SAMP14, a Novel, Acrosomal Membrane-associated, Glycosylphosphatidylinositol-anchored Member of the Ly-6/Urokinase-type Plasminogen Activator Receptor Superfamily with a Role in Sperm-Egg Interaction*

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We report a new member of the Ly-6/urokinase-type plasminogen activator receptor (uPAR) superfamily of receptors, SAMP14, which is retained on the inner acrosomal membrane of the human spermatozoan following the acrosome reaction and may play a role in fertilization. The SAMP14 sequence predicted a glycosylphosphatidylinositol (GPI)-anchored protein with a signal peptide, a transmembrane domain near the carboxyl terminus, and a putative transamidase cleavage site in the proprotein. Attachment of SAMP14 to the membrane by a lipid anchor was confirmed by its sensitivity to phosphatidylinositol phospholipase C. SAMP14 has a single functional domain similar to the Ly-6 and urokinase plasminogen activator receptor superfamilies of proteins, and the gene mapped to 19q13.33, near the PLAUR locus for uPAR at 19q13.2. Northern and dot blotting showed that SAMP14 expression was testis-specific. Indirect immunofluorescence and immunoelectron microscopy with antisera to purified recombinant SAMP14 localized the protein to outer and inner acrosomal membranes as well as the acrosomal matrix of ejaculated human sperm. Acrosome-reacted sperm demonstrated SAMP14 immunofluorescence, indicating its retention on the inner acrosomal membrane following the acrosome reaction. However, SAMP14 localized to the entire sperm when unwashed swim-up sperm from the ejaculate were stained, indicating that some SAMP14 is loosely associated with the plasma membrane. Antibodies against recombinant SAMP14 inhibited both the binding and the fusion of human sperm to zona free hamster eggs, suggesting that SAMP14 may have a role in sperm-egg interaction. SAMP14 represents a GPI-anchored putative receptor in the Ly-6/uPAR family that is exposed on the inner acrosomal membrane after the acrosome reaction.

Members of the Ly-6/urokinase-type plasminogen activator receptor (uPAR) family possess one or several repeats of a 100-amino acid Ly-6/uPAR domain that is defined by conservation of distinct disulfide bonds between 8 or 10 cysteine residues (1). The family can be divided into two subfamilies. One comprises GPI-anchored glycoprotein receptors with 10 cysteine residues. Members of this subfamily include uPAR, CD59, the Ly-6 family, thymocyte B cell antigen, and prostate stem cell antigen. Among these proteins, uPAR contains three tandem copies of the Ly-6/uPAR domain, whereas the others contain a single domain (2). The second subfamily contains snake and frog cytotoxins, principally three-finger neurotoxins. Its members lack the GPI-anchoring signal sequence and possess only eight cysteines. The Ly-6/uPAR receptors mediate several physiological functions including cellular activation, macrophage migration, trophoblast implantation, angiogenesis, and tumor cell invasion (3, 4).

During mammalian fertilization, the acrosomal and plasma membranes of the sperm interact with the egg vestments. Sperm surface molecules on the plasma membrane are involved in the recognition of the zona pellucida protein (ZP3) during the early process of sperm egg interaction (primary binding) (5). Following sperm binding to the egg the acrosomal membranes are involved in a critical cascade of events that includes the following: 1) the acrosome reaction, in which the outer acrosomal membrane fuses with the plasma membrane; 2) secondary binding of the inner acrosomal membrane to the zona pellucida; and 3) tertiary binding of the equatorial segment and subsequent fusion to the vitelline membrane. After the acrosome reaction, the inner acrosomal membrane becomes the limiting membrane on the external surface of the anterior principal acrosomal segment, whereas the plasma membrane remains continuous with the outer acrosomal membrane at the anterior tip of the equatorial segment (6). The acrosome contains many enzymes including proteases, such as acrosin (7), esterases, acid phosphatases, glycohydrolases, and aryl sulfatases. Enzymes released from the acrosome are believed to play an important role in digesting the zona and paving the way for penetration of the egg vestments by the sperm (8). Knowledge of the components of the acrosomal membranes and matrix is essential to understanding the biology of acrosomal

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF353721.

