Regulation of Protein Tyrosine Phosphorylation in Human Sperm by a Calcium/Calmodulin-Dependent Mechanism: Identification of A Kinase Anchor Proteins as Major Substrates for Tyrosine Phosphorylation

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Signal transduction pathways regulate various aspects of mammalian sperm function. When human sperm were incubated in a medium supporting capacitation, proteins became tyrosine-phosphorylated in a time-dependent manner. This phosphorylation was inhibited by genistein, a protein tyrosine kinase inhibitor. Phosphorylation was also reduced when sperm were incubated either in the presence of increasing concentrations of extracellular Ca²⁺ or in a medium containing the Ca²⁺ ionophore A23187. This Ca²⁺-induced dephosphorylation was calmodulin-dependent, suggesting that calcineurin was involved. In this regard, the calcineurin inhibitor deltamethrin inhibited the Ca²⁺ ionophore-induced dephosphorylation. A limited number of Mr 80,000–105,000 polypeptides were the most prominent phosphotyrosine-containing proteins present in human sperm. Unlike mouse sperm, which contains a tyrosine-phosphorylated isoform of hexokinase, a phosphotyrosine-containing hexokinase in human sperm was not detected. Most of the tyrosine-phosphorylated proteins were Triton X-100-insoluble and were localized to the principal piece of the flagellum, the region where the cytoskeletal fibrous sheath is found. Prominent phosphotyrosine-containing proteins of Mr, 82,000 and 97,000 were identified as the human homologues of mouse sperm AKAP82, the major fibrous sheath protein, and pro-AKAP82, its precursor polypeptide, respectively. These proteins are A Kinase Anchor Proteins, polypeptides that sequester protein kinase A to subcellular locations. Taken together, these results suggest that protein tyrosine phosphorylation may be part of a signal transduction cascade(s) regulating events pertaining to capacitation and/or motility in mammalian sperm and that an interrelationship between tyrosine kinase and cAMP signaling pathways exists in these cells.

INTRODUCTION

The intracellular signaling mechanisms regulating many sperm functions are poorly understood although it is clear that these events are likely to be regulated in a manner analogous to similar events in somatic cells (Kopf and Gerton, 1991). Cyclic nucleotides, in particular, cAMP, are important regulators of various maturation events in sperm including capacitation and motility (Garbers and Kopf, 1980). Sperm cAMP concentrations increase during capacitation in a number of mammalian species (White and Alt-
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Ken, 1989; Parrish et al., 1994). Furthermore, flagellar motility is initiated and maintained by cAMP (Tash and Means, 1983; San Agustin and Witman, 1994). However, while one of the major functions of cAMP is to stimulate protein kinase A (PKA), relatively little is known about either the regulation of these protein phosphorylation events or the protein substrates in sperm.

Although it has been known for more than 40 years that sperm must undergo a poorly defined, postepididymal maturation process termed capacitation before fertilization can occur (Chang, 1951; Austin, 1951), recent studies have suggested that capacitation involves the activation of second messenger systems (Duncan and Fraser, 1993; Parrish et al., 1994; Uguè et al., 1994; Visconti et al., 1995a,b). Capacitation occurs in a time-dependent manner either in the female reproductive tract in vivo or in defined media in vitro. This process has been correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility although it is unclear which, if any, of these events is obligate for capacitation (Yanagimachi, 1994). However, while the molecular basis underlying these events is poorly understood, they are consistent with a signal transduction cascade(s) being activated. In this regard, protein phosphorylation appears to be important in the capacitation of mammalian sperm. Recently, mouse sperm capacitation has been shown to be highly correlated with the tyrosine phosphorylation of a subset of proteins of M, 40,000–120,000, the phosphorylation of which is regulated by a cAMP-dependent pathway in a unique fashion (Visconti et al., 1995a,b).

While sperm become motile during epididymal maturation, during capacitation they undergo a qualitative change in motility called hyperactivation. For in vivo fertilization to occur, sperm motility is required for the passage both through the female reproductive tract (Overstreet and Katz, 1990) and through the egg investments (Katz et al., 1989). The quality of sperm motility can affect both of these steps. The initiation and maintenance of sperm motility involve the cAMP-dependent phosphorylation of proteins (Tash and Means, 1983; Lindemann and Kanous, 1989; San Agustin and Witman, 1994). Furthermore, motility can be inhibited by Ca2+ and this inhibition is correlated with the inhibition of protein phosphorylation via the calmodulin-dependent protein phosphatase calcineurin (Tash et al., 1988). Thus, it is thought that motility is mediated by a cascade of phosphorylation/dephosphorylation events that alter the activity of target proteins (Tash, 1989; Tash and Bracho, 1994). While the identities of these phosphorylated proteins that are involved in motility are, for the most part, not known, of particular interest is the finding that the tyrosine phosphorylation of M, 15,000 polypeptide is involved in the initiation of motility in trout sperm (Hayashi et al., 1987).

Signal transduction mechanisms regulating sperm motility undoubtedly involve flagellar proteins. The mammalian flagellum is composed of a number of cytoskeletal elements whose proper assembly is critical for the subsequent motility of the sperm (Oko and Clermont, 1990). The central core of the flagellum, the axoneme, is composed of the “9 + 2” array of microtubular doublets, associated dynein arms, and other cytoskeletal elements. The axoneme is surrounded in specific regions of the tail by several accessory structures termed the fibrous sheath (FS), the outer dense fibers (ODF), and the mitochondrial sheath. The FS is present only in the principal piece of the tail where it surrounds both the axoneme and ODF. While the precise function of this structure is unknown, FS sliding in a demembranated sperm model system is cAMP-dependent (Si and Okuno, 1993). Recently, the major mouse FS protein of M, 82,000 (p82) was shown to be a member of the A Kinase Anchor Protein (AKAP) family (Carrera et al., 1994). AKAPs tether PKA via its regulatory (RII) subunit and, thus, can sequester this protein kinase in close proximity to its physiological substrates (Rubin, 1994). The identification of this FS protein as an AKAP indicates that it is likely to be involved in the cAMP regulation of sperm motility.

