Identification of novel immunodominant epididymal sperm proteins using combinatorial approach

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Abstract

Functionally immature spermatozoa leave the testis mature during epididymal transit. This process of maturation involves either addition of new proteins or modification of existing proteins onto the sperm domains that are responsible for domain-specific functions. Epididymal proteins are preferred targets for immunocontraception. In an attempt to identify epididymis-specific sperm proteins, we used a novel combinatorial approach comprising subtractive immunization (SI) followed by proteomics. Following SI, sera of mice were used for immunoproteomics, which led to the identification of 30 proteins, of which four proteins namely sperm head protein 1, sperm flagella protein 2 (SFP2), SFP3, and SFP4 are being reported for the first time on sperm. Another group of four proteins namely collagen α-2 (I) chain precursor, homeodomain-interacting protein kinase 1, GTP-binding protein Rab1, and ubiquinol cytochrome c reductase core protein II although reported earlier in testis are being reported for the first time in epididymal sperm. Furthermore, seven out of these eight novel proteins could be validated using peptide ELISA. These data are a useful repository, which could be exploited to develop targets for post-testicular immunocontraception or biomarkers for infertility diagnosis and management.


Introduction

Spermatozoa leaving the testis are morphologically mature but they are immotile and unable to interact with the egg. The acquisition of fertilizing ability during the epididymal transit defines the concept of sperm maturation. The epididymal maturation events involve remodeling of the sperm plasma membrane by uptake of newer proteins from the epididymal luminal fluid, removal or post-translational modifications of existing proteins, or repositioning of proteins onto different domains (Jones 1998). These remodeling mechanisms lead to acquisition of specific functions by different domains of the spermatozoon.

Epididymal maturation is of strategic importance in the design of post-testicular methods of male contraception as well as elucidation of the causes of male infertility. A number of sperm antigens designated as contraceptive targets have been identified and most of them are of testicular origin (Suri 2004). However, targeting these testicular proteins will not only affect sperm production, but is also likely to lead to disruption of endocrine functions, loss of libido and autoimmune orchitis, which will not be reversible (Jones 1994, DePaolo et al. 2000). On the other hand, targeting epididymal sperm maturation events will not impede spermatogenesis and related testicular endocrine function and will provide early onset of infertility with rapid reversal (Cooper & Yeung 1999, Khole 2003).

The epididymis-specific proteins form a small percentage of the total sperm proteome and are likely to be masked by the abundance of testicular proteins that are highly immunodominant. Using the approach of subtractive immunization (SI), we reported a number of mAbs, one of which identified a sperm protein of 27 kDa (Joshi et al. 2003). Although this approach enabled us to obtain mAbs against epididymis-specific sperm proteins, it lacked a high throughput potential. In the present study, we have generated polyclonal antibodies (pAbs) that recognized epididymis-specific proteins in different domains of the sperm. These polyclonal sera have been used for immunoproteomics, which led to the identification of several epididymal sperm-specific proteins that include some novel proteins.

Results

Tolerized–immunized mice showed epididymis-specific immune response

Previous studies from our laboratory have successfully established the use of SI protocol for identification of epididymal sperm proteins. In this study, we employed the intact head/intact flagellum (group HI/FI) or soluble extracts of head/flagellum (group HS/FS) to elicit an immune response. Figure 1a represents immune response to different types of immunogens as measured by ELISA. Sera from mice tolerized with testicular proteins (post-tolerization (PT) sera) showed no reactivity to testicular proteins and epididymid sperm proteins, establishing a state of immune tolerance. Animals that were challenged PT with testicular protein (Fig. 1a, group T) also did not show any significant titer to the testicular antigen, confirming that the animals had been successfully tolerized. Subsequent immune challenge of tolerized mice with proteins from head or flagella of epididymal sperm showed a 9- to 11-fold increase in reactivity specific to epididymal sperm proteins (post-immunization (PI) sera). To assess reactivity of the PI sera with surface antigens on the sperm, cellular ELISA using rat caudal sperm was performed. Data depicted in Fig. 1b indicate that the PI sera reacted strongly with epididymal sperm as compared with PT sera, indicating surface presence of immunoreactive epitope. It was also noted that mice immunized with Tris buffered extract (HS, FS) showed better immune response as compared with mice immunized with intact fractions of either head/flagellum (HI, FI).

PI sera shows sperm domain-specific reactivity

Indirect immunofluorescence (IIF) was carried out using PI serum on cauda epididymal sperm to characterize the domain-specific immune reactivity. Fig. 2A represents the fluorescent images while Fig. 2B represents the corresponding DIC images. Sera of mice immunized with sperm head, either in intact or soluble form (HI/H S) showed the presence of antibodies recognizing epitopes on the acrosomal cap. Sera of mice immunized with testicular proteins (HI/HS) showed no reactivity to epididymal sperm proteins (post-immunization (PI) sera). To assess reactivity of the PI sera with surface antigens on the sperm, cellular ELISA using rat caudal sperm was performed. Data depicted in Fig. 1b indicate that the PI sera reacted strongly with epididymal sperm as compared with PT sera, indicating surface presence of immunoreactive epitope. It was also noted that mice immunized with Tris buffered extract (HS, FS) showed better immune response as compared with mice immunized with intact fractions of either head/flagellum (HI, FI).

