Cells Lacking Glycan Phosphatidylinositol-Linked Proteins Have Impaired Ability to Vesiculate

By Michael Whitlow, Kyoko Iida, Patricia Marshall, Robert Silber, and Victor Nussenzweig

Erythrocytes shed membrane vesicles in response to many stimuli. It has been previously demonstrated that glycan phosphatidylinositol-linked (GPI-linked) proteins such as decay accelerating factor and acetylcholinesterase are concentrated in these vesicles relative to the erythrocyte membrane. We have examined the requirement for GPI-linked proteins for the process of vesiculation. Erythrocytes that do not express GPI-linked proteins, obtained from patients with paroxysmal nocturnal hemoglobinuria (PNH), release between 10% and 50% of the quantity of vesicles as normal cells in response to the Ca\(^{2+}\) ionophore A23187. Platelets from the same patients produced 10% to 20% of the amount of vesicles as normal platelets. In addition, a mutant B-lymphoblastoid cell line that lacks GPI-linked molecules produces about half of the number of vesicles as compared with the wild-type cell line in response to the Ca\(^{2+}\) ionophore. Prior findings indicate that vesiculation is one of the mechanisms that the cell uses to remodel the plasma membrane, as well as protect itself from membrane-damaging agents such as the terminal complement components C5b-9. On the basis of the present results, we conclude that GPI-linked proteins play an important role in membrane vesiculation.

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MATERIALS AND METHODS

Buffers and solutions. All buffers were prepared with glass-distilled and ultrafiltered before use: phosphate-buffered saline (PBS), 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 7.4; dextrose, 50 mmol/L, veronal-buffered saline containing calcium, magnesium (DGVB\(^{2+}\)), veronal-buffered saline containing 71 mmol/L NaCl and 2.5 mmol/L sodium veronal, 0.1% gelatin, 1.05 mmol/L Ca\(^{2+}\), 1 mmol/L Mg\(^{2+}\), and 139 mmol/L dextrose; gelatin, veronal-buffered saline containing calcium, magnesium (GVBG\(^{2+}\)), veronal-buffered saline, pH 7.4, containing 142 mmol/L NaCl, 4.9 mmol/L sodium veronal, 0.1% gelatin, 0.15 mmol/L Ca\(^{2+}\), and 1 mmol/L Mg\(^{2+}\); GVB\(^{2+}\)-veronal-buffered saline, pH 7.4, containing 142 mmol/L NaCl, 4.9 mmol/L sodium veronal, 0.1% gelatin, 0.15 mmol/L Ca\(^{2+}\), and 1 mmol/L Mg\(^{2+}\).

Buffers were prepared with glass-distilled water and ultrafiltered before use: phosphate-buffered saline (PBS), 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 7.4; dextrose, 50 mmol/L, veronal-buffered saline containing calcium, magnesium (DGVB\(^{2+}\)), veronal-buffered saline containing 71 mmol/L NaCl and 2.5 mmol/L sodium veronal, 0.1% gelatin, 1.05 mmol/L Ca\(^{2+}\), 1 mmol/L Mg\(^{2+}\), and 139 mmol/L dextrose; gelatin, veronal-buffered saline containing calcium, magnesium (GVBG\(^{2+}\)), veronal-buffered saline, pH 7.4, containing 142 mmol/L NaCl, 4.9 mmol/L sodium veronal, 0.1% gelatin, 0.15 mmol/L Ca\(^{2+}\), and 1 mmol/L Mg\(^{2+}\); GVB\(^{2+}\)-veronal-buffered saline, pH 7.4, containing 142 mmol/L NaCl, 4.9 mmol/L sodium veronal, 0.1% gelatin, 0.15 mmol/L Ca\(^{2+}\), and 1 mmol/L Mg\(^{2+}\).

Antibodies. The monoclonal antibodies (MoAbs) against DAF, IA10, and IIH6 were described in Kinoshita et al.\(^{15}\) The MoAb against CD59, MEM-43, is a generous gift of Dr Irena Stefanova (Czechoslovak Academy of Sciences). Before use, the antibodies were purified from ascites fluid using Affi-gel protein A-Sepharose kit (Calbiochem, San Diego, CA).

