Phosphoproteome Analysis of Capacitated Human Sperm

Evidence of Tyrosine Phosphorylation of a Kinase-Anchor Protein 3 and Valosin-Containing Protein/p97 During Capacitation*

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Before fertilization can occur, mammalian sperm must undergo capacitation, a process that requires a cyclic AMP-dependent increase in tyrosine phosphorylation. To identify proteins phosphorylated during capacitation, two-dimensional gel analysis coupled to anti-phosphotyrosine immunoblotting and tandem mass spectrometry (MS/MS) was performed. Among the protein targets, valosin-containing protein (VCP), a homologue of the SNARE-interacting protein NSF, and two members of the A kinase-anchoring protein (AKAP) family were found to be tyrosine phosphorylated during capacitation. In addition, immobilized metal affinity chromatography was used to investigate phosphorylation sites in whole protein digests from capacitated human sperm. To increase this chromatographic selectivity for phosphopeptides, acidic residues in peptide digests were converted to their respective methyl esters before affinity chromatography. More than 60 phosphorylated sequences were then mapped by MS/MS, including precise sites of tyrosine and serine phosphorylation of the sperm tail proteins AKAP-3 and AKAP-4. Moreover, differential isotopic labeling was developed to quantify phosphorylation changes occurring during capacitation. The phosphopeptide enrichment and quantification methodology coupled to MS/MS, described here for the first time, can be employed to map and compare phosphorylation sites involved in multiple cellular processes. Although we were unable to determine the exact site of phosphorylation of VCP, we did confirm, using a cross-immunoprecipitation approach, that this protein is tyrosine phosphorylated during capacitation. Immunolocalization of VCP showed fluorescent staining in the neck of noncapacitated sperm. However, after capacitation, staining in the neck decreased, and most of the sperm showed fluorescent staining in the anterior head.

After ejaculation, sperm are able to move actively but lack fertilizing competence. They acquire the ability to fertilize in the female genital tract in a time-dependent process called capacitation (1). Capacitation is accompanied by a cAMP-protein kinase-dependent increase in tyrosine phosphorylation of a subset of proteins (2, 3). Because a protein kinase cascade is involved in the regulation of the sperm fertilizing ability, it is important to characterize the proteins that undergo phosphorylation and examine how these changes relate to capacitation.

Post-translational protein phosphorylation by protein kinases plays a role in many cellular processes including transduction of extracellular signals, intracellular transport, and cell cycle progression. The use of two-dimensional gel electrophoresis followed by tandem mass spectrometry (MS/MS) provides a comprehensive approach to the analysis of proteins involved in cell signaling (4). Specifically, changes in tyrosine phosphorylation can be monitored using two-dimensional gel electrophoresis followed by Western blot analysis with anti-phosphotyrosine (α-PY) antibodies (5). Proteins that undergo changes in tyrosine phosphorylation during cellular processes can then be isolated from a complementary gel and sequenced by MS/MS. In the present study, we have used this approach to identify several sperm proteins that undergo tyrosine phosphorylation during capacitation.

Identification of the site at which a particular protein is phosphorylated is important in identifying the physiologically relevant protein kinase involved in a particular pathway. In addition, to understand the function of a phosphoprotein, phosphorylation sites are excellent candidates for site-directed mutagenesis. Determination of individual phosphorylation sites in vivo often requires the purification to homogeneity and/or mutational analysis of the phosphoprotein. Recently, several methods for the selective detection and enrichment of phosphopeptides have been developed (6–8); however, most of these have been applied only on a protein-by-protein basis. In the present work, we have used Fe⁺³-imobilized metal affinity chromatography (IMAC) prior to MS/MS to enrich digests for peptides containing phosphoamino acids. To increase the selectivity of the IMAC column for phosphopeptides, we have used a recently developed modification of this technique in which acidic resi-

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*¶** The abbreviations used are: MS/MS, tandem mass spectrometry; AKAP, A kinase-anchoring protein; AP, alkaline phosphatase; α-PY, anti-phosphotyrosine; ESI, electrospray ionization; FITC, fluorescein isothiocyanate; FT-ICR, Fourier transform-ion cyclotron resonance; HPLC, high performance liquid chromatography; IMAC, immobilized metal affinity chromatography; LC, liquid chromatography; MS, mass spectrometry; NSF, N-ethylmaleimide-sensitive factor; PAS, protein A-Sepharose; PBS, phosphate-buffered saline; RP, reversed phase; SNARE, soluble NSF attachment protein receptors; VCP, valosin-containing protein.
dues are converted to methyl esters to block their binding to iron before IMAC is employed (9). Using this methodology, 5 sites of Tyr, 56 of Ser, and 2 of Thr phosphorylation have been characterized.

Although the combination of IMAC and MS/MS is ideally suited for the characterization of phosphorylation sites on proteins in complex mixtures, it is also important to determine which sites are phosphorylated in response to a particular stimulus. In the present paper, we have used differential isotopic labeling to quantify phosphorylation of defined sequences and to determine which sites were increased or decreased during phosphorylation during human sperm capacitation. The evaluation of differential phosphorylation, added to the knowledge of the exact phosphorylated sequence, goes beyond the sperm capacitation field and could be used to understand signaling mechanisms in a multiplicity of cell systems.

Identification of the tyrosine phosphorylation sites of AKAP-3 and AKAP-4 confirmed the previous findings using a two-dimensional gel approach, suggesting that these proteins are tyrosine phosphorylated in a capacitated sperm population. Nevertheless, because of the relative abundance of tyrosine-phosphorylated phosphopeptides compared with phosphoserine phosphopeptides, most of the tyrosine-phosphorylated sites remained unknown. Among them, sites in the NSF homolog VCP remained unidentified. Because VCP is associated with a role in membrane fusion in other cell types and because several aspects of sperm physiology required membrane fusion events, VCP was chosen for further investigation. Anti-VCP antibodies confirmed that this protein is tyrosine phosphorylated during capacitation. In addition, immunolocalization of VCP was determined and showed different localization patterns before and after human sperm capacitation.

