GPI-anchored Complement Regulatory Proteins in Seminal Plasma

An Analysis of Their Physical Condition and the Mechanisms of Their Binding to Exogenous Cells

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Abstract

We analyzed and compared the properties of three glycosylphosphatidylinositol (GPI)-anchored proteins, CD59, CD55 (both C regulators), and CDw52, and of the transmembrane C regulator CD46 in seminal plasma (SP). We demonstrated previously that anchor-intact SP CD59 is present on the membranes of vesicles (prostasomes) and that cells acquire this protein during incubation with SP. We now report that this acquisition is due partly to adherence of prostasomes to cells and partly to a second mechanism which may involve micellar intermediates. Using fluorescent labeling, ultracentrifugation, and density gradient centrifugation, virtually all CD46 was present on prostasomes whereas CD59, CD55, and CDw52 were also detected in a form which remained in the 200,000 g supernatant and equilibrated at higher density than prostasomes in gradients. All three GPI-linked proteins eluted at high molecular mass during size exclusion chromatography of this nonprostasome fraction. As documented by videomicroscopy and biochemical analysis, cells acquired new copies of the GPI-linked proteins during incubation with the nonprostasome fraction as well as with prostasomes. These data demonstrate the presence in SP of a stable population of membrane-free, GPI-linked proteins available for transfer to cells. Binding of these proteins to spermatozoa and pathogens in SP may confer new properties on their membranes including increased resistance to C attack. Finally, our data raise the possibility that lipid-associated GPI-linked proteins may be suitable for therapeutic applications. (*J. Clin. Invest.* 1996. 97:1675–1686.) Key words: CD46 • CD55 • CD59 • CD52 • prostasomes

Introduction

We demonstrated previously that cell-free seminal plasma (SP)[1] contains the glycosylphosphatidylinositol (GPI)-anchored late C regulatory protein CD59 in anchor-intact form and that cells, including human spermatozoa, acquire this protein during incubation with SP by a mechanism dependent on the anchor's integrity (1). This phenomenon may have clinical relevance since it suggests a mechanism by which spermatozoa and microbial pathogens may acquire membrane proteins from SP. We showed that most CD59 in SP is present on extracellular, vesicular organelles called prostasomes (1) which are believed to arise from prostatic epithelium (2). Further, we reported that cells acquired CD59 during incubation with partially purified prostasomes (1).

Here we report our continued investigation of prostasomes and of GPI-anchored proteins in SP. In addition to CD59, we studied two other GPI-anchored proteins, CD55 and CDw52, and a transmembrane protein, CD46, all of which have been identified in SP (3–6). CD55 (decay accelerating factor) and CD46 (membrane cofactor protein) are inhibitors of C activation (for review see reference 7). CDw52, a 20-kD protein of uncertain function, is abundantly expressed on human lymphocytes (8) and also present in the male reproductive tract, with particularly high levels in epididymis (4). We confirm that CD55 and CDw52 are carried on prostasomes (4) and demonstrate that these particles also possess CD46. We show that erythrocytes (E) acquire prostasome CD46 with comparable efficiency to CD59, indicating that the CD59 acquisition noted previously is due to association of whole prostasomes with the cells, rather than to transfer of CD59 between membranes; further, we present data from videomicroscopy of the interaction of fluorescent-labeled prostasomes with several cell types which establish that this association involves adherence of prostasomes to cells but not membrane fusion.

Using density gradient centrifugation we detected all three GPI-linked proteins studied not only on prostasomes but also in a nonprostasome fraction (NPF). These NPF proteins were anchor positive and could bind cells. They eluted at high molecular mass during size exclusion fast performance liquid chromatography (FPLC), suggesting that the anchor may be preserved by inclusion of these proteins in hydrophobic aggregates.

Methods

Reagents for SDS-PAGE and for Western blotting were obtained from Bio-Rad Laboratories (Richmond, CA). Protein concentrations were measured using a BCA assay kit (Pierce Chemical Co., Rock-

1. *Abbreviations used in this paper:* AP, alkaline phosphatase; BCECF-AM, acetomethoxy ester of 2′,7′-bis-(2 carboxyethyl)-5′-(and 6)-carboxyfluorescein; CHO, Chinese hamster ovary; DiI, 1′,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; E, erythrocytes; FPLC, fast performance liquid chromatography; GPI, glycosylphosphatidylinositol; GVB, veronal buffered saline containing 10% glycerol; HRP, horseradish peroxidase; NPF, nonprostasome fraction; SP, seminal plasma; VSP, vasectomy seminal plasma.

ford, IL). Unless otherwise noted chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies. Mouse mAb BRIC 229 (anti-CD59) was from Bioproducts Laboratories (Elstree, Herts, United Kingdom). Rat mAb CAMPATH IM (anti-CDw52) was from Harlan Bioproducts for Science, Inc. (Indianapolis, IN). Mouse mAb IIH6 (anti-CD55) was a gift from Dr. T. Kinoshita (Department of Immunoregulation, Research Institute for Microbiological Diseases, Osaka, Japan) and mouse mAb GB24 (anti-CD46) was a gift from Dr. B.L. Hsi (INSERM 210, Faculte de Medicine, Nice, France). Rabbit polyclonal Rb476 (anti-CD59) was a gift from Dr. A. Davies (Molecular Immunopathology Unit, University of Cambridge, United Kingdom) and rabbit polyclonal anti-MCP and anti-DAF antibodies were prepared in house.

FITC-conjugated sheep anti–rat Ig (SAR-FITC) and sheep anti–mouse IgG (SAM-FITC) were from Sigma Chemical Co. Alkaline phosphatase (AP)-conjugated goat anti–mouse IgG (GAM-AP) and goat anti–rabbit IgG (GAR-AP) were from Bio-Rad Laboratories and AP-conjugated rabbit anti–rat IgG (RAR-AP) was from Sigma Chemical Co. Horseradish peroxidase (HRP)-conjugated goat anti–mouse IgG (GAM-HRP) and goat anti–rabbit IgG (GAR-HRP) were from Sigma Chemical Co.

SDS-PAGE and Western blotting. Electrophoresis was performed on 15% gels which were then either silver stained or Western blotted to nitrocellulose. Unbound protein sites on the blots were blocked with 10% milk protein. Thereafter, specific proteins were detected by sequential incubations with the appropriate first antibody (mouse mAb Bric 229 1 μg/ml for CD59; mouse mAb IIH6, 2 μg/ml for CD55; rat mAb CAMPATH IM, 2 μg/ml for CDw52; rabbit polyclonal anti-CD46, 2 μg/ml for CD46) and AP-conjugated second antibody. Blots were developed using a chromogenic AP substrate (Bio-Rad Laboratories).

Phase separation in solution of Triton X-114. To determine whether proteins were amphophilic or hydrophilic, detergent partitioning experiments were conducted by a modification of the method of Bordier (9) as described previously (1).

Cells. Rat E were obtained by cardiac puncture under anesthesia. Chinese hamster ovary (CHO) cells were from the European Tissue Culture Collection (European Collection of Animal Cell Cultures, Porton Down, Salisbury, United Kingdom). Mouse macrophages were acquired by peritoneal lavage.

Collection and storage of SP and vasectomy SP (VSP). Samples of semen from healthy donors were liquefied at room temperature for 30 min. Cells and debris were then removed by centrifugation at 10,000 *g* for 10 min. Fractions of SP were used within 30–60 min of collection for functional experiments and either stored frozen at −20°C or analyzed immediately for analysis of the protein content. VSP was obtained from donors between 12 and 16 wk after vasectomy and examined by microscopy. Samples containing spermatozoa were discarded. Thereafter, VSP was treated as described for SP.

Fractionation of SP by ultracentrifugation and gel filtration. Crude separation of particulate from nonparticulate matter was achieved by centrifugation of cell-free SP (1-ml aliquots) at 200,000 *g* for 1 h at 4°C in a benchtop centrifuge (T100; Beckman Instruments, Inc., Fullerton, CA). Supernatant was decanted into a separate tube and the pellet was resuspended in a volume of PBS equal to the original volume of SP.

SP (1 ml) was applied to a 35-ml column of G-200 Sephadex (Sigma Chemical Co.) with PBS as the mobile phase. Flow rate was 0.25 ml/min and 1-ml fractions were collected.