Purification of DAF and CD59\(^{2+}\) erythrocytes. Blood from normal controls or PNH patients was collected in tubes containing EDTA.\(^{26}\) The blood of one patient was a generous gift of Dr Wendell Rosse (Department of Hematology, Duke University Medical Center, Durham, NC). Erythrocytes from PNH patients (10\(^8\)/mL in PBS, 0.1% bovine serum albumin [BSA], 0.02% azide [PBS-AZ]) were mixed with an equal volume of 5 mg/mL MEM-43, 5 mg/mL of IA10, and 5 mg/mL of IIH6. After incubation for 60 minutes on ice, cells were washed once with PBS-AZ, resuspended at 10\(^8\)/mL, and then loaded into a protein A-Sepharose CL-4B column (2 \times 10\(^8\) erythrocytes/mL packed gel; Pharmacia, Piscataway, NJ) equilibrated in the same buffer. The column was closed and kept for 15 minutes at room temperature. Unbound cells were collected by washing the column with five column volumes of the same buffer.\(^{26}\)

Fluorescence-activated cell sorter (FACS) analysis of normal and PNH erythrocytes. Samples of normal or PNH erythrocytes were
incubated with MoAbs to either DAF or MEM-43 for 15 minutes at 0°C. Control samples were incubated with irrelevant MoAb. The cells were washed, incubated with phycoerythrin-labeled antibodies to mouse IgG for 15 minutes at 0°C, and subjected to FACS analysis.

Vesiculation of erythrocytes. Blood from either normal volunteers or patients with PNH was collected in EDTA. Erythrocytes were washed, stored in DGVB1, and used within 3 days of collection. For vesiculation experiments, cells were washed and resuspended in GVB. Erythrocytes (5.3 x 10^8/mL) were incubated with A23187 (4 μmol/L) and calcium, or EDTA (3.3 mmol/L). To terminate vesiculation, EDTA (3.3 mmol/L) was added and the tubes were placed at 0°C.

Cells were then pelleted at 550g for 8 minutes and vesicles isolated from the supernatant by centrifugation at 30,000g for 15 minutes in a Beckman TL-100 (Beckman Instruments, Palo Alto, CA).

Calcium uptake. Erythrocytes (5.3 x 10^8/mL) were incubated with A23187 (4 μmol/L) at 37°C for 20 minutes. Calcium, containing a trace amount of 45Ca^{2+}, was then added. At various times, the medium was made 10 mmol/L in EDTA and spun through dibutyl phthalate. The cell pellet was analyzed for 45Ca^{2+} by 10% trichloracetic acid (TCA) precipitation and liquid scintillation counting. From the ratio of 45Ca^{2+} to cold calcium, the molecules of calcium per cell could be calculated.

Isolation and vesiculation of platelets. All procedures for platelet isolation were performed at room temperature. Whole blood (collected in the presence of EDTA) was centrifuged at 800g for 10 minutes. The plasma was then run over a Sepharose 2B column in Tyrode’s-EDTA buffer. The void volume containing the platelets was collected. Platelets were used for an experiment the same day the blood was drawn with the exception of CD59 negative controls. Vesicles were isolated by a variation on the procedure of Diaz et al. All manipulations were performed at 4°C. Cells were suspended in 20 mmol/L HEPES, pH 7.4, supplemented with 1.07 g/mL dextran 70 to block LDH leakage.