EXPERIMENTAL PROCEDURES
Preparation of Spermatozoa—The basic medium used for all experiments was modified human tubal fluid (Irvine Scientific, Santa Ana, CA). Semen samples were obtained by masturbation (approved by the UVA Human Investigation Committee). Individual semen samples were allowed to liquefy at room temperature (0.5–3 h), and mature sperm were purified by Percoll (Amersham Biosciences) density gradient centrifugation as described previously (5). Sperm present $>90\%$ motility were treated immediately to obtain a noncapacitated population after arcsin transformation (12).

Preparation of Samples for MS/MS and for Immunoprecipitation—Sperm were directly treated or capacitated for 18 h as described above; $1 \times 10^8$ sperm were centrifuged, washed in PBS, and then boiled in lysis buffer containing 150 mM NaCl, 0.1% SDS, and 25 mM Tris-HCl, pH 7.5, for 5 min. This treatment was efficient in solubilizing tyrosine-phosphorylated proteins. Proteins solubilized by this method were subsequently treated with trypsin or V8 protease (Glu-C) and tryptic peptides analyzed by MS/MS.

This solubilization procedure was also used for immunoprecipitation with $\alpha$-PY (4G10). Briefly, 10 $\mu$g of $\alpha$-PY antibody was added to the suspension and incubated at room temperature for 1 h. Then 100 $\mu$L of a 30% suspension of protein A-Sepharose (PAS) was added and incubated further for 1 h. The PAS was then washed five times by gentle centrifugation with lysis buffer. Finally the washed PAS beads were resuspended in 1 mM phenyl phosphate and incubated further for 1 h. The suspension was then centrifuged and the remaining supernatant divided; 90% of the sample was then used for MS/MS, and the remaining supernatant was analyzed by Western blot using $\alpha$-PY antibodies.

Isoelectrofocusing was performed in 15 $\times$ 0.15-cm acrylamide rods, using either the gel composition proposed by Hochstrasser et al. (11) or the gel composition proposed by Celis et al. (10). The carrier Ampholine (Amersham Biosciences) composition was 20% pH 7, 20% pH 7.5, and 8 M urea, and the remaining phosphate-buffered saline (PBS) at room temperature. The sperm pellet was then resuspended in the appropriate buffer depending on the experiment. For two-dimensional gel analysis, sperm were resuspended in Cells buffer (10). For immunoprecipitation and/or for direct MS/MS, cells were resuspended in 0.1% SDS, 25 mM Tris-HCl, pH 7.5, and boiled for 5 min, centrifuged at 20,000 $\times$ g for 5 min, and the supernatant recovered.

Two-dimensional Gel Electrophoresis and Western Blot—Sperm were routinely solubilized in a lysis buffer consisting of 2% (v/v) Nonidet P-40, 9.8 M urea, 1% dithiothreitol, 2% (v/v) Ampholines, pH 3.5–10, and as protease inhibitors 2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 5 mM EDTA, 3 mg/ml 1-chloro-3-tosylamido-7-amido-2-heptanone, 1.46 $\mu$L peptatin A, and 2.1 $\mu$L leupeptin. 5 $\times$ 10$^6$ cells/ml were solubilized by constant shaking at 4°C for 60 min. Insoluble material was removed by centrifugation.

Isoelectrofocusing was performed in 15 $\times$ 0.15-cm acrylamide rods, using either the gel composition proposed by Hochstrasser et al. (11) or that proposed by Celis et al. (10). The carrier Ampholine (Amersham Biosciences) composition was 20% pH 7, 20% pH 7.5, and 60% pH 3.5–10. 65 $\mu$L of sperm extract ($\sim 0.15$ mg of protein) was applied per rod. The tubes were filled gently overlaying the sample with a buffer consisting of 1% Ampholine, pH 3.5–10, 8 M urea, and 100 mM dithiothreitol. Focusing was conducted using voltage stepping: 2 h at 200 V, 5 h at 500 V, 4 h at 800 V, 6 h at 1,200 V, and 3 h at 2,000 V. Two-dimensional SDS-PAGE was carried out in 0.15-cm thick, 16 $\times$ 16-cm linear gradient gels (7.5–15%) in a Protean II xi Multi-Cell apparatus (Bio-Rad). Silver staining was performed according to Hochstrasser et al. (11). Electrophoresis and Western Blots were carried out as described previously (2).

Image Analysis of Two-dimensional Gels—Gel electrophoresis was performed concurrently to ensure equivalent electrophoretic conditions. Gels were stained with silver (5) or electrophoblot to polyvinylidene difluoride membrane and probed with $\alpha$-PY (clone 4G10, Upstate Biotechnology). The silver-stained gel and x-ray films (short and long exposure of ECL) were scanned at 300 dpi using a desktop Hewlett-Packard scanner. Digitized images were overlaid in Adobe Photoshop 6.0 using different percent of transparency. Using known “landmarks” such as fibrous sheath proteins and tubulins, the silver image was aligned with the ECL images. After marking all reactive spots on the ECL image with arrows, the ECL image was hidden and the arrows superimposed on the corresponding silver-stained spots. These were corefined from the silver-stained gel and submitted for mass spectrometry analysis.

Immunofluorescence Microscopy—Noncapacitated and capacitated sperm were air dried onto slides, washed three times with PBS, permeabilized with methanol, washed with PBS, and then blocked with 10% normal goat serum in PBS. Incubations were then carried out with $\alpha$-PY or $\alpha$-VCP/PG antibodies (1:250) diluted in PBS with 1% normal goat serum, washed, and incubated with FITC-conjugated F(ab')2 fragments of donkey $\alpha$-mouse IgG (1:200) or donkey $\alpha$-rabbit IgG (1:200) (Jackson ImmunoResearch) in normal goat serum in PBS. Slides were washed with PBS and mounted with Slow-Fade Light (Molecular Probes, Eugene, OR). Sperm were observed by differential interference contrast and epifluorescence microscopy using a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY). Results depicted in Fig. 1 represent the mean ± S.E. of three independent experiments with triplicate determination. Statistical differences between the groups were determined using Student's t test, comparing the capacitated with the noncapacitated population after arcsine transformation (12).

