognizing all HK1 isoforms (α-HK1) revealed the presence of the somatic isoform in many of these tissues (our unpublished results), consistent with their known expression patterns (Katzen, 1967). Taken together, these findings demonstrated that the α-sa, α-sb, and α-gcs antisera did not cross-react with the endogenous somatic HK1 isoform.

Tyrosine Phosphorylation of HK1-sc

Thus far, within the limits of detection of the antisera, only HK1-sc protein was present in developing germ cells and sperm. This finding was examined further using two-dimensional SDS-PAGE in an attempt to answer several questions. First, this technique would provide a method to examine the extent of tyrosine phosphorylation of HK1 in mouse sperm. Second, it would provide information regarding the presence or absence of the somatic HK1 isoform in mature sperm. Finally, it would corroborate the results of SDS-PAGE regarding the absence of HK1-sa and HK1-sb. It should be noted that the three potential germ cell-specific HK1 isoforms, as well as the somatic isoform, would be predicted to have differences in both apparent molecular weight and in isoelectric point (Table 2).

An immunoreactive signal migrating at an identical apparent molecular weight and an identical isoelectric point was visualized when blots were probed with α-gcs, α-HK1 (a polyclonal antisera recognizing all HK1 isoforms), and α-PY (Figure 6, A, B, and C, respectively). On certain blots, the endogenous α-gcs signal could be resolved into three spots (all of which immunoreacted with α-PY), migrating at the same molecular weight but having slightly different isoelectric points (our unpublished results). This pattern might be accounted for by small variations in charge of the same molecule (O'Farrell, 1975), possibly due to differences in degree of phosphorylation. Collectively, the data from these immunoblots demonstrated that the tyrosine-phosphorylated form of HK1 observed in mouse sperm was HK1-sc.

Given the differences in both the predicted molecular weights and isoelectric points between HK1-sc and the somatic isoform (Table 2), at least two distinct spots should be discerned if both of these isoforms were present, with all spots being detected by α-HK1, and only a subset of spots being detected by α-gcs. Yet the identical immunoreactive signal seen with the

