Absence of High-Affinity Band 4.1 Binding Sites From Membranes of Glycophorin C- and D-Deficient (Leach Phenotype) Erythrocytes

By Philippe Gascard and Carl M. Cohen

We investigated the role of glycophorins C and D in the association of band 4.1 with the erythrocyte membrane by measuring the binding of band 4.1 to erythrocyte inside-out vesicles stripped of endogenous band 4.1. Vesicles were prepared from either normal erythrocytes or erythrocytes completely lacking glycophorins C and D (Leach phenotype). Band 4.1 binding to vesicles from normal erythrocytes gave rise to a nonlinear Scatchard plot, indicative of two classes of binding sites: a low-capacity, high-affinity class of sites (about 10% of the total) and a high-capacity, low-affinity class of sites. Vesicles prepared from Leach erythrocytes had a binding capacity for band 4.1 that was, on average, 32% lower than that of vesicles from normal erythrocytes. This difference was caused by the complete absence of the high-affinity binding sites as well as by a decrease in the number of low-affinity binding sites. Reduction of membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) content by adenosine triphosphate depletion or activation of phosphoinositidase C resulted in a decrease in band 4.1 binding capacity to a similar extent in both control and Leach vesicles. The principal effect of PIP₂ depletion was a reduction in the number of low-affinity band 4.1 binding sites in control and Leach vesicles. The fact that PIP₂ depletion induced a decrease in band 4.1 binding to Leach vesicles shows that glycophorin C or D is not required for the formation of PIP₂-sensitive band 4.1 binding sites, and may not be involved in PIP₂-sensitive band 4.1 binding sites even when they are present. Our studies give new insights into the involvement of glycophorins and of PIP₂ in modulating cytoskeletal-membrane interactions.

The erythrocyte membrane skeleton has a great influence on the shape of the erythrocyte and the deformability of its membrane, and is composed of a self-assembled network of spectrin tetramers, short filaments of actin, and band 4.1. The membrane skeleton is anchored to the lipid bilayer via band 3 and the glycophorins, the major erythrocyte integral membrane proteins. Two linkage proteins are involved in the attachment of the skeletal network to the bilayer: ankyrin, which links spectrin to the cytoplasmic domain of band 3, and band 4.1, which binds to the cytoplasmic domains of band 4.1 and glycophorins. Whereas the role of the band 4.1-b band 3 association remains unclear, the binding of band 4.1 to glycophorins has been suggested to be essential in the interaction of the cytoskeleton with the bilayer.

Four glycophorins (A, B, C, and D) have been identified in the human erythrocyte membrane. Glycophorin C, although a minor glycophorin (50,000 to 100,000 copies per cell), has been proposed to be the principal attachment site for cytoskeletal band 4.1. Glycophorin C is the only glycophorin that remains bound to the cytoskeleton after detergent extraction of erythrocyte membranes, and is absent from cytoskeletons of band 4.1-deficient erythrocytes. Moreover, band 4.1 regulates membrane glycophorin C content. Finally, glycophorin C deficiency results in elliptocyte erythrocytes and in decreased membrane deformability and mechanical stability. By contrast, glycophorin A deficiency fails to induce such alterations.

However, other studies suggest that glycophorin A, which is the major glycophorin (500,000 to 1,000,000 copies per cell), may also be involved in the association of band 4.1 with the membrane. Although glycophorin A does not seem to interact with the cytoskeleton in its native state, band 4.1 can be cross-linked to glycophorin A as well as to glycophorin C in native membranes. A portion of glycophorins A and D remains bound to the cytoskeleton in band 4.1-deficient erythrocytes, and is absent from cytoskeletons of band 4.1-deficient erythrocytes. Moreover, band 4.1 regulates membrane glycophorin C content.

