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Clusterin Facilitates Exchange of Glycosyl Phosphatidylinositol-Linked SPAM1 Between Reproductive Luminal Fluids and Mouse and Human Sperm Membranes[1]

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ABSTRACT

Glycosyl phosphatidylinositol (GPI)-linked proteins, which are involved in post-testicular maturation of sperm and have a role in fertilization, are acquired on the sperm surface from both vesicular and membrane-free soluble fractions of epididymal luminal fluid (LF) and uterine LF. Herein, we investigate the mechanism of uptake of these proteins from the soluble fraction of LFs using sperm adhesion molecule 1 (SPAM1) as a model. Ultracentrifugation and native Western blot analysis of the soluble fraction revealed that SPAM1 is present in low-molecular-weight (monomeric) and high-molecular-weight (oligomeric) complexes. The latter are incapable of transferring SPAM1 and may serve to produce monomers. Monomers are stabilized by hydrophobic interactions with clusterin (CLU), a lipid carrier that is abundantly expressed in LFs. We show that CLU is involved in the transfer of SPAM1 monomers, whose delivery was decreased by anti-CLU antibody under normal and apolipoprotein-enhanced conditions. Coimmunoprecipitation revealed an intimate association of CLU with SPAM1. Both plasma and recombinant CLU had a dose-related effect on transfer efficiency: high concentrations reduced and low concentrations enhanced delivery of SPAM1 to human and mouse sperm membranes, reflecting physiological states in the epididymal tract. We propose a lipid exchange model (akin to the lipid-poor model for cholesterol efflux) for the delivery of GPI-linked proteins to sperm membranes via CLU. Our investigation defines specific conditions for membrane-free GPI-linked protein transfer in vitro and could lead to technology for improving fertility or treating sperm pathology by the addition of relevant GPI-linked proteins critical for successful fertilization in humans and domestic animals.

female reproductive tract, gamete biology, GPI anchor, GPI-linked proteins, lipid carriers, male reproductive tract, membrane modification, sperm, sperm hyaluronidase, sperm maturation

INTRODUCTION

During post-testicular maturation of sperm, a variety of glycosyl phosphatidylinositol (GPI)-linked proteins are added to the surface of the sperm as they traverse the male and female tract [1–3]. These proteins include receptors and immunoprotection molecules, as well as enzymes that participate in both sperm maturation and the fertilization processes [2, 3]. Most important, the proteins have a role in the remarkable degree of sperm surface modification and remodeling that occur during epididymal maturation and capacitation [2]. One GPI-linked protein that is present in both the epididymal and the uterine tract [2, 4, 5] and has been shown to be added to sperm in vitro is sperm adhesion molecule 1 (SPAM1) [2, 6]. Widely conserved and the major sperm surface hyaluronidase [7], SPAM1 is a multifunctional protein with essential roles in primate fertilization [8]. The effect of the in vitro uptake of SPAM1 by caudal mouse sperm from epididymal luminal fluid (ELF) and uterine luminal fluid (ULF) indicates that it is a marker of sperm maturation [3, 6]. Epididymal and uterine SPAM1, which is present during proestrus and estrus [5], are therefore ideal candidates for investigating the mechanisms of GPI-linked protein transfer to the sperm surface.

GPI-linked proteins are known to exist in luminal fluids (LFs) in both vesicular and soluble membrane-free fractions [9]. Recently, we investigated the mechanism of SPAM1 uptake by caudal mouse sperm from the vesicles of ELF (epididymosomes) and ULF (uterosomes) and showed that, while uptake occurs from both fractions, it was more efficient from the soluble fraction [10]. Similar to ELF and ULF, the fluid phase of the seminal plasma is known to carry GPI-linked proteins such as CD59, CD55, and CD52, which are transferred to the human sperm surface [11]. Also, soluble GPI-linked prion protein has been reported to be added to the sperm surface from the seminal plasma at ejaculation [12]. While it has been shown that vesicle docking mediates transfer from the vesicular fraction of LFs [10], the mechanism involved in the uptake of GPI-linked proteins from the liquid phase of any reproductive fluid has not been elucidated. Most important, it is unknown how GPI-linked proteins with their lipid anchors are stabilized and solubilized in the aqueous LFs. We thus considered the existence of ideal lipid carriers in the LFs and the seminal plasma.

Several multifunctional secretory lipoproteins, including clusterin (CLU [also referred to as apolipoprotein J or APOJ]), have been detected in the uterus and epididymis [13, 14]. Although its specific function in the epididymis is still unclear [15], CLU (which is a widely expressed and highly conserved chaperone-like protein) is known to bind both lipids and membrane-active proteins in a variety of body fluids [13, 16]. It is therefore an attractive candidate for solubilizing and stabilizing GPI-linked proteins. It also has been detected on the sperm surface, and epididymal CLU forms complexes with other proteins and/or lipids [16]. In conjunction with its receptor, LRP2, CLU is believed to have a role in the lipid exchange accompanying the remodeling of the sperm plasma membrane during sperm maturation in both the male and the female tract [14]. In the latter, LRP2 was detected in elevated levels during estrus and proestrus [14]. This parallels the secretion of SPAM1 [5], whose coexpression with CLU facilitates their association via hydrophobic interactions. Using SPAM1 as a model, the objectives of the present investigation

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were to determine the mechanism involved in the transfer of membrane-free GPI-linked proteins from the fluid phase of LFs to the sperm surface and the role that CLU may have in mediating the process.

MATERIALS AND METHODS

Animals and Reagents

The studies conform to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (publication 85–23 [revised 1985]) and were approved by the Animal Care Committee at the University of Delaware. The sexually mature 3- to 6-mo-old male and 4- to 6-wk-old female Institute of Cancer Research mice used throughout these studies were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The use of human sperm was approved by the Human Subjects Review Board of the University of Delaware, and informed consent was obtained from the subject studied.

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. The rabbit anti-mouse SPAM1 antiserum used throughout these studies is a polyclonal antipeptide ([C]NEKGMASRRKESSD in the C-terminus [#381–395]) custom made by Zymed (South San Francisco, CA), previously used and shown to be specific for SPAM1 via peptide inhibition [5, 17]. Preimmune serum (PIS) from the antibody (Ab) donor was also obtained from Zymed and used as a control to validate the experiments. The rabbit anti-rat CLU Ab was generously provided by the laboratory of Dr. Michael Griswold (Washington State University, Pullman, WA). The rabbit polyclonal anti-macaque Ab used in these studies to analyze human sperm SPAM1 was generated against the recombinant protein and was generously provided by the laboratory of Dr. James Overstreet (University of California, Davis, CA).