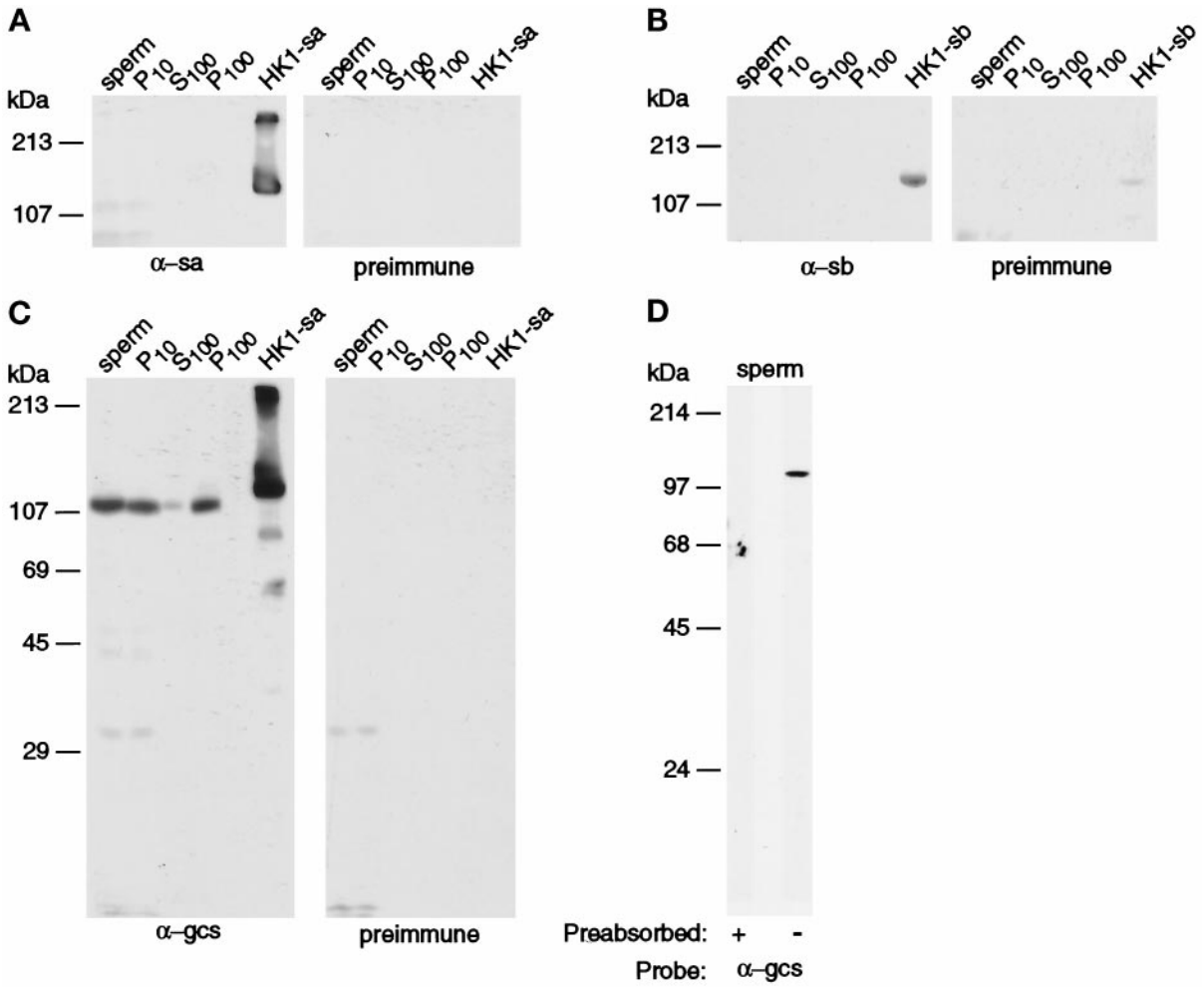

Figure 3. Expression of germ cell-specific isoforms of HK1 in sperm. Cauda epididymal mouse sperm were fractionated, and proteins from whole sperm extracts (20 μg, labeled "sperm"), a 10,000 $\times$ *g* sperm pellet (40 μg, labeled "P_{10}"), a 100,000 $\times$ *g* sperm supernatant (20 μg, labeled "S_{100}"), and a 100,000 $\times$ *g* sperm pellet (40 μg, labeled "P_{100}") were separated by SDS-PAGE and subjected to immunoblotting. (A) α-sa immune and α-sa preimmune antisera. (B) α-sb immune and α-sb preimmune antisera. (C) α-gcs immune and α-gcs preimmune antisera. In each case, the appropriate purified recombinant protein was used as a positive control. (D) Recognition of endogenous HK1-sc was demonstrated to be specific because immunoreactivity was lost when the α-gcs antiserum was preabsorbed with the peptide against which it was made.

α-gcs and the α-HK1 suggested that at the limits of detection, no somatic HK1 was observed. To confirm that this methodology had the resolving power to separate the HK1-sc from somatic HK1, samples of mouse sperm were spiked with 0.2 μg of purified rat brain HK1. When probed with α-HK1, these blots revealed two easily distinguishable populations (Figure 6D).

Behavior of HK1-sc as an Integral Membrane Protein

Previously, the HK1 population found in sperm membrane fractions was shown to behave biochemically as an integral membrane protein, both by its solubility characteristics and its partitioning into the detergent fraction of a Triton X-114 phase separation assay (Visconti *et al*., 1996). Those findings contrast with the behavior of the somatic HK1 isoform, which is found as a soluble protein or bound to the outer mitochondrial membrane via its PBD (Felgner and Wilson, 1977; Polakis and Wilson, 1985; Smith and Wilson, 1991).

To investigate the biochemical characteristics of the population of HK1-sc found in sperm membranes, the solubility of HK1-sc found in the P_{100} fraction was tested under a variety of conditions. HK1-sc behaved as an integral membrane protein, becoming solubilized only in the presence of 1% Triton X-100 (Figure 7A). It solubilized neither under conditions known to