In vitro, interaction between glycophorin A and band 4.1 has been shown to be strictly dependent on membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) which is tightly bound to glycophorin A. As observed in nucleated cells, human erythrocytes show an active turnover of PIP₂ that is catalyzed by a specific kinase and a specific phosphomonoesterase. Only a fraction (60%) of PIP₂ is accessible to these enzymes and to phosphoinositidase C, the enzyme responsible for PIP₂ hydrolysis. We have recently shown that this fraction of PIP₂ is the only one involved in the regulation of band 4.1 association with the membrane.

Here, we have used Leach phenotype human erythrocytes (referred to as Leach erythrocytes in the following), which are totally deficient in glycophorins C and D, to evaluate the involvement of glycophorins in the association of band 4.1 with the membrane. Our results show that lack of glycophorin C and D results in the absence of high-affinity membrane binding sites and in a decrease in low-affinity membrane binding sites for band 4.1. Our results show also that glycophorins C and D are not required for the formation of PIP₂-sensitive band 4.1 binding sites.
ERYTHROCYTE BAND 4.1 AND LEACH PHENOTYPE

MATERIALS AND METHODS

Materials
Adenine (A9126), calcium ionophore A23187 (C7522), diisopropyl fluorophosphate (DF0879), inosine (I4125), iodoacetamide (16125), leupeptin (L2884), phenylmethylsulfonyl fluoride (PMSF; P7626), sodium azide (S2002), and Triton X-100 were obtained from Sigma (St Louis, MO). Dithiothreitol (DTT) was from US Biochemicals (Cleveland, OH). Bovine serum albumin (BSA), fraction V (12659) was from Calbiochem (San Diego, CA). Tween 20 and reagents for gel electrophoresis were obtained from Bio-Rad (Richmond, CA). All other reagents used were of analytical grade. Silica gel 60-precoated plates were from Whatman (Maidstone, UK). X-Omat films were from Eastman Kodak (Rochester, NY). 125I-labeled Bolton-Hunter, 123I-protein A, and 32P-orthophosphoric acid were purchased from ICN Radiochemicals (Irvine, CA).

Methods
Preparation of band 4.1. Band 4.1 was prepared as described previously.54 In these conditions, band 4.1 is devoid of any endogenous or contaminating protein kinase activity, and contains < 0.1 mol of PO4/mol of band 4.1. Band 4.1 purity was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; not shown). Band 4.1 was iodinated with 125I-labeled Bolton-Hunter reagent as described.25

Preparation of erythrocytes. Human blood was withdrawn in heparinized tubes from either healthy volunteers or a Leach phenotype patient (J.C.) and was used within 2 days after withdrawal. Blood was centrifuged at 1,500g at 4°C for 10 minutes. Plasma anduffy coat were removed by aspiration and packed cells were washed twice in 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 7.8, 0.5 mmol/L EGTA, 10 mmol/L glucose and once in the same buffer supplemented with 1 mmol/L adenosine, 5 mmol/L inosine, 100 μg of streptomycin/mL, and 100 U of penicillin/mL (incubating buffer). Cells were resuspended in this buffer at a hematocrit of 40% and subsequently metabolically labeled. Unless indicated otherwise, all steps were performed at 4°C.

Metabolic labeling of erythrocytes. The cell suspension was pre-warmed at 37°C for 10 minutes and incubated in the presence of 32PPO4 (20 μCi/mL) of cells for 20 hours. Incubation was terminated by the addition of ice-cold incubating buffer. Cells were spun down and washed twice in buffers of various compositions depending on the subsequent treatment (see below).