Density gradient ultracentrifugation. This was performed using gradients of 5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N,N*′-bis(2,3-dihydroxypropyl)isophthalamide (Nycodenz; Sigma Chemical Co.) dissolved in solution of 0.25 M sucrose. Nycodenz is a nonionic derivative of triiodobenzoic acid which forms true solutions, allowing separation of organelles at relatively low speeds and short incubation times (10, 11). Discontinuous, median-loaded gradients were constructed as follows: SP was diluted 1:5 in Nycodenz solution so that

the final concentration of Nycodenz was 40% and 2 ml of this mixture was layered over a 50% Nycodenz cushion (2 ml) in a 13-ml tube. Thereafter, decreasing concentrations of Nycodenz were overlaid as follows: 35% (2 ml), 30% (2 ml), 25% (2 ml), 20% (1 ml), and 10% (1 ml). The gradients were centrifuged at 25,000 rpm for 2 h at 4°C in the TFT 41.4 swing-out rotor of a Centrikon T1170 ultracentrifuge (Kontron Instruments, Milan, Italy). Fractions were collected from the bottom of the tube by peristaltic action. Density of the fractions was determined by refractometry.

FPLC. Supernatant from ultracentrifugation of SP and the NPF from density gradient centrifugation of SP were fractionated on an FPLC system (Pharmacia, Uppsala, Sweden) using columns of Superose 12 or Superose 6. Flow rate was 0.3 ml/min. PBS was the mobile phase and 0.5-ml fractions were collected.

Fluorescent labeling of prostasomes. To examine the behavior of prostasomes during purification and during interaction with cells, the organelles were labeled with one of two fluorescent labels. First, the aceto methoxy (AM) ester of the soluble fluorochrome 2′,7′-bis-(2 carboxyethyl)-5′-(and 6)-carboxyfluorescein (BCECF-AM; Molecular Probes, Eugene, OR) diffuses passively across cell membranes. Cleavage of the AM moiety by cytoplasmic esterases renders the molecule highly fluorescent and membrane impermeant, so that it is
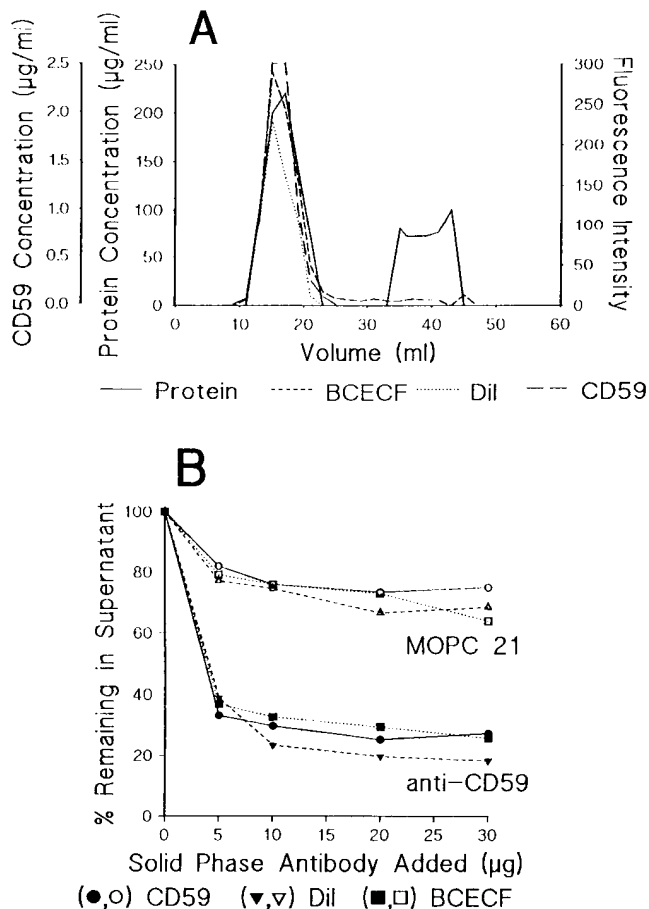

Figure 1. Purification of labeled prostasomes. (*A*) Gel filtration. The resuspended ultracentrifugation pellet from SP was labeled with one of two fluorochromes (DiI or BCECF) and gel filtered on G200 Sephadex. (*B*) Immunoprecipitation. The graph shows the percentage of material remaining in suspension of labeled prostasomes after incubation with antibody bound to solid phase and separation of bound material. Tests were performed in triplicate and results are the means of two experiments. *MOPC 21*, irrelevant monoclonal antibody.
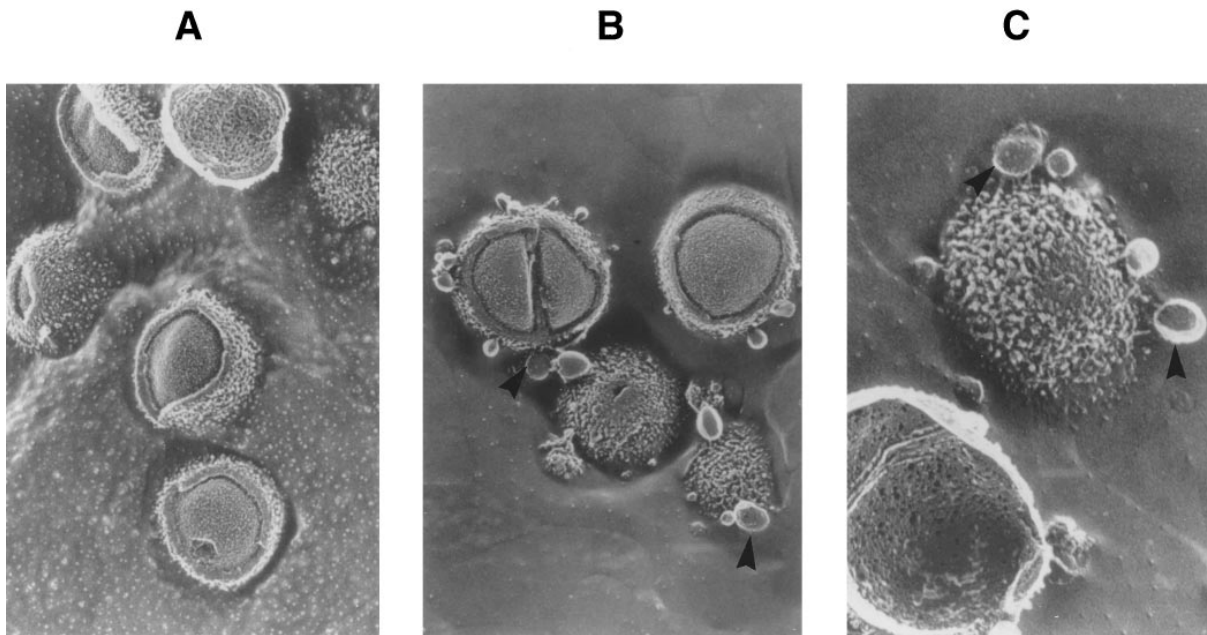
Figure 2. Electron microscopy of prostasomes. Fixed *S. aureus* cells coated with antibody were used to immunoprecipitate prostasomes from suspension. The precipitates were then examined by quick-freeze, deep-etch electron microscopy. (*A*) Control; *S. aureus* cells were coated with an irrelevant mAb, MOPC 21. The bacteria appear as spherical structures with a rough villous surface, but without any attached vesicles. (*B* and *C*) *S. aureus* cells were coated with mAb BRIC 229 (anti-CD59). This resulted in binding of closed, spherical membrane vesicles to the surfaces of the bacteria. A few of the vesicles display particles on their surface, suggesting the presence of protein aggregates in the membrane, whereas many others are smooth. *A* and *B*, ×25,000; *C*, ×50,000.

retained by the cell (12). We found that this probe could be used to label the internal aqueous contents of prostasomes. Second, the lipophilic probe 1′,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) inserts into the outer leaflet of lipid bilayers and has been used to monitor membrane fusion events (13–15). We used this probe to label the outer surface of prostasomes. To label purified prostasomes, cell-free SP was centrifuged at 200,000 *g* for 1 h in order to pellet prostasomes. The pellet was resuspended in 1 ml of PBS and labeled by incubation with BCECF (3 μM from a stock solution of 1 mM in DMSO) for 30 min at 37°C or DiI (5 μg/ml from a stock solution in DMSO) for 2 min at room temperature with vortex mixing. The suspension was then subjected to further separation techniques and prostasome-containing fractions were identified

by measurement of fluorescence in a Wellfluor plate-reading fluorimeter (Denley, Sussex, United Kingdom). Excitation was 480 nm and emission 530 nm for BCECF; excitation was 530 nm and emission 590 nm for DiI.