Collection of Epididymal and ULFs and Sperm

Caudal epididymides were finely minced in PBS at 37°C, and sperm were allowed to swim into solution for 10 min. After sperm dispersion in the suspension, tissue fragments were separated by gravity settling. The suspension was then centrifuged at 500 × *g* for 15 min to pellet the sperm without breaking their membranes. This method, ideal for optimal cell recovery while simultaneously retaining sperm motility [18], is used routinely for sperm washing [5, 16, 19]. The resulting fluid was further clarified by centrifugation at 16 100 × *g* for 20 min, and the supernatant was considered ELF. Sperm pellets were washed twice by centrifugation and resuspended in PBS.

ULF was obtained from mice artificially induced into estrus via i.p. injections of 7.5 IU of equine chorionic gonadotropin and 7.5 IU of human chorionic gonadotropin spaced 48 h apart. Uteri were removed 13.5–14 h later and flushed with either human tubal fluid (Chemicon International, Temecula, CA), a known capacitation medium, or PBS. Protein concentrations were obtained using a bicinchoninic acid assay (BCA kit; Pierce, Rockford, IL) after the LFs (2–3 ml) were subjected to centrifugation at 3500 × *g* for 10 min to pellet blood cells and excess tissue.

Collection of Human Spermatozoa

Freshly ejaculated sperm were collected from a fertile 25-yr-old male after a 3-day period of abstinence. Semen samples were allowed to liquefy at room temperature (RT) for 1 h and then placed into 15-ml tubes and layered with Hepes-BWW (Biggers, Whitten, and Whittingham) medium. Samples were incubated at 32°C for 1 h to allow sperm to swim out of the semen into the media. Media were collected and pooled, and the sperm concentration was determined using a hemocytometer.

Characterization of the Physical Nature of LFs by Ultracentrifugation

To separate the vesicular (120P) and nonvesicular (120S) fractions of ELF and ULF, samples were subjected to ultracentrifugation at 120 000 × *g* for 2 h at 4°C using a Beckman (Beckman Instruments, Columbia, MD) Optima L-70K ultracentrifuge and a Ti60 rotor. The supernatant (the soluble fraction) was then characterized via repeated ultracentrifugation steps at 150 000, 190 000, and 230 000 × *g* for 8, 16, and 24 h, respectively. All pellets were resuspended in the initial volume of 5 ml to determine the relative concentration and form of SPAM1 in each fraction. Equal volumes of each sample were subjected to native PAGE and Western blot analysis to determine the relative amounts of SPAM1 within them. The supernatant and pellet after the final 230 000 × *g* spin were subjected to analysis by transmission electron microscopy (TEM) as previously described [10].

Native Western Blot Analysis

Samples of ELF fractions collected via ultracentrifugation were subjected to Western blot analysis to detect the presence of SPAM1. Western blotting was visualized with the WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen, Carlsbad, CA), and all incubations were carried out according to the manufacturer's instructions (at RT, with gentle shaking at ~60 rpm). Samples of ~20–40 μg of protein from each fraction were subjected to 15% PAGE under nonreduced conditions [20] and transferred to a nitrocellulose membrane overnight (200 mA, 4°C, in transfer buffer [20]). Bovine serum albumin (BSA [2% w/v in PBS]) was used as a negative control in one lane of the gel. The membrane was incubated in blocking solution (2% BSA) for 30 min. After decanting the blocking solution, the membrane was rinsed twice in 20 ml of double-distilled (dd) H_2O for 5 min and incubated in 10 ml of SPAM1 antiserum (suspended in 2% BSA blocking solution [1:1000 dilution]) for 1 h. After washing, it was incubated in 10 ml of anti-rabbit IgG secondary Ab solution provided with the kit for 30 min, washed, and rinsed twice in 20 ml of ddH_2O. Signal was detected using the chemiluminescent substrate also provided with the kit.

Efficiency of Transfer of SPAM1 from the Soluble (230S) and Insoluble (230P) Subfractions of the Nonvesicular LF Fractions

Soluble and insoluble subfractions of the nonvesicular fractions of LFs were obtained by subjecting the 120S supernatant to ultracentrifugation at 230 000 × *g*. Caudal sperm were incubated in the supernatant or the pellet (or PBS plus BSA was used as a control) for 2 h at 37°C. After incubation, sperm were immunostained and analyzed for SPAM1 uptake via flow cytometry. Samples were incubated in SPAM1 antiserum (1:400) for 1 h at RT. They were washed thrice in PBS, incubated in fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:400) for 30 min in the dark at RT, and washed thrice in PBS. Samples were then analyzed using an FACSCalibur (Becton Dickinson, San Jose, CA) flow cytometer, which uses an argon laser at 488 nm with detectors for FITC (FL-1), and a CellQuest software package (Becton Dickenson). For each treatment, analysis of 50 000 cells was attempted. Unless otherwise specified, experiments were run in triplicate.

Identification of the GPI Anchor of SPAM1 in the Soluble 230S Supernatant of the Nonvesicular LF Fraction and Its Role in SPAM1 Transfer

Phospholipase C beta 1 (PLCB1, phosphatidylinositol specific) was added to a final concentration of 5 U/ml to remove the GPI anchor from SPAM1 and other proteins in the soluble 230S subfraction of the LFs. The negative control was the LFs treated with the vehicle for the enzyme. Digestion was performed at 30°C for 15 min, after which the enzyme was inhibited by treatment with manoalide (40 μg/ml) in ethylene glycol for 20 min at 37°C as previously described [21]. Manoalide was obtained from Wako Pure Chemicals (Mountain View, CA). After enzymatic inhibition, the fraction and its undigested vehicle control, also treated with manoalide, were incubated with caudal sperm to allow SPAM1 transfer. The latter was detected using flow cytometry.

The Effect of Plasma Lipoproteins Containing CLU on SPAM1 Transfer to Sperm from the 230S Liquid Phase of the LF

Lipoprotein isolation and effect of concentration on SPAM1 transfer from ELF. Rat or mouse serum lipoproteins were isolated by density ultracentrifugation. Rat blood was subjected to centrifugation at 2000 × *g* for 20 min to pellet red blood cells. The density of the resulting rat serum was increased to 1.21 g/ml by adding 1.41 g of NaBr to a final volume of 5 ml (adjusted with water to a final weight of 6.05 g) and ultracentrifuged at 230 000 × *g* for 48 h. The protein concentration of the resulting supernatant (shown to contain apolipoproteins via dot blot analysis for high-density lipoproteins and CLU [data not shown]) was determined using the BCA kit.

ELF 230S samples were supplemented with rat lipoproteins (concentrations ranging from 40 to 160 μg/ml) for 20 min before incubation with caudal mouse sperm; sperm incubated in ELF plus NaBr carrier or in BSA alone were used as controls. Sperm were analyzed for SPAM1 acquisition via immunostaining, followed by flow cytometry as already described.