Adenosine triphosphate (ATP) depletion. ATP depletion of the cells was performed as described with minor modifications.26 Briefly, 32P-prelabeled cells were washed twice in 154 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4, 6 mmol/L iodoacetamide, 10 mmol/L inosine, 1 mmol/L EGTA, resuspended in this medium at a hematocrit of 30%, and incubated at 37°C for 2 hours. It was previously shown that after 1 hour of incubation cell ATP content was already decreased by more than 95%.26 The reaction was terminated by addition of 20 vol of cold 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA. Cells were sedimented, washed twice in the same buffer, and hemolyzed as described.27

Phosphoinositide C activation in intact cells. Phosphoinositide C activation in intact cells was performed as previously described.26 Briefly, 32P-prelabeled cells were washed twice in 75 mmol/L KCl, 75 mmol/L NaCl, 0.15 mmol/L MgCl2, 10 mmol/L HEPES, pH 7.4, 10 mmol/L glucose. 20 mmol/L inosine and resuspended in this medium at a hematocrit of 10%. The cell suspension was divided into two samples, which were preincubated at 37°C for 10 minutes in the presence of EGTA or CaCl2 (1 mmol/L) before the addition of calcium ionophore A23187 (5 μmol/L), dissolved in dimethylsulfoxide as a 2 mmol/L stock solution. The incubation was performed for 20 minutes and terminated by the addition of 20 vol of ice-cold 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 8.0, 2 mmol/L EGTA. Cells were sedimented, washed twice in the same buffer, and hemolyzed as described.27

Extraction, thin layer chromatography (TLC) separation, and assays of membrane phospholipids. To measure changes in phospholipid content (or radioactivity) resulting from the different treatments described above, control and treated ghosts were submitted to an acidic lipid extraction as previously described.24 TLC of poly-phosphoinositides was performed as described.28 Phospholipids were located on TLC plates after staining in iodine vapor or by autoradiography. The quantification of their radioactivity (and/or content in some experiments) was performed as described.29 It has been described previously that, under the conditions we have used, changes in phospholipid radioactivity can be used to compute changes in their content.28

SDS-PAGE and immunoblotting. Membrane proteins of normal or Leach erythrocytes (60 μg protein) were analyzed by SDS-PAGE using 9% acrylamide according to the method of Laemmli.30 In some experiments, after electrophoresis, proteins were transferred onto nitrocellulose paper for detection of glycoporphin C or of band 3 by specific antibodies. The following steps were performed at room temperature. Blots were first stained with a 3% trichloracetic acid solution containing 0.1% Red Ponceau to monitor the efficiency of the transfer. After complete destaining in water, they were blocked in 150 mmol/L NaCl, 5 mmol/L sodium phosphate, pH 7.2, 0.5 g/L sodium azide (medium A) containing 5% BSA for 1 hour. They were subsequently incubated with the glycoporphin C or the band 3 antisera diluted 1:500 in blocking buffer for 18 hours. After washing four times with medium A containing 0.1% Tween 20 and once without Tween, blots were incubated in blocking buffer with 123I-labeled protein A (1 μCi/mL) for 3 hours. Finally, blots were washed as described above, dried, and exposed to X-ray film overnight at -70°C.

Preparation of stripped inside-out vesicles (sIOVs). White ghosts, prepared by hypotonic lysis of normal or of Leach erythrocytes, were stripped of all peripheral proteins by incubation in either potassium iodide (KI) or alkaline pH as described previously, with minor modifications.56,67 For KI stripping, ghosts were incubated in 40 vol of 1 mol/L KI, 10 mmol/L sodium phosphate, pH 8.0, 1 mmol/L EGTA, 1 mmol/L DTT, 0.02% Tween 20, 20 μg/mL PMSF, and 2 μg/mL leupeptin at 37°C for 30 minutes. The resulting KI sIOVs were washed once in 5 mmol/L sodium phosphate, pH 8.0, 0.5 mmol/L EGTA and passed three times through a 27-gauge needle to disrupt any aggregates.

For alkaline stripping, ghosts were incubated in 30 vol of 0.1 mmol/L EGTA, pH 8.5, at 37°C for 30 minutes. The resulting vesicles, depleted of spectrin and actin, were stripped of remaining peripheral proteins by incubation in 30 ghost vol of 0.1 mmol/L EGTA titrated to pH 11 with 0.1 mol/L NaOH at 25°C for 20 minutes. The vesicles were subsequently washed and passed through a needle as described above. We have previously shown that there is no selective loss of membrane phospholipids during vesicle preparation.6 These KI or alkaline sIOVs, referred to in the text as vesicles or stripped vesicles, were subsequently used for binding experiments.