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‡ These abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; EST, expressed sequence tag; PIMP, phosphatidylinositol-specific phospholipase C; Na, amino acid(s); recSAMP14, recombinant SAMP14; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; NHS, N-hydroxysuccinimide.
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ExPERIMENTAL PROCEDURES

Semen Samples—All semen samples for this study were obtained under informed consent using forms approved by the University of Virginia Human Investigation committee.

Animals—We used adult female Sprague-Dawley virgin rats of 6–9 weeks for immunization. All experiments were performed in accordance with the Guidelines for the Use and Care of Laboratory Animals (National Institutes of Health Publication 85-23).

Surface Labeling, Phase Partitioning, and Two-dimensional Gel Electrophoresis—Human semen samples were obtained from healthy donors as described earlier (11). Triton X-114 phase partitioning was employed to enrich potential membrane proteins from human spermatozoa. This protein, named SAMP14 (sperm acrosomal membrane-associated protein 14) on the basis of its localization, appears to be a member of the Ly-6 and urokinase plasminogen activator receptor family and play a role in sperm-egg binding and fusion.

One-dimensional SDS-PAGE and Western Blot—Protein electrophoresis was performed on discontinuous polyacrylamide gels (4% stacking and 15% separating gel) on a Bio-Rad minigel apparatus or in 0.15-cm-thick 16 × 16-cm slab gels. Gels were stained with Coomassie Blue or blotted. For Western blots, proteins were transferred to nitrocellulose membrane and processed as described previously (10).

Reverse Transcription-PCR—Oligonucleotide primers designed from an expressed sequence tag (EST) (AA778671) were manufactured by Invitrogen. The forward primer (5′-TACCATATTTCTAGGACTACTAAAGTG-3′) and reverse primer (5′-CTGGGTCTAAGCAAAAAGGTCGCG-TG-3′) were utilized to generate a 475-bp probe. For subsequent PCRs, human testicular RNA (Clontech, Palo Alto, CA) was reverse transcribed as described by Wolkowicz et al. (14). 1-μl aliquots of the cDNA solution were amplified for 40 cycles with denaturation at 94 °C, annealing at 50 °C for 30 s, and extension at 68 °C for 3 min, as specified by the manufacturer of the recombinant Thermophilus thermophilus polymerase (PerkinElmer Life Sciences). Sequencing was performed by the University of Virginia Biomolecular Research Facility.

Northern and Dot Blot Analysis—Human multiple tissue northern membranes containing eight tissues and multitissue array RNA dot blots containing 76 tissues were purchased from Clontech. The PCR-amplified 172-bp cDNA corresponding to the open reading frame of SAMP14 was used as a probe for both analyses. Both multiple tissue Northern and multitissue array RNA dot blot analysis were done as described previously (15).

Screening of a cDNA Library—A DR2 cDNA library from human testis was purchased from Clontech and screened according to the user manual and as described earlier (10). A 475-bp cDNA fragment was PCR-amplified from the human testicular cDNA using two primers based on GenBank™ sequence AA778671 and used to probe filters. Probes were labeled with [32P]deoxycytidine triphosphate by the random primer method using an oligolabeling kit (Amer sham Biosciences).

Filters were washed once in 2× SSC, 0.2% SDS at 22 °C, once in 0.2× SSC, 0.2% SDS at 42 °C; and once in 0.2× SSC, 0.2% SDS at 50 °C, each for 20 min. After washing, filters were exposed to x-ray film at −80 °C for 24–48 h. Secondary screening was repeated as above to isolate single, pure phage plaques. Finally, the cDNA inserts were recovered from the phage particles as plasmids in the pBR2 vector by transforming the Escherichia coli, AM1 strain. Sequencing was performed by the University of Virginia Biomolecular Research Facility in both directions, and the nucleotide and amino acid data were analyzed using the Genetics Computer Group and SEQWeb (Madison, WI) program.