Peptide Synthesis—Peptides were synthesized as described (15). Resins, Fmoc-protected amino acids, and phosphoamino acids were purchased from Calbiochem. Peptides were purified by RP-HPLC, and sequences were verified by MS/MS experiments.
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0.1% acetic acid. Peptide mixtures, pH ∼ 3.5, were loaded onto the IMAC column at a rate of 1 µl/min. Nonbinding peptides were removed by rinsing with 25 µl of acetonitrile/water/acetic acid (25/75/1 v/v/v) containing 100 mM NaCl (Aldrich). The IMAC column was re-equilibrated with 10–20 µl of 0.1% acetic acid and then was connected to a second fused silica column (360 × 75-µm inner diameter or 360 × 100-µm inner diameter, fused silica). Phosphopeptides were eluted with 5 µl (360 × 75-µm inner diameter IMAC column) or 8 µl (360 × 100-µm inner diameter IMAC column) of 50 mM Na2HPO4, pH 9.0. The reversed phase column was then disconnected and rinsed with 0.1% acetic acid to remove salts before subsequent MS analysis.

General LC/MS Parameters—All HPLC experiments employed a gradient of 0–60% B in 40 min (unless noted otherwise) composed of solvent A (0.1 M acetic acid) and solvent B (70% acetonitrile with 0.1 M –casein (50 pmol/µl) dissolved in 100 mM NH4HCO3, pH 8.5 (500 µl of 0.1% acetic acid). All microcapillary column connections were made with 1 cm of 0.152 × 0.03 cm inner diameter Teflon tubing (Zeus, Orangeburg, SC). In experiments performed with IMAC enrichment, peptides were loaded onto an IMAC column and selectively eluted to a C18 microcapillary column (see above). In experiments performed without IMAC enrichment, peptides were loaded directly onto a C18 microcapillary column.

LC/MS Parameters on the LCQ Fourier Transform-Ion Cyclotron Resonance (FT-ICR) Mass Spectrometer—Peptide mixtures were analyzed as described (16, 17). A C18 microcapillary column containing peptides of interest was connected to an analytical column with an integrated ESI emitter tip (1–5-µm diameter). Peptides were gradient eluted into an LCQ quadrupole ion trap mass spectrometer (spray voltage = 1.6 kV). All MS/MS scans (both targeted and data-dependent) were performed with an isolation window of 3 Da (precursor m/z ± 1.5 Da). For data-dependent analyses, the dynamic exclusion option was selected with a repeat count of 1, a repeat duration of 0.5 min, and exclusion duration of 1 min.

LC/MS Parameters on the Fourier Transform-Ion Cyclotron Resonance (FT-ICR) Mass Spectrometer—Peptide mixtures were also analyzed on a home-built FT-ICR mass spectrometer (17). C18 microcapillary columns containing the peptide of interest were connected to analytical columns with integrated ESI emitter tips. Peptides were eluted into the mass spectrometer with the above gradient. Full scan mass spectra (500–20,000 mass units) were acquired at a rate of ~1 scan/s. Mass resolving power ranged from 5,000 to 10,000.

Phosphoproteome Analysis of Capacitated Sperm Total Protein Digests—Aliquots containing 700 µg and 2 mg of capacitated sperm total protein were digested with trypsin and Glu-C (1:20 enzyme:substrate ratio) for 18 h at 37 °C. The protein digest of capacitated sperm was treated with d7-methanolic DCl, whereas the protein digest of noncapacitated sperm was treated with d7-methanolic HCl. Peptide methyl esters were dissolved in 1:1:1 acetonitrile/methanol/0.1% acetic acid, and 100 µg of each digest was loaded onto a 360 × 200-µm inner diameter IMAC column. Phosphopeptides were eluted with 360 × 100-µm inner diameter C18 columns and gradient eluted into the mass spectrometer. In another experiment, equal portions of each digest were combined, and 40 µg of total peptide was loaded onto the IMAC column and gradient eluted into a FT-ICR instrument.

Data Base Searching of Phosphopeptide MS/MS Spectra and Sequence Assignments—MS/MS spectra were matched to sequences in various protein data bases using SEQUEST (18). Spectra from sperm protein analyses were searched against the nonredundant protein data base (NRFP) from NCBI and with a subdata base of proteins from NRPF which contained the phrase testis-specific. In these searches, differential modification of 80 Da to Ser, Thr, and Tyr residues was not selected for phosphorylation. Phosphopeptide spectra recorded during analysis of peptide methyl esters, a differential modification of 14 Da to Glu and Asp acid and a static modification of 14 Da to the C terminus were also selected. Rapid identification of phosphopeptide candidates from the 1,000 MS/MS spectra acquired during a typical HPLC gradient was accomplished with an in-house computer program (neutral loss tool) (16). This program screens MS/MS spectra for losses characteristic of phosphorylated peptides (98, 49, and 32.6 Da from single, double, and triple charged precursor phosphopeptides, respectively). Neutral loss of phosphoric acid from the peptide precursor mass is a common feature of ion trap MS/MS spectra (19). For all phosphotyrosine-containing peptides and one peptide containing two phosphoserine residues, synthetic phosphopeptide standards were experimentally obtained for comparison of MS/MS spectra, confirming our assignments. For all sequences reported, spectra were manually validated and contained sufficient information to assign not only the sequence but also the site of phosphorylation (unless otherwise noted).

RESULTS

Characterization of Proteins That Undergo Tyrosine Phosphorylation during Capacitation—To identify the proteins that serve as substrates for tyrosine phosphorylation during capacitation, human sperm proteins were extracted before and after overnight capacitation and separated by two-dimensional gel electrophoresis. As described previously (20), after transfer of the two-dimensional gels to Immobilon P, a capacitation-associated increase in protein-tyrosine phosphorylation was observed by Western blot using α-PY (Fig. 1A). Immunofluorescence experiments also showed an increase in α-PY fluorescent staining, indicating an increase in tyrosine phosphorylation (Fig. 1B). Before capacitation, 18% of the sperm displayed a low intensity α-PY signal in the tail only. After overnight capacitation, there was a significant increase in the number of sperm that show fluorescent staining of the head. Although we did not use an imaging program to quantify fluorescent intensity, it was possible to observe qualitatively an increase in the fluorescence of the tail in capacitated sperm compared with a noncapacitated sperm population as observed in Fig. 1B.