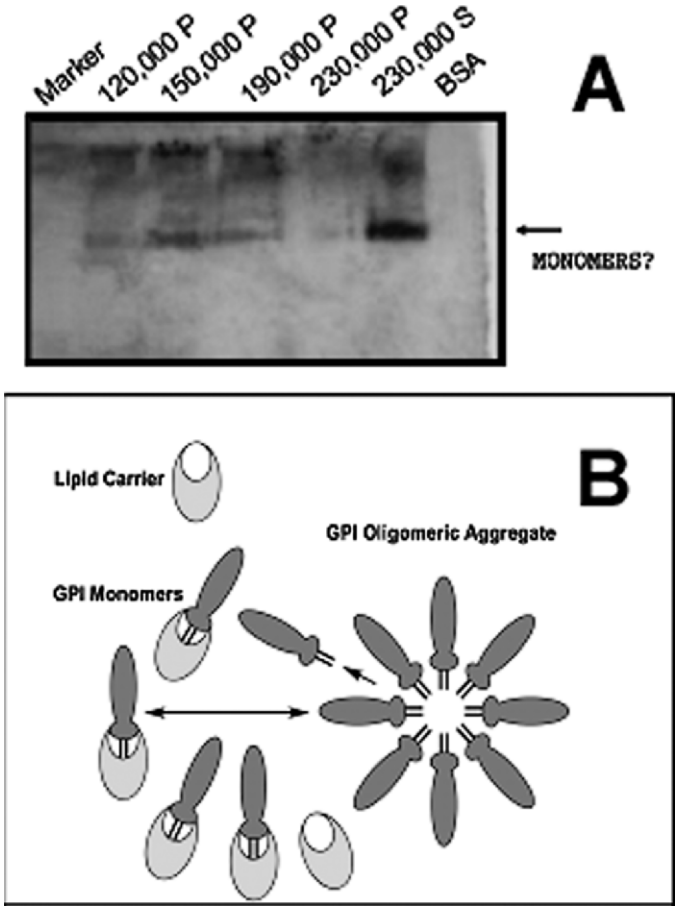

FIG. 1. The soluble membrane-free fraction of ELF contains SPAM1 in complexes with varying MWs. **A**) Native Western blot analysis showed that low-MW complexes of SPAM1 were most abundant in the 230S supernatant (S) (monomeric SPAM1, arrow) after repeat ultracentrifugation. The pellets (P) at 120 000, 150 000, 190 000, and 230 000 × *g* had most of the SPAM1 in smears, reflecting the presence of oligomeric aggregates, which were diminished in the last pellet. Lane 7 had BSA used as a control to show the specificity of anti-SPAM1 Ab. **B**) A model is shown depicting a possible dynamic equilibrium (double-headed arrow) between oligomeric aggregates and monomers, whose hydrophobic tails are stabilized by apolipoproteins (lipid carriers) in LF 230S.

Lipoprotein removal of SPAM1 from mouse and human sperm and delivery to human sperm. The results of the previous experiment suggested that higher apolipoprotein concentrations removed SPAM1 from the sperm surface. Thus, very high concentrations of lipoproteins were used for incubating mouse (800 μg/ml) and human (8 mg/ml) sperm at 37°C for 15–20 min. To confirm the removal of SPAM1, a fraction of the lipoprotein supernatant recovered after pelleting sperm was subjected to Western blot analysis and showed that SPAM1 was present. The remainder of the lipoprotein supernatant containing GPI-linked sperm proteins was diluted (to 8, 16, or 80 μg/ml) with PBS. These diluted samples were used for incubation of fresh populations of human sperm for the delivery of SPAM1. Following incubation for delivery of human SPAM1 to human sperm, as was the case with its removal from sperm membranes, cells were immunostained for flow cytometric analysis of SPAM1. Sperm incubated in PBS plus 1.21 g/ml of NaBr (lipoprotein carrier) or in PBS plus 80 μg/ml of fresh lipoproteins served as negative controls.

The Effect of Ab Inhibition of Endogenous CLU in 230S on SPAM1 Transfer

To determine if endogenous CLU is involved in SPAM1 transfer from ELF and ULF 230S, CLU was inhibited with anti-CLU Abs added to the 230S subfraction 30 min before incubation of sperm. Thus, caudal sperm were incubated at 37°C in PBS, LF 230S plus PIS (1:1000) as the controls, or LF 230S plus CLU Ab (1:1000) for 2 h. After incubation, samples were washed twice in PBS and stripped of the CLU Ab from the sperm surface. This step was necessary because both the CLU and SPAM1 Abs are rabbit polyclonals and would bind to the same secondary Ab. Stripping of CLU Ab with 3 M acetic acid was shown to be ineffective; therefore, all samples were stripped of CLU Ab with 1 M KCl (pH 7.2) for 15 min at RT, after which they were washed three times in PBS, immunostained for SPAM1, and subjected to flow cytometric analysis.

The Effect of Low Concentrations of Lipoprotein Supplements and CLU Ab Inhibition on SPAM1 Transfer from LF 230S

The effect of low concentrations (5, 10, 15, and 20 μg/ml) of rat or mouse lipoprotein on SPAM1 delivery from ULF 230S was investigated. To determine if the enhancement of uptake was partially due to an interaction of CLU with SPAM1, the lipoprotein supplement was CLU Ab inhibited before introduction to LF and sperm in a parallel set of samples. Thus, lipoprotein samples were subjected to CLU Ab or PIS (1:1000) for 30 min before their introduction to ULF 230S. Caudal sperm (5×10^4) were incubated in each sample for 2 h at 37°C. After incubation, samples were exposed to 1 M KCl treatment to strip the CLU Ab, followed by SPAM1 immunostaining and flow cytometry as already described.

Coimmunoprecipitation of CLU and SPAM1 in ULF and ELF

To determine the presence of an association between SPAM1 and CLU in LFs, coimmunoprecipitation was performed on the ELF and ULF 230S fractions. Samples (1 ml) were treated with PIS (1:1000), CLU Ab (1:1000), or SPAM1 Ab (1:1000) overnight at 4°C before incubation with 125 μl of Seize X protein A beads (Pierce) overnight at 37°C. Beads were washed three times in 1× PBS, and equivolume (20 μl) samples were treated with 100 mM dithiothreitol in sample loading dye and heated to 60°C for 5 min to extract immunoprecipitated proteins. Immunodetection of SPAM1 and CLU (1:1000) was performed via Western blot analysis as already described.

Dose Effects of Recombinant Human CLU on SPAM1 Transfer from ELF 230S

To validate the role of CLU in SPAM1 transfer, ELF 230S samples were supplemented with recombinant human CLU (Prospec Tany TechnoGene Ltd., Rehovot, Israel). According to the manufacturer, *N*-linked glycosylated protein was expressed in human cells (293 HEK) and therefore was likely to have the correct glycosylation pattern. Varying concentrations of human CLU (purity >95%) ranging from 10 to 100 ng/ml and solubilized in deionized water were added to the samples before their incubation with caudal mouse sperm. Control samples received only PBS-BSA or the unsupplemented ELF 230S. Experiments were performed in duplicate.