Expression of Recombinant Protein and Production of Antiserum—To express SAMP14 in E. coli, a DNA fragment encoding amino acids (aa) 20–100 (lacking both the putative signal peptide and COOH-terminal domain) of the open reading frame was amplified by PCR. The amplified fragment was fused in frame with His tag at the COOH terminus of PET 28b using the Ncol and XhoI sites. The plasmid was then transformed into the host strain E. coli BL21 DE3. The recombinant protein was affinity-purified on a Ni2+-nitrilotriacetic acid column under denaturing conditions (16). The recombinant protein was further purified on a model 491 Prep Cell (Bio-Rad) to a single band, which was verified by SDS-PAGE and used for immunizations.

Female virgin Lewis rats (three rats per each group), weighing 160–200 g, were injected with purified recombinant SAMP14 (recSAMP14) in PBS emulsified with equal amount of Freund’s complete adjuvant. Each rat received subcutaneously 100 μg of protein in 0.3 ml of PBS for the primary injection and each subsequent injection 3 and 6 weeks later. Antibody titers were checked by Western blot analysis 10 days after each injection, and the animals were sacrificed 10 days after the last boost. Specificity of the rat antisera for SAMP14 was tested by Western bloting against recSAMP14 as well as human sperm protein extracts.

Immunofluorescence and Electron Microscopic Analysis—Spermatozoa were purified by the swim-up method as described by Mandal et al. (16) and were air-dried onto poly-L-lysine-coated slides (Polysciences, Warrington, PA). Following three washes with PBS for 10 min each, slides were blocked in PBS containing 10% normal goat serum for 1 h with or without 0.1% saponin for permeabilization. The sperm were then incubated with a 1:200 dilution of anti-SAMP14 primary anti-serum or preimmune serum for 1 h at 22 °C, followed by washing in PBS three times for 10 min each at 22 °C. Following incubation with the secondary goat anti-rat IgG conjugated with TRITC at 1:100 dilution for 1 h, the slides were washed in PBS, incubated for 5 min with equilibration buffer followed by Slow Fade (Molecular Probes, Eugene, OR), and mounted with coverslips. Images were captured with an Axiovision fluorescence microscope (Zeiss, Oberkochen, Germany).

Processing of pooled sperm for electron microscopy was done as described earlier (15). To stain the sections, they were first blocked in undiluted normal goat serum for 15 min at 22 °C. They were then incubated for 16 h at 4 °C with either preimmune or rat anti-SAMP14 primary antibody dilution 1:500 in 3% normal goat serum albumin, and 0.1% Tween 20. After washing, the sections were incubated with a 1:100 dilution of 5-nm gold-conjugated goat-anti-IgG (Goldmark Biologicals, Phillipsburg, NJ) for 1.5 h at 22 °C. The sections were washed in distilled water, stained with uranyl acetate, and ob-
overnight capacitation, 2 mg/ml human serum albumin (Sigma) in 15-ml centrifuge tubes. Sperm were capacitated at a concentration of 20 million sperm heads in 8-ml volumes of BWW medium containing 5 mg/ml human serum albumin (Sigma) for 15-ml centrifuge tubes. Sperm were capacitated at a concentration of 20 × 10^6 sperm/ml overnight in 250-µl drops of BWW containing 30 mg/ml human serum albumin. Hamster ova were obtained from Golden Syrian hamsters by superovula-
tion (18). Cumulus cells were removed by treating eggs with 0.05% hyaluronidase (Sigma) for 3 min. The oocytes were then pooled and washed through 20-µl drops of medium using a pulled, heat-polished Pasteur pipette. Zona pellucidae were removed by treating eggs with 1 mg/ml trypsin for 30 s followed by five washes. The eggs were then randomly allotted into treatment groups.