To identify tyrosine-phosphorylated proteins, extracts from capacitated human sperm were separated by two-dimensional gel electrophoresis. In each experiment, two gels were run in parallel. One gel was stained with Coomassie Blue and subsequently with silver, and the other was transferred and probed with α-PY (Fig. 1C). Both the silver-stained gel and the Western blot were scanned and compared. Protein spots showing α-PY staining were excised, digested, and sequenced. The results of this analysis are summarized in Table I and their exact
Phosphoproteome Analysis of Capacitated Human Sperm Cells—Identification of phosphorylation substrates using two-dimensional gel electrophoresis and immunoblotting is a powerful approach. However, this methodology has several limitations. First, it is difficult to identify proteins that undergo phosphorylation on serine or threonine residues because antibodies against those phosphoamino acids are not sensitive enough to detect most proteins phosphorylated on these residues. Second, MS/MS of proteins obtained from polyacrylamide gels has detection limits several orders of magnitude higher than MS performed on proteins not embedded in gels. Third, the use of this approach strongly suggests that a given protein is phosphorylated on tyrosine residues; nevertheless, a full demonstration requires the use of an independent method (e.g. cross-immunoprecipitation, direct sequencing, mutagenesis analysis). Fourth, although in some cases it is possible to obtain the exact site of phosphorylation of a candidate protein, in general the phosphorylation site remains elusive because of the aforementioned lack of sensitivity. In addition, to determine the site of phosphorylation is, in most cases, a very important goal in a phosphorylation study and the strongest demonstration that a protein is phosphorylated on a particular amino acid.

As shown above, several proteins undergo tyrosine phosphorylation during capacitation. However, no sites of phosphorylation were defined. To understand further the role of phosphorylation in human sperm capacitation, we have analyzed the phosphoproteome of capacitated human sperm. Toward this goal, we have improved the enrichment of phosphopeptides by IMAC converting acidic residues to methyl esters and adapting the use of this approach strongly suggests that a given protein is phosphorylated on tyrosine residues; nevertheless, a full demonstration requires the use of an independent method (e.g. cross-immunoprecipitation, direct sequencing, mutagenesis analysis). Fourth, although in some cases it is possible to obtain the exact site of phosphorylation of a candidate protein, in general the phosphorylation site remains elusive because of the aforementioned lack of sensitivity. In addition, to determine the site of phosphorylation is, in most cases, a very important goal in a phosphorylation study and the strongest demonstration that a protein is phosphorylated on a particular amino acid.

As shown above, several proteins undergo tyrosine phosphorylation during capacitation. However, no sites of phosphorylation were defined. To understand further the role of phosphorylation in human sperm capacitation, we have analyzed the phosphoproteome of capacitated human sperm. Toward this goal, we have improved the enrichment of phosphopeptides by IMAC converting acidic residues to methyl esters and adapting this technology to the analysis of phosphorylation sites directly from capacitated human sperm total protein extracts.

Phosphorylated peptides were identified by screening MS/MS spectra for an abundant neutral loss of phosphoric acid from the peptide precursor mass. This process is commonly observed in ion trap MS/MS spectra of phosphorylated peptides (19). When peptides (1 mg) were analyzed by this method, more than 200 distinct phosphorylated species were detected. Manual and SEQUEST (18) interpretation of MS/MS spectra led to the identification of 18 sites of Ser phosphorylation and a single site of tyrosine phosphorylation on a total of 7 different proteins (Table II, trypsin). Fragment ions in the spectra allowed unambiguous assignment of the phosphorylation sites. MS/MS spectra recorded on synthetic peptides confirmed the sequence assignment of the phosphotyrosine-containing peptide (data not shown). Although 200 phosphopeptides were detected, a majority of the spectra (>75%) showed multiple (up to four) losses of phosphoric acid from the precursor mass and were difficult to interpret.

Because trypsin digestion can generate phosphopeptides that are too large or too small to be compatible with RP (C18) chromatography, another protease with a different specificity was used. V8 protease (Glu-C) cleaves substrate proteins on the C-terminal side of Asp and Glu. Using this protease combined with IMAC and MS/MS, 40 phosphopeptides were detected. Data base searching and de novo sequencing efforts elucidated new sites of phosphorylation including 11 on Ser, 1 on Thr, and 2 on Tyr (Table II, Glu-C). Fragment ions in spectra allowed phosphorylated residues to be assigned unambiguously. Alternative enzymes (i.e. chymotrypsin) may be necessary to define the entire capacitated human sperm phosphoproteome. No nonphosphorylated sperm peptides were detected in either analysis. This illustrates that conversion of the sample to peptide methyl esters prior to IMAC increases significantly the selectivity of this technique toward phosphorylated peptides.

**IMAC Analysis of Immunoprecipitated Protein Digests—**
IMAC/MS analyses of total protein extracts facilitated the discovery of three sites of tyrosine phosphorylation. Western blotting (Fig. 1), however, suggests that many more proteins are tyrosine phosphorylated. In an effort to identify sites of tyrosine phosphorylation selectively, total capacitated human sperm protein extracts were immunoprecipitated with α-PY antibodies (clone 4G10). Elution was performed using 1 mM phenyl phosphate to ensure selective elution from the α-PY antibody after precipitation with PAS. Immunoprecipitated proteins were digested separately with trypsin or Glu-C and digested peptides were separated by reversed-phase HPLC and subjected to mass spectrometry (MS/MS). To assign a particular protein to the respective cut band, at least five peptides from a single spot matched the database sequence for the assigned protein.