Immunoprecipitation of prostasomes. Fixed *Staphylococcus aureus* cells (protein A cell suspension; Sigma Chemical Co.) were used as solid-phase support for antibodies. mAb BRIC 229 anti-CD59 or an irrelevant mouse mAb (MOPC 21) were incubated with the solid phase (2 mg IgG/ml of a 10% suspension of cells in PBS) for 1 h at 4°C. After washing, the cell suspension was incubated with PBS/2% BSA for 30 min at 4°C to block unbound sites, washed three times in PBS, and resuspended at a density of 10% in PBS. Aliquots of fluorescent-labeled prostasomes partially purified by gel filtration (200 μg
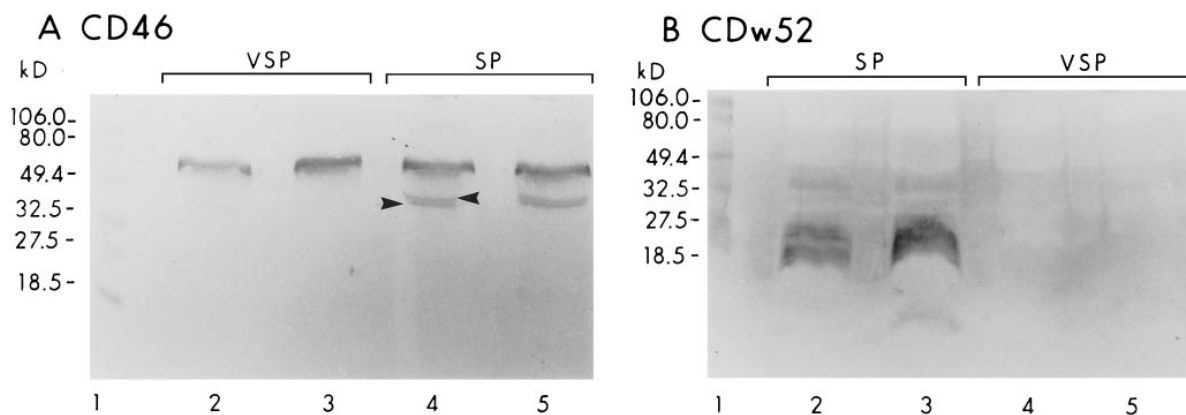

Figure 3. CD46 and CDw52 in SP and VSP. (*A*) Western blot stained for CD46. *Lane 1*, markers; *lanes 2* and *3*, two samples of VSP; *lanes 4* and *5*, two samples of SP. A 60-kD band was detected with comparable intensity in the four samples; SP also contained two lighter bands of 40 and 42 kD which were absent from VSP (*arrows*). (*B*) Western blot stained for CDw52. *Lane 1*, markers; *lanes 2* and *3*, SP; *lanes 4* and *5*, VSP. A broad band (18–25 kD) was detected in SP samples but was absent from samples of VSP.

Table I. Complement Inhibitor Concentrations in SP and VSP

Sample No.	Protein	CD59	CD55	CD46
	mg/ml	*μg/ml*	*ng/ml*	*ng/ml*
SP				
1	32.0	30.0	1600	640
2	29.2	30.0	1700	640
3	29.2	23.5	900	640
4	25.6	25.0	900	400
5	39.2	46.0	480	1080
6	21.2	18.0	900	700
7	48.0	18.5	660	700
8	36.8	23.0	900	560
9	33.7	25.5	1700	800
10	38.0	28.5	900	660
Mean	33.3	26.8	1064	682
SD $(n-1)$	7.6	7.9	439	174
Range	21.2–48.0	18.0–46.0	480–1700	400–1080
VSP				
1	25.6	16.0	420	640
2	29.2	16.0	470	600
3	22.0	9.5	540	860
4	18.8	18.0	580	400
5	13.6	10.0	380	440
6	41.6	16.5	540	500
7	24.0	9.5	360	600
8	30.8	20.0	560	540
9	21.2	16.5	360	560
10	23.8	15.0	900	560
Mean	25.1	14.7	511	562
SD $(n-1)$	7.6	3.7	161	134
Range	13.6–41.6	9.5–20.0	360–900	400–860

Samples of unfractionated SP and VSP from 10 donors were assayed in duplicate for total protein and by ELISA for CD59, CD55, and CD46. In three cases, protein measurements were performed before and after freezing and the results were similar. These ELISA measurements indicate higher levels of CD59, CD46, and CD55 in SP than suggested by Western blotting. Anchor cleavage may partly explain this discrepancy (less efficient detection of anchor-negative CD59 and CD55 by Western blot than by ELISA has been noted by other authors [17]). The presence of variably sized proteolytic products of the proteins may also contribute.

total protein in 1 ml PBS) were incubated with varying concentrations of the solid-phase antibody preparations for 30 min at 4°C. The solid phase was then pelleted by centrifugation at 200 *g* for 3 min and supernatants and pellets were assayed for their content of CD59 and fluorescent label. The pelleted solid phase was washed three times by centrifugation and resuspension in PBS before fixation and preparation for electron microscopy.

Electron microscopy. Samples were prepared for electron microscopy by the quick-freeze, deep-etch technique, described in detail elsewhere (16). This technique involves abrupt application of a cryogenically cooled copper block to the sample to freeze it, then freeze-fracture and vacuum "etching" or freeze-drying of its surface, followed by platinum replication of the etched surface and examination of the replica with a standard transmission electron microscope.

ELISA for CD59, CD55, and CD46. Microtiter plates were coated with first antibody (mAb Bric 229 anti-CD59; 1 μg/ml) overnight at 4°C and unbound protein sites were blocked by incubation with 10% dried milk powder for 1 h at 37°C. After washing five times in PBS/0.1% Tween, standards and samples (diluted in PBS/0.5% NP-40) were applied in duplicate and incubated for 2 h at 37°C. Wells were washed five times, incubated with second antibody (polyclonal Rb476 anti-CD59; 5 μg/ml) for 1 h at 37°C, washed a further five times, incubated with third antibody (GAR-HRP, 1 μg/ml), and washed six more times before development with 1,2-orthophenylenediamine (Sigma Chemical Co.).

CD55 ELISA was conducted as the ELISA described above for CD59 with the following changes: first antibody was mouse mAb IIH6 (anti-CD55; 1 μg/ml) and second antibody was rabbit polyclonal anti-CD55 (1 μg/ml).

CD46 ELISA was as described above for CD59 with the following changes: first antibody was rabbit polyclonal anti-CD46 (1 μg/ml), second antibody was mouse mAb GB24 (anti-CD46; 1 μg/ml), and third antibody was GAM-HRP (1 μg/ml).

Video-enhanced fluorescence microscopy. Murine bone marrow–derived macrophages, differentiated in vitro by standard techniques of cultivation with CSF-1, as well as CHO cells grown as monolayer cultures by standard techniques, were visualized by phase-contrast and fluorescence microscopy using a $63 \times$ lens (Leitz) projecting into a Namamatsu C-2700 "SIT" television camera. To minimize light damage to the cells, the illuminator was kept below 10 W and the television original was improved by 8-frame averaging and constant enhancement with an Argus 10 processor, before storage on a Panasonic TQ 3038 optical memory disk recorder. Final prints for study and publication were made directly from this analogue-stored video data with a Sony 5.5″ thermal printer.

Cell-binding assay (flow cytometry). To determine whether proteins present in fractions of SP had the potential to bind cell membranes, rat E were prepared as a 2% suspension in veronal buffered saline containing 10% glycerol (GVB), and CHO cells were suspended at a density of 1×10^7/ml in GVB. 100-μl aliquots of the cell suspensions were incubated with the fractions to be tested (100 μl) at 37°C for varying times. Cells were then washed three times by centrifugation at 400 *g* and resuspension in cold GVB (1 ml) to remove unbound protein. They were stained by incubation with first antibody (mouse mAb BRIC 229 anti-CD59 at 1 μg/ml or rat mAb CAMPATH IM anti-CDw52 at 5 μg/ml) for 30 min at 4°C, washed three times and incubated with second antibody (SAM-FITC or SAR-FITC diluted according to instructions) for 30 min at 4°C, before final washing and flow cytometric analysis on a FACS® 440 (Becton Dickinson, Abingdon, Oxon, United Kingdom).

Cell-binding assay (ELISA). Rat E (10% in GVB) were incubated with the fraction to be tested for 12 h at 37°C to maximize binding. Cells were then separated from unbound material by centrifugation at 400 *g* and washed three times in cold GVB before solubilization and dilution in PBS containing 0.5% NP-40 and analysis by ELISA for CD59 and CD46.