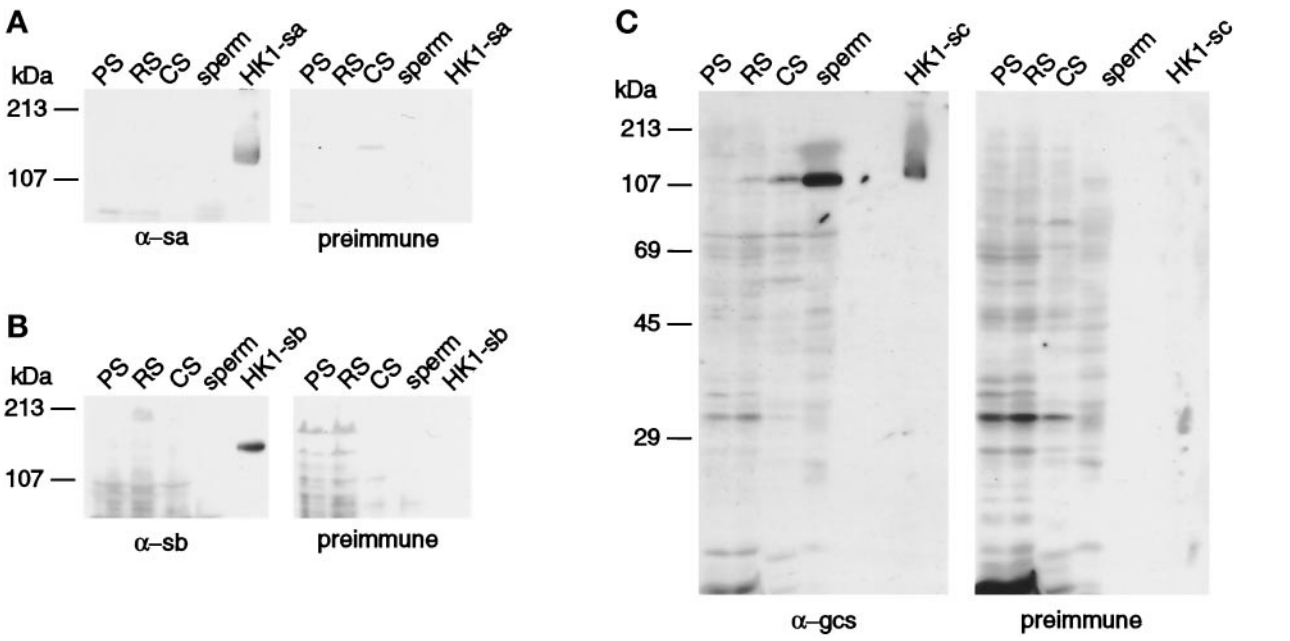

Figure 4. Expression of germ cell-specific isoforms of HK1 during spermatogenesis. Proteins (40 μg) from pachytene spermatocytes (PS), round spermatids (RS), condensing spermatids (CS), and whole sperm ("sperm") were separated using SDS-PAGE. The appropriate purified recombinant proteins were used as positive controls. (A) α-sa immune and α-sa preimmune antisera. (B) α-sb immune and α-sb preimmune antisera. (C) α-gcs immune and α-gcs preimmune purified antisera. The first appearance of HK1-sc in round spermatids matches the previously established expression pattern of HK1-sc mRNA (Visconti *et al.*, 1996).

release peripheral proteins [0.5 M NaCl or 0.1 M Na_2CO_3 at pH 11 (Fujiki *et al.*, 1982)] nor under conditions that have been shown to dissociate somatic HK1 from mitochondrial porin [2 mM glucose-6-phosphate (Felgner and Wilson, 1977)]. An alternative method of solubilizing somatic HK1 [1 mM ATP, 1 mM DTT (Arora and Pederson, 1988)] also failed to release HK1-sc (our unpublished results). Corroborating the Triton X-100 extraction pattern were the results from Triton X-114 phase separation assays. As seen in Figure 7B, HK1-sc partitioned into the amphiphilic detergent phase, but not into the hydrophilic aqueous phase. These experiments indicated that the major population of the HK1-sc found in sperm membranes behaved biochemically as an integral membrane protein despite the absence of a clear signal sequence and transmembrane domain (Mori *et al.*, 1993).

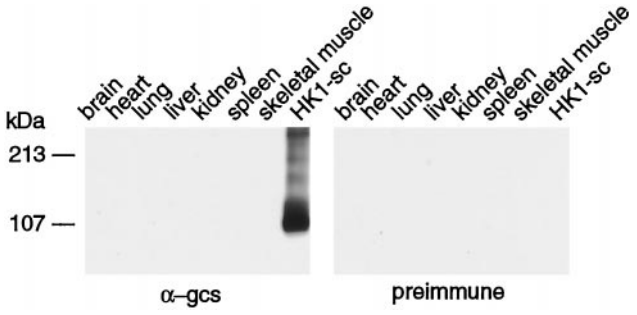

Figure 5. Expression of germ cell-specific isoforms of HK1 in somatic tissues. Forty micrograms of protein extracts from various somatic tissues were prepared and separated using SDS-PAGE and then subjected to immunoblotting with the α-gcs immune and α-gcs preimmune antisera. Immunoblots with the α-sa and α-sb antisera are not shown but yielded identical results. Reprobing of these blots with the α-HK1 antiserum capable of recognizing the somatic isoform revealed that the somatic isoform was detectable in a variety of tissues (our unpublished results).