One goal of our laboratories is to understand the role of signal transduction pathways involved in mammalian sperm capacitation and motility. We demonstrate that ejaculated human sperm displayed a time-dependent increase in protein tyrosine phosphorylation under conditions conducive to capacitation in vitro. This tyrosine phosphorylation was sensitive to the protein tyrosine kinase inhibitor genistein. Protein tyrosine dephosphorylation was both Ca2+ and calmodulin-dependent and appeared to be mediated by calcineurin. A distinct subset of polypeptides of M, 80,000–105,000 were the most prominent phosphotyrosine-containing proteins present in sperm. The majority of the tyrosine-phosphorylated proteins were localized to the principal piece of the sperm flagellum, the region where the FS is located. Furthermore, the prominent Triton X-100-insoluble phosphotyrosine-containing proteins of M, 82,000 and 97,000 were identified as the human homologues of mouse sperm AKAP82 (the major FS protein) and pro-AKAP82 (its precursor polypeptide). In accordance with the nomenclature proposed for RII anchoring proteins (Hirsch et al., 1992), we have renamed p82, AKAP82, and its M, 97,000 precursor (p97), pro-AKAP82, respectively. Similar to their mouse counterparts, human AKAP82 and human pro-AKAP82 are AKAPs, proteins that tether PKA to specific subcellular locations.

MATERIALS AND METHODS

Antibodies. Anti-phosphotyrosine (anti-pY) is a monoclonal antibody [Upstate Biotechnology, Inc., Lake Placid, NY (clone 4G10)] which has been used previously to characterize phosphotyrosine-containing proteins in mouse sperm (Visconti et al., 1995a,b). Anti-hexokinase (anti-HK) is a polyclonal antiserum raised against rat brain hexokinase, type I [kindly provided by Dr. John Wilson (Michigan State University)], and has been used to characterize mouse sperm hexokinase (Kalab et al., 1994). Anti-AKAP82 is a polyclonal antiserum raised against electrophoretically purified AKAP82, the major mouse sperm FS protein, and has been used to characterize this protein in mouse germ cells and mature sperm (Carrera et al., 1994).

Preparation of sperm. Samples of human semen were obtained
from normal healthy donors. After semen liquefaction (~30 min), the sperm ejaculates were loaded onto a 40–80% step Percoll gradient in modified HTF medium (Quinn et al., 1985) with 0.2 mg/ml of BSA (Fraction V; Sigma Chemical Co., St. Louis, MO). The sperm were washed through the gradient at 160g for 30 min, collected from both the pellet and the 80% Percoll layer, and then washed again by centrifugation with HTF to remove the Percoll. These washing procedures took ~45 min to perform. Sperm (1 x 10^6/ml) were then incubated in HTF media under various conditions in a humidified incubator maintained in 5% CO2 in air at 37°C. In some experiments, the washed sperm were solubilized directly in SDS sample buffer (Laemmli, 1970) and the resultant supernatant was designated as total sperm proteins. Alternatively, the sperm were extracted for 10 min at 4°C in a buffer containing 20 mM β-glycerophosphate, pH 7.4, 10 mM 4-nitrophenylphosphate, 0.2 mM sodium orthovanadate, 4 mM EGTA, 1 mM DTT, 1% Triton X-100, 10 μg/ml leupeptin, and 10 μg/ml aprотinin. The supernatant, designated as the Triton-soluble fraction, was recovered after centrifugation at 10,000g. The pellet, designated as the Triton-insoluble fraction, was solubilized in SDS sample buffer. Mouse sperm, when required, was obtained from the cauda epididymides.

In experiments that examined the effect of Ca2+ on protein tyrosine phosphorylation, the medium and Percoll gradients were prepared without Ca2+. Typically, sperm were incubated in the medium containing various concentrations of Ca2+ at 37°C for 2 hr. In some experiments, the Ca2+ ionophore A23187 was added to the medium to a final concentration of 10 μM with 2 mM Ca2+. The cells were incubated for an additional 30 min at 37°C and then centrifuged at 10,000g for 3 min. In experiments that examined the effect of various protein kinase inhibitors on protein tyrosine phosphorylation, sperm were prepared and incubated in HTF medium with a particular inhibitor at 37°C for 1 hr. In experiments that examined the effect of either calmodulin (Calbiochem Corp., San Diego, CA) or phosphatase inhibitors (dextran sulfate; BioMol, Plymouth Meeting, PA); all others were from LC Labs, Woburn, MA), the particular reagent was added to sperm in HTF medium at 37°C for 1 hr. A23187 was then added to a final concentration of 10 μM and the incubation was allowed to continue for 30 min. In all experiments, sperm were collected and solubilized in SDS sample buffer and the proteins analyzed by SDS–PAGE.

To isolate the FS, sperm were washed with PBS and centrifuged at 400g for 5 min (Jassim et al., 1992). The sperm then were washed at 4°C in a buffer containing 50 mM Tris–HCl, pH 8.8, 20 mM DTT, 0.2 mM PMSF, 1% Triton X-100 and extracted with 4 M urea, 50 mM Tris–HCl, pH 8.8, 20 mM DTT, and 0.2 mM PMSF at 4°C for 1 hr. Sperm morphology was checked every 10 min by phase contrast microscopy to assess the disappearance of the middle piece (typically, within 40–50 min). Heads of decapitated sperm were removed by centrifugation at 3300g for 20 sec. The resulting supernatant then was centrifuged at 10,000g for 10 min and the pellet was washed with PBS. For biochemical characterization, the washed FS pellets were dissolved in SDS sample buffer containing 40 mM DTT and boiled for 5 min. The amount of protein in each sample was determined by the Amido Black procedure (Schaffner and Weissman, 1973).

All of the experiments were performed at least four times and representative experiments are shown.

Immunocytochemistry of sperm. Human sperm were washed and extracted with Triton X-100 as described above. The sperm then were smeared onto glass slides, dried, and fixed with 100% methanol for 30 sec. The sperm were incubated with anti-pY (5 μg/ml) for 30 min at room temperature, washed, and visualized after incubation with a fluorescein-conjugated anti-mouse IgG.