Measurement of band 4.1 binding to vesicles. This measurement was performed as described.123I-band 4.1, used at the concentrations indicated in the figures, was incubated with 18 μg/mL vesicles in 100 mmol/L KCl, 5 mmol/L sodium phosphate, pH 8.0, 1 mmol/L DTT, 1 mg/mL BSA for 1 hour at 22°C. The vesicles were centrifuged at 20,000 rpm for 20 minutes in a Beckman 42.2 Ti rotor (Beckman Instruments, Palo Alto, CA). The supernatant...
was carefully aspirated and the $^{125}$I in the pellet was measured in a Gamma counter. To account for nonspecific band 4.1 sedimentation or sticking to tubes, control samples containing all of the components except vesicles were treated identically, and the $^{125}$I counts of these samples were subtracted from the corresponding samples with vesicles. Each data point represents the mean of duplicate determinations. Control experiments showed that binding equilibrium was reached after 1 hour of incubation (data not shown).

**Data analysis.** All binding data were analyzed using the Enzfit computer program (Elsevier-Biosoft), using either a one- or a two-site binding model.

**Antisera.** Rabbit polyclonal antiserum against an hexadecapeptide (residues 113-128) of the cytoplasmic domain of glycophorin C was a generous gift from Dr Philip S. Low and Dr Christian R. Lombardo (Purdue University, West Lafayette, IN). Rabbit polyclonal antiserum was raised against the 43-kD cytoplasmic domain of band 3 prepared as previously described.29

**Other procedures.** Protein concentration was determined by the method of Bradford,30 using BSA as a standard.

**RESULTS**

Glycophorin C has been suggested to be the principal attachment site of band 4.1 to the erythrocyte membrane.2,8,13-15 However, other studies suggest that glycophorin A may also be involved in this association.2,24,7,9-11 To determine the importance of glycophorin C in the binding of band 4.1 to the erythrocyte membrane, we compared the binding of band 4.1 to sLOVs prepared from either control or Leach erythrocytes.

**Comparison of Band 4.1 Binding to Control and Leach Vesicles**

Previous studies have shown that glycophorin C migrates on SDS gels with an apparent molecular mass of 31 to 34 kD and that the Leach phenotype patient studied here lacks glycophorin C.15 A more recent study34 has shown that this patient lacks both glycoporphins C and D because of a gene defect that deletes the entire transmembrane and cytoplasmic domains of these glycoporphins. We confirmed by immunoblotting that glycophorin C was absent from the erythrocyte membranes of the Leach patient J.C. Whereas control membranes showed two reactive bands of approximately 35 and 25 kD, Leach membranes showed neither of these bands (data not shown).

Figure 1 shows that the binding capacity of vesicles from Leach membranes was 26% lower than that of control vesicles. Scatchard analysis (inset) of the binding data showed several differences between Leach and control vesicles. (1) Leach vesicles showed a complete absence of a class of high-affinity binding sites detected in control vesicles (Fig 1, inset), which, in this experiment, contributed 5% of the total band 4.1 binding capacity of vesicles (see legend to Fig 1). (2) Compared with normal vesicles, Leach vesicles had a 22% reduction in the binding capacity of low-affinity band 4.1 binding sites (the only class of sites distinguished on Leach vesicles). Also, in four separate experiments, the Scatchard plot of band 4.1 binding to Leach vesicles was convex. One possible explanation for a convex Scatchard plot is positive cooperativity in band 4.1 binding, but we have no independent evidence to support this interpretation.