Localization of HK1-sc in Mature Sperm

The previous localization of HK1 in mouse sperm was accomplished with indirect immunofluorescence using a monoclonal antibody that would recognize both the somatic HK1 isoform and the germ cell-specific isoforms (Visconti *et al.*, 1996). Because HK1-sc was the only germ cell-specific HK1 isoform detectable in mature sperm and was distributed into multiple compartments after sperm fractionation, it became important to localize HK1-sc at a higher resolution. Therefore, immunoelectron microscopy using the purified α-gcs IgG fraction was employed. This antiserum was shown previously not to cross-react with endogenous, somatic HK1 (Figure 5).

As seen in Figure 8A, very strong staining occurred in the region of the mitochondrial membranes in the

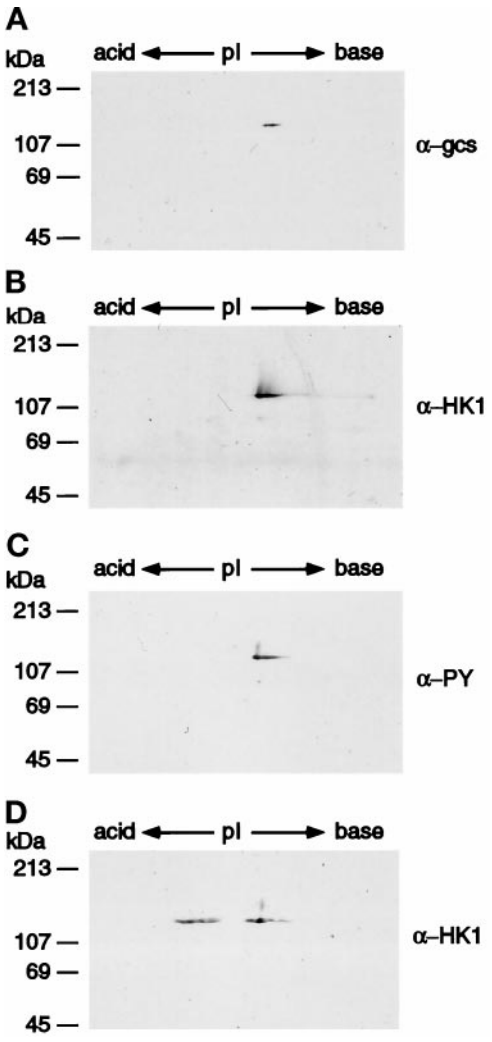

Figure 6. Tyrosine phosphorylation of HK1-sc. Sperm proteins (10 μg) were separated by two-dimensional gel electrophoresis and probed with α-gcs (A), α-HK1 (B), and α-PY (C). An identical pattern of immunoreactivity was seen with all three antisera. Sperm proteins spiked with 0.2 μg of purified rat brain hexokinase were also separated by two-dimensional gel electrophoresis and probed with α-HK1 to confirm the resolving power of the system (D). These results suggested that all HK1-sc was tyrosine phosphorylated, and that the somatic HK1 isoform was not detectable in mature sperm.

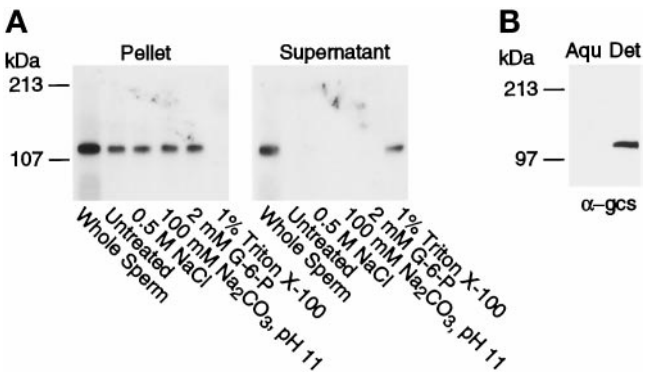

Figure 7. Solubilization characteristics of sperm membrane HK1-sc. (A) A membrane fraction (P_{100}) from cauda epididymal mouse sperm was incubated in different extraction conditions as follows: 1) buffer (untreated); 2) 0.5 M NaCl; 3) 100 mM Na_2CO_3 at pH 11; 4) 2 mM glucose-6-phosphate; or 5) 1% Triton X-100. Only Triton X-100 resulted in the solubilization of HK1-sc. B) Triton X-114 phase separation of membrane-bound HK1-sc. A membrane fraction (P_{100}) prepared from cauda epididymal mouse sperm was subjected to Triton X-114 phase separation (Bordier, 1981). HK1-sc immunoreactivity partitioned into the amphiphilic detergent phase and not the hydrophilic aqueous phase.