Results

Fluorescent labeling, immunoprecipitation, and electron microscopy of prostasomes. The ultracentrifugation pellet of SP was labeled either with the lipophilic probe DiI or with BCECF-AM and then subjected to gel filtration on G-200 Sephadex. Both labels coeluted with CD59 and with a major protein peak in the void volume (Fig. 1 *A*). The presence of DiI indicates that this peak contained lipid bilayers while that of BCECF indicates closed membrane vesicles. When these fractions were ultracentrifuged at 200,000 *g* for 1 h, > 95% of fluorescence was in the pellet; however, if treated with 0.1% NP-40 before ultracentrifugation, < 5% was found in the pellet and > 95% in supernatant. Thus, BCECF was contained within closed vesicles and was released by lysis.
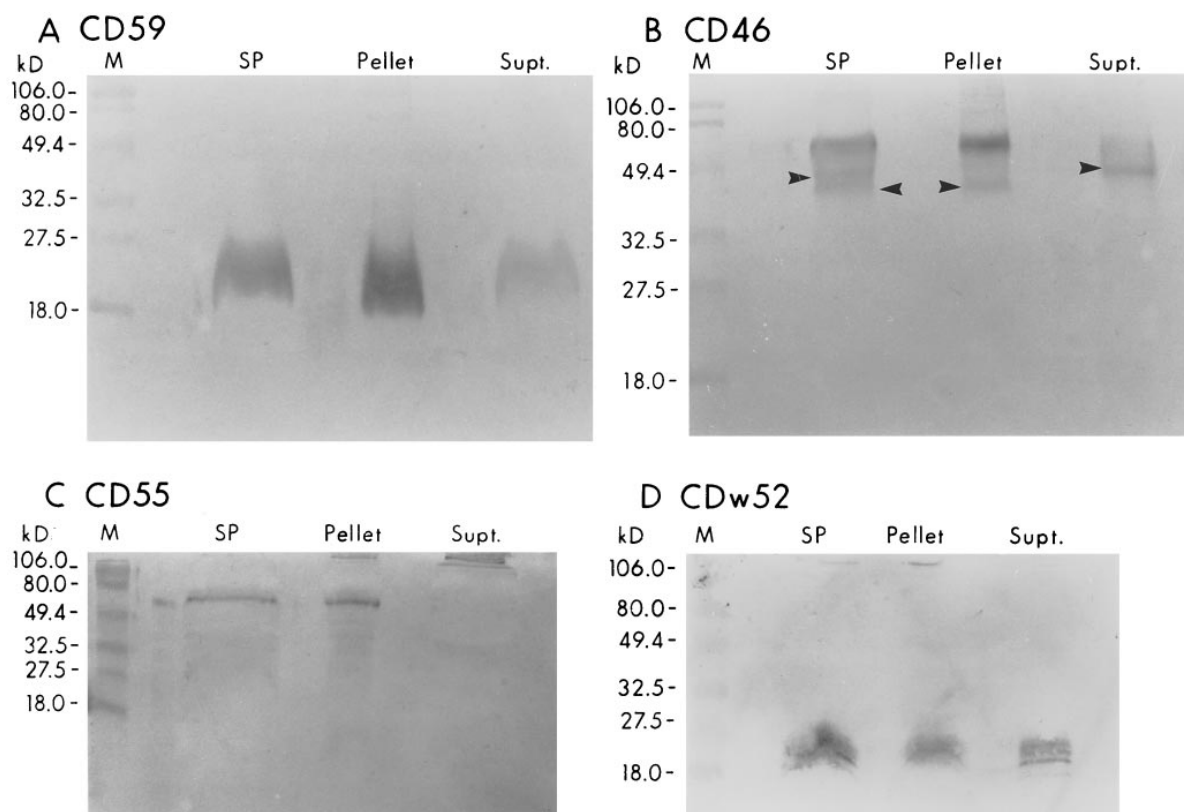
Figure 4. Ultracentrifugation of SP. SP was ultracentrifuged at 200,000 g for 1 h, the supernatant was removed and the pellet was resuspended in PBS equal to the original volume of SP. The input sample, pellet, and supernatant were examined by Western blotting. *M*, markers; *Supt.*, supernatant. (*A*) CD59 was detected as a strong band in SP and pellet. (*B*) CD46. SP contained a strong 60-kD band and two weaker bands of 42 and 40 kD (*arrows*). The pellet contained a strong 60-kD band and a weak 42-kD band (*arrow*) whereas the supernatant contained only a weak 40-kD band (*arrow*). (*C*) CD55 was detected in SP and in the pellet; no specific staining was observed in supernatant. (*D*) CDw52 was detected in SP, pellet and supernatant.

Gel filtration–purified prostasomes, labeled with DiI or BCECF, were incubated with mAb anti-CD59 bound to fixed *S. aureus* cells. CD59 and both fluorochromes were bound with comparable efficiency (Fig. 1 *B*), whereas < 30% was removed by a control mAb. These data confirm that prostasomes possess CD59 on their outer surface and indicate that labeling with both fluorochromes was uniform. Electron microscopy of the bound prostasomes revealed variably sized membrane vesicles (Fig. 2).

DiI could also be used to label unpurified prostasomes present in crude, unfractionated SP. When SP was incubated with DiI and then subjected to ultracentrifugation at 200,000 g, > 95% of DiI fluorescence was present in the pellet, indicating that most membrane fragments present in SP were pelleted during the procedure. BCECF-AM is not useful in unfractionated SP because of degradation of the ester moiety by SP enzymes.

Detection and distribution of CD59, CD46, CD55, and CDw52 in SP. Total protein, CD59, CD46, and CD55 were measured in 10 samples of SP and VSP (Table I). Protein and CD46 concentrations of the two groups were comparable. In contrast, the concentrations of CD55 and CD59 in VSP were ∼ 50% of those measured in SP. In both groups the concentration of CD59 was ∼ 20-fold greater than that of either CD46 or CD55.

CD46 was detected as a 60-kD band which had comparable intensity in SP and VSP (Fig. 3 *A*); less intensely stained bands
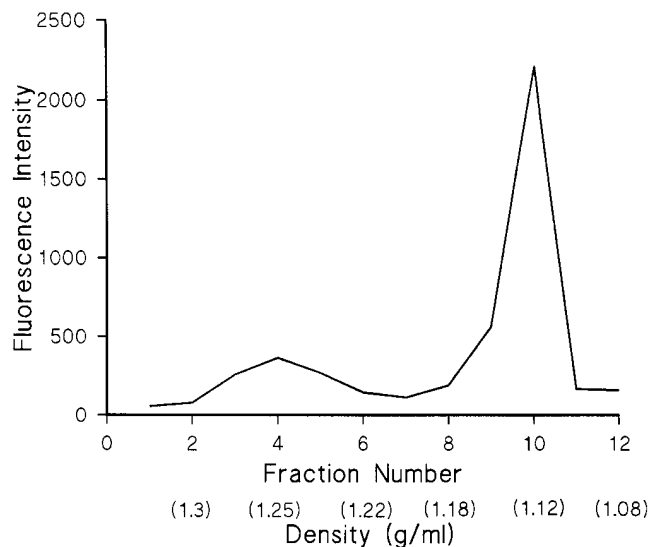

Figure 5. Density gradient centrifugation of DiI-labeled SP. DiI-labeled SP was fractionated by centrifugation in a median loaded, discontinuous Nycodenz gradient. 1-ml fractions were collected. The figure records intensity of DiI fluorescence in the fractions; numbers in parentheses are the density of the fractions in grams per milliliter.
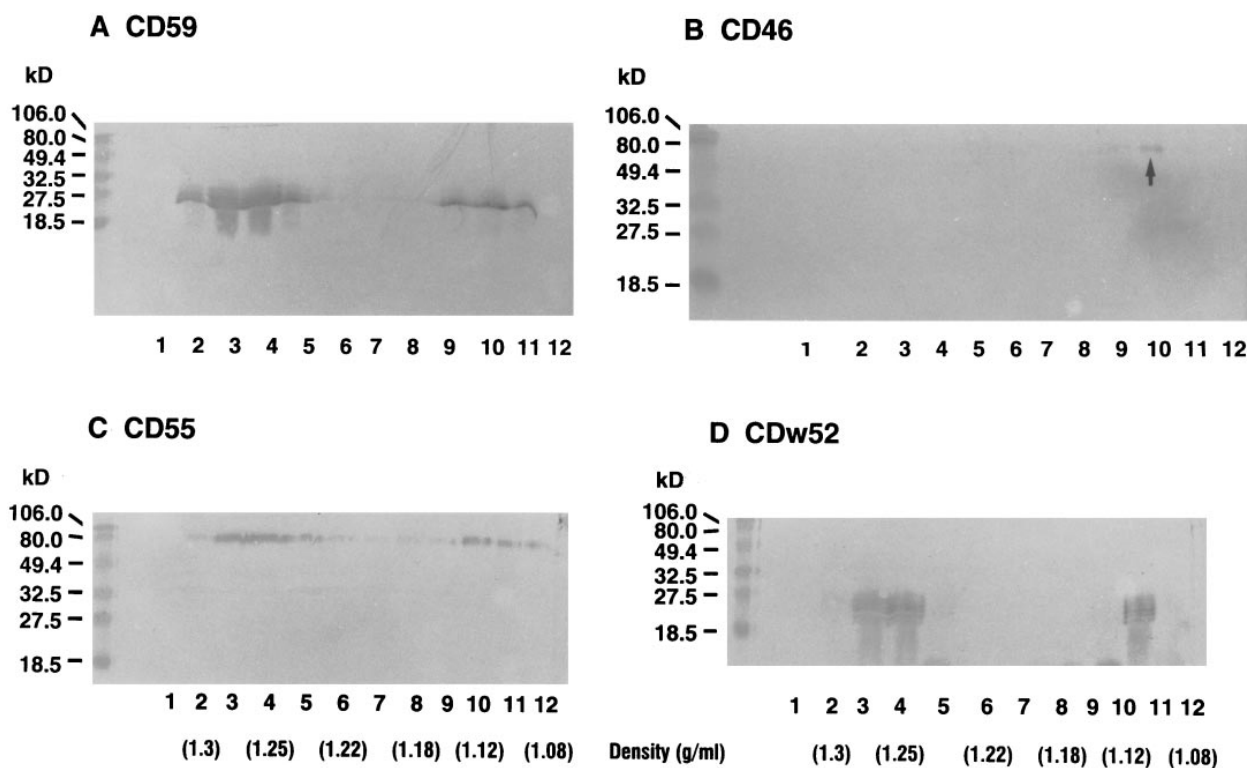
Figure 6. Western blots of fractions from density gradient centrifugation of SP. Fractions from density gradient centrifugation of SP (conducted as for Fig. 5) were Western blotted (15 μl/lane). Lanes are labeled with the fraction numbers; densities of the fractions were as recorded in Fig. 5. (A) CD59 was detected in fractions 9–11 (1.12 grams/ml) and with comparable intensity in fractions 3–5 (density 1.24–1.27 grams/ml). (B) CD46. A 60-kD band was present in fraction 10 only (arrow). (C) CD55 was detected in fractions 10 and 11 and also in fractions 3 and 4. (D) CDw52 was detected in fraction 10 and in fractions 3–5.