### Table I

| Protein assigned from blast search of sequenced peptides NCBI nr.12.5.2001 | Accession no. |
|---------------------------------------------------------------|-------------|
| Voltage-dependent anion select channel 2                      | 1172554     |
| Keratins                                                       | 547499, 88054|
| Phospholipid hydroperoxide glutathione peroxidase              | 13124748    |
| Ubiquitin cytochrome c, Reductase 1                           | 4507841     |
| Glutamate ammonia ligase                                       | 2144562     |
| Pyruvate dehydrogenase β                                       | 4505687     |
| F-actin capping protein β                                      | 4826659     |
| A kinase anchor protein 3                                      | 5454076     |
| A kinase anchor protein 4                                      | 4502017     |
| TRAP-1 (tumor necrosis factor type 1 receptor-associated protein) | 6175069     |
| N-Acetyl-aminocarboxyamide amidohydrolase                      | 9951917     |
| VCL-containing protein (VCP or pr97)                           | 6005942     |
| HSP 70                                                         | 5729877     |
| HSP 90α                                                        | 123678      |
| Proacrosin-binding protein (sp 32)                             | 14210496    |
| Glutathion s-transferase M3                                    | 4504177     |
| Outer dense fiber 1 (ODF 1)                                    | 16162058    |
| α-Tubulin                                                      | 2843123     |
Post-IMAC AP Treatment of Phosphopeptides—Many MS/MS spectra recorded during IMAC analysis of sperm peptides had peaks at m/z values corresponding to multiple losses of phosphoric acid from the precursor mass. Such spectra contain few ions indicative of amino acid sequence because amide bond cleavage cannot effectively compete with gas phase deposphorylation during MS/MS. To assess whether phosphate removal prior to MS analysis would facilitate peptide identification, we converted sperm tryptic peptides to methyl esters. After IMAC enrichment, phosphopeptides were eluted on-line to an AP column before capture on C18 particles and MS/MS. For comparison, a similar analysis was performed without AP treatment. These parallel analyses each detected several peptides (Tables III and IV). The MS/MS spectrum of SVESVK, recorded during analysis of AP-treated peptides, contains peaks at m/z values corresponding to y4, b4, and b5 ions, whereas the phosphorylated analog, pSVESVK, does not (Fig. 2, A and B, respectively). This illustrates that phosphate removal prior to MS analysis aids in peptide sequence determination. Although sequences of dephosphorylated peptides themselves cannot reveal where or to what extent a peptide is phosphorylated, complementary information provided by phosphopeptide MS/MS spectra obtained through parallel analysis without AP treatment may provide enough information to assign the phosphorylation sites.

The peptides PLASSPPPR and VSGSSQSPSNLK were also detected after AP treatment. These peptides are derived from PLAapSpSPPR and VSGpsSpSPPSNLK observed in analyses of trypsin-digested sperm proteins (see Table II). Not all phosphorylated peptides detected without AP treatment were detected after AP treatment. This is probably the result of incomplete elution of phosphopeptides from the IMAC column in the AP experiment because phosphate was not included in the IMAC elution buffer to avoid inhibition of the AP enzymatic activity. In contrast, the use of the AP column allows analysis of peptides not amenable to positive ion MS in their phosphorylated form. For example, the peptide SPSSPAPKPPSTQR, detected during MS analysis of dephosphorylated sperm peptides (Table IV), was not found in samples without AP treatment. Retention of this peptide by IMAC indicates that this stretch of amino acids was phosphorylated within the parent protein (AKAP-4). The phosphorylated form of this peptide may not ionize well in the positive ion mode, preventing the identification of this peptide during analysis performed without the use of AP. These results suggest that dephosphorylation of phosphopeptides after IMAC enrichment prior to MS analysis is a useful tool to derive sequences of multiple phosphorylated peptides.

Quantitative Phosphorylation Analysis: Protein Standards—We have identified 56 sites of phosphorylation in several capacitated human sperm proteins. Although this information describes phosphorylation in sperm on a global scale, it would be useful to discern sites of phosphorylation induced during capacitation. This goal could be achieved by comparison of the ratio of any particular phosphopeptide present in digests of proteins from capacitated and noncapacitated sperm cells. To adapt IMAC/MS methodology to display phosphopeptides differentially from different sperm capacitated states, we utilized an isotopic labeling strategy (Fig. 3A). Tryptic peptides from two samples of cells are converted to peptide methyl esters with deuterated (d6) and nondeuterated (d4) methanol, respectively. Both samples are then mixed in equal proportions and the mixture purified by IMAC to ensure that only phosphopeptides were retained. Signals for phosphopeptides present in both samples appear as doublets separated by n(3 Da)/z (where n is the number of carboxylic acid groups in the peptide and z is the charge on the peptide). The ratio of the two signals in the doublet changes as a function of expression level of the particular phosphoprotein in each sample. Peptides of interest are then targeted for sequence analysis subsequently performed on the ion trap instrument.

To validate this approach, we used β-casein because tryptic

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**Table III**

| Sequence | Parent protein |
|----------|----------------|
| (QPK) ENEqSpr | CABYR |
| (CLK) IDEDpYK | Infertility-related protein |
| (EMR) GTGEpSR | AKAP 4 |
| (AIK) IgpSEK | AKAP 4 |
| (FKR) DgpSEPpK | AKAP 3 |
| (ASK) ApSpSmNR | AKAP 4 |
| (SKK) pSVEpsVK | CABYR |

* Three residues N-terminal to the peptide in the parent protein are shown in parentheses.
* Also identified in first analysis of tryptic sperm peptides.

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**Table IV**

| Sequence | Parent protein |
|----------|----------------|
| (CER) PLASSPPPR | AKAP 3 |
| (GDR) VSGSSQSPSNLK | AKAP 3 |
| (SEK) SVESVK | Calcium Binding Protein-86 |
| (NNQ) SPSSPAPKPPSTQR | AKAP 4 |

* Three residues N-terminal to the peptide in the parent protein are shown in parentheses.
digestion of this protein produces a phosphorylated peptide, FQpSEEQQQTEDELQDK, which is detectable by positive ion electrospray. Briefly, 50 and 250 pmol of tryptic peptides from the phosphorylated protein β-casein was converted to d0-tryptic peptide methyl esters, and 500 pmol of tryptic peptides from the same protein was converted to d3-methyl esters. Equal portions of deuterated and nondeuterated tryptic peptide methyl esters were combined to produce known concentrations of the phosphorylated peptide FQpSEEQQQTEDELQDK, which is detectable by positive ion electrospray. Using this method, the peptide pSVEpSVK (derived from capacitated and noncapacitated digests) were then mixed in equal proportions, and an aliquot was analyzed by IMAC/RP-HPLC/ESI/MS on a home-built FT-ICR instrument. The data were examined manually to identify singlet peaks, i.e., phosphopeptide species unique to the capacitated or noncapacitated sample. Parallel IMAC/RP-HPLC/ESI/MS experiments on a quadrupole ion trap mass spectrometer were used to define the sequences of these peptides.