midpiece. This result demonstrated that in the absence of a classical PBD, HK1-sc could still localize to the mitochondria. Strong labeling was also seen throughout the fibrous sheath, including both the longitudinal columns and the circumferential ribs (Figure 8, B and C). On many cross-sections (e.g., Figure 8D), immunoreactivity in the head appeared to be clustered into discrete units and was in the region of the membranes of both the anterior and posterior head (Figure 8E). Staining in the region of the acrosomal membranes was less consistent, although the region of the inner acrosomal membrane often stained with a higher intensity than the region of the membranes overlying the acrosome (Figure 8E and our unpublished results). In addition to the structures found in mature sperm, heavy labeling was seen in cytoplasmic droplets associated with the midpiece (Figure 8F). Minimal background staining was seen when α-gcs was preabsorbed with the peptide against which it was made (Figure 8, G, H, and I), indicating that the immunoreactivity was specific. Similarly, preimmune purified IgG at a high concentration (1:10 vs. 1:200 for the immune α-gcs) also gave minimal background staining (our unpublished results).

DISCUSSION

Mouse sperm contain an HK1 that differs from its somatic counterpart in localization and biochemical properties (Katzen, 1967; Kalab *et al*., 1994; Visconti *et al*., 1996). A possible explanation for these differences is that three germ cell-specific HK1 isoforms are transcribed (Mori *et al*., 1993) and may be translated into distinct proteins. Specifically, the mRNAs encoding these potential isoforms of HK1 all lack a classical amino-terminal PBD (Mori *et al*., 1993) through which the somatic HK1 isoform interacts with porins in the outer mitochondrial membranes (Felgner and Wilson, 1977; Polakis and Wilson, 1985; Smith and Wilson, 1991).

To determine which of the possible HK1 isoforms were translated into proteins, and where they might be localized in sperm, antisera specific for HK1-sa and