Hamster Egg Penetration Assay—Gamete incubations were carried out in microdrops under paraffin oil at 37 °C and 5% CO2. The swim-up sperm prepared as described above were washed twice by centrifuga-
tion (8 min at 600 × g) in 8-ml volumes of BWW medium containing 5 mg/ml human serum albumin (Sigma) in 15-ml centrifuge tubes. Sperm were capacitated at a concentration of 20 × 10^6 sperm/ml overnight in 250-µl drops of BWW containing 30 mg/ml human serum albumin. Hamster ova were obtained from Golden Syrian hamsters by superovula-
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Rat serum IgG was isolated using Nab™ protein G spin chromatog-
raphy kit (Pierce) according to the manufacturer’s protocol. Protein concentration was measured by reading the OD at 280 nm. Following incubation, 2 mL of sperm suspension was treated for 1 h in a 20-µl drop containing 100, 50, 20, 10, or 5 µg/ml of IgG from rat anti-SAMP14 serum in BWW containing 30 mg/ml human serum albumin. One hundred micrograms/ml of preimmune IgG was used as a negative control and 100 µg/ml of SAMP32 (15) IgG was used as a positive control. Following sperm/antibody incubation, zona free hamster oocytes were added directly to the sperm suspension, and the gametes were co-
incubated for 3 h. Following gamete co-incubation, loosely bound sperm were removed from the oocytes by gentle pipetting.

The eggs were then treated with 1 mM acridine orange in 3% Me2SO (Sigma) for 15 s to stain the chromatin and washed through three 20-µl drops. To quantitate binding, oocytes were placed between a mi-
crodrop and an elevated coverslip, and the number of sperm bound per oocyte was recorded. The number of sperm fused per egg was scored by counting the number of acridine orange-stained decondensed sperm heads within each oocyte using fluorescent microscopy. The assay was repeated at least three times for each concentration of IgG used. Specific effect of the antibody was verified by preabsorbing the antibody with an excess amount of recombinant SAMP14. Experimental and control group averages were reported as means ± S.E. Student’s t test was used to identify differences in the mean numbers of bound or fused spermatozoa. p < 0.05 was considered to be statistically significant.

Treatment of Spermatozoa with PIPLC—Percol-harvested sperm (200 × 10^6) were incubated with 1.0 unit/10^8 sperm/ml of PIPLC from Bacillus cereus (Molecular Probes, Inc., Eugene, OR) without enzyme for 20 min at 22 °C. At the end of the incubation time, sperm were pelleted by centrifugation at 1000 × g for 30 min. To prevent proteol-
ysis, 2 mM phenylmethylsulfonyl fluoride and 5 mM EDTA were added to collect supernatants, which were collected and dialyzed against distilled water at 4 °C overnight. The dialyzed solution was concentrated by lyophilization, and the proteins were reconstituted in Celsi lysis reagent (19) before loading for isoelectric focusing.