Using this method, the peptide pSVEpSVK from a novel Ca2+-binding protein, CABYR, was present in the capacitated and noncapacitated sperm total protein digests at about the same level (Fig. 3C). In contrast, the peptide INApSTDpSLAK, derived from AKAP-4, was found at a level 23 times greater in capacitated sperm (Fig. 4C), indicating a capacitation-dependent phosphorylation of this peptide. The charge state of these peptides can be determined by the difference in mass between the C12 and C13 isotope peaks observed by MS analysis. Because the actual mass difference between these isotope peaks is 1 Da, the observed mass difference of 0.5 Da between peaks in the isotopic envelopes of pSVEpSVK and INApSTDpSLAK (data not shown) indicates that both peptides were double charged. Using Equation 1, it was deduced that both phosphopeptides (pSVEpSVK and INApSTDpSLAK) possessed two free carboxyl groups because double charged isotope envelopes corresponding to deuterated and nondeuterated analogs were separated by 3 Da (Fig. 4D and data not shown). Total ion chromatograms were acquired by plotting the sum of the intensities of ions within a small mass window i.e. 418.67 ± 0.1 Da, versus time (data not shown). Single ion chromatograms were obtained by plotting the sum of the intensities of ions within a small mass window (i.e. 418.67 ± 0.1 Da, versus time) derived from MS analysis of differentially methyl ester-modified tryptic sperm peptides using an FT-ICR mass spectrometer. Double charged ions corresponding to deuterated (d2) and nondeuterated (d0) forms of peptides P1 (pSVEpSVK) and P2 (INApSTDpSLAK) were observed. Note that both peptides contain two carboxyl groups so that derivatized analogs differ in mass by 6 Da. Peptide sequences were derived from parallel MS/MS experiments performed on an ion trap mass spectrometer.

in our experiments) of FT-ICR allows for quick correlation of related species. The number of carboxyl groups can be calculated by Equation 1, where Δmass is the mass difference between deuterated and nondeuterated isotopic distributions of the same charge state.

No. of carboxyl groups = (Δ mass × charge state)/3 (Eq. 1)

The information gained by FT-ICR analysis of phosphopeptides (accurate mass and number of carboxyl groups) can be coupled with the complementary information provided by data-dependent or targeted ion trap MS/MS analyses (fragment ions, sequence tags, and minimum number of phosphorylated residues from neutral losses of phosphoric acid) to identify peptide sequences from protein data bases rapidly.

To identify capacitation-associated sites of phosphorylation, 2 × 10^6 human sperm were separated in equal aliquots, one aliquot was immediately used, the remaining sperm aliquot was capacitated overnight, and then protein extracts were obtained. Because sperm are unable to synthesized proteins de novo and both samples contained the same amount of sperm, quantitation of phosphopeptides in each sample reflected changes in phosphorylation which occurred during capacitation. Both noncapacitated and capacitated human sperm extracts were then digested with trypsin, and the resulting peptides were converted to the corresponding peptide methyl esters using d0 and d3 methanol, respectively. Peptide pools (derived from capacitated and noncapacitated digests) were then mixed in equal proportions, and an aliquot was analyzed by IMAC/RP-HPLC/ESI/MS on a home-built FT-ICR instrument. The data were examined manually to identify singlet peaks, i.e. phosphopeptide species unique to the capacitated or noncapacitated sample. Parallel IMAC/RP-HPLC/ESI/MS experiments on a quadrupole ion trap mass spectrometer were used to define the sequences of these peptides.

Using this method, the phosphopeptide pSVEpSVK from a novel Ca2+-binding protein, CABYR, was present in the capacitated and noncapacitated sperm total protein digests at about the same level (Fig. 3C). In contrast, the peptide INApSTDpSLAK, derived from AKAP-4, was found at a level 23 times greater in capacitated sperm (Fig. 4C), indicating a capacitation-dependent phosphorylation of this peptide. The charge state of these peptides can be determined by the difference in mass between the C12 and C13 isotope peaks observed by MS analysis. Because the actual mass difference between these isotope peaks is 1 Da, the observed mass difference of 0.5 Da between peaks in the isotopic envelopes of pSVEpSVK and INApSTDpSLAK (data not shown) indicates that both peptides were double charged. Using Equation 1, it was deduced that both phosphopeptides (pSVEpSVK and INApSTDpSLAK) possessed two free carboxyl groups because double charged isotope envelopes corresponding to deuterated and nondeuterated analogs were separated by 3 Da (Fig. 4D and data not shown). Total ion chromatograms were acquired by plotting the sum of the intensities of ions within a small mass window i.e. 418.67 ± 0.1 Da, versus time (data not shown). Single ion chromatograms were obtained by plotting the sum of the intensities of ions within a small mass window (i.e. 418.67 ± 0.1 Da, versus time) derived from MS analysis of sperm tryptic peptide methyl esters using an FT-ICR mass spectrometer (Fig. 3E).

Approximately 500 peptide species were observed in FT-ICR MS analysis of IMAC-enriched modified peptides. Most of these species were observed as doublets, indicating similar levels of phosphorylation between capacitated and noncapacitated sperm populations; 20 unique species were differentially phosphorylated in these populations. Most of these spectra showed neutral losses characteristic of phosphopeptides, however, pep-
The concentration of nonimmune antibody was unable to immunoprecipitate VCP/p97 or AKAP-3 (data not shown). As described previously (20, 24), total protein extracts of noncapacitated as well as of capacitated sperm protein were separated by 8% PAGE, transferred to Immobilon P, and Western blots were performed using α-PY (clone 4G10), α-VCP (S), donated by Dr. Samelson (23), and α-VCP (T) donated by Dr. Tonks (26), as indicated in the figure. B, noncapacitated and capacitated human sperm proteins were extracted as described under “Experimental Procedures” and then immunoprecipitated with α-PY (clone 4G10: 1:50) (left panel) or with α-AKAP-3 (21) (right panel) for 1 h. PAS was then added for another h. PAS beads were then washed five times using the same buffer, and proteins bound to the PAS were eluted boiling in sample buffer. Immunoprecipitated proteins were then separated by 8% PAGE, transferred, and probed with α-PY (RC20), α-VCP (S) (29), or α-AKAP-3 as indicated in the figure. * indicates the localization of the VCP band, and # indicates the localization of AKAP-3. The band shown with the symbol ~ is likely to be an unprocessed form of AKAP-3 because it is recognized by both α-PY and α-AKAP-3 antibodies. Western blots shown in A and B have been exposed for ~2 min in each case. The same concentration of nonimmune antibody was unable to immunoprecipitate VCP/p97 or AKAP-3 (data not shown).