For the colocalization of AKAP82 and phosphotyrosine-containing proteins, sperm were attached to poly-L-lysine (1 mg/ml)-coated slides, permeabilized in PBS containing 1% Triton X-100 at 4°C for 1 hr, and then fixed with 95% ethanol at room temperature for 30 sec. The samples were incubated with anti-AKAP82 (1:100) at 37°C for 45 min. After washing in PBS, the samples were incubated at 37°C for 45 min with a secondary biotinylated anti-rabbit IgG (1:1000) and anti-pY (1:100). The slides were washed with PBS and then incubated with avidin–fluorescein (1:1000) and a secondary Texas red anti-mouse IgG (1:250) at 37°C for 45 min. Finally, the slides were washed and mounted using Fluoromount-G.

Slides were examined using a Zeiss Photomicroscope III equipped with epifluorescence and photographed with Kodak T-Max P3200 film. For controls, some of the following was substituted for the primary antibody: (1) the preimmune serum for anti-AKAP82 (1:100), (2) anti-pY preadsorbed with 20 mM phosphotyrosine at room temperature for 1 hr, and (3) PBS–goat serum.

Electrophoresis and immunoblotting. For one-dimensional gel electrophoresis, sperm proteins were resolved by SDS–PAGE using 10% polyacrylamide either in the presence or in the absence of reducing agents (Laemmli, 1970). Either equal numbers of sperm or equal amounts of protein were analyzed in each lane. For immunoblot analysis, the proteins were transblotted onto Immobilon P membrane (Millipore Corp., Bedford, MA) (Towbin et al., 1979). Sperm proteins were probed with anti-pY (5 μg/ml) or anti-HK (1:5,000) and then subjected to enhanced chemiluminescence according to the manufacturer’s instructions (ECL; Amersham Corp., Arlington Heights, IL).

For two-dimensional gel electrophoresis, sperm were washed in PBS and the pellet was solubilized in 9.5 M urea, 2% ampholines (3–10), 40 mM DTT, and 8% NP-40 (Ames and Nikaido, 1976). The freshly solubilized proteins were quantitated and separated in the first dimension by the NEPHGE technique (O’Farrell et al., 1977). Gels contained 9.5 urea, 4% acrylamide, 2% NP-40, 2% ampholines (3–10) and were electrophoresed for 1 hr at 400 V and then for 4.5 hr at 800 V for a total of 4000 V-hr. The gels were equilibrated in SDS sample buffer for 20 min and then loaded onto a 10% SDS–polyacrylamide gel. Following electrophoresis, the second dimension gel was either stained with silver or transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Sperm proteins were probed with anti-AKAP82 (1:100) or anti-pY (1:100) and then detected by enhanced chemiluminescence.

Ligand blotting procedure. The regulatory subunit (RlI) of the CAMP-dependent protein kinase from bovine heart was labeled by phosphorylation with [γ-32P]ATP in the presence of the catalytic subunit and CAMP at 4°C for 30 min (Rubin et al., 1979). Proteins from human sperm were separated by two-dimensional electrophoresis, blotted to nitrocellulose membranes, and probed with labeled RlI (Carrera et al., 1994). The membranes were dried and exposed to film. To control for specificity of binding, duplicate filters were preincubated with a 50-fold excess of nonlabeled RlI prior to the addition of labeled RlI.

RESULTS

Human ejaculated sperm display a time-dependent increase in protein tyrosine phosphorylation under conditions conducive to capacitation. Previously, mouse sperm capacitation was shown to be tightly correlated with the tyrosine phosphorylation of specific proteins of M, 40,000–120,000 (Visconti et al., 1995a). It is not possible to assess
capacitation in human sperm by the ability to fertilize an egg due to obvious experimental limitations. Therefore, in order to examine whether a similar correlation exists in human sperm between capacitation and protein tyrosine phosphorylation, the experiments in this paper were performed under conditions described by others to support capacitation (Yanagimachi, 1994). Sperm were purified by Percoll washing and incubated for up to 5 hr in a medium (HTF) conducive to capacitation. Aliquots were collected at 1-hr intervals and the presence of phosphotyrosine-containing proteins was analyzed by immunoblotting with anti-pY (Fig. 1). At time 0, a limited number of proteins of M, 80,000–105,000 contained a small amount of phosphotyrosine. However, after 1 hr, these polypeptides showed a dramatic increase in their phosphotyrosine content; this level then remained relatively constant over time. Significant tyrosine phosphorylation of additional proteins was not detected during this incubation period. To demonstrate that this increase in protein tyrosine phosphorylation was correlated with conditions conducive to sperm capacitation, sperm were also incubated in HTF medium lacking BSA and NaHCO₃, two components that are absolute requirements for capacitation (Yanagimachi, 1994). Under these conditions no increase in tyrosine phosphorylation levels was observed (Fig. 1). Immunoreactivity was abolished when the antibody was initially absorbed with 20 mM phosphotyrosine at room temperature for 1 hr prior to the immunoblot analysis (data not shown).

**Protein kinase inhibitors affect the level of protein tyrosine phosphorylation in human sperm.** To examine the regulation of this protein tyrosine phosphorylation process, live sperm were incubated with the protein tyrosine kinase inhibitor genistein at various concentrations. Proteins then were analyzed by immunoblotting with anti-pY. Genistein inhibited the in vivo tyrosine phosphorylation of proteins of M, 80,000–105,000 (and some additional polypeptides) in a concentration-dependent manner (Fig. 2A). An inhibitory effect was initially observed at 2 μM with an IC₅₀ of less than 20 μM based on densitometry analysis (data not shown). Diadzien, a weakly active genistein analog, showed, as would be predicted, only a slight effect even at concentrations of 200 μM. Another protein tyrosine kinase inhibitor, tyrphostin RG50864 (kindly provided by Dr. Alain Schreiber, Rorer Chemicals), was less effective than genistein and did not display an inhibitory effect on protein tyrosine phosphorylation at concentrations lower than 100 μM (data not shown).