RESULTS

Native Western blot analysis showed that the BSA control gave no signal, while both low- and high-molecular-weight (MW) forms of SPAM1 complexes were detected in subfractions of the LFs with each ultracentrifugation. However the proportions varied after each spin. The low-MW SPAM1 complex was most and least abundant in the 230 000 × *g* supernatant (230S) and pellet (230P), respectively (Fig. 1A), which are likely monomeric- and oligomeric-rich subfractions, respectively. TEM analysis revealed that LF 230S was entirely membrane free (data not shown), while LF 230P contained irregularly shaped and sized micellar objects (Supplemental Fig. S1 available at www.biolreprod.org). A model proposing a dynamic equilibrium between monomers and aggregates in LFs is shown in Figure 1B.

Caudal sperm incubated in ELF 230S acquired demonstrable levels of SPAM1 compared with those incubated in BSA as seen by an increase in fluorescence intensity (Fig. 2, A and B), unlike those incubated in 230P under identical conditions (Fig. 2B). This indicates that the primary form of SPAM1 transferred to the sperm surface from the soluble fraction of LFs is found in the 230S subfraction and not the 230P subfraction. Most

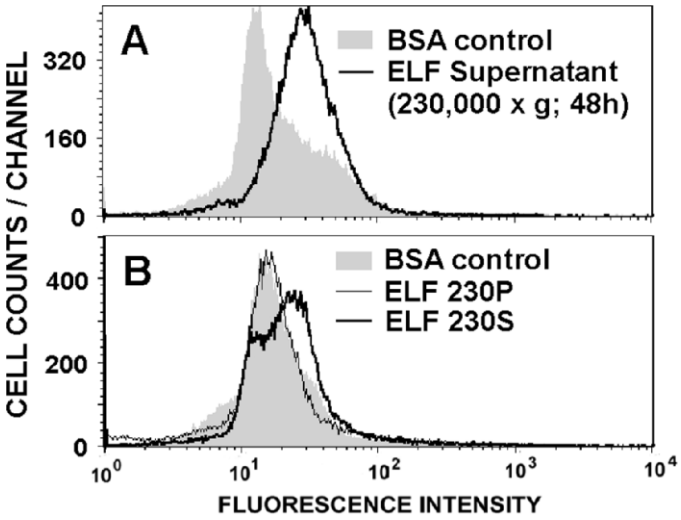

FIG. 2. SPAM1 uptake occurs only from the soluble ELF 230S monomeric fraction. Flow cytometric analysis of the acquisition of SPAM1 from 230S (**A** and **B**) and 230P (**B**) oligomeric fractions shows that fluorescence intensity increased (shifted to the right) compared with the BSA control only for 230S.

important, we demonstrated that the 230S monomeric fraction contains an intact GPI anchor, which when enzymatically cleaved dramatically reduces uptake from ELF (Fig. 3). Similar results were obtained for ULF 230S (data not shown).

When 40–160 μg/ml of rat plasma lipoproteins was added to the 230S ELF, the level of SPAM1 transfer varied with the concentration. While 40 μg/ml showed an increased uptake with respect to the carrier control, the 80- and 160-μg/ml samples showed a dose-related inhibition of SPAM1 transfer (Fig. 4). This demonstrates that lipoproteins are involved in the acquisition of SPAM1 on the sperm surface in a dose-related manner.

Uptake of SPAM1 was shown to be decreased by addition of CLU Ab to both ELF and ULF 230S (Fig. 5), suggesting that CLU has a role in SPAM1 delivery to the sperm plasma membrane. Further support for this comes from the results of Ab inhibition of CLU in rat serum lipoprotein supplements before introduction to ULF 230S (Fig. 6, v–viii). The fact that addition of CLU Ab negated lipoprotein-mediated enhance-

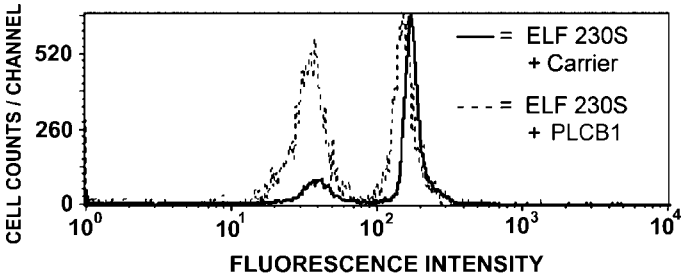

FIG. 3. SPAM1 transfer from 230S is diminished by cleavage of the GPI anchor. After PLCB1 or carrier treatment of ELF 230S fractions, followed by addition of manoalide for inactivation of the enzyme before incubation of samples with sperm, transfer was analyzed by flow cytometry. In the bimodal distribution of SPAM1 quantities, fluorescence intensities of both subpopulations shifted to the left for the enzyme-treated samples, representing a decrease in delivery of SPAM1 compared with the carrier-treated sample. There was a >7-fold increase in the number of sperm from the subpopulation with lower SPAM1 quantities after PLCB1 treatment compared with the carrier-treated sample.

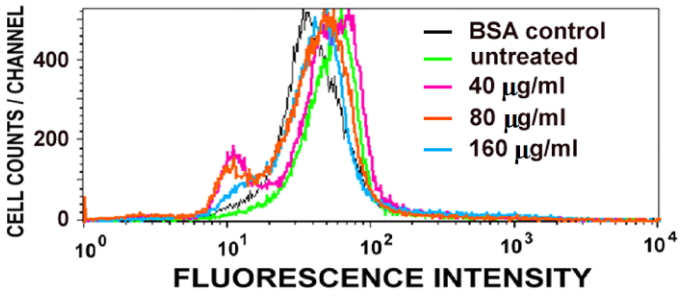

FIG. 4. SPAM1 uptake was related to the concentration of exogenous lipoproteins added to ELF 230S. While the lowest concentration (40 μg/ml) enhanced uptake (compare pink with green, which was untreated ELF plus NaBr carrier), the higher concentrations had the opposite effect in a dose-related manner (compare green with orange and aqua) as revealed by flow cytometric analysis.

ment of SPAM1 uptake (compare v–viii with i–iv in Fig. 6) demonstrates that CLU has a major role in the transfer of monomeric SPAM1 from the reproductive LFs. The results show that 5–20 μg/ml of lipoproteins in a dose-dependent manner increased SPAM1 uptake from ULF by caudal sperm, with the maximal uptake seen at 10 μg/ml. Higher doses reversed this effect, explaining the inhibitory effects seen with the 80- and 160-μg/ml samples for the ELF subfraction in Figure 4. Thus, lipoprotein concentration operates bimodally with an increase in SPAM1 transfer with dose, followed by a decrease with increasing doses.