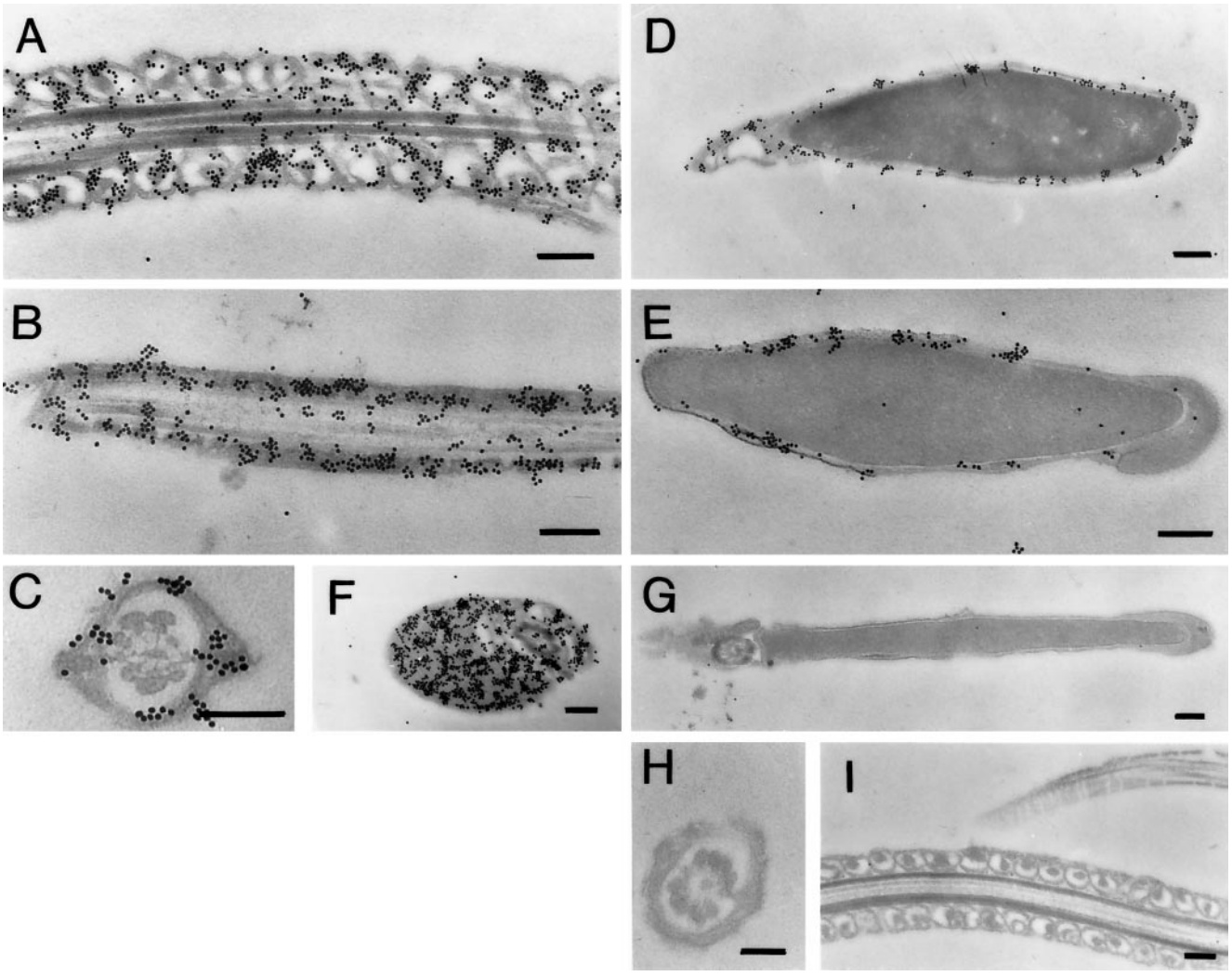

Figure 8. Immunoelectron microscopic localization of HK1-sc in mouse sperm. Sperm were collected and prepared as described in MATERIALS AND METHODS. Panels A–F are micrographs of sections probed with a 1:200 dilution of purified α-gcs IgG. Panels G–I are micrographs of sections probed with a 1:200 dilution of purified α-gcs IgG preabsorbed with the peptide against which it was made. In all cases, a secondary goat anti-rabbit antibody conjugated to 18-nm colloidal gold particles was used for labeling. (A) A longitudinal section of the midpiece of the flagellum showing labelling associated with the mitochondria. (B) A longitudinal section of the principal piece of the flagellum showing labelling associated with the fibrous sheath. (C) A cross-section of the principal piece of the flagellum showing labelling associated with the fibrous sheath. (D) An oblique section through the posterior head showing labelling in the region of the membranes, which often appeared organized into discrete clusters. (E) A transverse section through the head, including a portion of the equatorial region, showing labelling associated with the membranes of the anterior and posterior sperm head. (F) A cross-section of the midpiece of the flagellum showing labelling of a cytoplasmic droplet. (G) A longitudinal section of a preabsorbed control of the sperm head. (H) A cross-section of a preabsorbed control of the principal piece. (I) Longitudinal sections of a preabsorbed control of the midpiece and fibrous sheath. Bar, 250 nm, in all panels.

HK1-sb were created (called "α-sa" and "α-sb," respectively). Because of sequence identity among the potential germ cell-specific isoforms, no antibody could be generated that would be specific for HK1-sc. Instead, the α-gcs antiserum was designed to recognize the germ cell-specific sequence encoded by all three mRNAs, but not encoded by the somatic isoform. Using these antisera, no evidence of HK1-sa or HK1-sb was found in whole sperm extracts or in various sperm fractions. Furthermore, no immunoreactivity was found with the α-sa or α-sb antisera when proteins from spermatogenic cells of different stages were examined, indicating that neither HK1-sa nor HK1-sb was present during development. The concept that germ cell transcripts can exist with little or no subsequent translation into proteins has ample precedent (e.g., Kew *et al*., 1989; Capel *et al*., 1993; Kleene, 1996). Alternatively, HK1-sa and/or HK1-sb might be

expressed in amounts below the limits of detection, expressed and rapidly degraded, or expressed and modified after translation. For example, the HK1-sa isoform might be translated but have its amino-terminal end removed by proteolytic cleavage, thereby effectively leaving the HK1-sc isoform as the mature product.

It could be argued that another type of modification, such as a phosphorylation event, might mask the antigenicity of the HK1-sa or HK1-sb epitopes, thereby making these two antisera ineffective. However, the pattern of immunoreactivity seen with the α-gcs antiserum, which was shown to recognize all three recombinant protein-positive controls, makes this possibility unlikely. As seen in Table 1, the three potential germ cell-specific isoforms have predicted differences in both apparent molecular weights and isoelectric points. If the HK1-sa and/or HK1-sb antisera were ineffective and these isoforms were present, the α-gcs antiserum should detect additional immunoreactive signals. Yet on immunoblots of proteins from sperm or from cells at various stages of spermatogenesis, the α-gcs antiserum produced only one immunoreactive band at a consistent apparent molecular weight. This finding suggested that only one isoform, most likely HK1-sc, was translated.