Among other protein kinase inhibitors tested, staurosporine, an inhibitor of various protein kinases including protein kinase C, also effectively inhibited the tyrosine phosphorylation of sperm proteins at a concentration of 100 nM (Fig. 2B). In contrast, the more selective inhibitor of

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**FIG. 1.** Protein tyrosine phosphorylation of human sperm proteins under conditions conducive to capacitation. Sperm were prepared and incubated in HTF medium containing 2 mM Ca²⁺ for up to 5 hr. Aliquots were taken at 1-hr intervals, and the sperm collected and solubilized in SDS sample buffer. Equal numbers of sperm/lane were resolved by SDS–PAGE and analyzed by immunoblotting with anti-pY. A dramatic increase in the level of phosphotyrosine-containing proteins was seen in a subset of proteins of M, 80,000–105,000 after 1 hr. This level of tyrosine phosphorylation was maintained for up to 5 hr. Sperm were also incubated in HTF medium containing Ca²⁺ but lacking BSA and NaHCO₃, two components that are absolute requirements for capacitation (Yanagimachi, 1994). Under these conditions no increase in protein tyrosine phosphorylation was observed during the time course of the experiment. Numbers to the left of the blot represent the positions of the molecular weight standards (×10⁻³).

**FIG. 2.** Effect of protein kinase inhibitors on protein tyrosine phosphorylation in human sperm. Sperm were prepared and incubated in HTF medium with various concentrations of protein kinase inhibitors for 1 hr at 37°C. Sperm then were collected and solubilized in SDS sample buffer. Equal numbers of sperm/lane were separated by SDS–PAGE and analyzed by immunoblotting with anti-pY. (A) Immunoblot analysis using anti-pY after treatment of sperm with staurosporine and chelerythrine chloride. While staurosporine inhibited protein tyrosine phosphorylation, chelerythrine chloride had no effect. Numbers to the left of the blots represent the positions of the molecular weight standards (×10⁻³).
protein kinase C, chelerythrine chloride, did not inhibit tyrosine phosphorylation (Fig. 2B), suggesting that the staurosporine effect is not mediated through PKC.

**Effects of extracellular calcium on protein tyrosine phosphorylation in human sperm.** Since extracellular Ca\(^{2+}\) plays a role in various aspects of sperm physiology, we examined whether Ca\(^{2+}\) was important for the tyrosine phosphorylation of human sperm proteins. The tyrosine phosphorylation of proteins of M, 80,000–105,000 was significantly enhanced when sperm were incubated in HTF media containing no added Ca\(^{2+}\) when compared with sperm incubated in HTF media containing either 0.2 or 2.0 mM Ca\(^{2+}\) (a concentration typically used in capacitation media) (Fig. 3A). In addition, when the Ca\(^{2+}\) ionophore A23187 was added to sperm in complete HTF media to induce a further Ca\(^{2+}\) uptake, an additional decrease in the level of protein tyrosine phosphorylation was observed when compared to the phosphorylation level in HTF media alone (Fig. 3B). The decreases in the levels of tyrosine phosphorylation observed under various conditions did not represent a loss of protein substrates due to the sperm acrosome reaction as the major tyrosine-phosphorylated polypeptides were Triton X-100 insoluble and were found in the principal piece of the flagellum (see below). Quantification of these differences (Fig. 3C) indicated that Ca\(^{2+}\) had a dramatic inhibitory effect on protein tyrosine phosphorylation. It should be noted that the addition of EGTA to the Ca\(^{2+}\)-depleted media to lower the free Ca\(^{2+}\) concentration did not further result in a greater enhancement of protein tyrosine phosphorylation (data not shown).

The calcium-stimulated dephosphorylation of human sperm phosphorytyrosine-containing proteins is inhibited by the calmodulin antagonist calmidazolium and the phosphatase inhibitors deltamethrin and okadaic acid. As the dephosphorylation of phosphorytyrosine-containing proteins in human sperm appeared to be Ca\(^{2+}\)-dependent, we examined whether it was also calmodulin-dependent. Sperm were first preincubated with the calmodulin antagonist calmidazolium at various concentrations for 60 min and then incubated either with or without the Ca\(^{2+}\) ionophore A23187. In the absence of A23187, increasing concentrations of calmidazolium resulted in an increase in protein tyrosine phosphorylation (Fig. 4). In the presence of A23187, 10 \(\mu\)M calmidazolium inhibited the effect of the Ca\(^{2+}\) ionophore on protein tyrosine dephosphorylation (Fig. 4), indicating that protein tyrosine phosphorylation appeared to be both Ca\(^{2+}\) - and calmodulin-dependent.

The calmodulin-dependent tyrosine dephosphorylation of sperm proteins suggests the involvement of the Ca\(^{2+}\) - and calmodulin-dependent protein phosphatase 2B (calcineurin). This enzyme has been localized to the sperm flagellum (Tash
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et al., 1988) where some of the more prominent phosphotyrosine-containing proteins are located (see below). To examine whether calcineurin was involved in protein tyrosine phosphorylation, human sperm were incubated with various phosphatase inhibitors and then treated with the Ca$^{2+}$ ionophore A23187. The calcineurin inhibitor dexamethasone inhibited the Ca$^{2+}$ ionophore-induced dephosphorylation at a concentration of 10 nM (Fig. 5). In contrast, permethrin, a less active inhibitor analog, did not display this property. Okadaic acid, a potent inhibitor of phosphatases, was a weak inhibitor of calcineurin, also inhibited the A23187-stimulated dephosphorylation at a concentration of 100 nM (Fig. 5).

Limited proteolysis is an alternative pathway which could result in the activation of calcineurin (and other calmodulin-dependent enzymes) (Kim et al., 1986). Since both the zona pellucida-induced and the ionophore-induced acrosome reactions lead to the activation and release of the trypsin-like protease acrosin, 4-acetamidophenyl guanidino-benzonitrile (AGB) was used to examine whether released acrosin is responsible for some of the Ca$^{2+}$-stimulated protein tyrosine dephosphorylation observed in sperm. However, AGB, a potent trypsin and acrosin inhibitor, did not affect the Ca$^{2+}$- and A23187-induced dephosphorylation of human sperm proteins (Fig. 5).