RESULTS

Erythrocytes lacking GPI-linked proteins vesiculate less than normal erythrocytes. We compared erythrocytes from normal individuals and CD59- and DAF-negative erythrocytes from two PNH patients (S.B. and L.D.) with regard to their ability to vesiculate. In the case of S.B., erythrocytes that were negative for CD59 and DAF were purified as outlined; these cells were totally negative for DAF and CD59 as measured by FACS analysis. Ten percent of the erythrocytes from L.D. were positive for DAF and CD59 by FACS analysis, and were used without purification. Erythrocytes were incubated with A23187 and calcium for 1 hour, a time when vesiculation reached a endpoint. Controls consisted of erythrocytes incubated with A23187 and EDTA. The cells were pelleted and vesicles isolated from the supernatant. Vesicles were quantitated by measuring either protein (Fig 1) or lipid phosphate (Fig 2). In some of the subsequent experiments, only protein was measured, as protein assays are simpler and more reproducible than lipid phosphate determinations.
Figure 1. Vesicle protein released by normal and PNH erythrocytes. Erythrocytes from normal controls or PNH patients were treated with 4 μmol/L A23187 and 80 μmol/L CaCl₂ at 37°C for 60 minutes. Vesicle protein was quantitated as indicated in the text. Normal control data are expressed as mean ± standard deviation. P values reflect the significance of the patient’s erythrocyte vesiculation compared with normal erythrocyte vesiculation.

The results demonstrate that GPI-negative erythrocytes from the PNH patients vesiculate significantly less than the normal erythrocytes when either vesicle lipid or vesicle protein is measured. In normal individuals, 12.2% of the total lipid phosphate is shed, whereas only 1.0% of the protein is released into the supernatant. In patient L.D., 0.8% of the lipid and 0.23% of the protein is shed, whereas in patient S.B., 4.5% of the lipid and 0.49% of the protein is released as vesicles. The higher proportion of lipid than protein most likely reflects a much higher surface/volume ratio of the vesicles compared with the intact erythrocytes.

We next wished to examine whether separating PNH and normal erythrocytes by density would effect the ability of the cells to vesiculate. For this experiment, we separated either normal erythrocytes or erythrocytes from patient L.D. on a Percoll gradient before vesiculation. It has been previously shown that erythrocytes increase in density and decrease in volume as they age.35 In addition, in erythrocytes from PNH patients, there is a progressive increase in GPI-positive cells as the erythrocytes age. As can be seen in Table 1, all three fractions of L.D.’s erythrocytes show significantly less vesiculation than does the normal control. In addition, as the percentage of GPI-positive erythrocytes increase, the amount of vesiculation also increases. As this decreased ability to vesiculate is present in all fractions, including the youngest (lightest) erythrocytes, it indicates that the defect in vesiculation is an inherent defect in the PNH erythrocytes, not a consequence of prior in vivo vesiculation by the PNH erythrocytes.

Platelets that lack GPI-linked proteins vesiculate less than normal platelets in response to calcium loading. We compared vesiculation in platelets from two PNH patients that were totally deficient in GPI-anchored proteins. Figure 3 shows that the PNH platelets vesiculate significantly less than the normal platelets when incubated with calcium and ionophore A23187.

A lymphoblastoid cell line that lacks GPI-linked molecules vesiculates less than the wild-type cells. The JY25 cell line expresses GPI-linked molecules, whereas JY5, a mutant of JY25, does not. In experiments not shown, we found that JY25 cells treated with A23187 and calcium produced lipid vesicles with the same density as vesicles produced by erythrocytes, and that these vesicles were enriched for DAF 10-fold relative to the untreated plasma membrane. The vesicles contain 3.26 × 10⁻⁷ ng DAF/nmol lipid phosphate and the

Table 1. Vesiculation of Density-Separated Erythrocytes

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>MCV (%)</th>
<th>% GPI (+)*</th>
<th>Vesicle Lipid (nmol cell × 10⁻⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.D. Top</td>
<td>152</td>
<td>4.4</td>
<td>0.127</td>
</tr>
<tr>
<td>Middle</td>
<td>110</td>
<td>9.5</td>
<td>0.208</td>
</tr>
<tr>
<td>Bottom</td>
<td>111</td>
<td>16.7</td>
<td>0.238</td>
</tr>
<tr>
<td>Control</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Top</td>
<td>109</td>
<td>100</td>
<td>3.16</td>
</tr>
<tr>
<td>Middle</td>
<td>91.7</td>
<td>100</td>
<td>3.23</td>
</tr>
<tr>
<td>Bottom</td>
<td>98.9</td>
<td>100</td>
<td>2.28</td>
</tr>
</tbody>
</table>

The normal and PNH erythrocytes were separated into three fractions, based on density using Percoll gradient centrifugation as described in Materials and Methods. The top fraction is the least dense and the bottom fraction the most dense.