of 42 and 44 kD were also present in most samples of SP but were faint or absent from VSP. CDw52 was detected by Western blot as a 20–25-kD band. This protein was present in all samples of SP examined but was not detected in VSP (Fig. 3 B). CD59 appeared on Western blots as an 18–25-kD band (Fig. 4 A) and CD55 as a major band at 70 kD (Fig. 4 C). Western blotting also indicated that CD59 and CD55 were lower in concentration (see Table I) in VSP than in SP.

After ultracentrifugation, most CD59 in SP was in the pellet (Fig. 4 A). The quantity in the supernatant never exceeded 20% and was usually undetectable. For CD46, the 60- and 40-

kD bands were restricted to the pellet, whereas the 42-kD band appeared only in the supernatant (Fig. 4 B). CD55 detected in supernatant by this technique was more variable, ranging from undetectable in some cases (Fig. 4 C) to approaching 50% of the total in others. A significant amount of CDw52 (at least 50%) was present in supernatant in all cases (Fig. 4 D). The CDw52, CD55, and CD59 in the supernatant partitioned (> 90%) into the detergent phase of Triton X-114, indicating that each possessed a GPI anchor (not shown).

Anchor-positive proteins are present in an NPF. The data presented in Fig. 4 suggest that some GPI-anchored proteins in SP
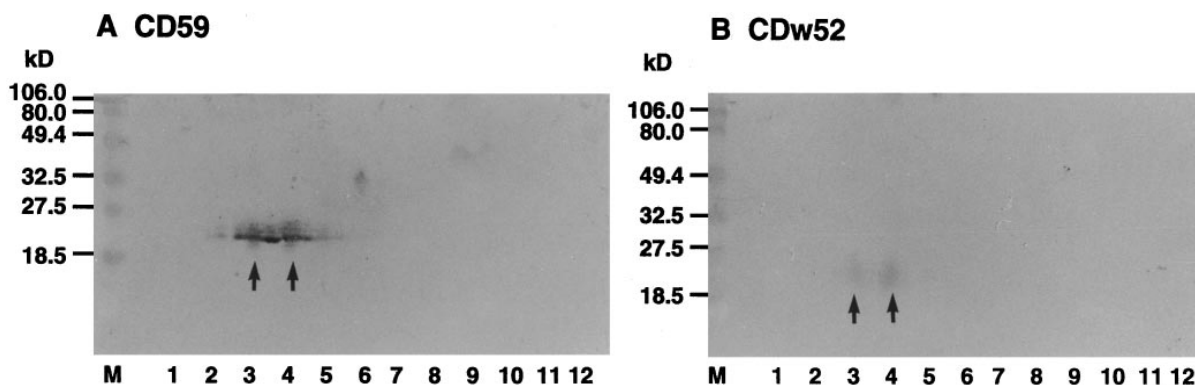

Figure 7. Density gradient centrifugation of the ultracentrifugation supernatant of SP. The supernatant from ultracentrifugation of SP was separated by density gradient centrifugation and fractions were analyzed by Western blot. CD59 (A) and CDw52 (B) were detected in fractions 3–5 but were absent from the lower density fractions 10–12.
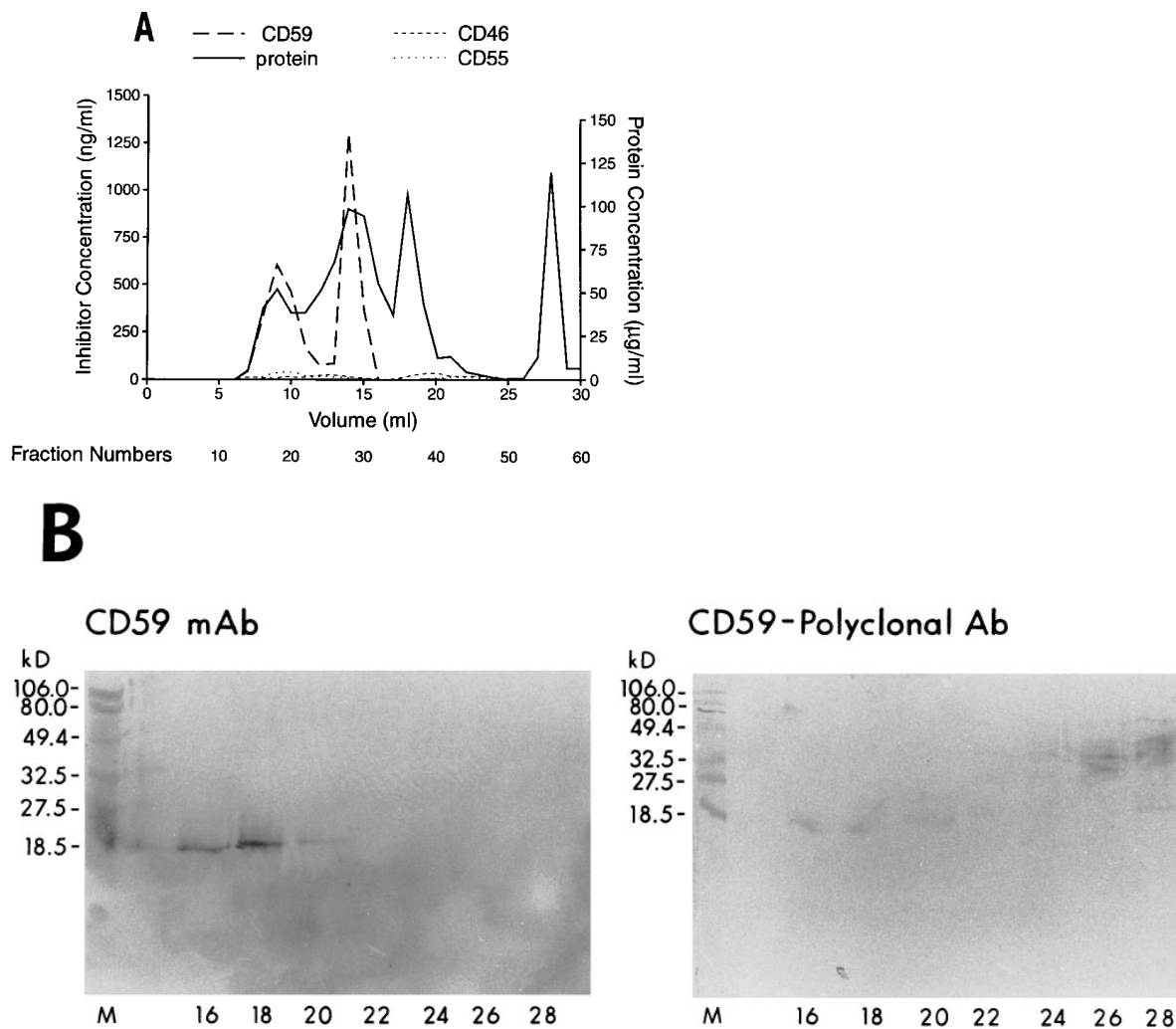
Figure 8. Gel filtration FPLC of the NPF on Superose 12. Fractions 3–5 (density 1.24–1.27 grams/ml) from density gradient centrifugation of SP were pooled, dialyzed against PBS, concentrated to 0.3 ml, and then fractionated by FPLC on a column of Superose 12. (*A*) Biochemical analysis of fractions. CD59 and CD55 (detected by ELISA) coeluted with the first major protein peak (fractions 16–22; 8–11 ml). For each protein a second, later peak was also detected (13–15 ml for CD59 and peak 21 ml for CD55). CD46 immunoreactivity was detected at very low levels throughout the elution profile. The elution profile of the ultracentrifugation supernatant of SP was identical, except for variations in peak size. (*B*) Western blotting of fractions. CD59 (molecular mass 20 kD) was detected in fractions 16–22 (8–11 ml only). Using polyclonal anti-CD59, 20-kD CD59 was detected in fractions 16–22 and several higher molecular mass bands were detected in fractions 26–30 (13–15 ml).