PI sera identifies epididymis-specific antigens

To address the issue of epididymis-specific reactivity, we performed immunohistochemical localization with serum from different groups of mice on rat testis and sagittal epididymal tissue section (Fig. 3). Sera from all groups displayed no reactivity to testis (A1, B1, C1, and D1).
in line with its epididymis-specific reactivity as seen by ELISA in Fig. 1a. Figure 3A2–A6 shows reactivity of serum from mice immunized with intact sperm head (HI) with different regions of the epididymis. It is seen that reactivity first appears from distal caput specifically in supranuclear region of the principal cells (PC) in the epididymal epithelium as well as the sperm in the lumen (LS). In corpus epididymis (A4–A5), there was a gradual increase in reactivity on both the PC and the sperm from proximal to distal region. The distal corpus showed prominent reactivity in the supranuclear region of the PC and the luminal sperm. In cauda, the serum shows prominent localization on the microvilli (MV) and the luminal sperm. A similar pattern of reactivity was seen in the group of mice immunized with either FI (B2–B6) as well as soluble head (C2–C6). Sera of mice immunized with FS showed appearance of reactivity from proximal caput onwards specifically the supranuclear area of the PC in epididymal epithelium with no reactivity on the luminal sperm (D2). In subsequent regions from distal caput (D3) to distal corpus (D5) and cauda (D6), the sera showed a staining pattern similar to that seen with HI, HS, and FI sera. No staining was seen with PT sera or buffer control (secondary alone), which were used as negative controls (data not shown). Overall, the data show a characteristic epididymis-specific pattern of localization. Immunohistochemical analysis using PI sera with various rat somatic tissues such as brain, heart, liver, kidney, spleen, and thymus showed no positive staining (data not shown) suggesting that the sera identified only epididymis-specific antigens.

Identification of immunoreactive proteins using PI sera

Preparative SDS-PAGE for separation of rat testicular and cauda epididymal sperm proteins, followed by western blotting using PT and PI sera, is shown in Fig. 4. Sera of all animals showed negligible reactivity with testicular proteins (data not shown) whereas they reacted with 31 bands with molecular masses ranging from 21 to 180 kDa in caudal sperm proteins. The immunoreactive bands were aligned with the corresponding gel and labeled as R1–R31 as shown in Fig. 4a. The detailed information of the immunoreactive bands is listed in Fig. 4b. Sera from group HI identified four proteins, group HS sera identified three proteins, group FI sera identified 13 proteins, and FS sera reacted with 18 proteins. PT sera did not show any reactivity with caudal proteins. Buffer control also did not show any reactivity (data not shown). The 31 gel sections were manually excised and subjected to in-gel tryptic digestion.

Protein sequencing of immunoreactive proteins

The digested proteins were subjected to proteomic analysis. Following MALDI-TOF mass spectrometry and tandem TOF/TOF mass spectrometry, combined mass spectrometry (MS) and MS/MS spectra were used to search against the taxa Mammalia (mammals) in the Mass Spectrometry protein sequence DataBase (MSDB) using the GPS software running Mascot search algorithm for peptide and protein identification. Of the 31 bands, seven bands had two protein matches each (14 proteins) while 16 bands gave a single match (16 proteins), and the remaining eight bands did not give any significant protein match leading to the identification of total 30 as reported in Table 1 of Supplementary Data 1, which can be viewed online at www.reproduction-online.org/supplemental/. Please refer to Supplementary Data 2 and 3, which can be viewed online at www.reproduction-online.org/supplemental/ for protein identification details.

The data were categorized as proteins identified using head-specific sera (HI and HS, three proteins), flagella-specific sera (FI and FS, 24 proteins), and those identified by sera from both the head/flagella group (HI, HS, FI, and FS, three proteins). For further analysis, proteins identified by both the domain sera were added to their...
respective groups. Therefore, six (3 + 3) proteins were identified on the head region and 27 (24 + 3) proteins in the flagellar region.

Single entry search was performed to get more information about the identified proteins from NCBI, Universal Protein Resource (UniProt) and Expert Protein Analysis System (Expasy) proteomics server of the Swiss Institute of Bioinformatics (SIB). Table 2 of Supplementary Data 1 represents common and abbreviated names of identified proteins.