The reduced SPAM1 levels on sperm exposed to high concentra-

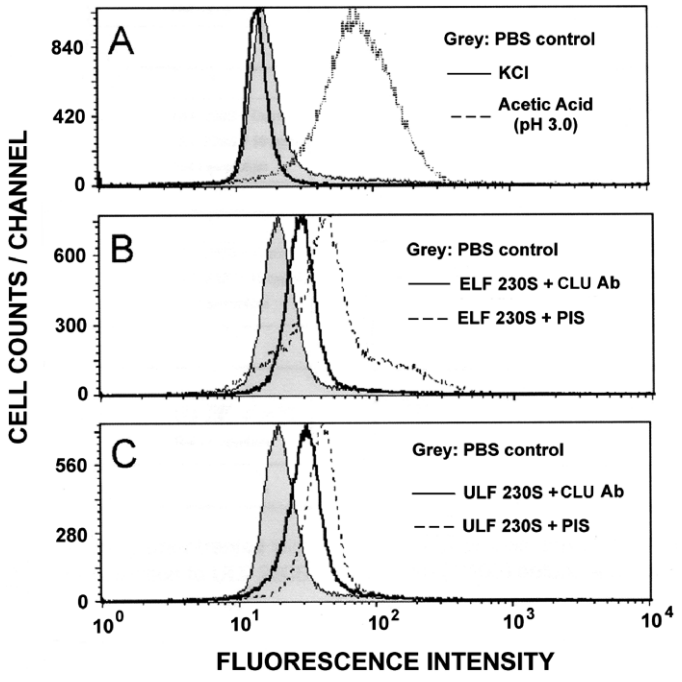

FIG. 5. CLU Ab-inhibited 230S has markedly reduced levels of SPAM1 transfer. **A**) Flow cytometric analysis demonstrates that, when sperm were incubated in PBS containing CLU Ab (1:1000) for 2 h, the CLU Ab could be removed with 1 M KCl, unlike acetic acid (pH 3.0) treatment, in which the CLU fluorescence was shifted to the right of the control (grey). Thus, KCL was used to strip sperm of the rabbit polyclonal CLU Ab before immunostaining of bound SPAM1 with a rabbit anti-SPAM1 Ab, preventing cross-reactivity of the secondary rabbit IgG. **B** and **C**) Addition of CLU Ab (1:1000) to ELF and ULF 230S before incubation with sperm inhibited SPAM1 transfer as detected by SPAM1 fluorescence.

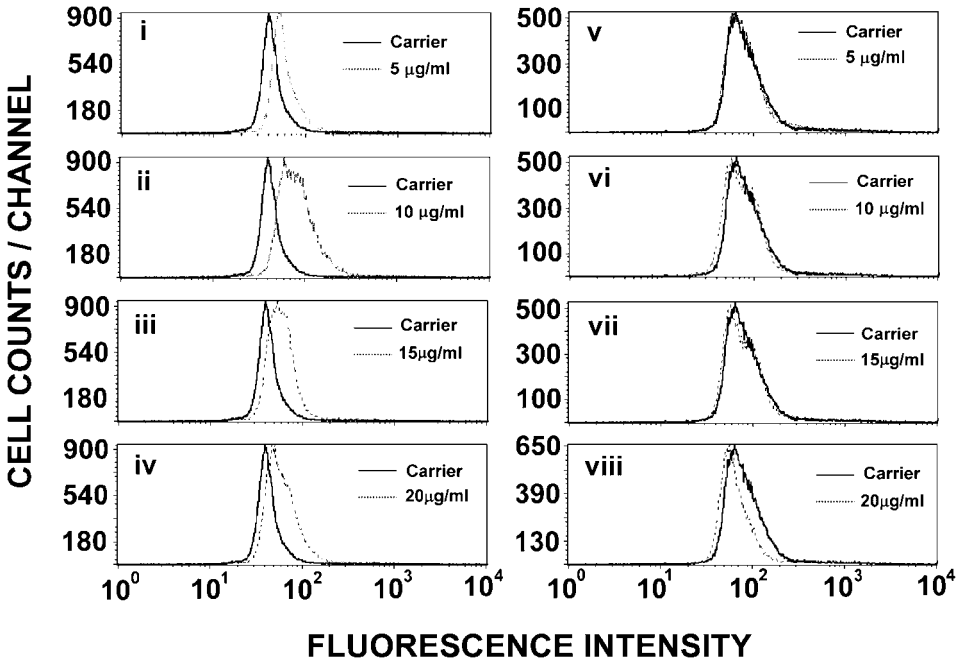

FIG. 6. CLU is responsible for lipoprotein-mediated enhancement of SPAM1 uptake from 230S. Increased uptake from ULF 230S supplemented with low doses of lipoproteins is negated after CLU Ab inhibition. **i**–**iv**) Rat serum lipoproteins (5–20 μg/ml) treated with PIS (1:100) before addition to ULF enhanced uptake, with the maximal increase seen at 10 μg/ml. **v**–**viii**) Lipoprotein-enhanced uptake was eliminated in the presence of CLU Ab (1:100), which was stripped from the sperm before immunostaining for SPAM1 as described in the legend for Figure 5.

tions of CLU may result from the removal of SPAM1 already present on the sperm plasma membrane. This is supported by the finding that very high concentrations (800 and 8000 μg/ml) of plasma lipoproteins are able to remove considerable amounts of SPAM1 from human and mouse sperm (Fig. 7A). Western blot analysis showed that the lipoprotein supernatant recovered after pelleting human sperm revealed the presence of the 64-kDa human SPAM1 (data not shown). Most important, when the recovered lipoprotein supernatant with solubilized human SPAM1 was diluted with PBS to ~80, 16, and 8 μg/ml and incubated with fresh human sperm, SPAM1 acquisition occurred (Fig. 7B), indicating that SPAM1 and other GPI-linked proteins are removed from the sperm plasma membrane by lipoproteins with their lipid anchors intact. Sperm incubated in ~16 μg/ml of lipoprotein supernatant acquired the most SPAM1 compared with those incubated in 80 and 8 μg/ml. This is consistent with the findings for mouse sperm, which showed maximal enhancement of SPAM1 uptake at lipoprotein concentrations of 10–15 μg/ml (Fig. 6).