* Percent of erythrocytes that express CD59 and DAF.
GPI-NEGATIVE CELLS HAVE LESS MEMBRANE VESICULATION

plasma membrane contains $3.20 \times 10^{-3}$ ng DAF/nmol lipid phosphate. The amount of membrane that is released as vesicles is very small. Nevertheless, when we incorporated the fluorescent cationic membrane probe DiI into the plasma membrane of JY5 and JY25, and measured the fluorescence of the vesicles released into the supernatant, reproducible measurements of vesiculation were obtained. Figure 4 illustrates the results of a kinetic experiment in which we measured the percent of total incorporated DiI released as vesicles after incubation of the cells with ionophore for different periods of time. As shown, the wild-type JY25 cells release two to three times more vesicles than the mutant JY5 cells line. At the various time points, the proportion of dead JY5 and JY25 cells as measured by LDH release was not significantly different (Table 2).

We also removed GPI-linked proteins from the surface of JY25 cells in an attempt to obtain confirmatory evidence for the role of GPI in vesiculation. For this purpose, JY25 cells were treated with phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus thuringiensis. This procedure led to the removal of 80% of DAF and CD59 from the JY25 cell surface. Nevertheless, the vesiculation experiments with the PIPLC-treated cells were inconclusive, because the combination of PIPLC and calcium ionophore treatments was toxic to the cells, leading to an unacceptable proportion of dead cells at the end of the incubation.

PNH GPI-negative erythrocytes vesiculate less than GPI-positive erythrocytes. Next, we wished to compare vesiculation in GPI-negative and GPI-positive erythrocytes from the same PNH patient. Although the negative cells could be purified using a very mild procedure (see Materials and Methods), unfortunately we could not isolate the positive cells without damaging them. For example, after treatment of the mixture with acidified serum to lyse the GPI-negative cells, the remaining cells showed excessive lysis when incubated with calcium and ionophore. For this reason, we could only compare vesiculation of normal erythrocytes (with unseparated erythrocytes from PNH patient S.B.) and of GPI-negative erythrocytes from the same patient (negative for DAF and CD59 as determined by FACS analysis). This was performed on two separate occasions. About 70% of her cells were negative for DAF, CD59, and AChE, and about 30% were positive for these molecules. In one experiment, the "negative" cell population contained 4% GPI-positive cells.

Based on our previous observations, we expected that the cells that were negative for GPI-linked proteins would vesiculate less than red blood cells from a normal individual, and that the unseparated PNH cells would show intermediate...

Table 2. A23187-Induced Cytotoxicity

<table>
<thead>
<tr>
<th>Inophor</th>
<th>Cell</th>
<th>2 min</th>
<th>6 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>+A23187</td>
<td>JY5</td>
<td>4.3%</td>
<td>11.0%</td>
<td>11.1%</td>
</tr>
<tr>
<td>+A23187</td>
<td>JY25</td>
<td>6.8%</td>
<td>10.6%</td>
<td>11.0%</td>
</tr>
<tr>
<td>-A23187</td>
<td>JY5</td>
<td>6.6%</td>
<td>10.1%</td>
<td>8.3%</td>
</tr>
<tr>
<td>-A23187</td>
<td>JY25</td>
<td>7.0%</td>
<td>10.0%</td>
<td>10.6%</td>
</tr>
</tbody>
</table>

The JY5 and JY25 cells in Fig 4 were assayed for cell death by the release of LDH into the supernatant. Total releasable LDH was measured by incubation of the cells with mellitin. The cells were preincubated with DiIC1(3) and stimulated to vesiculate with 3 μmol/L A23187 and 1 mmol/L CaCl2.
levels of vesiculation. The results of this experiment (Fig 5) fully confirm these predictions. The unseparated and GPI-negative erythrocytes from patient S.B. shed fewer vesicles than the normal cells, and the GPI-negative cells shed fewer vesicles than the unseparated cells. Furthermore, there is a linear relationship between the proportion of GPI-positive cells and the amounts of vesicles produced, suggesting strongly that the GPI-positive cells from S.B. vesiculate to the same extent as normal erythrocytes.