The binding capacity of Leach vesicles for band 4.1 was found to be reduced by an average of 32% ± 15% relative to that of normal vesicles in four independent experiments. The high-affinity binding sites, which accounted for an average of 10% of the total number of binding sites on normal vesicles, were absent from Leach vesicles in all experiments. Because of the small contribution of the high-affinity sites to the total binding capacity of the vesicles, most of the difference in band 4.1 binding capacity between Leach and normal vesicles was caused by the smaller number of low-affinity sites in Leach vesicles. In addition to this difference in binding capacity, the apparent k<sub>d</sub> of the low-affinity binding sites was twofold higher in Leach vesicles than in control vesicles in the three other experiments not shown here.

**Effects of PIP<sub>2</sub> Depletion on Band 4.1 Binding Capacity of Control and Leach Vesicles**

PIP<sub>2</sub> has been shown to be involved in the regulation of band 4.1 association with some of its membrane receptors.6,10,11 To rule out the possibility that the lower band 4.1 binding capacity of Leach vesicles was caused by a lower PIP<sub>2</sub> content, we assayed membrane PIP<sub>2</sub> in control and Leach membranes. The results showed that the amounts of membrane PIP<sub>2</sub> were similar in both types of cells (4.05 and 5.35 nmol PIP<sub>2</sub>/mg membrane protein in controls and 4.05 and 4.70 nmol PIP<sub>2</sub>/mg membrane protein in Leach; results of two independent experiments) and were in accordance with previous studies.35,36 Thus, the lower band 4.1 binding capacity of Leach vesicles did not result from a lower PIP<sub>2</sub> content.

To investigate further the role of PIP<sub>2</sub> in regulating the association of band 4.1 with glycoporphin C and D, we determined the effects of PIP<sub>2</sub> depletion on band 4.1 binding to control and Leach vesicles. Two methods were used to alter membrane PIP<sub>2</sub> content of erythrocytes: ATP depletion or activation of the endogenous phosphoinositidase C in intact cells. ATP depletion alters phosphoinositide content by inhibition of phosphoinositide kinases,36 displacing the metabolic equilibrium from PIP<sub>2</sub> to phosphatidylinositol 4-monophosphate and phosphatidylinositol. Activation of phosphoinositidase C, achieved by incubating erythrocytes with Ca<sup>2+</sup> in the presence of the ionophore A23187, induces hydrolysis of PIP<sub>2</sub>.37 The PIP<sub>2</sub> depletions obtained after ATP depletion or activation of phosphoinositidase C in control cells were in accordance with previous studies.6,26,35-37 However, the depletion was always enhanced in Leach erythrocytes compared with control erythrocytes (Table 1).

Figure 2 shows that depletion of PIP<sub>2</sub> by activation of phosphoinositidase C resulted in decreased band 4.1 binding to vesicles from normal erythrocytes, as previously reported6,11 (see Table 1). Scatchard analysis (Fig 2, inset) showed that the PIP<sub>2</sub>-depleted vesicles had a decreased band 4.1 binding capacity caused principally by a reduction in the number of low-affinity binding sites. The binding of band 4.1 to the high-affinity sites was not significantly altered by PIP<sub>2</sub> depletion, although the k<sub>d</sub> was increased by fourfold. This increase in the k<sub>d</sub> of the high-affinity site was seen in three other experiments.

Vesicles prepared from Leach erythrocytes also showed a
erythrocyte band 4.1 and leach phenotype

and Leach cells were measured as described under Methods. The results shown are from three independent experiments. The data for the Leach vesicles might have resulted from a degradation of band 4.1, which might have adversely affected the protein composition of the resulting vesicles.

decrease in their band 4.1 binding capacity after phosphoinositidase C-induced PIP₂ depletion (Fig 3 and Table 1). PIP₂ depletion resulted in a decrease in the binding capacity of the single class of binding sites identified in the Leach vesicles without any significant alteration of their apparent kₐ (Fig 3, inset). The Scatchard plot still showed evidence of convexity in the low band 4.1 region of the curve (more evident in the three other experiments not shown here). These results show that band 4.1 binding sites other than those on glycophorin C or D are regulated by PIP₂.