Phosphorylation of VCP/p97 and AKAP-3 during human sperm capacitation. A, total protein extracts of noncapacitated (Non) and capacitated (Cap) human sperm were separated in 10% PAGE, transferred to Immobilon P, and Western blots were performed using α-PY (clone 4G10), α-VCP (S), and α-VCP (T) donated by Dr. Tonks (26), as indicated in the figure. B, noncapacitated and capacitated human sperm proteins were extracted as described under “Experimental Procedures” and then immunoprecipitated with α-PY (clone 4G10: 1:50) (left panel) or with α-AKAP-3 (21) (right panel) for 1 h. PAS was then added for another h. PAS beads were then washed five times using the same buffer, and proteins bound to the PAS were eluted boiling in sample buffer. Immunoprecipitated proteins were then separated by 8% PAGE, transferred, and probed with α-PY (RC20), α-VCP (S) (29), or α-AKAP-3 as indicated in the figure. * indicates the localization of the VCP band, and # indicates the localization of AKAP-3. The band shown with the symbol ~ is likely to be an unprocessed form of AKAP-3 because it is recognized by both α-PY and α-AKAP-3 antibodies. Western blots shown in A and B have been exposed for ~2 min in each case. The same concentration of nonimmune antibody was unable to immunoprecipitate VCP/p97 or AKAP-3 (data not shown).

The tyrosine-phosphorylated sequence of VCP is present in both capacitated and noncapacitated human sperm (Fig. 6). Similarly, AKAP-3 localized in the flagellum in both capacitated and noncapacitated human sperm (Fig. 6). Because capacitation prepares the sperm to undergo a ligand-dependent exocytosis and VCP/p97 has a role in membrane fusion events in other biological systems, our findings suggest that the regulation of VCP/p97 might be a link between capacitation and the acrosome reaction.

**DISCUSSION**

The physiological changes that render mammalian sperm able to fertilize are collectively known as capacitation. Capacitation has been correlated with the increase in tyrosine phos-
mediated fusion of Golgi membranes (21, 34). Although VCP/p97 and NSF are highly homologous, they appear to act in distinct fusion events, presumably because of additional specific cofactors (35). VCP/p97 has a role as a chaperone and aids in the assembly, disassembly, and functional operation of protein complexes. VCP/p97 undergoes tyrosine phosphorylation during T-cell activation, and although this phosphorylation did not alter its ATPase activity (23), tyrosine phosphorylation regulates the subcellular localization of this protein (25). Moreover, a membrane fusion process such as the transitional endoplasmic reticulum assembly in vitro requires tyrosine phosphorylation of VCP/p97 (36). We have demonstrated that VCP/p97 undergoes tyrosine phosphorylation during capacitation. In addition, we have shown that prior to capacitation VCP/p97 localizes in the neck of human sperm, whereas, after overnight incubation, immunofluorescence experiments showed a decreased staining in the neck and the appearance of this protein in the anterior head of capacitated sperm. At least three hypotheses can be made to explain these different immunofluorescent staining patterns. First, modifications of the sperm during capacitation allowed VCP/p97 and NSF to enter the anterior head and mediate the fusion of Golgi membranes (21, 34). Although VCP/p97 and NSF are highly homologous, they appear to act in distinct fusion events, presumably because of additional specific cofactors (35). VCP/p97 has a role as a chaperone and aids in the assembly, disassembly, and functional operation of protein complexes. VCP/p97 undergoes tyrosine phosphorylation during T-cell activation, and although this phosphorylation did not alter its ATPase activity (23), tyrosine phosphorylation regulates the subcellular localization of this protein (25). Moreover, a membrane fusion process such as the transitional endoplasmic reticulum assembly in vitro requires tyrosine phosphorylation of VCP/p97 (36). We have demonstrated that VCP/p97 undergoes tyrosine phosphorylation during capacitation. In addition, we have shown that prior to capacitation VCP/p97 localizes in the neck of human sperm, whereas, after overnight incubation, immunofluorescence experiments showed a decreased staining in the neck and the appearance of this protein in the anterior head of capacitated sperm. At least three hypotheses can be made to explain these different immunofluorescent staining patterns. First, modifications of the sperm during capacitation allowed VCP/p97 antibodies to enter the anterior head of the sperm. Although possible, experiments showing that SP-10 can be recognized before and after capacitation added to the observation of a decrease in fluorescent staining in the neck of capacitated sperm argue against this explanation. Second, the epitopes recognized by two α-VCP antibodies are unmasked in the anterior head, but they are masked in the neck of capacitated sperm. Third, there is a translocation of VCP dur-

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Fig. 5. Immunolocalization of VCP/p97 in human sperm before and after capacitation. Left panels show the immunofluorescence (IF) of air-dried human sperm at ×40 magnification using two different rabbit anti-VCP/p97 antibodies, α-VCP (S) and α-VCP (T), before (Non) and after (Cap) capacitation. The antibodies were visualized using FITC α-rabbit secondary antibody as described under “Experimental Procedures.” Right panels are the corresponding bright fields. Insets in the upper right bright fields show the α-VCP immunofluorescence in either noncapacitated or capacitated sperm at higher magnification (×100). Controls were performed using normal rabbit serum.