Of the six proteins from head group, sperm head protein 1 (SHP1; accession no P85300) similar to uncharacterized protein c10orf 58 homolog precursor CJ058 is being reported in this study for the first time on sperm (Table 1). One of the protein ubiquinol cytochrome c reductase core protein II, mitochondrial precursor (UQCRC2), is known in testis and is reported in this study for the first time in epididymal sperm (Table 2). Four proteins, voltage-dependent anion-selective channel protein 2 (VDAC2), glyceraldehyde 3-phosphate dehydrogenase type 2 (GAPDHS), citrate synthase a mitochondrial precursor (CISY) and ADP-ATP translocase (ADT4), are known to be present on testis and epididymis/ejaculated sperm.

The flagella group proteins comprised 27 proteins of which three were novel proteins, sperm flagella protein (SFP) 2 (Acc no P85301) similar to bullous pemphigoid antigen 1, SFP3 (Acc no Q5BK63) similar to NADH dehydrogenase ubiquinone 1-α subcomplex (NDUFA9) and SFP4 (Acc no Q06647) similar to H+ -transporting two sector ATPase α chain (ATP5O) are being reported in this study for the first time on sperm (Table 1). Four proteins were identified twice (eight proteins) in separate 1D bands (R9 and R10, R13 and R14, R15 and R16, R29 and R30), which are GAPDHS, 3-oxoacid CoA transferase 2A (OXCT2A), H+ -transporting two sector ATPase-α chain (ATPA), and sperm mitochondrial cysteine-rich protein (SMCP). Of the remaining 16, four proteins collagen α-2(I) chain precursor (COL1A2), homeodomain-interacting protein kinase 1 (HIPK1), GTP-binding protein Rab1 (RAB1), and ubiquinol cytochrome c reductase core protein II mitochondrial precursor (UQCRC2) are known in testis but are being reported here for the first time in epididymal sperm (Table 2), while remaining 12 have been shown to be present in testis and epididymis/ejaculated sperm.
Validation of novel proteins using peptide ELISA

To validate that all eight novel proteins (Tables 1 and 2) identified by the proteomic analyses actually represented the immunoreactive species, peptide ELISA was carried out. Table 3 summarizes the details of the peptides synthesized for each protein. Figure 5a represents peptide ELISA results of pooled peptide of novel proteins namely R27, R1, R21, R29 as antigen with PT and PI sera of respective group (HI/HS/FI/FS). Figure 5b represents peptide ELISA results of pooled peptide of proteins namely R18, R3, R25a, R25b with PT and PI sera of respective group (HI/HS/FI/FS). Out of these eight proteins, with the exception of protein R1, the remaining seven proteins showed significantly high reactivity with PI sera as compared with PT sera, which confirmed the specific reactivity and validated the earlier proteomic analysis results. Only protein R1 did not show significant reactivity with PI sera as compared with PT sera.

Discussion

Epididymal sperm proteins are preferred targets for immunocontraception. In order to achieve 100% contraceptive effect, the proteins on different domains of sperm need to be targeted, as every domain is responsible for a function leading to successful fertilization. It has been

Table 1 List of novel proteins.

<table>
<thead>
<tr>
<th>Domain-specific sera used</th>
<th>Sample ID</th>
<th>Protein name</th>
<th>Abbreviation</th>
<th>Name submitted in database</th>
<th>Assigned accession number</th>
<th>Other tissues reported in literature</th>
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<tr>
<td>Head</td>
<td>R27</td>
<td>Uncharacterized protein C10orf58 homolog precursor</td>
<td>C1058</td>
<td>Sperm head protein 1 (SHP1)</td>
<td>P85300</td>
<td>Not known</td>
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<tr>
<td>Flagella</td>
<td>R1</td>
<td>Bullous pemphigoid antigen 1, isoforms 1/2/3/4</td>
<td>BPA</td>
<td>Sperm flagella protein 2 (SFP2)</td>
<td>P85301</td>
<td>Neuron, heart, muscle, brain, fetal skin and spinal cord</td>
</tr>
<tr>
<td></td>
<td>R21</td>
<td>NDUFA9 protein (fragment)</td>
<td>NDUFA9</td>
<td>Sperm flagella protein 3 (SFP3)</td>
<td>Q5BK63</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>R29</td>
<td>ATP synthase O subunit, mitochondrial precursor</td>
<td>ATPSO</td>
<td>Sperm flagella protein 4 (SFP4)</td>
<td>Q06647</td>
<td>Pituitary</td>
</tr>
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</table>
suggested that immunization with a multivalent vaccine containing multiple sperm antigens can generate a greater anti-fertility effect than a single sperm antigen (Kurth et al. 2008). As part of our effort to identify putative protein targets for the development of post-testicular male contraceptives, we exploited combinatorial approach of SI followed by proteomics to delineate potential targets from different sperm domains.