RESULTS

Phase Partitioning and Surface Labeling—Among several proteins that were selectively partitioned into the Triton X-114 detergent phase, a major protein spot of 14 kDa and pI 4.8 was identified on an avidin-ECL blot of human sperm proteins after surface-labeling the sperm with sulfo-NHS-LC-biotin. C, two-dimensional blot of human sperm proteins probed with rat serum against purified recSAMP14. The 14-kDa region (pI 4.8) originally microsequenced bound the antibody (arrow). In addition, the antibody reacted with several spots on the basic and acidic sides of the original excised spot, indicating the existence of SAMP14 charge vari-
ants in sperm. The preimmune serum did not react with any proteins (data not shown). D and E, two-dimensional gel electrophoresis followed by Western blotting of supernatants from human sperm treated with PIIPLC using rat anti-recSAMP14. Sperm purified by swim-up were separated into two groups (150 × 10^6 sperm/group) and treated with or without 3 units/10^8 sperm of PIPLC. The control group that was not exposed to PIPLC (D) released a small amount of one isoform of immunoreactive SAMP14 into the medium. However, treatment with PIPLC (E) released additional SAMP14 isoforms into the medium in-
cluding several more charge variants (arrowheads). An acidic shift in the pH of the PIPLC-released isoforms accompanies loss of the GPI anchor (arrowheads in E correspond to the location of different isoforms in C).
sequence embedded within and a contiguous open reading frame encoding 124 amino acids (Fig. 2). The predicted molecular mass and pI of 13 kDa and 5.4, respectively, closely matched the molecular weight and pI of the excised spot. The peptide microsequence ATSCGLEEPVSYR obtained by mass spectrometry was present in the cloned protein. A comparison of the deduced sequence with the GenBank™ data base using BLAST and FASTA as well as Prosite and Prodom revealed that the predicted protein had the closest amino acid similarity to proteins from the Ly-6 and uPAR superfamily. Fig. 3 shows a multiple sequence alignment of SAMP14 and other Ly-6/uPAR superfamily members with highest homology to SAMP14. One of the unique features of these molecules is the presence of a cysteine-rich domain that is conserved among all of the members (see asterisks). The highly conserved motif Cys-Cys-Xaa-Xaa-Asp-Leu-Cys-Asn, which is a signature of proteins belonging to this family, was located at the COOH terminus of many of these sequences, including SAMP14. Alignment of SAMP14 with three tandem Ly-6 domains of uPAR (uPAR-1, uPAR-2, and uPAR-3) showed that two residues (tyrosine and leucine) located in the uPAR-1 functional epitope involved in ligand binding (20) are conserved in SAMP14, suggesting that SAMP14 may be closely related to uPAR.

**SAMP14 Is a Predicted Membrane Protein Putatively Anchored through a GPI Moiety** — Running the algorithms provided in the Simple Modular Architecture Tool on the ExPASy server revealed several features: 1) a signal peptide cleavage site.
Expression of SAMP14 and Western Blot Analysis—The cDNA sequence encoding a processed SAMP14 protein from residue 20 to 100 (lacking the signal peptide and putative transmembrane domain) was cloned into the bacterial expression vector pET28a. After induction with isopropyl-1-thio-

galactopyranoside for 4 h, recSAMP14 with a molecular size of 

$10.5 \text{kDa}$ was produced (data not shown). The recombinant protein was affinity-purified with a Ni$_2^+$-nitrilotriacetic acid column under denaturing conditions. The affinity-purified recSAMP14 was further purified on a Prep Cell and used as an immunogen to inoculate rats. The resulting antibody to recSAMP14 recognized the "native" SAMP14 present in sperm extracts. When the two-dimensional blots containing human sperm Triton X-114 detergent phase extracts were probed with recSAMP14 antibody (Fig. 1C), the protein spot that was cored for microsequencing was recognized. The antiserum also recognized several spots on either side of the original cored spot, which are probably charge variants of SAMP14. Interestingly, these "charge variants" were poorly stained on a silver-stained gel (see Fig. 1A), indicating that they may be present in low abundance.

SAMP14 Is Associated with the Human Sperm Acrosome—Indirect immunofluorescence analysis of fresh air-dried, ejaculated swim-up human spermatozoa using rat serum against recSAMP14 localized SAMP14 to the entire sperm when sperm were washed only once after the swim-up procedure (Fig. 5B). However, when the sperm were washed at least 3–5 times after the swim-up, the immunofluorescent staining was restricted to the acrosomal region (Fig. 5P) with some sperm showing more intense staining at the equatorial region (arrows). This experiment suggested that some of the antigen is loosely associated with the sperm surface and removed during the washing procedure.
Double staining showed that acrosome-reacted sperm, as determined by more intense staining with ConA (Fig. 6B, boxed sperm), also showed a cap-shaped reactivity with recSAMP14 antibody (Fig. 6C, boxed sperm). This suggested that a substantial portion of SAMP14 is intra-acrosomal and that a population of SAMP14 is retained on the inner acrosomal membrane of acrosome-reacted sperm. At the ultrastructural level, the antisera stained mainly the outer and inner acrosomal membranes, although some gold particles were also seen in the acrosomal matrix (Fig. 7A). In the majority of the cases, the gold particles were lined up along the inner acrosomal membrane when acrosome-reacted sperm were examined (Fig. 7B).