are not bound to prostasomes. We investigated this possibility using median loaded equilibrium density gradient centrifugation. DiI-labeled, purified prostasomes and CD59 immunoreactivity equilibrated in a discrete band at a density of 1.12 grams/ml (not shown). These data indicate that if all GPI-anchored proteins in SP were prostasome bound, they should equilibrate at 1.12 grams/ml regardless of prostasome size. Unfractionated, DiI-labeled SP was subjected to density gradient centrifugation. Approximately 80% of DiI fluorescence rose to equilibrate at 1.12 grams/ml while the remainder was detected in the sample loading zone (1.22–1.30 grams/ml; Fig. 5). The 60-kD CD46 detectable by Western blot was localized to the prostasome fraction (Fig. 6 *B*). In contrast, CD59, CD55, and CDw52 (Fig. 6, *A*, *C*, and *D*) were detected in the prostasome fraction and in the sample loading zone, referred to as the NPF. In both the prostasome fraction and the NPF, CD59, CD55, and CDw52 were GPI anchored, based on partitioning

in Triton X-114 (not shown).

The ultracentrifugation supernatant was next investigated by this technique. CD59 (Fig. 7 *A*), CDw52 (Fig. 7 *B*), and CD55 (not shown) were detected in the sample loading zone (or NPF) but were not detected in the lower density fraction (1.12 grams/ml) expected for prostasomes, indicating that the ultracentrifugation supernatant was prostasome free.

FPLC analysis of proteins in the NPF. The above data demonstrate the unexpected finding that a high-density NPF of SP, which remains in supernatant after ultracentrifugation, contains intact GPI-anchored proteins. Therefore, the proteins in the ultracentrifugation supernatant and the NPF from density gradient centrifugation were subjected to FPLC. Using Superose 12, protein eluted in four major peaks (Fig. 8 *A*).

By ELISA, CD59 was detected in two peaks, a smaller, early peak which coeluted with the first protein peak between 7 and 11 ml (fractions 16–22) with peak concentration at 9 ml
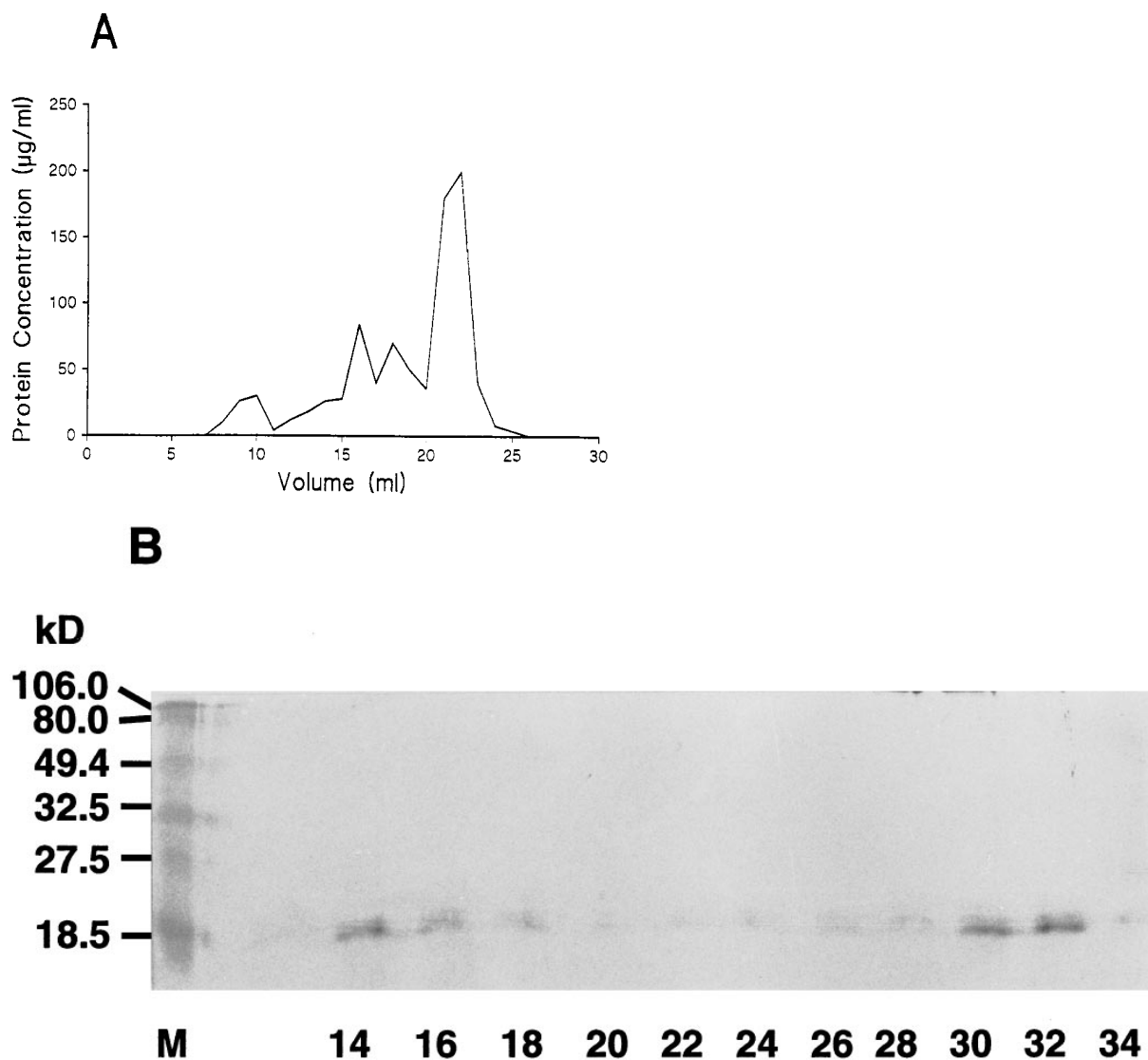
Figure 9. Gel filtration FPLC of the NPF on Superose 6. (*A*) The figure shows the concentration of total protein in fractions from the column. (*B*) Western blot stained for CD59 using mAb anti-CD59. CD59 was detected in fractions 30–32 (15–16 ml) with high intensity and with lower intensity in fractions 14–29 (7–14.5 ml).

(void volume was 7 ml) and a taller one (fractions 26–30) which eluted with peak concentration at 14 ml. Amounts of CD59 were 0.82 μg in peak 1 and 0.84 μg in peak 2 (the predicted molecular mass of a protein eluting in this position is 50 kD) (Fig. 8 *A*). By Western blotting using mAb anti-CD59 (Fig. 8 *B*), a 20-kD band was detected in fractions of the first peak, whereas no anti-CD59 material was detected in fractions 26–30.

By Western blotting, CD55 and CDw52 also eluted in fractions 16–22 (not shown). As expected, no CD46 was detected by Western blot in these fractions. When Western blots were stained with polyclonal anti-CD59 antibody, 20-kD CD59 was again detected in fractions of the first peak (Fig. 8 *B*). In addition this antibody detected a "ladder" of anti-CD59 reactive material with a predicted molecular mass of 30–50 kD in fractions 26–30.

Based on partitioning in solution of Triton X-114, CD59 immunoreactivity in the first peak was hydrophobic, whereas CD59 immunoreactivity in the second peak was hydrophilic,

indicating that CD59 immunoreactivity in the second peak was due to anchor-negative CD59 fragments or to some other cross-reacting species.

The above data establish that all GPI-anchored CD59, CD55, and CDw52 coeluted with the first protein peak and were therefore present as part of a high molecular mass species.