The approach used in the present study was modified to overcome the lack of high throughput capacity of the earlier approach. The approach differs in terms of several aspects from the studies reported earlier (Ensrud & Hamilton 1991, Khole et al. 2000). First, in this study, we used extracts from different sperm domains, i.e. the head and flagellum separately, rather than whole sperm as immunogen. This enabled us to generate response against proteins on individual functional domains of sperm simplifying their characterization. Secondly, we changed the extraction procedure of both tolerogen and immunogen wherein Tris extraction buffer was used instead of PBS so as to get a better qualitative and quantitative yield. We could not use other harsher extraction procedures to avoid toxic effects on the immunized mice, which were day 0 and day 5 old. Finally, we chose to directly characterize the proteins using pAbs from the mice rather than generating mAbs to reduce the time and increase the throughput.

It was seen that the serum from the immunized mice localized antigens specifically in the sperm regions with which they were immunized proving the feasibility of this approach in the identification of domain-specific epididymal sperm maturation antigens. It may be noted that although the sperm were sonicated for domain-specific immunogen preparation, the reactivity of the resulting antiserum (PT) with intact sperm in IIF rules out the possibility of loss of all proteins of the plasma

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**Table 2** List of known proteins reported for the first time in epididymis.

<table>
<thead>
<tr>
<th>Domain-specific sera used</th>
<th>Sample ID</th>
<th>Protein name</th>
<th>Abbreviation</th>
<th>Accession number</th>
<th>Testis</th>
<th>Other tissues reported in literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head/flagella</td>
<td>R18</td>
<td>Ubiquinol-cytochrome c reductase complex core protein 2, mitochondrial (precursor)</td>
<td>UQCR2C</td>
<td>Q5XR3_RAT</td>
<td>Transcript</td>
<td>Spinal cord</td>
</tr>
<tr>
<td>Flagella</td>
<td>R3</td>
<td>Collagen α-2(I) chain precursor</td>
<td>COL1A2</td>
<td>AAD41775</td>
<td>Transcript</td>
<td>Spermatogonia</td>
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<tr>
<td></td>
<td>R25</td>
<td>Homeodomain-interacting protein kinase 1/nuclear body associated kinase 2b</td>
<td>HIPK1</td>
<td>Q8C642_MOUSE</td>
<td>Transcript</td>
<td>Not known</td>
</tr>
<tr>
<td></td>
<td>R25</td>
<td>Ras-related protein Rab-1A GTP-binding protein Rab1</td>
<td>RAB1A</td>
<td>TVRTYP</td>
<td>Spermatid</td>
<td>Prostate</td>
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**Table 3** List of synthesized peptides of proteins listed in Tables 1 and 2.

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<th>Serial no.</th>
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<th>Protein name</th>
<th>Amino acid sequence</th>
<th>No of AA</th>
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<td>Novel proteins</td>
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</tr>
<tr>
<td>2</td>
<td>R27</td>
<td>SHP1</td>
<td>DRVNLSSVEAIVKK</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>R1</td>
<td>SFP2</td>
<td>ADQLVERWQSVHVQI</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>R1</td>
<td>SFP2</td>
<td>ASSILTYQVTGGVHS</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>R1</td>
<td>SFP2</td>
<td>ISHSYEDLGLLKDKVIEL</td>
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<tr>
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<td>R1</td>
<td>SFP2</td>
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<tr>
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<td>SFP3</td>
<td>RSVSGVVTAVFGA</td>
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</tr>
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<tr>
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<td>FEDVFVNIPTAIAQ</td>
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<td>SFP4</td>
<td>QGVISAFSTIMS</td>
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<td>R3</td>
<td>COL1A2</td>
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</tr>
<tr>
<td>18</td>
<td>R3</td>
<td>COL1A2</td>
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<td>R25A</td>
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membrane and outer acrosomal membrane. It was also observed that serum of mice immunized with soluble flagellar proteins identified sperm antigens on both the acrosome and the flagellar domains. This could have been due to exposure of cryptic epitopes of the same antigens following solubilization or as a result of the sharing of epitopes of different proteins on the two domains. In context of the former possibility, we have recently reported identification of TSA-70 a sperm autoantigen that is present on the tip of acrosome and also the principal piece (Wakle et al. 2005). Ubiquitin and EPPIN are among the few epididymal proteins that are known to be present on both the sperm domains (Richardson et al. 2001, Sutovsky et al. 2001). In case of spermatozoa, the presence of same protein on two different domains is indicative of a dual role for the protein, as every domain is endowed with a specific function. Proteins with multiple functions are probably evolved so as to conserve a great deal of energy required for growth and reproduction.