**Phosphotyrosine-containing residues are not detected in human sperm hexokinase.** The identity of the human sperm proteins that become tyrosine-phosphorylated in a capacitation-dependent manner is of considerable interest. It has been proposed that the sperm receptor for the zona pellucida glycoprotein ZP3 is a phosphotyrosine-containing protein kinase of M₉, 95,000 under nonreducing conditions in mouse sperm (Leyton and Saling, 1989) and M₉, 95,000 under reducing conditions in human sperm (Burks et al., 1995). In mouse sperm, the major tyrosine-phosphorylated protein of M₉, 95,000 under nonreducing conditions has been shown to be a unique form of hexokinase (Kalab et al., 1994). Since hexokinase has a molecular weight similar to that of the human proteins which become tyrosine-phosphorylated after incubation in medium supporting capacitation (see Fig. 1), we looked for the presence of a phosphotyrosine-containing form of hexokinase in human sperm. After extracting human sperm with 1% Triton X-100, hexokinase was found only in the Triton-soluble fraction, it did not contain phosphotyrosine. The majority of the phosphotyrosine-containing proteins was present in the Triton X-100-insoluble fraction. (B) Human (Hu) and mouse (Mo) sperm were prepared and extracted with 1% Triton X-100. Proteins from the Triton X-100-soluble fraction were separated by SDS–PAGE and analyzed by immunoblotting with anti-HK (HK) and anti-pY (pY). While hexokinase was found only in the Triton-soluble fraction, it did not contain phosphotyrosine. The majority of the phosphotyrosine-containing proteins was present in the Triton X-100-insoluble fraction. (B) Human (Hu) and mouse (Mo) sperm were prepared and extracted with 1% Triton X-100. Proteins from the Triton X-100-soluble fraction were separated by SDS–PAGE under both nonreducing (−DTT) and reducing (+DTT) conditions and analyzed by immunoblotting with anti-HK and anti-pY. While both the mouse and the human hexokinases migrated at their expected molecular weights under reducing (M₉, 116,000) and nonreducing (M₉, 95,000) conditions, only mouse sperm hexokinase contained detectable levels of phosphotyrosine. Since human sperm appear to contain less immunoreactive hexokinase than mouse sperm, twice the number of human sperm/lane compared to mouse sperm/lane were analyzed. Numbers to the left of the blots represent the positions of the molecular weight standards (×10⁻³).

**FIG. 5.** Effect of phosphatase and protease inhibitors on the A23187-induced protein tyrosine dephosphorylation in human sperm. Sperm were incubated in the absence (0) or presence of various phosphatase inhibitors (10 nM dexamethasone (D), 10 nM permethrin (P), 100 nM okadaic acid (Ok)) or the trypsin inhibitor (10 μM) AGB (A) at 37°C for 1 hr. A23187 was added to a final concentration of 10 μM and the incubation allowed to continue for 30 min. Sperm were then collected and solubilized in SDS sample buffer. Equal numbers of sperm/lane were separated by SDS–PAGE and analyzed by immunoblotting with anti-pY. Dexamethasone and okadaic acid inhibited the Ca$^{2+}$ ionophore-induced dephosphorylation of proteins of M₉, 80,000–105,000 but permethrin and AGB showed no effect. Numbers to the left of the blots represent the positions of the molecular weight standards (×10⁻³).

**FIG. 6.** Fractionation of phosphotyrosine-containing proteins and hexokinase in sperm. (A) Human sperm were prepared and extracted with 1% Triton X-100. The Triton-insoluble fraction was solubilized in SDS sample buffer. Equal numbers of sperm/lane from both the Triton-soluble (Ts) and the insoluble fractions (Ti) were separated by SDS–PAGE and analyzed by immunoblotting with anti-HK (HK) and anti-pY (pY). While hexokinase was found only in the Triton-soluble fraction, it did not contain phosphotyrosine. The majority of the phosphotyrosine-containing proteins was present in the Triton X-100-insoluble fraction. (B) Human (Hu) and mouse (Mo) sperm were prepared and extracted with 1% Triton X-100. Proteins from the Triton X-100-soluble fraction were separated by SDS–PAGE under both nonreducing (−DTT) and reducing (+DTT) conditions and analyzed by immunoblotting with anti-HK and anti-pY. While both the mouse and the human hexokinases migrated at their expected molecular weights under reducing (M₉, 116,000) and nonreducing (M₉, 95,000) conditions, only mouse sperm hexokinase contained detectable levels of phosphotyrosine. Since human sperm appear to contain less immunoreactive hexokinase than mouse sperm, twice the number of human sperm/lane compared to mouse sperm/lane were analyzed. Numbers to the left of the blots represent the positions of the molecular weight standards (×10⁻³).
photophosphorylated proteins of human sperm were Triton-insoluble suggests that they were associated with the cytoskeleton fraction.

To further support the conclusion that human sperm hexokinase does not contain detectable phosphorysine residues, proteins from Triton X-100-extracted mouse and human sperm were analyzed under nonreducing and reducing conditions. Previously, mouse sperm hexokinase had been shown to migrate at M, 95,000 under nonreducing conditions and M, 116,000 under reducing conditions, presumably due to the presence of intramolecular disulfide bonds (Kalab et al., 1994). As expected, a protein from mouse sperm of the correct molecular weight under both electrophoretic conditions immunoreacted with both anti-HK and anti-pY (Fig. 6B). In contrast, human sperm hexokinase displayed a similar mobility shift under reducing conditions, but did not appear to contain detectable phosphorysine residues as seen by a lack of immunoreactivity with anti-pY (Fig. 6B).

Tyrosine-phosphorylated proteins are localized to the principal piece and neck of the flagellum. As shown in Fig. 6A, a majority of the phosphorysine-containing proteins of human sperm fractionated into the Triton X-100-insoluble fraction, indicating that they are associated with a cytoskeletal component(s) of the sperm. To localize these proteins further, the Triton X-100-insoluble sperm proteins were fractionated into a nuclear fraction and a FS component, a cytoskeletal structure found in the principal piece of the sperm flagellum where it surrounds the axoneme and ODF. This protocol solubilizes the plasma membrane, axoneme, and other accessory structures of the flagellum including the ODF and mitochondrial sheath while leaving the FS relatively intact (Jassim et al., 1992). After immunoblotting with anti-pY, the majority of the immunoreactive proteins was seen in the FS fraction (Fig. 7C). The pattern of immunoreactivity observed was similar to that seen in the Triton X-100-insoluble fraction, i.e., the major phosphorysine-containing proteins were of M, 80,000–105,000 (Fig. 6A). However, the phosphorysine-containing proteins in the FS fraction were more prominent when compared to the tyrosine-phosphorylated proteins in the starting Triton X-100-insoluble fraction (an equal amount of protein was loaded/lane), suggesting that most of these polypeptides were enriched in the FS. In contrast, very few phosphorysine-phosphorylated proteins were detected in the nuclear fraction.