Using two-dimensional SDS-PAGE of proteins from mature sperm, only one immunoreactive band could be seen with α-gcs, as well as with an antiserum that is predicted to recognize all HK1 isoforms, and with an anti-phosphotyrosine antibody. Taken together, these findings suggested that not only is HK1-sc the only HK1 isoform present in mature sperm, but also that virtually all the HK1-sc is phosphorylated on tyrosine residues. Although it is possible that the somatic HK1 isoform exists as a minor population, HK1-sc is clearly the major form of this enzyme in sperm. The observation that the immunoreactive protein on blots from two-dimensional gels could be resolved into three closely spaced spots, regardless of whether the α-gcs, α-HK1, or α-PY antisera was used, could most easily be explained by charge heterogeneity (e.g., degree of phosphorylation) within the HK1-sc population.

Immunoelectron microscopic localization of HK1-sc using the α-gcs antiserum offered an explanation for the apparent lack of somatic HK1; HK1-sc was shown to localize to the mitochondria in the midpiece of the sperm flagellum. In essence, the PBD found in somatic HK1 is not necessary for hexokinase to localize to the mitochondria in sperm. This finding is in contrast to the essential role of the PBD in the targeting of somatic HK1 to mitochondria (Smith and Wilson, 1991; Gelb *et al*., 1992) and raises at least three possibilities. First, it is possible that the common, germ cell-specific sequence that replaces the classical PBD could function identically as this highly conserved N-terminal region does in somatic HK1. Second, the germ cell-specific sequence could function as an alternative PBD by interacting with different isoforms of porin not present in somatic cells. A recent study reports that there are at least three porin cDNAs in the mouse (Sampson *et al*., 1997), suggesting that family members of porin could exist in particular cell types. Complementation analysis in yeast mutants lacking their endogenous porin suggests that at least one of these porins may have an alternative physiologic function (Sampson *et al*., 1997). The third possibility raised by the localization of HK1-sc to mitochondria is that HK1-sc binds to the mitochondria of sperm via a completely different interaction. Sperm mitochondria are surrounded by a keratinous mitochondrial capsule (Cataldo *et al*., 1996), which might even necessitate a different means of localization. Regardless of mechanism, the finding that an isoform of HK1 can localize to the mitochondria without a classical PBD is intriguing and deserves further examination.

In addition to the midpiece, extremely heavy staining was seen associated with both the longitudinal columns and circumferential ribs of the fibrous sheath, whereas in the head, staining was seen in discrete clusters on the membranes of the anterior and posterior head. Staining in the membranes over- and underlying the acrosome was variable in amount and pattern. How HK1-sc localizes to other regions of the sperm is also not yet understood. Our results suggested that the HK1-sc found in the sperm membrane fraction becomes solubilized only by the presence of Triton X-100, thereby behaving biochemically as an integral membrane protein. Kyte-Doolittle analysis of the HK1-sc sequence revealed that no substantial hydrophobic region exists that could easily be interpreted as a transmembrane domain (our unpublished results). While there have been reports of proteins targeted to the plasma membrane that lack a signal sequence (Cooper and Barondes, 1990; Rapoport, 1992), HK1-sc may alternatively be linked to the membranes via a covalent interaction with another protein(s). In this regard, it is intriguing to note that preliminary results with the HK1-sc found in the P_{10} fraction revealed a different solubility pattern, resistant to even Triton X-100 (our unpublished results). Moreover, another glycolytic enzyme recently has been reported to be bound to an organelle of sperm through a covalent linkage (Westhoff and Kamp, 1997, discussed below).

If localization of HK1-sc is determined by its interactions with porins, then the staining seen in the head and principal piece of the flagellum, both regions lacking mitochondria, suggests that porins may localize to these regions as well. Alternatively, HK1-sc present in these regions of the sperm may interact with other proteins. Both these possibilities will be explored in future studies. Ultimately, the

finding that one isoform of HK1 is located in such diverse areas of the cell as the plasma membrane, mitochondria, and fibrous sheath suggests that this enzyme plays a role in glycolytic energy production throughout the cell or that it has alternative functions in different regions.