In Figure 8, we show an association or interaction between SPAM1 and CLU in both ELF and ULF using reciprocal coimmunoprecipitation. Most important, Western blot analysis shows that the major 67-kDa isoform of mouse SPAM1 that is present in ELF and ULF 230S can be immunoprecipitated from these fluids by CLU Ab but not PIS (Fig. 8A). Similarly, when SPAM1 Ab was used for immunoprecipitation and CLU Ab for

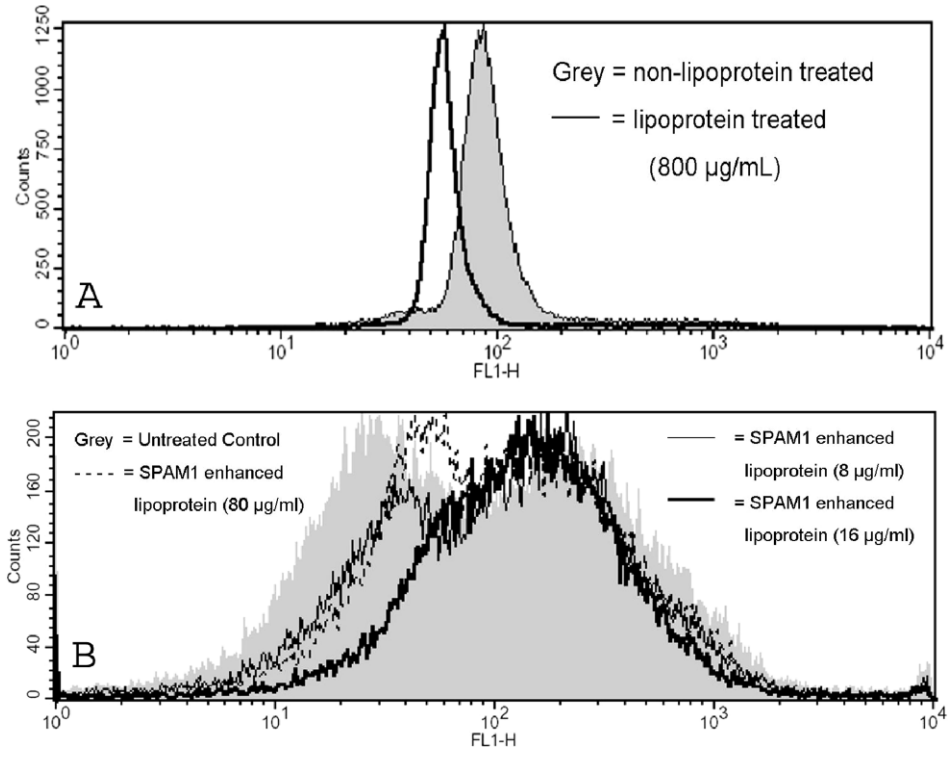

FIG. 7. High and low concentrations of exogenous lipoproteins remove and deliver SPAM1, respectively, from the sperm plasma membrane. **A**) Decreased level of SPAM1 on mouse sperm after treatment with 800 μg/ml of lipoproteins compared with the PBS-treated control (grey). Western blot analysis of the lipoproteins collected after treatment revealed the presence of SPAM1 in the proteins removed from human sperm (data not shown). **B**) SPAM1 quantities on fresh human sperm incubated in varying concentrations (8–80 μg/ml) of lipoproteins with solubilized SPAM1 from human sperm plasma membrane. Compared with controls (grey) incubated purely in lipoproteins (solvent), SPAM1 was increased in all test samples, with transfer efficiency highest and lowest at 16 and 8 μg/ml, respectively. In **A** and **B**, 50 000 cells were analyzed for each sample by flow cytometry. FL1-H indicates fluorescence intensity.

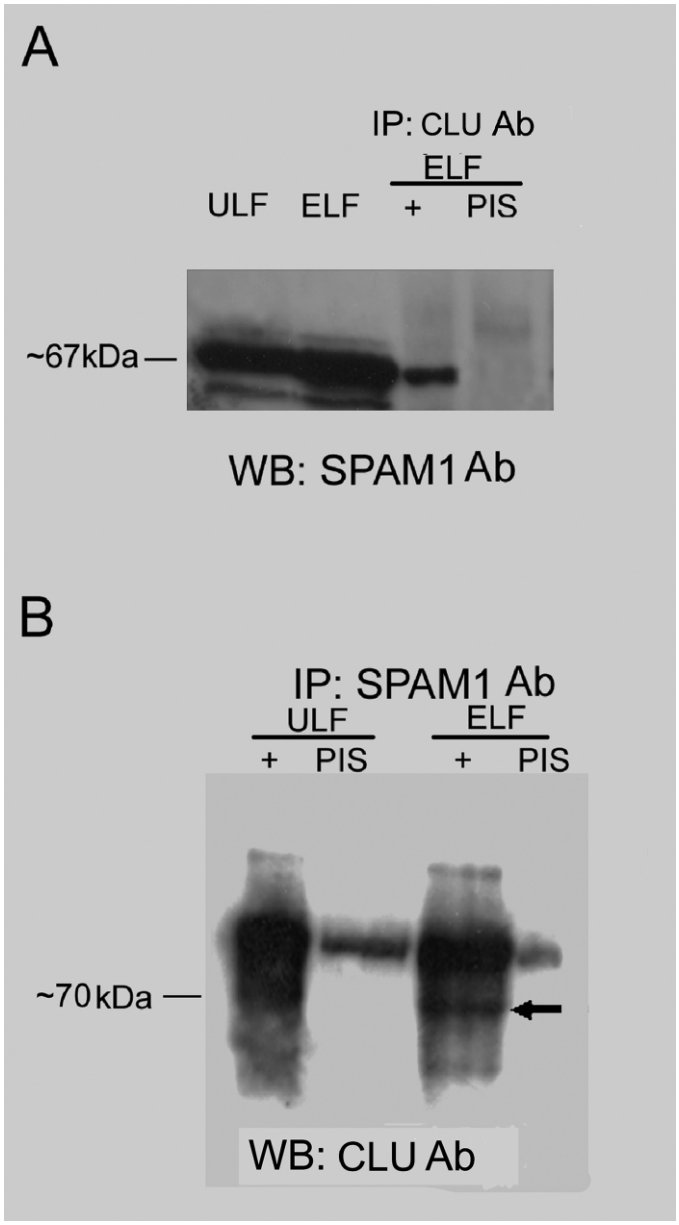

FIG. 8. Coimmunoprecipitation (co-IP) reveals that SPAM1 and CLU physically associate in ELF and ULF. Indicated are the presence (+) of anti-CLU and anti-SPAM1 Ab, control PIS, and the Ab used for Western blots (WB). **A**) Shown is the 67-kDa mouse SPAM1 band in ULF and ELF, and co-IP of SPAM1 with anti-CLU Ab is seen for ELF. **B**) An ~70-kDa CLU band (arrow) is seen after co-IP with anti-SPAM1 Ab in both ULF and ELF. Proteins in B are unreduced to resolve the 70-kDa CLU band.

Western blot analysis, CLU was detected (Fig. 8B). Taken together, these findings strongly support a role for CLU in the transport of SPAM1 monomers to the sperm surface.

Finally, when purified recombinant human CLU was added to ELF 230S before incubation with caudal mouse sperm, there was a dose-dependent effect on SPAM1 transfer. Compared with the untreated samples, uptake increased steadily in sperm incubated in the presence of recombinant CLU at concentrations of 10–60 ng/ml. The latter resulted in sperm with the highest amount of SPAM1 transfer (Fig. 9, A and B). At doses >60 ng/ml of CLU, the level of SPAM1 on sperm decreased, and at 100 ng/ml it was similar to that seen at 10 ng/ml (Fig. 9B).