To rule out the possibility that the decrease in band 4.1 binding capacity after activation of the phosphoinositidase C might have resulted from a degradation of band 3 and/or glycophorin molecules, we monitored these proteins by Western blotting. The results clearly showed that the treatment did not induce any detectable proteolysis of band 3 in both types of cells or of glycophorin C in control cells (data not shown). The results of the phosphoinositidase C experiment, presented in Figs 2 and 3 and in Table 1, were confirmed in two other independent experiments in which PIP₂ reduction was induced by ATP depletion of intact cells (Table 1). The ATP depletion was performed in the presence of EGTA to prevent metabolic depletion-induced increase in cytosolic Ca²⁺, which might have adversely affected the protein composition of the resulting vesicles.

**DISCUSSION**

Our data show that membrane vesicles prepared from erythrocytes of the Leach phenotype have a binding capacity for band 4.1 that is on average 32% less than that of vesicles from normal erythrocytes. This lower binding capacity is reflected in the complete absence of a class of high-affinity binding sites as well as in a 30% (average) deficit of low-affinity binding sites. The results suggest that all of the high-affinity sites and about 30% of the low-affinity sites are composed exclusively of glycophorin C or D or require glycophorin C or D as a component. Even though membrane vesicles from Leach erythrocytes lack glycophorin C and D, 20% to 40% of their band 4.1 binding sites are PIP₂ sensitive. This is nearly the same range of PIP₂-sensitive sites as is found in vesicles from normal cells. In vesicles from normal cells the effect of PIP₂ depletion on the properties of the high-affinity band 4.1 binding site (which must involve glycophorin C or D) is a fourfold increase in kₐ with no effect on the binding capacity. We conclude that, whereas the PIP₂-induced change in the kₐ of

---

**Table 1. Changes in the PIP₂ Content and in the Band 4.1 Binding Capacity of Control or Leach Vesicles on Phosphoinositidase C Activation or ATP Depletion**

<table>
<thead>
<tr>
<th></th>
<th>Control Vesicles</th>
<th>Leach Vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change in PIP₂ Content</td>
<td>Change in Band 4.1 Binding Capacity</td>
</tr>
<tr>
<td>Phosphoinositidase C activation in intact cells</td>
<td>57⁺</td>
<td>36⁺</td>
</tr>
<tr>
<td>ATP depletion of intact cells (2 h)</td>
<td>58</td>
<td>27</td>
</tr>
<tr>
<td>58</td>
<td>40</td>
<td>81</td>
</tr>
</tbody>
</table>

Values shown are the percent decrease. PIP₂ membrane content was altered by phosphoinositidase C activation in intact cells or by 2 hours of ATP depletion of intact cells as described in Methods. Phospholipid radioactivity and band 4.1 binding to vesicles prepared from untreated or treated control and Leach cells were measured as described under Methods. The results shown are from three independent experiments.

* Results of the experiment presented in Figs 2 and 3.
Glycophorin
of dependent binding of band 4.1

We show that band 4.1 binding which phosphoinositidase treated Leach erythrocytes phosphoinositidase (which accounts for approximately 65% of band 4.1 binding sites) removal of the cytoplasmic domain of band 4.1 by trypsinization of stripped vesicles hardly changes the number of PIP₂-affinity sites must involve other membrane components. Two likely candidates are band 3 and glycophorin A. In Gascard et al.,⁶ we show that band 4.1 binding to band 3 (which accounts for approximately 65% of band 4.1 binding sites) is unlikely to be sensitive to PIP₂. This is because removal of the cytoplasmic domain of band 3 by trypsinization of stripped vesicles hardly changes the number of PIP₂-sensitive sites on the vesicles. Thus, the PIP₂-sensitive low-affinity sites must involve other membrane components. Glycophorin A is the most logical candidate because PIP₂-dependent binding of band 4.1 to glycophorin A has been shown previously.⁵,¹¹