After the initial characterization of the antibody produced by the modified SI approach, we set out to determine the origin of the cognate antigens in the different regions of the epididymis. The regions of epididymis viz. the caput, corpus, and cauda have different patterns of gene and protein expression (Kirchhoff 1999). Several proteins such as CST8 (cystatin-related gene), SPINT4 (serine protease inhibitor), RNASE9 and RNASE10 (RNase, RNase A family 9 and 10), and others have been shown to be expressed in defined areas of the epididymis (Cornwall et al. 1992). Immunohistochemistry (IHC), using PI sera of all the four groups, demonstrated intense reactivity starting from distal caput with three of the four sera (HI, HS, and FI) and proximal caput with the serum of the fourth group (FS). Active synthesis and incorporation of proteins in the sperm has been shown to take place in the proximal region of the epididymis, i.e. caput. AEG, an acidic epididymal glycoprotein, was shown to originate in the caput epididymis and coat sperm during their transit through this region (Leo et al. 1978). Klinefelter & Hamilton (1985) have shown that mammalian sperm on leaving the testis acquire fertilizing ability after interacting with epididymal secretory products especially in the caput region. Report from our earlier study has also indicated that the 29 kDa protein appeared from distal caput and increased in the corpus region (Khole et al. 2001). Our present data also indicate similar pattern of reactivity. The PC of the corpus are known to be actively involved in endocytosis and secretion of a wide variety of proteins with discrete functions some of which play an important role in sperm maturation (Flickinger 1981, Cooper 1986, Hermo et al. 1998).

A primary requirement of a potential sperm-based contraceptive vaccine is its specificity. None of the sera showed any reactivity to multiple somatic tissues. This suggests that the cognate proteins identified by the antibodies are specific to epididymis and sperm and these proteins, if found to have any functional role, could serve as ideal targets for immunocontraception. The molecular mass of proteins identified by PI sera was in the range 21–182 kDa. It was seen that sera from mice immunized with intact head or flagella identified fewer proteins as compared with those immunized with solubilized proteins. This is probably because following solubilization more proteins are accessible to the immune system for antigen presentation. It was also observed that, irrespective of the state of immunogen used, sera of animals immunized with head proteins identified fewer proteins than those immunized with flagellar proteins. It is possible that majority of the head proteins are synthesized during spermatogenesis, which takes place in the testis and are therefore not picked up by SI approach. It is also likely that the protein acquired on the head may be deeply embedded and may be less antigenic as opposed to flagellar proteins. Indeed, it is known that the head, especially the acrosome, is rich in lipids (Alvarez & Storey 1995), which are likely to mask several proteins. Several sperm proteins are known to be glycosylated and are likely to

Figure 5 (a) Peptide ELISA showing reactivity of post-tolerization (PT) and post-immunization (PI) sera of respective group with pooled peptide of novel proteins namely R27, R1, R21, R29, which is represented by bars. The dilutions used were 1:50 and 1:25. Values shown are mean ± S.E.M. from three readings. (b) Peptide ELISA showing reactivity of post-tolerization (PT) and post-immunization (PI) sera of respective group with pooled peptide of proteins reported first time in epididymis namely R18, R3, R25a, R25b, which is represented by bars. The dilutions used were 1:50 and 1:25. Values shown are mean ± S.E.M. from three readings.
be less immunogenic due to the fact that glycosylated proteins are more stable towards misfolding and aggregation (Weert & Møller 2008).

We identified a total of 30 proteins, of which group 1 consisted of four novel proteins as they are reported for the first time on sperm and group 2 consisted four proteins that have been shown for the first time on epididymal sperm and the remaining 22 proteins have been reported earlier. Of the four novel proteins, a protein, namely SHP1 (accession no. P85300), is being reported on head domain and another three proteins, SFP2 (accession no. P85301), SFP3 (accession no. Q5BK63), and SFP4 (accession no. Q06647), are on flagella domain of the sperm. SFP4 similar to ATP5O protein was submitted to Uniprot Knowledgebase and assigned a new accession no. Q06647 as it was novel when submitted. However, recently Puri et al. (2008) have also reported this protein.

The four proteins from group 2 namely ubiquinol cytochrome c reductase core protein II (UQRC2), COL1A2, HIPK1, and RAB1A are being shown for the first time in epididymal sperm. Of these, the first three were reported in testis at transcript level (The MGC Project Team 2004, He et al. 2005) while the fourth one was reported at protein level (Emkey et al. 1991).