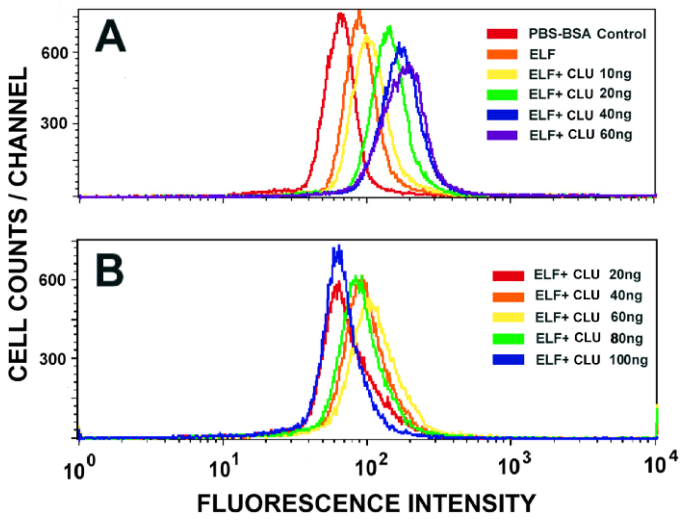

FIG. 9. Purified recombinant human CLU shows a dose-related effect on SPAM1 transfer to the sperm surface. **A**) SPAM1 uptake is increased when sperm are incubated in 230S ELF compared with PBS-BSA controls, and there is a direct relationship between the amount of transfer and the concentration of CLU added to ELF 230S up to 60 ng/ml. **B**) At CLU concentrations higher than 60 ng/ml of sperm, SPAM1 is decreased in a dose-dependent manner. This is consistent with the formation of complexes of CLU and SPAM1 in the liquid phase and (after saturation) the sequestration of sperm SPAM1 at high CLU concentrations.

DISCUSSION

Following the removal of vesicles from LFs, ultracentrifugation at >120 000 × *g* and native Western blot analysis revealed that in the soluble membrane-free fraction SPAM1 exists in high- and low-MW complexes. At 230 000 × *g*, these low- and high-MW complexes were predominantly present in the supernatant and the pellet, respectively. The latter was shown by TEM to consist of micellar oligomeric aggregates, while the low-MW component showed no structure and is likely to be monomeric. As suggested by the model shown in Figure 1B, hydrophobic interactions of the GPI anchors of SPAM1 are likely responsible for stabilizing the molecules in oligomeric aggregates.

While the insoluble aggregates were unable to deliver SPAM1 to the sperm surface, under identical conditions the monomeric fraction was able to do so. This was also the case for insoluble vesicles, which were previously shown to transfer the protein [10]. Because the vesicles were reported to dock on the sperm membrane at specific sites (lipid raft-associated domains) during transfer, it is likely that docking is a receptor-mediated event [10]. Thus, vesicles may carry receptors [10] that are not present on oligomeric aggregates, which are therefore not targeted to the membrane. When aggregates were resuspended in PBS and Western blot analysis was performed on native gel, there was solubilization as detected by low-MW SPAM1, while SDS-PAGE showed the 67-kDa mouse protein (data not shown). We also noted that these aggregates could be solubilized in lipoproteins, suggesting that oligomeric SPAM1 may serve as a source or pool of monomers. By doing so, they may regulate GPI-linked protein transfer to the membrane to effect maturation in an incremental and timely manner.

The model shown in Figure 1B proposes that a population of GPI-linked monomers might be stabilized and solubilized in an aqueous solution by forming hydrophobic interactions with a lipid carrier. If these carriers are targeted to the sperm surface, as is the case with CLU [16], they would be ideal vehicles for transporting GPI-linked proteins to the plasma membrane,

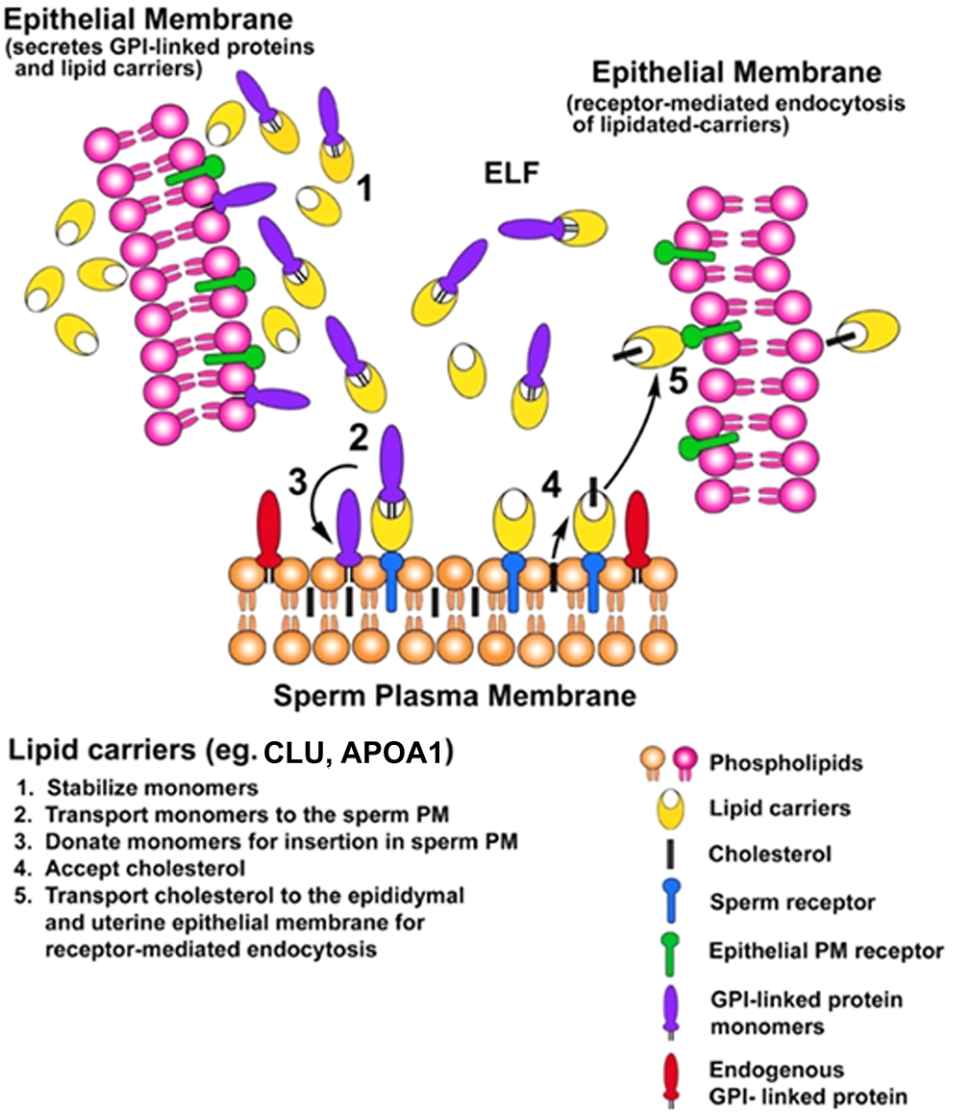

FIG. 10. A model of lipid exchange at the surface of sperm within the epididymis and uterus is shown. Included are the potential dual roles of CLU and other lipid carriers (e.g., APOA1 in ELF and ULF); they serve as GPI-linked protein monomer stabilizers and donors to the sperm plasma membrane (PM), in addition to being acceptors of cholesterol. Therefore, our model extends the lipid-poor model for cholesterol efflux [29, 30]. Details of the five steps are shown at the bottom left.