Indirect immunofluorescence analysis of Triton X-100 permeabilized sperm with anti-pY demonstrated prominent immunoreactivity in the principal piece, the region where the FS is located, and in the neck region of the flagellum (Fig. 7B). Neither the midpiece of the flagellum nor the sperm head displayed detectable staining. Of particular interest was the finding that the staining was heterogeneous within any given sperm population, i.e., not every sperm displayed positive immunoreactivity. We estimated that ~10–15% of the sperm were stained in any given population. However, it should be emphasized that if a particular sperm was stained after incubation with anti-pY, only the principal piece and neck region showed immunoreactivity.

The human sperm phosphorysine-containing proteins of M, 82,000 and 97,000 are homologues of the mouse FS AKAPs. The major FS protein of the mouse sperm flagellum, AKAP82, is synthesized as a M, 97,000 precursor (pro-AKAP82) during spermiogenesis (Carrera et al., 1994). Similarities in the solubility characteristics (Triton X-100 insoluble), cellular location (the principal piece), and molecular weights (M, 80,000–105,000) between these polypeptides and the phosphorysine-containing proteins described in this report suggested that some of these tyrosine-phosphorylated proteins might be the human homologues of mouse pro-AKAP82 and AKAP82. When human sperm were resolved by two-dimensional gel electrophoresis, a complex array of polypeptides were detected (Fig. 8A). Two prominent polypeptides at M, 97,000 and 82,000 were detected when these proteins were analyzed by immunoblotting with anti-AKAP82 (an antibody which recognizes both pro-AKAP82 and AKAP82 in the mouse) (Fig. 8B). Both polypeptides had similar molecular weights and pIs (predicted from the mouse cDNA clone) when compared to their mouse counterparts; hence, they will be referred to as human pro-AKAP82 and human AKAP82. These two polypeptides were relatively abundant and were broadly distributed in the second dimension, suggesting that they may be posttranslationally modified, e.g., phosphorylated.

When immunoblots of human sperm proteins were probed with anti-pY, numerous proteins were detected, including those corresponding to AKAP82 and pro-AKAP82 (Fig. 8C). The immunoreactivity of pro-AKAP82 was particularly intense and broadly distributed. To eliminate the possibility of nonspecific immunoreactivity, anti-pY was first preabsorbed with phosphorysine. Under these conditions, human pro-AKAP82 and AKAP82 immunoreactivity disappeared while many of the other spots remained (Fig. 8D), indicating that, at least for human pro-AKAP82 and AKAP82, the immunoreactivity was specific. Another prominent phosphorysine-containing protein migrated slightly more slowly than human pro-AKAP82 (occasionally, a doublet in this region of a one-dimensional SDS-containing gel was resolved). This protein has not yet been identified. These results indicated that the human homologues of mouse AKAP82 and pro-AKAP82 were present in human sperm and, in addition, were tyrosine-phosphorylated. By their ability to bind the RII subunit of PKA in a ligand blotting assay, mouse AKAP82 and pro-AKAP82 are members of the AKAP family (Carrera et al., 1994), proteins that sequester PKA to specific subcellular locations. To examine whether the human homologues also bind RII, human sperm proteins were analyzed via a ligand blotting assay. Even though a heterogeneous array of proteins were present (Fig. 8A), only a limited number of bands bound RII (Fig. 8E). The most predominant was human pro-AKAP82 while human AKAP82 bound less RII. An excess of unlabelled RII competed with 32P-RII for binding to human pro-AKAP82 and AKAP82 (data not shown), indicating that binding to
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FIG. 7. Localization of phosphotyrosine-containing proteins in human sperm. (A, B) Sperm were prepared, extracted with 1% Triton X-100, smeared onto glass slides, and fixed with 100% methanol. The fixed sperm were incubated with anti-pY and then with a fluorescein-conjugated anti-mouse IgG antibody. While only a small percentage of sperm displayed positive immunoreactivity, this reactivity was restricted to the principal piece and neck region of the flagellum (B). A is the corresponding phase contrast image. (C) Sperm were extracted with 1% Triton X-100 and the detergent-insoluble fraction (Ti) was collected and further fractionated into a fibrous sheath-enriched fraction (F) and a nuclear fraction (N). Proteins from all three samples were prepared, and an equal amount of protein/lane was separated by SDS-PAGE and analyzed by immunoblotting with anti-pY. The vast majority of the immunoreactivity was seen in the FS fraction when compared to the nuclear fraction. Numbers to the left of the blot represent the positions of the molecular weight standards (kDa). RII was specific. These results indicate that both human pro-AKAP82 and AKAP82 bind RII. Finally, preliminary sequencing of a human cDNA clone corresponding to AKAP82 confirmed its homology to the mouse counterpart (data not shown).

AKAP82/pro-AKAP82 and phosphotyrosine-containing proteins colocalize to the principal region of the human sperm flagellum. Mouse AKAP82 is localized specifically to the principal piece of the sperm flagellum (Carrera et al., 1994). As shown previously (Fig. 7), when human sperm were incubated with anti-pY, staining was localized to two distinct regions: (1) the entire length of the principal piece of the flagellum, and (2) a small region near the neck of the sperm. Incubation of human sperm with anti-AKAP82 and anti-pY simultaneously demonstrated a colocalization to the entire length of the principal piece of the flagellum (Figs. 9B and 9C). However, it is not possible to determine whether human pro-AKAP82 and AKAP82 were differentially localized within the principal piece. As expected, anti-AKAP82 stained every sperm while anti-pY stained only a subpopulation (10–15%).

DISCUSSION

The activation of various signal transduction pathways is thought to be critical for a number of sperm functions including capacitation and motility. Although capacitation is an ill-defined process, many of the events associated with this maturational event (e.g., changes in intracellular ion concentrations, plasma membrane fluidity, and motility) have properties consistent with intracellular signaling processes. In this regard, changes in tyrosine phosphorylation of specific mouse sperm proteins have been shown to occur under conditions that support capacitation (Leyton and Saling, 1989), although a correlation or cause-and-effect relationship between these two parameters was not examined. Recently, mouse sperm capacitation has been shown to be tightly correlated with the tyrosine phosphorylation of specific proteins of Mr 40,000–120,000 and the two events (capacitation and phosphorylation) are regulated by a cAMP-dependent pathway (Visconti et al., 1995a,b).