Fig. 6. Immunolocalization of AKAP-3 and SP-10 in human sperm before and after capacitation. Left panels show the immunofluorescence (IF) of air-dried human sperm using a rat α-AKAP-3 (×40) antibody or a mouse monoclonal α-SP-10 (×100) antibody, α-VCP (S) and α-VCP (T), before (Non) and after (Cap) capacitation. The antibodies were visualized using FITC α-rat or FITC α-mouse secondary antibody as described under “Experimental Procedures.” Right panels are the corresponding bright fields. Controls were performed using normal rat serum or normal mouse serum and showed no immunofluorescence staining (data not shown).

phorylation of several proteins (1). With the exception of two members of the AKAP family (13, 27) and CABYR (28), proteins that undergo tyrosine phosphorylation during capacitation have not yet been characterized. Capacitation prepares the sperm to undergo the acrosome reaction and also is associated with changes in sperm motility (e.g. hyperactivation) in a number of species (1). Therefore, one may postulate that components of the sperm exocytotic and motility machinery are modified during capacitation (e.g. phosphorylation of specific proteins, changes in protein localization, and/or modification of protein–protein interactions). In the present work, we have analyzed the phosphoproteome of capacitated human sperm using 1) two-dimensional gels followed by MS/MS sequencing of phosphopeptides in total protein extracts.

To understand the link between capacitation and the acrosome reaction, an increased knowledge of the mechanisms that regulate this exocytotic event in sperm is necessary. Sperm homologs of SNARE (29) as well as SNARE-associated proteins have been detected in sea urchin (30) and mammalian sperm (31, 32). These observations support the idea that the sperm acrosome reaction might be regulated in ways similar to the exocytotic processes in somatic cells. Among the proteins that undergo tyrosine phosphorylation during capacitation, we identified VCP/p97 in this study. VCP/p97, a member of the AAA family (ATPases Associated with various cellular Activities) (33), along with NSF and the Golgi t-SNARE syntaxin 5,
ing capacitation from the neck to the anterior head of human sperm. Ideally this hypothesis should be tested by a direct measurement of VCP in the neck and the anterior head after separation of these subcellular compartments; however, the impossibility of separating the neck from the anterior head prevented us from performing this experiment. Considering the role of VCP in the neck and tyrosine phosphorylation of this protein could have a role as a link between capacitation and the acrosome reaction. Alternatively, this protein can act as a chaperone, bringing relevant membrane fusion proteins to the site of the acrosome reaction.

Capacitation is also linked to events that occur in the sperm flagellum. AKAPs represent a growing family of scaffolding proteins that function to tether the regulatory subunits of protein kinase A and other enzymes to organelles or cytoskeletal elements. These proteins permit the precise control of signal transduction in discrete regions of the cell (37). In the present work, we have confirmed tyrosine phosphorylation of AKAP-3 and AKAP-4 during human sperm capacitation and mapped eight phosphorylation sites of these proteins. Among these sites, the AKAP-4 phosphopeptide INApSSTDpSLAK was found to be 23 times more abundant in capacitated sperm, suggesting that this phosphorylation site might be involved in the regulation of AKAP-4 function during capacitation.

Multiple methodologies have been used to study phosphorylation. Recently, mass spectrometry has become the preferred method to identify phosphopeptides because of its speed and high sensitivity. Nevertheless, because phosphorylation sites are usually substoichiometric, it has been necessary to devise new methods for selective detection and enrichment of phosphopeptides. Recently, although several methods have been developed, most have been applied only on a protein-by-protein basis (8) with a few notable exceptions (38, 39). None of these methods has been used successfully to identify phosphotyrosine residues from complex mixtures probably because of multiple steps of derivatization that reduced the final recovery. On the other hand, IMAC has been used previously to enrich digests for phosphorylated peptides (6, 16); however, the selectivity of this technique is poor because peptides containing acidic residues (i.e. Glu and Asp) bind to the immobilized iron atoms (40). To solve this lack of specificity, acidic residues were converted to methyl esters, eliminating the binding of nonphosphorylated species to the IMAC column. This procedure does not generate diasteromers and is compatible with phosphorylated Ser, Thr, and Tyr residues. This methodology was used successfully to map 60 sites of phosphorylation.

The sites of phosphorylation identified here defined in vivo sites of phosphorylation resulting from normal phosphorylation events in capacitated human sperm and were not obtained by kinase overexpression or by kinase activators or phosphatase inhibitors. Although most of the sites described in the present work have not been observed previously, phosphorylation of the C-terminal protein kinase A catalytic subunit peptide IRVpSINE has been demonstrated to be necessary for the catalytic activity of recombinant protein kinase A (41). All other observations have been obtained from the capacitated human sperm phosphoproteome that is four of the five tyrosine-phosphorylated sequences contained phosphoserine in the proximity of the phosphotyrosine. This result raises the possibility that a dual specificity kinase is involved in the capacitation process and/or that phosphoserine is part of the substrate recognition motif for a sperm tyrosine kinase. Because the identity of tyrosine kinases present in sperm is at present not known, peptide sequences found to be tyrosine-phosphorylated can be used as substrates to purify tyrosine kinases from sperm.

After enrichment of phosphorylated peptides, the next step is to derive their sequences and define the sites of phosphorylation. In many cases, identification of the exact phosphorylation sequence was precluded because of the complexity of the phosphopeptide MS/MS spectra. To simplify interpretation of phosphopeptide spectra, we performed on-line AP dephosphorylation of IMAC phosphopeptide enrichment. AP treatment has been shown to enhance detection of multiply phosphorylated peptides (42). Analysis of phosphopeptides followed by AP treatment and reanalysis has been demonstrated as a technique for phosphopeptide identification based on 80-Da differences (43). This method allowed identification of one additional peptide that was not detected without AP treatment.

In summary, 16 proteins that are recognized by α-PY antibodies in two-dimensional Western blots were determined. From these proteins, VCP was also detected in α-PY immunoprecipitates and showed different immunolocalization patterns before and after capacitation. In addition, we have successfully employed IMAC enrichment of phosphopeptides coupled to MS/MS analysis to isolate and sequence phosphopeptides from total protein digests of human capacitated sperm. This is the first study in which this methodology has been used to map phosphopeptides in a human cell type. Moreover, we have coupled this methodology with differential isoform labeling and IMAC enrichment to derive quantitative information on phosphorylation events that occur during capacitation. This technique goes beyond the field of reproductive biology and could potentially be used to map and compare sites of phosphorylation in a variety of biological systems.

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