For a target to be considered suitable for a vaccine approach, immunogenicity is very crucial and therefore separate studies have been specially designed by investigators to establish immunogenicity of otherwise well-characterized proteins such as ESP, SLIP-1, SPACA1, ACRV1, and SPACA4 (Kurth et al. 2007). Proteins such as ENO1, GAPDHS, ATPA, GPX4, TEKT3, AKAP4 and VDAC2 reported in the present study were also identified in recent proteomic studies carried out in human and rodent sperm (Cao et al. 2006, Martinez-Heredia et al. 2006, Stein et al. 2006, Domagała et al. 2007). Proteins such as ENOL, malate dehydrogenase (MDHM), GAPDHS, dihydrolipoamide dehydrogenase (DLAT) have been reported to be immunogenic in humans as these are also detected using sera of infertile (Carrera et al. 1994, Westhoff & Kamp 1997). Force and his coworkers have shown that sperm-specific enolase (ENO-S) exists as different isoforms in the different stages of sperm maturation wherein a prominent S2 isoform is epididymis specific (Stein et al. 2006, Domagała et al. 2007). Force and his coworkers have shown that sperm-specific enolase (ENO-S) exists as different isoforms in the different stages of sperm maturation wherein a prominent S2 isoform is epididymis specific (Force et al. 2004). However, nothing is known about the molecular identity of this epididymis-specific form. The enzymes citrate synthase, malate dehydrogenase and carnitine acetyltransferase have been reported at higher levels in epididymal sperm and also indicated to be androgen dependent with the exception of malate dehydrogenase (Marquis & Fritz 1965, Brooks 1978).
predominantly on the flagella. These proteins are immunogenic in nature, conserved in rodents. These data are a useful repository that could be exploited for mining proteins with important physiological function in sperm. Validation of epididymal specificity of the identified proteins and the elucidation of the relevance of these proteins in sperm function is essential and is the current focus of the research in our laboratory.

Materials and Methods

Materials

Adjuvants, BSA, o-phenylenediamine dihydrochloride, sucrose, 3,3’diaminobenzidine, p-phenylenediamine free base were obtained from Sigma. Protease inhibitor cocktail was obtained from Roche Diagnostics and non-fat dry milk from Amul (Mumbai, India). Molecular weight marker, nitrocellulose membranes, ECL detection kit, protein silver staining kit, and Percoll were obtained from Amersham Biosciences, conjugates from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), and Dako Cytomation (Glostrup, Denmark). ELISA plates were purchased from Nunc (Roskilde, Denmark). Reagents for trypsin digestion and MALDI TOF/TOF were obtained either from Sigma or Applied Biosystems (Foster City, CA, USA). M-PER Mammalian protein extraction reagent was procured from Pierce (Rockford, IL, USA); Ready Gel precast polyacrylamide gels 4–15% Tris HCl were procured from Bio-Rad Laboratories. All other reagents were procured from Qualigens (Mumbai, India), SRL India Ltd (Mumbai, India), and Hi-Media (Mumbai, India).

Animals

Animals were housed with food and water available ad libitum. All the experimental protocols were approved by the Institutional Ethics Committee for Care and Use of Laboratory Animals for Biomedical Research. Inbred Balb/C neonates at day 0 of birth as well as adult mice 8–12 weeks and adult inbred male Holtzman rats weighing 180–220 g were used in the study.

Sperm collection

Rats were killed; testes and epididymides were removed and placed in 0.01 M PBS (pH 7.4). Testes were used for the preparation of tolerogen as described below. The cauda epididymides were minced in PBS and incubated at 37 °C for 30 min. The supernatant containing sperm was centrifuged at 500 g for 20 min to get the pellet. To the pellet, PBS was added and centrifuged at 500 g for 20 min, this step was repeated thrice. The pellet was then resuspended in 1 ml Tris buffer (pH 7.4; 40 mM Tris HCl, 5 mM MgSO₄, and complete protease inhibitor cocktail). This suspension was sonicated at 4 °C (Ralsonic Ultrasonic Processor, Model RP-120-122, India) at 100% output for 1 min bursts at 30-second intervals for total 5 min. The sonicated sample was then centrifuged at 10,000 g at 4 °C for 10 min. Protein was quantified in the supernatant by Lowry assay (Lowry et al. 1951).

Preparation of immunogen

Isolation and enrichment of epididymal sperm head and flagellum fraction for immunization

The fractions of sperm head and flagella were prepared as described earlier (Oko 1988). Briefly, cauda epididymal sperm pellet was resuspended in PBS and sonicated at 4 °C as described above. This treatment resulted in 95% decapitation of spermatozoa, as judged by the phase contrast microscopy. The sonicated suspension was layered onto sucrose gradients 65, 70, and 75% (w/v; 7 ml of each) and centrifuged at 4 °C at speed 100 000 g for 60 min in Sorvall Pro 80 swinging bucket rotor. The sperm flagellar fraction was collected from the 65 to 70% interface while the isolated heads were collected from the bottom of 75% gradient. The isolated heads and flagellum were washed twice with PBS. Percentage of purity was determined by counting number of flagella in the head fraction and vice versa. Fractions with >95% purity was used for immunization. Immunization was carried out either by using intact or soluble extract of sperm head or flagellum. For soluble proteins, fraction was suspended individually in Tris extraction buffer (pH 7.4), sonicated for 5 min at 4 °C, and centrifuged at 4 °C 10 000 g for 10 min. The supernatant was used as immunogen. Protein was quantified by Lowry assay (Lowry et al. 1951).