In this report, we demonstrated that ejaculated human sperm also displayed a time-dependent increase in the tyrosine phosphorylation of a distinct subset of proteins of Mr 80,000–105,000 under conditions that support capacitation. Other investigators have reported the tyrosine phosphorylation of human sperm proteins of similar sizes (e.g., see Luconi et al., 1995). Previously, Aitken et al. (1995) demonstrated that human sperm also contain phosphotyrosine-containing proteins under oxidizing conditions which stimulate sperm function. However, with the exception of a Mr 82,000 protein, these polypeptides are considerably larger than those we routinely observed. The reason for this discrepancy is unknown although the incubation conditions for the sperm were different. We do see higher molecular weight proteins when immunoblots are overexposed (see Figs. 3B and 4), but these proteins clearly are not the predominant tyrosine-phosphorylated substrates. Regardless, the correlation between capacitation and/or conditions conducive to capacitation and the tyrosine phosphorylation of sperm proteins appears to be a general phenomenon as it has now been shown in mouse (Leyton and Saling, 1989; Visconti et al., 1995a), human (this report), and bovine (Galantino-Homer, Visconti, and Kopf, unpublished results). Taken together, these results suggest that protein tyrosine phosphorylation may represent an important regulatory pathway essential for the development of the capacitated state.
Increased extracellular Ca\(^{2+}\), as well as a rise in intracellular Ca\(^{2+}\) concentration, resulted in the dephosphorylation of phosphotyrosine-containing human sperm proteins, indicating that Ca\(^{2+}\) regulates protein tyrosine phosphorylation in some manner. While a rise in extracellular Ca\(^{2+}\) has been reported to be associated with human sperm capacitation (DasGupta et al., 1993), it is possible that such changes in intracellular Ca\(^{2+}\) may serve to limit, and thus control, protein tyrosine phosphorylation in order to ensure appropriate timing of the capacitation response. The Ca\(^{2+}\)-induced protein tyrosine dephosphorylation was calmodulin-independent, suggesting that calcineurin was involved. While potent calcineurin inhibitors such as deltamethrin blocked dephosphorylation, so did okadaic acid, a weak inhibitor of this enzyme. These findings suggest that dephosphorylation may not necessarily be mediated via calcineurin. Alternatively, dephosphorylation may be regulated by a unique form of this phosphatase. In this regard, it is interesting to note that a testis-specific isoform of both the catalytic (Muramatsu and Kincaid, 1992) and the regulatory (Nishio...
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FIG. 9. Colocalization of pro-AKAP82/AKAP82 and phosphotyrosine-containing proteins in human sperm. Sperm were attached to slides, extracted with 1% Triton X-100, and fixed with ethanol. Sperm were then incubated with both anti-AKAP82 (B) and anti-pY (C), followed by the appropriate secondary antibodies, and examined. Both antibodies colocalized to the entire length of the principal piece of the sperm flagellum (delimited by arrows). The corresponding phase contrast micrograph is shown (A).

et al., 1992) subunits of calcineurin has been reported, suggesting that testicular calcineurin may have different pharmacological properties (e.g., response to okadaic acid) compared to neuronal calcineurin. Thus, although dephosphorylation of tyrosine-containing proteins is initiated by an increase in Ca\(^{2+}\) and is calmodulin-dependent, the role of calcineurin in these events requires further examination.

The regulation of CaMP-dependent phosphorylation/dephosphorylation events controlling sperm motility presumably occurs at multiple levels. The finding that a Ca\(^{2+}\) and calmodulin-regulated cyclic nucleotide phosphodiesterase is a component of both the rat and the bovine sperm flagella suggests that more than one calmodulin-regulated enzyme may be associated with the sperm flagellum and may play a role in the phosphorylation/dephosphorylation of sperm proteins (Wasco et al., 1989). In addition, PP1, a major serine/threonine protein phosphatase (inhibited by okadaic acid), is present in fowl sperm, presumably at or near the axoneme (Ashizawa et al., 1994). This phosphatase appears to be involved in the inhibition of motility via a Ca\(^{2+}\)-dependent mechanism. While PP2A also acts on serine/threonine residues, it has a low basal level of phosphotyrosyl phosphatase activity. This activity is stimulated by an activator whose mRNA is found at high levels in rabbit testis (Cayla et al., 1994).

The identity and localization of the protein substrates for tyrosine phosphorylation are important for dissecting the various signal transduction pathways in sperm. One candidate for a phosphotyrosine-containing protein in human sperm is hexokinase, which is tyrosine-phosphorylated in mouse sperm (Kalab et al., 1994). However, while hexokinase was present in human sperm, it did not contain detectable amounts of phosphotyrosine when probed with anti-pY (Fig. 6). A second candidate phosphotyrosine-containing protein is a M, 95,000 human sperm protein proposed to be a receptor for ZP3 (Burks et al., 1995), although the identity and function of this protein as a receptor protein tyrosine kinase are controversial (Bork, 1996; Tsai and Silver, 1996). In addition, while more than one tyrosine-phosphorylated protein of M, ~95,000 appeared to be present (see Fig. 8C), our data do not support the conclusion that the protein described by Burks et al. (1995) is the major phosphotyrosine-containing protein in human sperm. While receptor tyrosine kinases are usually membrane-bound and thus soluble in nonionic detergents such as Triton X-100, the vast majority of phosphotyrosine-containing proteins in human sperm including those of M, ~95,000–97,000 was Triton X-100-insoluble, suggesting an association with a cytoskeletal element(s). This association with the cytoskeleton is supported by the indirect immunofluorescence of sperm probed with anti-pY, which showed prominent immunoreactivity in the principal piece of the flagellum, the site of the cytoskeletal FS.