Although extramitochondrial energy production is perhaps the most likely function of HK1-sc, the possibility that HK1-sc may play a role in sperm-ZP binding cannot be dismissed. Although variable in intensity and distribution, immunolabeling in the region of the acrosomal membranes and/or plasma membranes overlying the acrosome was seen. Originally proposed to be a primary receptor in sperm-zona binding, p95 was thought to be involved in the signal transduction cascade leading to acrosomal exocytosis (Leyton and Saling, 1989; Leyton *et al.*, 1992). Given the immunolabeling on the membranes of both the anterior and posterior head, perhaps a more likely function in this regard could be that of a nonspecific, lectin-like tethering molecule that would help keep the motile spermatozoan adherent to the ZP. Supporting the concept that HK1-sc could possibly be involved in this capacity is the fact that *N*-acetylglucosamine, a component of ZP3, is a recognized substrate of HK1 (Wilson, 1995). An alternative candidate for a sperm surface ZP3 receptor is another carbohydrate-binding enzyme, β-galactosyltransferase (Shur and Hall, 1982). This molecule has been proposed to act as a lectin, and to bind *N*-acetylglucosamine residues on the ZP without acting enzymatically (Benau and Storey, 1988; Benau *et al.*, 1990). It would be interesting from an evolutionary perspective if two metabolic enzymes utilized their carbohydrate-binding properties to recognize carbohydrate epitopes on the ZP.

Alternatively, the fact that these cells are highly differentiated and contain little cytoplasm suggests that HK1-sc plays its normal role as the initial enzyme in the Embden-Meyerhof pathway, particularly in the tail, which is the major site of ATP consumption in the sperm. The utilization of glucose is crucial both for the maturation of sperm and for fertilization. Glycolysis is the major source of energy required for the capacitation of epididymal mouse sperm (Hoppe, 1976). Glucose is also needed by mouse spermatozoa for successful fusion to the plasma membrane of the egg (Urner and Sakkas, 1996). The appearance of HK1-sc throughout the cell suggests that cellular ATP is provided by glycolysis in regions lacking mitochondria.

In support of this suggestion is the recent finding that an isoform of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is covalently bound to the fibrous sheath (Westhoff and Kamp, 1997). GAPDH was not found in the midpiece; rather, it was removed from that region during release of cytoplasmic droplets (Westhoff and Kamp, 1997), which have been demonstrated previously to contain many metabolic enzymes (Garbers *et al.*, 1970; Harrison and White, 1972). The lack of GAPDH in the midpiece led to the proposal that glycolysis is carried out in the principal piece of the flagellum, whereas respiration alone occurs in the midpiece of mature sperm (Westhoff and Kamp, 1997). Our finding of HK1-sc in the cytoplasmic droplets, as well as in the midpiece of mature sperm, can be interpreted in several ways. In keeping with the above authors' suggestion that glycolysis does not occur in the midpiece of mature sperm, the HK1-sc found in that region may represent a nonfunctional, residual population of enzymes. Alternatively, the HK1-sc may have a different function or may act in a glycolytic pathway that employs an isozyme of GAPDH that was not immunoreactive with the antiserum used in the study of Westhoff and Kamp (1997).

The present study is the first to demonstrate that sperm hexokinase, the initial upstream enzyme in the Embden-Meyerhof pathway, is associated with the fibrous sheath, a major structural component of the principal piece. The findings that GAPDH is bound to the fibrous sheath (Westhoff and Kamp, 1977) and that all the enzymes of the pathway downstream from GAPDH remain bound to the cell structure after demembranation (Storey and Kayne, 1975) lead us to the hypothesis that the fibrous sheath acts as a scaffold for all the enzymes of the glycolytic pathway. The same scaffold arrangement for the glycolytic pathway enzymes has been demonstrated for skeletal muscle (Arnold and Pette, 1968). This scaffold arrangement allows rapid flux through the pathway, leading to rapid production of ATP at precisely the site where it is utilized: in sperm, the flagellar ATPase; in skeletal muscle, the myosin ATPase.

Of particular relevance to this hypothesis for the sperm cell is the recent demonstration that the fibrous sheath acts as a scaffold for proteins involved in the regulation of motility. The major protein of this structure, AKAP82, is a member of the A-kinase anchor protein (AKAP) family of polypeptides (Carrera *et al.*, 1994). AKAPs anchor protein kinase A (PKA) to the cytoskeleton or subcellular organelles via the regulatory (RII) subunit of the kinase. Thus AKAP82 in the fibrous sheath presumably acts as a scaffolding protein for the subcellular localization of PKA in the flagellum. This localization is noteworthy because motility is thought to be mediated by a series of phosphorylation events of target proteins in the tail (Tash, 1989). It is an intriguing possibility that glycolysis could be localized to a region of the sperm where the resultant ATP could be utilized both by PKA to regulate motility as well as by the dynein ATPases, which function as the flagellar motors.

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