Calcium-dependence of the vesiculation process. Next, we compared the ability of normal and PNH erythrocytes to vesiculate in the presence of various concentrations of calcium. The patient's (L.D.) erythrocytes were 90% negative for GPI-linked proteins and were used without further purification. In a preliminary experiment, we found that the kinetics of calcium uptake of the PNH and normal erythrocytes was identical and that a steady state was reached after 30 minutes.

Normal and PNH erythrocytes were then incubated with 4 \( \mu \)mol/L A23187 and various concentrations of CaCl₂ and vesiculation was measured after 60 minutes of incubation. To measure calcium uptake, a separate experiment was performed simultaneously in which the erythrocytes were incubated for 30 minutes with medium containing the same concentrations of ionophore and calcium, but including trace quantities of \(^{45}\)Ca²⁺. In Fig 6, we plotted the calculated intracellular Ca²⁺ concentrations versus quantities of vesicles released. As shown, the PNH erythrocytes show significantly less vesiculation than normal erythrocytes at equivalent amounts of calcium uptake. In addition, we observed that vesicle release was inhibited in both erythrocytes at high Ca²⁺ concentrations, a phenomenon observed by Allan and Thomas.²⁶

**DISCUSSION**

We studied here vesiculation of cells lacking GPI-linked proteins in response to an increase in intracellular calcium. When erythrocytes take up calcium, a number of biochemical events occur, including potassium efflux and consequent cell shrinkage (Gardos effect),³⁷ breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate with a consequent increase in 1,2-diacylglycerol and phosphatidate,³⁸ transglutaminase-catalyzed protein cross-linking,³⁹ and proteolysis of some cytoskeletal proteins.⁴⁰ Nevertheless, how these biochemical events lead to the release of erythrocyte vesicles is unknown.

Our main finding is that in three different GPI-deficient cell types, ie, erythrocytes and platelets from PNH patients, and in a mutant lymphoblastoid cell line, the defect was associated with a substantial decrease in the amount of plasma membrane vesicles released during incubation with ionophore A23187 and calcium. As documented in Fig 6, the defect was observed at equivalent levels of intracellular Ca²⁺ accumulation in the GPI-positive and -negative cells.

It is also apparent that factors other than GPI-linked proteins play a role in membrane vesiculation. As can be seen in Figs 1 and 2, even though 10% of the erythrocytes from L.D. are GPI-positive, they vesiculate less than the erythrocytes from S.B., which are negative for GPI-linked proteins. However, from the data in Table 1 and Fig 5, when cells

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**Fig 5.** Vesiculation of normal erythrocytes, unseparated PNH erythrocytes, and GPI-negative PNH erythrocytes from S.B. Erythrocytes were incubated with 4 \( \mu \)mol/L A23187 and either 80 \( \mu \)mol/L CaCl₂ or 10 mmol/L EDTA at 37°C for 60 minutes. Percent GPI-positivity of cells is estimated by FACS analysis. Vesicles were isolated and quantitated as outlined in the text. The correlation coefficient (\( r \)) and significance (\( p \)) were calculated using the method of least squares.

**Fig 6.** Calcium-dependence of vesiculation. To measure vesiculation, normal (N = 2) or PNH erythrocytes from L.D. were incubated at 37°C for 60 minutes with 4 \( \mu \)mol/L A23187 and increasing concentrations of CaCl₂, or with 3 mmol/L EDTA. In a separate experiment to measure calcium uptake, the erythrocytes were incubated for 30 minutes with 4 \( \mu \)mol/L A23187 and the same concentrations of CaCl₂, but containing trace quantities of \(^{45}\)Ca²⁺. Normal erythrocytes (mean ± standard deviation); (○) PNH erythrocytes.
from an individual are studied, as the percentage of cells that express GPI-linked proteins increases so does the vesiculation.