Subtractive immunization

SI protocol used earlier (Khote et al. 2000) was modified for the present study and is diagrammatically depicted in Fig. 6. Briefly, female Balb/C neonates were injected intraperitoneally with 20 μg tolerogen in 50 μl Tris buffer within 24 h of birth, followed by another injection later on day 5. On day 21, blood samples were collected to obtain PT sera samples. The tolerized animals were divided into five groups namely T, HI, HS, FI, and FS. Each group containing three animals was immunized with different immunogen as described in Fig. 6. Blood samples were collected through the retro-orbital plexus to obtain the tolerized and immunized (PI) serum samples. Immunogenicity of tolerogen was checked by immunizing adult mice with tolerogen and checking titer after the final booster.

Immunochromical characterization

PI sera were used for immunochromical characterization by ELISA, IIF, IHC, and western blot.
incomplete adjuvant. Immunogen. FCA, Freund’s complete adjuvant; FIA, Freund’s incomplete adjuvant.

PI indicates post-immunization sera collected after two boosters of tolerogen (100 μg/100 μl in Tris buffer). PT indicates post-tolerization sera collected on day 21 and PI indicates post-immunization sera collected after two boosters of immunogen. FCA, Freund’s complete adjuvant; FIA, Freund’s incomplete adjuvant.

Figure 6 Diagrammatic representation of modified subtractive-immunization protocol (SI). Tolerogen: T, testicular protein extract; immunogens: HI, represents intact rat sperm head fraction; FI, intact sperm flagellar fraction; HS, soluble protein of sperm head fraction; FS, soluble protein of sperm flagellar fraction. Group T animals were immunized with tolerogen (100 μg/100 μl in Tris buffer). Group HI and FI were immunized with intact sperm head fraction and intact flagellar fraction (1 x 10^6/100 μl in Tris buffer) respectively. Group HS and FS were immunized with solubilized proteins of head and flagellum respectively (100 μg/100 μl in Tris buffer). PT indicates post-tolerization sera collected on day 21 and PI indicates post-immunization sera collected after two boosters of immunogen. FCA, Freund’s complete adjuvant; FIA, Freund’s incomplete adjuvant.

Soluble ELISA

Reactivity of PI sera with rat testis and cauda epididymal sperm proteins extracted in 1% (w/v) SDS was carried out as described earlier (Khole et al. 2000). Sera from all animals were diluted 1:100 while secondary antibody Rabbit anti mouse conjugated HRP (Dako) was diluted 1:1000. PT sera were actually the preimmunization sera, while PBS was used as buffer control. Each sample was assayed in duplicates. The optical density (OD) of buffer control was deducted from all the OD readings of PT and PI sera.

Cellular ELISA

ELISA was employed to detect the reactivity of PT as well as PI sera with rat caudal sperm coated onto microtiter plate as described earlier (Wakle et al. 2005).

Indirect immunofluorescence

Immunolocalization of the cognate proteins on rat caudal sperm was performed in suspension using PI and PT sera of all groups as described earlier (Joshi et al. 2003). PBS buffer was used as the negative control.

Immunochemistry

Rat testis, whole epididymis, and various somatic tissues such as brain, heart, liver, kidney, spleen, and thymus were fixed in Bouin’s fixative for 24–48 h and processed for paraffin embedding. Serial sections of 5 μm were cut and placed onto clean glass slides. Immunochemical localization was done using 1:25 diluted PT and PI sera of all groups as described earlier (Khole et al. 2000). For negative control, sections were incubated with buffer instead of sera.

SDS-PAGE and western blot

Proteins from rat testis and cauda epididymal sperm were extracted in 1% (w/v) SDS as described earlier (Wakle et al. 2005) and 1000 μg protein was resolved on 1 mm thick preparative 10% SDS–PAGE gel (16 x 20 cm, Protean II XL module, Bio-Rad Laboratories). Transfer of proteins from the gel onto the nitrocellulose membrane was performed using current of 400 mA for 2 h as described by Towbin et al. (1979). The gels were washed thoroughly with water and then silver stained using commercial silver staining kit (Amersham Biosciences). The blots were then stained with Ponceau S (0.5 mg Ponceau in 0.01% (v/v) acetic acid) and destained with distilled water to determine the quality of SDS-PAGE separation of sperm proteins. The membrane with blotted proteins was processed for immunodetection as described earlier (Wakle et al. 2005). PT and PI sera at 1:250 dilution while secondary antibody 1:40 000 dilution of HRP conjugated goat anti mouse IgG were used. PT sera were actually the preimmunization sera, while PBS was used as buffer control.