Superose 6 was used to determine the molecular mass of the GPI-anchored protein-containing species in the NPF. Protein eluted in four major peaks (Fig. 9 *A*). By Western blotting CD59 (Fig. 9 *B*), CD55 (not shown) and CDw52 (not shown) were detected with highest intensity in fractions 30–32 (15–16 ml), indicating a molecular mass of 4×10^5 kD. CD59 was also detected with lower intensity in earlier fractions. The high intensity of CD59 reactivity in fractions 14–16 (7–8 ml) reflects the fact that these fractions are just within the column's void volume and cannot be interpreted as a discrete peak of homogeneous complexes. These data indicate that the NPF contains a population of 4×10^5 kD, CD59-containing complexes, to-
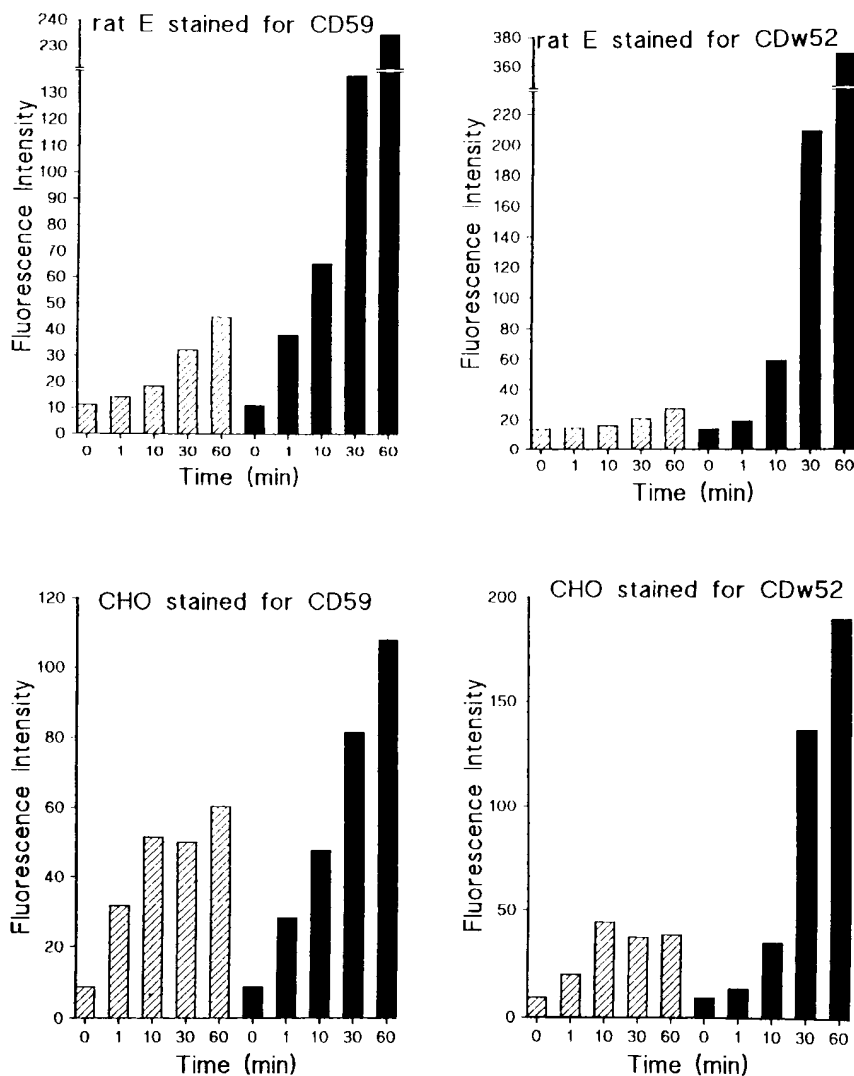
Figure 10. Cellular uptake of proteins from prostasomes (*hatched bars*) and from the NPF (*black bars*). Rat E or CHO cells were incubated for varying times with prostasomes or with the NPF from density gradient centrifugation of SP, stained for CD59 or CDw52 and analyzed by flow cytometry.

gether with a heterogeneous population of larger, variably sized CD59-containing complexes, possibly aggregates of the 4 × 10^5 kD units. The CD59-, CD55-, and CDw52-containing fractions were examined by negative staining transmission electron microscopy. No prostasomes were present. Variably sized, irregularly shaped structures which excluded stain were observed but were not further identified.

Binding of GPI-anchored proteins on prostasomes and in the NPF to cells. Both the prostasome fraction and the NPF from density gradient centrifugation of SP donated CD59 and CDw52 to rat E and CHO cells in a time-dependent manner (Fig. 10). These cells acquired lower amounts of both proteins from prostasomes than from the NPF. CHO cells acquired slightly greater levels of both proteins from prostasomes than from the NPF for the first 10 min of incubation. Thereafter, no further increase in protein levels was seen in cells incubated with prostasomes, whereas those incubated with the NPF continued to acquire increasing levels of protein with time. The incorporated proteins were stably bound and sensitive to removal by phosphatidyl inositol–specific phospholipase C.

The interaction of DiI- and BCECF-labeled prostasomes with adherent CHO cells and macrophages in vitro was exam-

ined using fluorescence-enhanced videomicroscopy. Prostasomes were visible as discrete, spherical, fluorescent particles exhibiting Brownian motion. These were observed to contact and adhere to the surface of cells; however, the fluorescence remained prostasome associated and was neither transferred to the cytoplasm (as would have occurred if BCECF-labeled vesicles fused with the cells) nor transmitted to the plasma membrane (as would have occurred if DiI-labeled vesicles fused). After 15–30 min, the fluorescent prostasomes were internalized by the macrophages and CHO cells and became associated with the cellular endosomal system (Fig. 11). This internalization may explain the plateau phase of CD59 and CDw52 acquisition by CHO cells incubated with prostasomes, since internalized material is not detected by the surface immunostaining technique. Rat E do not internalize prostasomes, therefore no plateau phase was observed when these cells were used.

When rat E were incubated with prostasomes, washed, solubilized, and assayed for their content of CD59 and CD46, their uptake of prostasome CD46 was found to be comparable with that of CD59, suggesting that the cells acquired transmembrane and GPI-anchored proteins from prostasomes with
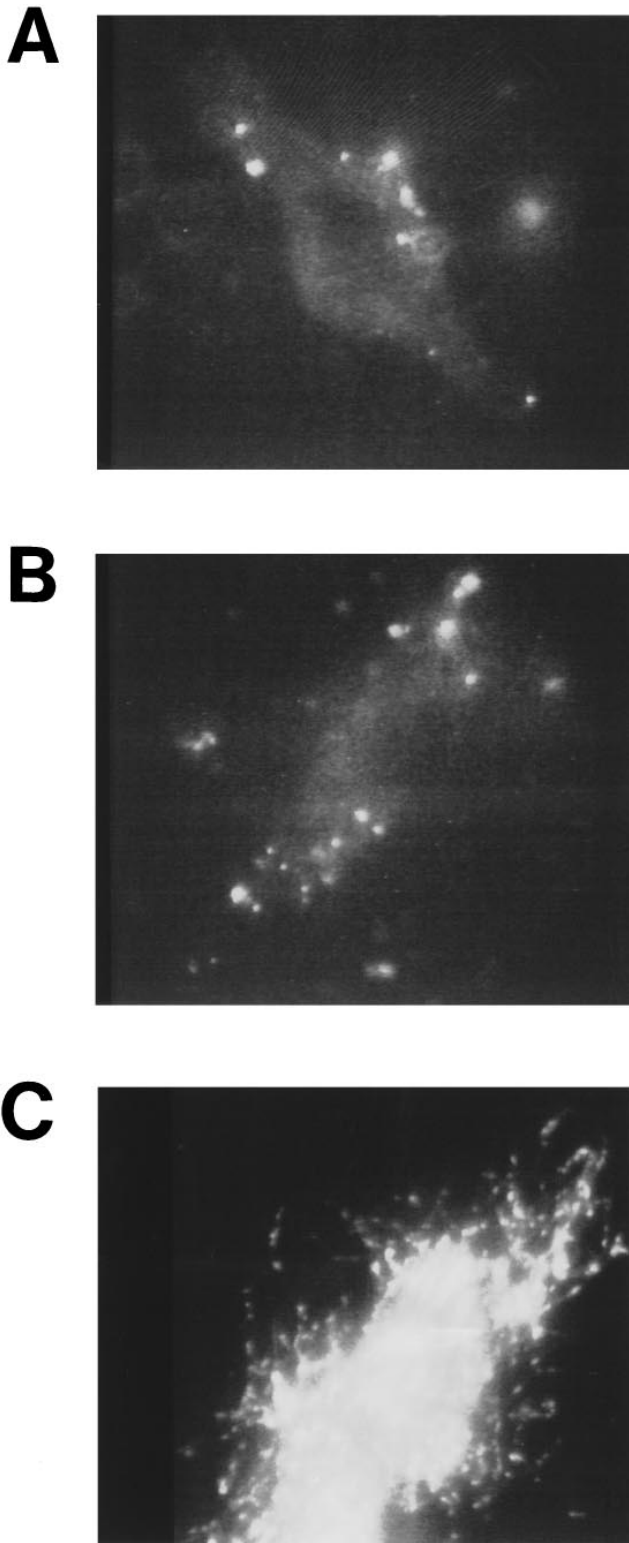
Figure 11. Interaction of DiI-labeled prostasomes with cells. The interaction of DiI-labeled prostasomes with macrophages attached to glass coverslips was observed by fluorescence microscopy. Labeled prostasomes appeared as discrete, fluorescent particles which initially moved with Brownian motion. However, after 10 min (*A*) or 12 min (*B*) several prostasomes had adhered to the cell surface. By 30 min (*C*) the adherent prostasomes were internalized by the cells, apparently by endocytosis. ×1,000.