PIPLC Releases SAMP14 from Human Sperm—Bioinformatic analysis of SAMP14 predicted a protein anchored to a membrane through a GPI moiety. To test this prediction, a biochemical assay was performed in which fresh ejaculated sperm were harvested by swim-up and treated with PIPLC to see whether the enzyme would result in cleavage and release of a population of SAMP14. Fig. 1E shows the two-dimensional Western blot analysis using anti-recSAMP14 antibody on the supernatant obtained after treatment of the sperm with PIPLC. Compared with the untreated control (Fig. 1D), PIPLC treatment released more SAMP14, including several charge variants not evident in the controls (Fig. 1E), supporting the hypothesis that at least three of the SAMP14 isoforms are GPI-anchored. Further, a shift in the pI of the released forms was noted by comparing the PIPLC-treated blot (Fig. 1E) to immunoblots of control (Fig. 1D) and whole (Fig. 1B) extracts of sperm, indicating that after loss of the lipid anchor the variants migrate further toward the acidic end of the gel.

Rat Anti-recSAMP14 Serum Blocks the Penetration of Zona Free Hamster Egg by Human Sperm—To assess the possible role(s) of SAMP14 in sperm-egg interaction during fertilization,
we tested whether anti-recSAMP14 IgG would block penetration of zona free hamster eggs by capacitated human sperm (Fig. 8). A rat antibody to SAMP32 (15), an acrosomal antigen previously shown to inhibit the hamster egg penetration test, was used as a positive control in the assay. This control showed a significant inhibition of binding (48%) and fusion (57%) to the eggs at 100 \( \mu \text{g/ml} \) concentration. Significant effects on binding were observed at 100, 50, and 20 \( \mu \text{g/ml} \) rat anti-SAMP14 IgG and on fusion at 100 and 50 \( \mu \text{g/ml} \). This dependence on antibody dose suggests specificity of the inhibition. In three independent assays, for example, the antibody suppressed sperm binding to hamster eggs by roughly 47% \((p < 0.001)\) at the 50 \( \mu \text{g/ml} \) concentration, and blocking of sperm fusion was more pronounced at about 63% inhibition \((p < 0.001)\). The inhibition of binding was completely abolished when the antibody was preincubated with a 10-fold excess of recombinant protein.

**DISCUSSION**

The results of this study indicate that SAMP14 is an acrosomal membrane-associated member of the GPI-anchored Ly-6/uPAR family of glycoprotein receptors. In its NH\(_2\) terminus, SAMP14 contained a single Ly-6/uPAR domain including 10 cysteine residues. At the COOH end of this domain, the highly conserved sequence of this family, Cys-Cys-Xaa-Xaa-Asp-Leu-Cys-Asn, was present with arginine substituted for aspartic acid at position 94. PIPLC treatment released a series of
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SAMP14 charge variants, indicating that several SAMP14 isoforms are GPI-anchored. This finding is in concert with the presence of a canonical transamidase cleavage site, a characteristic of GPI-anchored proteins, between amino acids 97 and 98.