In-gel tryptic digestion

The bands in the stained gel were perfectly aligned with the bands on the adjacent blots and individual protein band of interest were manually excised from the silver-stained gels in duplicate. BSA gel plugs were used as a protein standard for each set of in-gel tryptic digestion experiment. The gel bands were destained using a 1:1 mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. The protein bands were typically digested according to the protocol described earlier by Shevchenko et al. (1996) with minor modifications, using sequencing grade trypsin 0.01 μg/μl (Applied Biosystems) for 16 h at 37°C. The tryptic peptides were extracted with trifluoroacetic acid (TFA). The extracts were pooled and dried completely by centrifugal lyophilisation. The peptides were reconstituted in sample diluent and mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid, 10 mg/ml in 70% v/v ACN, 1% v/v TFA) and spotted in duplicate on a target plate and allowed to air dry. Trypsin digested β-galactosidase Escherichia coli (βgal) and CHCA mixture was also spotted on MALDI plate and used as a known standard for each set of MS analysis. Peptides were analyzed by MALDI-TOF/TOF.
MALDI-TOF-TOF

All the proteomics work reported in this paper was carried out at the central proteomics facility of National Institute for Research in Reproductive Health by the first two authors. Mass spectrometry analyses were performed using the Applied Biosystems 4700 Proteomics Analyzer (MALDI-TOF–TOF; Foster City, CA, USA) in reflector mode for positive ion detection. The laser wavelength was 355 nm. All the MS spectra resulted from accumulation of at least 1000 laser shots. MALDI target plate was internally calibrated and default updated using the [M + H] ion from 4700 proteomics analyzer calibration mixture (4700 Cal Mix, Applied Biosystems) as per the manufacturer’s instructions. βgal (β-galactosidase digested), a known standard, was used to validate mass accuracy of mass spectrometer. In case of samples, the mass spectra were calibrated using the three trypsin auto digest products: fragment 92-99 ([M + H]+ = 805.416 Da), fragment 50–69. ([M + H]+ = 2163.056 Da), and fragment 70–89 ([M + H]+ = 2273.159 Da). Most of the observed peptide peaks of trypsin autolysis and keratin were validated and subsequently excluded from monoisotopic precursor ion list generated for tandem MS/MS analysis. A maximum of the 25 strongest precursor ions per sample were chosen for tandem MS/MS analysis. In the TOF1 stage, all ions were accelerated to 1 kV under conditions promoting metastable fragmentation. The peak detection criteria used were; S/N of 10 and local noise window width of 200 (m/z).

Mass spectrometric data analysis

Combined MS and MS/MS spectra were used to search against the taxonomy Mammalia (mammals) in the MSDB Release 20063108; 3239079 sequences; 1079594700 residues) using the GPS software (version 3.5, Applied Biosystems) running Mascot search algorithm (version 2.0, Matrix Science, Boston, MA, USA) for peptide and protein identification. Searches were performed to allow for carbamidomethylation (C), oxidation (M), trypsin as an enzyme, and a maximum of one missed trypsin cleavage. A mass tolerance of 100 ppm and 0.25 Da (m) for precursors and fragment ions respectively. Known contaminant ions (keratin) and autolysed trypsin peaks were excluded. The confident identification had statistically significant (P ≤ 0.05) protein score (based on combined MS and MS/MS spectra) and best ion score (based on MS/MS spectra). Redundancy of proteins that appeared in the database under different names and accession numbers was eliminated.

Design and synthesis of peptides

All eight proteins identified as novel (Tables 1 and 2) in this study were further validated by peptide ELISA. The sequences of those eight proteins were subjected to determine antigenic peptides using tools available at http://immunax.dfc..harvard.edu/Tools/antigenic.pl based on the method of Kolaskar & Tongaonkar (1990). These predicted peptides that matched with peptides obtained by sequencing were synthesized at commercial USV peptide synthesis facility (India). Two to four peptides were synthesized for each of the proteins.

Peptide ELISA

Peptides of the individual proteins were pooled together to perform peptide ELISA. The protocol followed was as described earlier (Jagtap et al. 2007). Briefly, peptides of the individual proteins with concentration 2 μg each were adsorbed overnight at 4 °C in carbonate buffer (pH 9.0). The nonspecific binding sites were blocked using 1% (w/v) gelatin in PBS (pH 7.4) for 2 h at 37 °C. PT and PI sera from the group HI/HS/FI/FS of animals diluted 1:50 and 1:25 in PBS were incubated overnight at 4 °C. The wells were then washed five times with PBS containing 0.05% (v/v) Tween20 (PBS–T20). Rabbit anti-mouse secondary antibody conjugated HRP diluted 1:1000 in 1% (w/v) PBS was added and incubated for 1 h at 37 °C. Finally, wells were washed with PBS–T20, prior to incubation with 100 μl 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. The reaction was terminated by addition of 2 M H2SO4 and the color was measured at 450 nm on Universal Micro plate Reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Each sample was assayed in duplicate.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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