A novel feature of the results shown here is that the Scatchard plots are biphasic, a feature not detected in our previous work,⁵,⁶ but suggested in one other previous study.⁴ A possible explanation for this difference is that in this study we added several binding measurements at very low concentrations of band 4.1 to increase the resolution of our Scatchard plots. It should also be noted that the band 4.1 binding capacities of the vesicles in the present study were considerably lower than those reported in our previous study⁶ but similar to those reported in Danilov et al.⁵ Possibly related to these points is the fact that the range of reduction in band 4.1 binding caused by PIP₂ depletion was higher in the present study (27% to 40%) than in our previous work (5% to 22%). We considered the possibility that the conditions used to prepare the stripped vesicles (e.g., pH 11

![Figure 2](image1.png)

Fig 2. Effect of phosphoinositidase C activation in intact cells on ¹²⁵I-band 4.1 binding to control KI sIOVs. The indicated concentrations of ¹²⁵I-band 4.1 were incubated with 18 μg/mL vesicles prepared from untreated erythrocytes (●) or vesicles prepared from erythrocytes in which phosphoinositidase C had been activated (□). Procedures of vesicle preparation, of phosphoinositidase C activation in intact cells, and of band 4.1 binding measurement are described under Methods. Inset, Scatchard plot of binding of band 4.1 to vesicles prepared untreated erythrocytes (n, kₐ = 2.3 ± 5.9 × 10⁻⁸ mol/L, capacity₁ = 20 ± 10 μg/mg vesicle protein; kₐ = 1.9 ± 0.3 × 10⁻⁷ mol/L, capacity₂ = 325 ± 10 μg/mg vesicle protein) or from erythrocytes in which phosphoinositidase C had been activated (C, kₐ = 0.9 ± 2.7 × 10⁻⁸ mol/L, capacity₁ = 25 ± 50 μg/mg vesicle protein; kₐ = 1.4 ± 0.7 × 10⁻⁷ mol/L, capacity₂ = 195 ± 40 μg/mg vesicle protein) control erythrocytes. In this experiment, 57% of membrane PIP₂ was hydrolyzed by phosphoinositidase C activation.

![Figure 3](image2.png)

Fig 3. Effect of phosphoinositidase C activation in intact cells on ¹²⁵I-band 4.1 binding to Leach KI sIOVs. The indicated concentrations of ¹²⁵I-band 4.1 were incubated with 18 μg/mL vesicles prepared from untreated Leach erythrocytes (●) or from Leach erythrocytes in which phosphoinositidase C had been activated (□). Procedures of vesicle preparation, of phosphoinositidase C activation in intact cells, and of band 4.1 binding measurement are described under Methods. Inset, Scatchard plot of binding of band 4.1 to vesicles prepared from untreated Leach erythrocytes (n, kₐ = 2.0 ± 0.4 × 10⁻⁷ mol/L, capacity = 255 ± 15 μg/mg vesicle protein) or from Leach erythrocytes in which phosphoinositidase C had been activated (C, kₐ = 1.6 ± 0.3 × 10⁻⁷ mol/L, capacity = 150 ± 10 μg/mg vesicle protein). In this experiment, 75% of membrane PIP₂ was hydrolyzed by phosphoinositidase C activation. As in Fig 1, the lowest three data points from the binding to untreated Leach vesicles (●) were not included in the fit because of the appearance of positive cooperativity (see Results).
treatment) were responsible for the different binding capacities in the several studies. However, because pH 11 treatment was used in some of the experiments reported here and in all of the studies reported in Danilov et al. 2 and Gascard et al. 6 it is unlikely that this treatment is responsible for the different binding capacities found in the several studies. Moreover, we found no consistent differences in the binding characteristics of vesicles prepared by pH 11 extraction and those prepared by KI extraction.