In humans, several members of the Ly-6 family containing a single Ly-6/uPAR domain map to chromosome 8, whereas the uPAR (CD87) locus PLAUR, encoding three tandem Ly-6/uPAR domains, is found at 19q13.2 (22). SAMP14 mapped to the SPACA4 locus at 19q13.33, close to the PLAUR locus. Further, the transcript size of SAMP14, as shown by Northern blot analysis, is ~1.3 kb. This is larger than other human Ly-6 family members such as E48 with a transcript size of 0.8 kb (23) or prostate stem cell antigen with a transcript size of ~1 kb (24) and is closer to the uPAR transcript of 1.4 kb (25). Last, 2 of the 4 residues of the functional epitope of uPAR that were previously identified to be involved in ligand binding (20) are also conserved in the Ly-6 domain of SAMP14. All of these observations suggest that SAMP14 is more closely related to uPAR than other family members, and functions assigned to uPAR should be considered in hypotheses regarding the role of SAMP14 in acrosomal biology. uPAR binds to urokinase plasminogen activator through the NH2 terminus (2), the region conserved in SAMP14. uPAR has a role in generating proteolytic activity that is spatially restricted to the plasma membrane, is involved in cell-cell and cell matrix interactions, and participates in signal transduction events (26). Of further interest is the fact that neutrophils contain reservoirs of uPAR in intracellular granules that are translocated to the plasma membrane upon activation (27), a process that may be considered analogous to the exposure of SAMP14 on the inner acrosomal membrane following the acrosome reaction. Together these considerations suggest that SAMP14 may function as an acrosomal membrane receptor in the orphan class, with its ligand yet undefined, and may participate in proteolytic or adhesive events involving the acrosomal membrane.

A search of the Riken mouse genome data base revealed a putative orthologous gene at chromosome 7B2 and a cDNA from mouse testis. Comparison of the mouse and human SAMP14 sequences suggests that both have a Ly-6/uPAR domain with a potential GPI anchor at the COOH terminus, and evolutionarily there is little change in this sequence. The tight linkage of all other murine Ly-6 proteins on chromosome 15 in contrast to the location of murine uPAR on chromosome 7, which is syntenic to human chromosome 19, further confirms that SAMP14 is closely related to uPAR.

One GPI-anchored Ly-6 family member, CD59, has been identified previously on the human sperm surface (28). In lymphocytes, CD59 is a multifunctional protein involved in complement regulatory activity and co-stimulatory activity for human T cells mediated via the CD2 T cell antigen. Apart from having a probable function of protection of sperm from complement-mediated damage, CD59 is also predicted to be involved in cell-cell adhesion during gamete interaction (29). Another Ly-6 family member that is specifically expressed in testis is SP-10, an intra-acrosomal protein that is involved in secondary binding to the egg (30–32). Among acrosomal membrane proteins, PH-20 is the only GPI-anchored protein characterized previously (33–38). On acrosome-intact human sperm, PH-20 is located on the plasma membrane over the entire head. However, after the acrosome reaction, PH-20 is also located on the inner acrosomal membrane (38). There are also several GPI-anchored proteins reportedly acquired by the sperm membrane during post-testicular transport. Among them is CD52, an antigen that is secreted by epithelial cells of the distal epididymis and ductus deferens and acquired by sperm during their passage through the tract (39, 40). Several other GPI-anchored antigens found on the cells of the immune system (namely CD62, CD55, and CD73) are also found anchored to the sperm surface (41). The functions of these GPI-anchored molecules in sperm is largely unknown.

A Northern blot of eight human tissues initially indicated that the RNA transcript is about 1.3 kb long and that SAMP14 is expressed only in testis. This testis specificity of SAMP14 expression was further reinforced by our comprehensive study of RNA expression by RNA dot blot hybridization to 76 human tissues. In agreement with this designation of SAMP14 as a testis-specific gene, EST data base searches during the re-

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IgG from anti-SAMP32 (15) serum at a 100 µg/ml concentration was used as a positive control. Bars represent means ± S.E. of 3–5 individual experiments. n = total number of oocytes per group. *, p ≤ 0.05; **, p ≤ 0.001 (Student’s t test).

Antibody to recSAMP14 reacted with the original protein spot cored for peptide sequencing on two-dimensional Western blots. This result, coupled with the finding of one identical microsequence in the protein sequence derived from the cloned cDNA, indicates that the protein corresponding to the microsequenced spot has been successfully cloned. However, lack of the other microsequence in the predicted protein sequence suggests that there may be another protein co-migrating with SAMP14. The antiserum to SAMP14 recognized several additional bands on the basic and acidic sides of the original cored spot that appear to be charge variants of SAMP14, suggesting that post-translational modifications of SAMP14 are likely. Based on the two-dimensional gels of PIPLC-treated sperm, at least three SAMP14 isoforms appear to be GPI-anchored. These were not visualized on a Coomassie- or silver-stained gel, indicating that they are present in lower amounts compared with the major form of SAMP14 at pI 4.8.