Based on immunoreactivity, solubility characteristics (Triton X-100 insolvability), cellular location (the principal piece of the flagellum), relative molecular weights, and pIs, we have shown that two of the tyrosine-phosphorylated polypeptides in human sperm are the homologues of mouse AKAP82 and its precursor protein, pro-AKAP82. AKAP82 is a major component of the mouse FS, a cytoskeletal element of the sperm flagellum (Carrera et al., 1994), while pro-AKAP82 is present in the FS of testicular sperm (Johnson et al., manuscript submitted for publication). While pro-AKAP82 is synthesized during mouse spermiogenesis, the predominant form in epididymal sperm is AKAP82, indicating that the processing of pro-AKAP82 to the mature AKAP82 is nearly complete. However, since both pro-AKAP82 and AKAP82 were present in human sperm, it appears that the precursor was not processed completely.

In a typical human sperm ejaculate, a significant percentage
of abnormal sperm is present; many of these cells display defects in motility. It will be of interest to examine whether human pro-AKAP82 is present only in those sperm exhibiting such defects.

Previously, a human Mr 97,000 polypeptide of the FS was shown to be phosphorylated, although the nature of the particular phosphoamino acid(s) was not determined (Jassim et al., 1991). Progesterone induces the tyrosine phosphorylation of human sperm proteins; in one study, a protein of Mr 94,000 is tyrosine phosphorylated (Tesarik et al., 1993), while in a second study, polypeptides of Mr 97,000 and 75,000 become phosphorylated (Bonaccorsi et al., 1995). Although similar in molecular weight, it is not known whether these proteins correspond to either human pro-AKAP82 or AKAP82. Finally, while the major Mr 80,000 protein of the rat FS [whose mouse homologue was subsequently identified as AKAP82 (Carrera et al., 1994)] is phosphorylated on serine residues (Brito et al., 1989), we have not detected the presence of phosphotyrosine in mouse sperm AKAP82 with anti-pY (Carrera, Gerton, and Moss, unpublished observations).

In the mouse, both capacitation and protein tyrosine phosphorylation are regulated at the level of PKA (Visconti et al., 1995b). It has been suggested that the changes in cAMP levels that occur in the sperm regulate PKA activity which, in turn, leads to the tyrosine phosphorylation of protein substrates by either the activation of sperm tyrosine kinases and/or the inhibition of phosphoprotein phosphatases. The inhibitory effects of both genistein and staurosporine on protein tyrosine phosphorylation support the idea that a protein tyrosine kinase is present and active in human sperm. Furthermore, the connection between PKA and these tyrosine phosphorylation events is of particular interest considering that among the human sperm protein substrates for tyrosine phosphorylation are human pro-AKAP82 and AKAP82, members of the AKAP family of polypeptides. Since AKAPs bind the RII subunit of PKA, thereby sequestering the protein kinase near its physiological substrates, this suggests that additional interrelationships between PKA and tyrosine kinase signaling pathways are possible. While the major rat Mr 80,000 FS polypeptide contains phosphoserine (Brito et al., 1989) and mouse AKAP82 and pro-AKAP82 are phosphorylated on serine/threonine sites (Johnson et al., manuscript submitted for publication), this is the first report of AKAPs that are tyrosine-phosphorylated.

Since cAMP-dependent phosphorylation via PKA is critical for the initiation and maintenance of sperm motility (Tash and Means, 1983; San Agustin and Witman, 1994), the presence of proteins in the tail that can bind the regulatory subunit of PKA offers an attractive mechanism for regulating protein phosphorylation. In this regard, the RII subunit of PKA is localized to both the fibrous and the mitochondrial sheaths of bovine and rat sperm (Lieberman et al., 1988; MacLeod et al., 1994). A similar localization pattern has been seen in human sperm; in addition, a human-specific (but not testes-specific) isoform of RIIα is detected on the axonemal microtubular wall (Pariset and Weinman, 1994). Recently, the neuronal AKAP79 has been shown to bind calcineurin (Coglian et al., 1995) and PKC (Klauck et al., 1996) (in addition to the RII subunit of PKA), indicating that multifunctional kinases and phosphatases can be targeted to subcellular locations via their anchoring to the same protein. As calcineurin is present in the flagellum of sperm and most likely involved in sperm motility (Tash et al., 1988), it will be important in the future to examine whether these AKAPs can bind this phosphatase. It is possible that the action of calcineurin is upstream of these protein tyrosine phosphorylation events. Alternatively, although calcineurin typically is a serine/threonine protein phosphatase, it also can dephosphorylate phosphotyrosine-containing proteins (Kincade et al., 1986).

Sperm motility must be activated and then maintained. While the maintenance of motility relies on a cascade of phosphorylation/dephosphorylation events that alter the activity of target proteins, the initiation of motility presumably does not need to be reversible. Although the mechanism that initiates motility is not known, a Mr 15,000 tyrosine-phosphorylated polypeptide that is found in the flagellum of trout sperm has been shown to be important (Hayashi et al., 1987). In addition, a tyrosine kinase inhibitor inhibits the motility activation of Ciona sperm (Dey and Brokaw, 1991). Moreover, Berruti and Martegani (1989) have suggested that the three phosphotyrosine-containing proteins that they observe in boar sperm may be associated with the axoneme although their precise location and function have not been assessed. While the molecular weights and solubility characteristics of these boar sperm proteins are different from those of the human pro-AKAP82 and AKAP82, such differences may be species-specific or reflect differences in the incubation conditions under which the sperm were prepared. Regarding the former possibility, while mouse (Visconti et al., 1995a), bovine (Gallino-Homer, Visconti, and Kopf, unpublished results), and human (this report) sperm display an increase in protein tyrosine phosphorylation under conditions supporting capacitation, different subsets of phosphotyrosine-containing proteins are observed.

The tyrosine phosphorylation of human pro-AKAP82 and AKAP82 increases dramatically when sperm are incubated in a medium conducive to capacitation (an event that follows the activation of motility but is associated with sperm hyperactivation) and remains relatively constant. Thus, the phosphorylation of these proteins may be important for some nonreversible event regarding motility, e.g., initiation of hyperactivation. While less than 20% of human sperm showed immunoreactivity in the principal piece region of the flagellum when incubated with anti-pY, it is not known whether there are motility differences between these two populations. In the future, we will need to examine whether the inhibition of protein tyrosine phosphorylation of human pro-AKAP82 and AKAP82 affects various parameters of sperm motility, in particular, hyperactivation.

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