We previously proposed (see Gascard et al. 8 for further discussion) that differences in band 4.1 binding capacity could result from changes in the intermolecular or intramolecular associations of band 3 and the glycoporphins, leading to exposure of more or fewer sites on both molecules in different experiments. This hypothesis is supported by the fact that the proportion of trypsin-sensitive (largely or exclusively band 3 associated) to trypsin-insensitive sites (including glycoporphins) was always similar, regardless of the total band 4.1 binding capacity in a particular experiment. Several lines of evidence suggest that some band 3 and glycoporphin molecules may be associated in the membrane. 3,8,39 Such associations could account for the observations that some antisera to band 3 or glycoporphins block a higher percentage of membrane binding sites than would be expected from the separate contribution of these proteins to vesicle binding capacity. 11

Erythrocytes deficient in glycoporphin C and D have a nearly normal morphology, although approximately 10% of the cells have been reported to be elliptocytic. 14 However, membranes from glycoporphin C- and D-deficient erythrocytes have been reported to have a 50% reduced mechanical stability and a 40% reduced deformability. 15 Several lines of evidence suggest that the principal function of glycoporphin C is to anchor some fraction of membrane skeletal band 4.1 to the membrane. 2,8,12-15 It has also been proposed that the abnormal mechanical properties of Leach erythrocytes arise from defective or reduced skeletal-membrane linkages. 15,40 If these hypotheses are true, then the mechanical abnormalities of Leach membranes could be caused by (1) the absence of the high-affinity band 4.1 binding sites, (2) the deficit in the low-affinity binding sites and consequent reduction in total membrane band 4.1 binding capacity, or (3) a combination of (1) and (2).

Recent results (A.H. Chisti, personal communication, June 1993) indicate that membranes from Leach erythrocytes have about 20% less band 4.1 than do membranes from normal erythrocytes. It is possible that this deficit could account for the abnormal mechanical properties of the Leach membranes cited above. It is also tempting to relate this observation to the lower than normal band 4.1 binding capacity of Leach vesicles found here. However, such comparisons must be made with caution because in this and previous studies the band 4.1 binding capacity of stripped vesicles (from both normal and Leach erythrocytes) was found to be greater than the endogenous band 4.1 content of ghosts. For example, we calculate that a binding capacity of 340 µg band 4.1/mg stripped vesicle protein (Fig 1) is equivalent to approximately 120 µg band 4.1/mg ghost protein, or about 2.4 times the endogenous band 4.1 content of ghosts (see Gascard et al. 8 for further discussion).

In summary, our results show that glycoporphin C and/or D is involved in the formation of high-affinity low copy number band 4.1 binding sites in normal erythrocyte membranes. These glycoporphins also contribute to the formation of more numerous low-affinity band 4.1 binding sites. Our data show that membrane components other than glycoporphin C or D (likely glycoporphin A) can bind band 4.1 in a PIP2-sensitive manner, and suggest that, except for a twofold to threefold alteration in kd, binding of band 4.1 to glycoporphin C and D may be independent of PIP2. Regulation of band 4.1-membrane associations is important not only in the erythrocyte, but also in nonerythroid cells that contain analogues of both band 4.1 41 and glycoporphin C (the only glycoporphin species found in nonerythroid cells). 2,42,43 Our results support the idea that membrane skeletal linkages involving band 4.1-like proteins in nonerythroid cells may be remodeled by agonist-induced changes in membrane PIP2, but leave open the question of the identity of the band 4.1 binding sites involved.

ACKNOWLEDGMENT

We thank Lucille Paul for editing the manuscript and Donna-Marie Mironchuk for the preparation of the figures. We also thank Dr Joel Chasis for help in obtaining the Leach erythrocytes.

REFERENCES


Absence of high-affinity band 4.1 binding sites from membranes of glycophorin C- and D-deficient (Leach phenotype) erythrocytes

P Gascard and CM Cohen