Table II. Uptake of Prostasome Proteins by Erythrocytes

	Incubation		
	1 min RT	12 h 4°C	12 h 37°C
Percent CD59 bound	5.6	5.1	31.7
Percent CD46 bound	6.8	8.1	29.7

Rat E were incubated with purified prostasomes under the conditions stated (*RT*, room temperature). After separation from unbound material and washing, the erythrocytes were solubilized and their content of CD59 and CD46 was measured by ELISA (expressed as a percentage of the total). Tests were done in duplicate and values are means of two experiments.

equal efficiency (Table II). These data, together with the videomicroscopy data reported above, point out that cells acquire prostasome proteins as a result of adherence of whole prostasomes to their membranes.

Discussion

We here report our investigation in SP of the distribution and cell-binding potential of three GPI-anchored proteins, CD59, CD55, and CDw52, and of one transmembrane protein, CD46. The late complement inhibitor CD59 was present in SP at higher levels (26.8 μg/ml) than the C3 convertase inhibitors CD55 (1.06 μg/ml) and CD46 (0.682 μg/ml). These values are comparable with previously reported values of 20 μg/ml for CD59 (1) and 500 ng/ml for CD46 (3). We were not able to measure CDw52 by two-site ELISA but data from Hale et al. (4) indicate that SP contains high concentrations of this protein.

We reported previously that SP CD59 is anchor intact and is present on membrane vesicles called prostasomes (1). We demonstrate here that CD55 and CDw52 in SP are also anchor intact. Further, we report the surprising finding that at least 50% of anchor-intact CDw52, a smaller percentage of anchor-intact CD59, and a variable percentage of anchor-intact CD55 are not prostasome bound. Our data indicate that the GPI anchors of proteins in this NPF are preserved in the absence of lipid bilayers.

Although most CD59 is pelleted with prostasomes during ultracentrifugation, at least 50% of CDw52 and a variable percentage of CD55 remain in the supernatant (Fig. 4). To examine the behavior of prostasomes during separation procedures we used the lipophilic probe DiI to label their outer membranes and the AM ester of BCECF to label their internal aqueous contents. Prostasomes retained BCECF during centrifugation, gel filtration chromatography (Fig. 1 *A*), and immunoprecipitation (Fig. 1 *B*), demonstrating that they are closed membrane vesicles. Anti-CD59 mAb precipitated CD59, DiI, and BCECF from suspensions of purified prostasomes with equal efficiency, indicating that all prostasomes in the suspension possessed CD59 (Fig. 1 *B*), and electron microscopy of immunoprecipitated prostasomes revealed a population of variably sized (average 150 nm) membrane vesicles (Fig. 2).

BCECF-AM, which is vulnerable to cleavage by a number of enzymes present in body fluids, could be used to label prostasomes only after they had been purified. In contrast, when unfractionated SP was incubated with DiI, most of the probe partitioned selectively into the membrane component and

could therefore be used as a prostasome marker during separation procedures. During ultracentrifugation of SP, > 95% of DiI was detected in the pellet, indicating that virtually all membrane present in SP was pelleted, and during density gradient centrifugation of SP most DiI rose to equilibrate at 1.12 grams/ml (Fig. 5), the equilibration density, determined here, of purified prostasomes.

SP CD46 pelleted with prostasomes during ultracentrifugation of SP, whereas significant amounts of the three GPI-anchored proteins remained in the supernatant (Fig. 4). Density gradient centrifugation of SP established that CD46 was associated with the prostasome fraction whereas CD59, CD55, and CDw52 equilibrated both in the prostasome fraction and in a separate fraction of higher density (1.24–1.27 grams/ml), referred to here as the NPF. When the ultracentrifugation supernatant of SP was separated by density gradient centrifugation, CD59, CD55, and CDw52 were detected in the NPF and not at the density expected for prostasomes, confirming that the ultracentrifugation supernatant was prostasome free (Fig. 7). CD59, CD55, and CDw52 present in ultracentrifugation supernatant and in the NPF partitioned into the detergent phase of Triton X-114 and bound cell membranes (Fig. 10), demonstrating that they were anchor intact. Collectively, these data establish that some GPI-anchored proteins in SP are not prostasome bound. The density of the NPF indicates a high protein/lipid ratio which, together with the failure of proteins in this fraction to pellet during ultracentrifugation, suggests that these proteins were not associated with organized membrane. The presence of some DiI ($\sim$ 20% of the total) in the 1.24–1.27 grams/ml fraction from density gradient centrifugation of SP (Fig. 5) indicates that this fraction contained lipid which might permit preservation of the hydrophobic anchor.

GPI-anchored proteins in the NPF eluted at high molecular mass ($> 4 \times 10^5$ kD) during size exclusion FPLC (Figs. 8 and 9) and could bind cell membranes (Fig. 10). Examination of these FPLC fractions by negative staining transmission electron microscopy (which allowed good visualization of prostasomes) revealed irregular, variably sized structures which were not further identified, but no identifiable organelles. We propose that GPI-linked proteins in the NPF are contained within hydrophobic aggregates and as a result are available for incorporation into cell membranes.

We reported previously that cells acquired CD59 during incubation with partially purified prostasomes, but we did not determine the mechanism by which this acquisition occurred (1). Our finding that rat E acquired prostasome CD46 and CD59 with comparable efficiency (Table II) indicates that cells acquire prostasome proteins by binding whole prostasomes, rather than by intermembrane transfer of the GPI anchor. Prostasomes have previously been noted to associate with cells (18) although the nature of this association was unclear. Using videomicroscopy we determined that the organelles adhere to, but do not fuse with, cells (Fig. 11).

As a first step in elucidating the relationship of the NPF with prostasomes, we partially localized the origins of the four proteins investigated here by comparing their concentrations in SP with VSP. Mean concentration of total protein in VSP was only slightly lower than that in SP, in keeping with our knowledge that most SP protein originates in seminal vesicles and prostate (19, 20). Levels of CD46 in VSP were also comparable with those in SP, indicating that most CD46 arises from below the point of transection of the ductus deferens. SP CD46

appeared predominantly as a 60-kD band on Western blotting as noted by others (21, 22) and, since prostatic epithelium expresses a 60-kD form of CD46 exclusively (22), these data suggest that SP CD46 is associated with membrane particles derived from the prostate. The lower molecular mass forms of CD46 detected in SP but not in VSP likely derive from spermatozoa which express CD46 of comparable molecular mass (23).

Levels of CD59 and CD55 in VSP were approximately half those seen in SP, indicating that these proteins derive from sites both proximal and distal to the point of transection of the ductus deferens. CD59 is widely expressed in human tissues (24) and might arise from any organ of the male reproductive tract. SP CD55 is unlikely to arise from testis (which expresses CD55 of lower molecular mass than the 70- and 76-kD species identified here [22, 25]) or from prostate, which expresses CD55 very poorly (22); Therefore, likely sites of CD55 production are epididymis and seminal vesicles. CDw52 was absent from VSP, indicating that this protein arises proximal to the point of transection, most likely from the epididymis, where it is very strongly expressed (5).

A number of cell types release vesicles rich in GPI-anchored proteins (26, 27) and such vesicles might release GPI-anchored proteins to the fluid phase more easily than the membranes of whole cells. A second possibility is that micelles containing GPI-anchored proteins are released from cells as a consequence of stress to the plasma membrane during vesiculation. Investigation of these issues is complicated by mixing and biochemical interaction of secretions from several organs within SP before the material reaches the laboratory. Animal models and tissue culture systems are required and we are pursuing both approaches.

Prostasomes and GPI-containing aggregates in SP represent two mechanisms by which spermatozoa (which do not synthesize protein) may acquire new proteins, thus altering their antigenicity, resistance to immune attack, and other surface properties. Sperm are at risk of C attack within the female reproductive tract (28) and C inhibitors may be of importance for fertility. Further, sperm acquire new proteins during passage through the epididymis (29). It will be of interest to determine whether these acquired proteins are GPI anchored and whether a micellar intermediate is involved in transfer. The GPI-anchored proteins reported here might also be acquired by microbial pathogens in the male reproductive tract with important clinical consequences including increased resistance to C attack. Finally, this description of a stable population of membrane-free, GPI-anchored proteins available for transfer to cells within a body fluid raises the possibility of developing lipid-associated, GPI-anchored proteins suitable for therapeutic use.

Acknowledgments

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