What is the explanation for the association between the two defects? They may not be causally related; nevertheless, some observations are compatible with the idea that the GPI-anchored proteins participate in the process leading to membrane vesiculation. GPI-linked proteins are greatly enriched in the erythrocyte vesicles released from the Ca\textsuperscript{2+}-ionophore\textsuperscript{17} or from complement-treated erythrocytes (Whitlow, Iida, and Marshall, unpublished observations). In addition, our data with the JY25 vesicles show an even more dramatic concentration of DAF relative to the plasma membrane. There are also indications that GPI-anchored molecules are associated with each other and with other membrane proteins, and that they participate in signaling intracellular events.\textsuperscript{18-23} Cross-linking of GPI-linked proteins such as Ly-6, DAF, and CD59 can lead to the activation of T cells under appropriate conditions.\textsuperscript{20,40-42} In the case of Ly-6, this activation requires the presence of the glycolipid anchor.\textsuperscript{20} GPI-linked membrane proteins are associated with protein kinases, and cross-linking of GPI-anchored molecules leads to phosphorylation of cellular proteins.\textsuperscript{23}

Perhaps GPI-linked proteins rapidly accumulate in areas of Ca\textsuperscript{2+} influx, but this would necessitate specific signaling events, as well as mechanisms for the directional transport of the GPI proteins. Alternatively, domains of cellular membranes that are enriched in GPI-linked proteins may be preferentially vesiculated and shed. In support of this idea, lipid domains with compositions different from the bulk membrane have been shown in erythrocytes by fluorescence imaging microscopy,\textsuperscript{43} but the partition of GPI-anchored proteins between these domains is not known. Differently from most other integral membrane proteins, those that are GPI-linked are poorly solubilized by nonionic detergents such as triton X-100.\textsuperscript{44} In polarized epithelial cells, GPI-anchored molecules are expressed exclusively on the apical surface,\textsuperscript{45} and may form protein-glycosphingolipid microdomains in the plasma membrane.\textsuperscript{46,47} Folate receptors, which are GPI-linked, are clustered in the membrane.\textsuperscript{48} The forces that lead to folate receptor clustering are not known; however, removal of cholesterol causes these clusters to disperse.\textsuperscript{48} On the basis of these reports, it is conceivable that the GPI-anchored proteins partition preferentially in plasma membrane domains enriched in certain membrane constituents such as cholesterol and glycosphingolipids. The functional consequences of this association are not clear, but perhaps it is important in membrane remodeling events such as vesiculation and some forms of endocytosis.

Studies on the Ca\textsuperscript{2+}-dependent vesiculation of cells during attack by the terminal complement components C5b-9 also highlight the selectivity of the shedding process.\textsuperscript{5,13,14,49} C9 is increased in concentration several fold in the vesicles relative to the remaining plasma membrane, indicating that the areas surrounding, or in close proximity to, the open channels are preferentially released as vesicles. Perhaps the assembly and/or insertion of the membrane attack complex C5b-9 is not random and leads to channel formation more readily when it occurs in the putative GPI-enriched microdomains of the plasma membrane.

In short, we speculate that vesiculation may represent a facet of the normal process of turnover and repair of the plasma membrane in response to focal damage, and that domains of the membrane enriched in GPI-anchored molecules are preferentially released as vesicles. The triggering event may be Ca\textsuperscript{2+} influx at the site of disturbance of the lipid bilayer, and GPI may participate in the process. If this hypothesis is correct and there is a unique biochemical pathway leading to membrane vesiculation, our findings suggest that this pathway involves GPI and imply that cells from PNH patients should be more susceptible to destruction by membrane-perturbing agents other than complement.

An increase in intracellular calcium and very vigorous plasma membrane blebbing characterizes the initial stages of apoptosis and cell death.\textsuperscript{50} In myocardial infarction, the myocytes within areas of ischemia are deficient in DAF.\textsuperscript{51} Perhaps the blebbing that occurs during cell death sheds GPI-linked proteins preferentially. It is also possible that preferential association of GPI-linked proteins and membrane internalization plays a role in receptor-mediated endocytosis by caveolae.\textsuperscript{48}

REFERENCES


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