In the initial experiments, SAMP14 was enriched in the detergent phase, suggesting that it is a hydrophobic protein and thus possibly associated with a membrane. Upon cloning the cDNA, a putative transmembrane domain (aa 101–124) was found in the predicted protein, and bioinformatic analysis of this domain revealed a potential transamidase cleavage site predicting a GPI-anchored protein. Further, SAMP14 was found to be sensitive to PIPLC, confirming that it is a GPI-anchored protein, as are several other members of the Ly-6/uPAR family. However, treatment with PIPLC did not release all SAMP14, since some of it remained in the sperm pellet following the separation of the supernatant (data not shown), suggesting that 1) there may be a population of GPI-anchored SAMP14 that is resistant to PIPLC; 2) all of the SAMP14 is not accessible to the enzyme; or 3) a non-GPI-anchored population exists. The presence of some soluble SAMP14 in the control samples in the PIPLC release studies suggests a non-GPI-anchored population of SAMP14. Regarding the presence of PIPLC-resistant SAMP14, it is well documented that many of the GPI-anchored proteins have a PIPLC-resistant subpopulation (42).

Immuno fluorescence studies of ejaculated sperm, immunolocalization on enzyme-dissociated testicular cells, and electron micrographic studies all showed that SAMP14 is mainly located in the acrosome. However, indirect immuno fluorescence study of unwashed, air-dried sperm showed the presence of the protein on the entire surface of the sperm. There are at least two possible explanations for this surface staining. First, SAMP14 might be produced in another location along the male reproductive system so that it coats the sperm when they traverse the tract. Second, SAMP14 may be expressed only in the acrosome, but some of the protein may leach into the seminal plasma due to sperm damage or during spontaneous acrosome reactions. In any case, coating the sperm with SAMP14 in this way is in concert with the known adhesive property of many members of the Ly-6 and uPAR family of proteins. The labeling of the SAMP14 spot with biotin may be due to this population of SAMP14 on the plasma membrane.

The hamster egg penetration assay demonstrated that antibody against SAMP14 significantly inhibited both the binding and fusion of sperm with zona free hamster eggs, which suggests that SAMP14 may have a role in one or more events leading to fertilization. It is well known that the equatorial region of the sperm contacts the vitelline membrane prior to sperm internalization. Further, following the acrosome reaction, the inner acrosomal membrane is known to come directly in contact with the egg, leading to the later events of egg penetration. Considering that the concentrations of SAMP14 were highest in the posterior acrosome close to the equatorial region and that it is retained and localized in the inner acrosomal membrane, it is not surprising that the antibody to SAMP14 inhibited both sperm-egg binding and fusion. It is appropriate to add here that another acrosomal membrane protein SAMP32, which was discovered recently (15), also localized to the inner acrosomal membrane of the principal piece and equatorial segment. Further, anti-SAMP32 blocked the penetration of zona free hamster eggs by human sperm.

Although a large number of potential candidate binding partners for sperm-egg interaction have been described, including galactyltransferase, Sp56, zona receptor kinase, spermadhesins, PH20, proacrosin, Sp38, and Sp17, the precise roles of these molecules have not been firmly established (43). Hence, the identification of additional molecules that participate in gamete interactions, particularly those retained after the remodeling events of acrosomal exocytosis, could greatly enhance the understanding of this process. SAMP14 is an attractive addition to this group. SAMP14 may be a signaling molecule like PH20 (44), or a SAMP14 ligand may exist on the oolemma. By analogy to uPAR, SAMP14 may play a role in spatial restriction of proteolytic processes at the surface of the inner acrosomal membrane. As a Ly-6/uPAR family member,
SAMP14 merits further evaluation as a GPI-linked receptor that is retained on the inner acrosomal membrane after acrosomal exocytosis.

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