where the GPI anchors could be inserted into the outer layer of the lipid bilayer [22] via hydrophobic insertion. Most important, the monomeric fraction of SPAM1, which was shown to have the GPI anchor accessible to enzymatic cleavage, also had the anchor in a form in which it could be inserted into the sperm plasma membrane as evidenced by the fact that SPAM1 transfer from 230S was markedly reduced after treatment with PLCB1. Thus, the monomeric form of GPI-linked proteins, and not aggregates, is the primary form from which protein transfer occurs via the soluble membrane-free fraction of the luminal secretion.

The efficiency of SPAM1 transfer from the ELF monomeric fractions increased in the presence of rat serum lipoprotein concentrations that were $\leq$40 μg/ml. This is consistent with the increase in SPAM1 uptake from ULF supplemented with 5–15 μg/ml of rat serum lipoproteins (Fig. 6, i–iv), as well as that from ELF supplemented with the same concentration of mouse serum lipoproteins (data not shown). The identical findings with mouse and rat serum lipoproteins suggest that there were no cross-species effects with respect to the source of lipoproteins that enhanced SPAM1 uptake. When high concentrations (>40 to 160 μg/ml) of lipoproteins were added to ELF 230S, the amount of SPAM1 on sperm was markedly reduced (Fig. 4), suggesting that high lipoprotein concentrations sequester SPAM1 from the sperm plasma membrane. This was confirmed when ~800 μg/ml of rat serum lipoproteins was shown to dramatically remove SPAM1 from the membranes of mouse sperm (Fig. 7A). Similarly, SPAM1 could be solubilized from human sperm with high concentrations of lipoproteins as detected via Western blot analysis (data not shown). When the lipoproteins with solubilized human SPAM1 were diluted to16 μg/ml and incubated with a population of fresh human sperm, SPAM1 transfer was optimal (Fig. 7B). These findings, which clearly demonstrate how lipoprotein concentration is important in both the delivery and removal of SPAM1 from the sperm plasma membrane, argue for an important role of lipid carriers present in the LFs other than their role in cholesterol efflux [23, 24].

CLU has been implicated in lipid efflux from the sperm plasma membrane during capacitation [14]. Law and Griswold [16] have shown that in the epididymal fluid it has a major fraction that is free or loosely associated with sperm, while a smaller fraction is tightly associated with the lipid bilayer. It is possible that the free fraction of CLU is involved in stabilizing GPI-linked protein monomers, while the fraction that is tightly associated with the sperm membrane is in the process of delivering GPI-linked proteins to the sperm. When CLU was Ab inhibited in lipoprotein supplements for the 230S fraction, enhancement of SPAM1 transfer was negated (Fig. 6, v–viii). This demonstrates its involvement in SPAM1 transfer. The

finding that Ab inhibition of epididymal and uterine CLU in the monomeric fractions also diminished transfer confirms the involvement of this lipid carrier in SPAM1 delivery to the sperm surface. Further confirmation of its role in transferring SPAM1 was obtained by the results of coimmunoprecipitation that showed an intimate association between SPAM1 and CLU. This association is consistent with the report that CLU forms hydrophobic complexes with the GPI-linked prion protein, which is found in the soluble phase of ELF of rams [25]. Therefore, prion protein in rams may potentially be another protein delivered to the sperm surface via CLU.

The role of CLU in the transfer of SPAM1 to the sperm surface was validated by the use of purified recombinant protein. As was the case with rat serum lipoproteins, there was a dose effect of the addition of purified recombinant human CLU. The direct relationship between increasing concentration ($\leq$60 ng) of CLU and SPAM1 transfer, followed by a leveling off of transfer after saturation (Fig. 9), is consistent with the formation of a complex between CLU and SPAM1. Furthermore, the decrease in sperm SPAM1 after sperm incubation with $>$60 ng/ml of CLU reflects the removal of SPAM1 from the sperm plasma membrane in the formation of a complex with the recombinant protein at high concentrations.

The finding that CLU concentration modulates removal and delivery of SPAM1 has physiological relevance and practical implications. Sylvester et al. [26] have demonstrated that the level of rat epididymal CLU that is secreted only in the caput is $\sim$7-fold higher in the epididymal fluid from the caput compared with that in the cauda. This is consistent with the fact that in the caput SPAM1 and other GPI-linked proteins are removed from the sperm plasma membrane [1, 27], while in the caudal epididymis they are added to the sperm [25, 28]. In the case of the prion protein and other GPI-linked proteins associated with the soluble phase, addition to the sperm surface occurs before [11] or after [25] ejaculation from the seminal plasma, where the level of CLU would be lower than that in the caput and cauda. From a practical standpoint, this work has the potential of leading to advances in technology for treating sperm pathology via the delivery of membrane-free GPI-linked molecules to enable sperm to effect fertilization in vitro and after intrauterine insemination.

In conclusion, our studies show that the soluble form of GPI-linked proteins, exemplified by SPAM1, is transported through an aqueous solution by CLU. Implicated in cholesterol efflux from the sperm surface during capacitation [14], as well as in other functions [22], CLU has now been shown to have a definitive role in the delivery of GPI-linked proteins to sperm. To date, there has been no clear evidence for a definitive role. Another apolipoprotein that could perform the same function as CLU is APOA1, which is also expressed in both the male and the female tract and is also implicated in cholesterol efflux [14]. Notably, it shares with CLU the same receptor and coreceptor, LRP2/cubilin, on the epithelial membrane of the epididymal and uterine tract [14], where it mediates endocytotic removal of lipidated proteins [13, 14].

Thus, we put forward an expansion of the lipid-poor apolipoprotein model proposed to carry out cholesterol efflux on the plasma membrane of somatic cells and sperm [29, 30] as a model for lipid exchange involving GPI anchors. As shown in Figure 10, we propose that CLU (and possibly APOA1) solubilizes and stabilizes GPI-linked monomers from the epithelial membranes and transports and donates them to the sperm surface, where they accept cholesterol. The lipidated proteins are then endocytosed via a receptor-mediated mechanism at the epithelial cell lining [13, 14, 16]. Therefore, the findings of this study present a more efficient interaction of CLU and the sperm membrane and its role in membrane remodeling than previously envisaged, while revealing the mechanism by which soluble GPI-linked proteins are delivered to the sperm surface.

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