Glycophorin C Content of Human Erythrocyte Membrane Is Regulated by Protein 4.1

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Human erythrocyte transmembrane sialoglycoprotein, glycophorin C, plays a functionally important role in maintaining erythrocyte shape and regulating membrane material properties, possibly through its interaction with protein 4.1. Moreover, it has previously been shown that membranes deficient in protein 4.1 exhibit decreased content of glycophorin C. To further define the relationship between protein 4.1 and glycophorin C, a series of studies were performed using both protein 4.1- and glycophorin C-deficient erythrocytes. Quantitation by flow cytometry showed that the glycophorin C content of cells totally deficient in protein 4.1 was 9% of normal and that of cells partially deficient in protein 4.1 was 44% of normal. Interestingly, while homozygous glycophorin C-deficient cells had no detectable levels of this sialoglycoprotein, cells from obligate heterozygotes had normal levels. Protein 4.1 content of membranes of these glycophorin C-deficient cells was also normal. These data suggest that glycophorin C may be synthesized in excess by erythroid cells and its membrane content regulated by protein 4.1. To investigate if this regulation is due to association between protein 4.1 and glycophorin C, we examined the retention of glycophorin C in membrane skeletons (Triton shells) prepared from normal membranes, protein 4.1-deficient membranes, and protein 4.1-deficient membranes reconstituted with exogenous protein 4.1. Glycophorin C is retained by Triton shells prepared from normal membranes, whereas Triton shells prepared from protein 4.1-deficient membranes are totally devoid of this sialoglycoprotein. However, reconstitution of protein 4.1-deficient membranes with purified protein 4.1 resulted in retention of glycophorin C with the Triton shells. This finding suggests that protein 4.1 is necessary for association of glycophorin C with the membrane skeleton. Furthermore, these data suggest that through its interaction with glycophorin C, protein 4.1 may play a role in regulating the membrane content of this sialoglycoprotein in mature human erythrocytes.

FOUR DISTINCT transmembrane sialoglycoproteins have been identified in the human erythrocytes. Three of these sialoglycoproteins (glycophorins A, C, and D) have extracellular, transmembrane, and cytoplasmic domains, while the fourth (glycophorin B) has only extracellular and transmembrane domains. The genes coding for glycophorins A, B, and C have recently been isolated and characterized. It has been shown that these three sialoglycoproteins are products of three distinct genes. Erythrocytes that lack either glycoprotein A or B or both these sialoglycoproteins have normal discoid shape and normal membrane material properties, while erythrocytes that lack glycophorin C exhibit elliptocytic morphology and also decreased membrane deformability and mechanical stability. Based on these findings, it has been suggested that the interaction between glycophorin C with the erythrocyte membrane skeleton may play a functionally important role in regulating cell shape and membrane mechanical properties. Some previous studies have provided indirect evidence to suggest that glycophorin C interacts with the membrane skeletal component, protein 4.1, while other in vitro studies have led to the suggestion that glycophorin A interacts with protein 4.1. The aim of this study is to obtain more direct support for the interaction between erythrocyte sialoglycoproteins and the membrane skeleton in the mature erythrocyte membrane.

MATERIALS AND METHODS

Blood samples were obtained from a previously studied Algerian family in whom we found elliptocytosis associated with protein 4.1 deficiency, and also from other unrelated families with partial deficiency of protein 4.1. Blood samples from individuals who are presumed heterozygotes for the expression of glycophorin C were obtained from six children (V-2, V-3, V-4, V-5, V-6, V-7) of two sisters (IV-2, IV-4) whose red blood cells had previously been shown to lack glycophorin C. Blood was collected into acid citrate dextrose, stored at 4°C, and used within 4 days.

Monoclonal antibodies (MoAbs) were gifts from D.J. Anstee, Southwestern Regional Transfusion Centre, Bristol, UK, and from R. Jensen, Lawrence Livermore Laboratories, Livermore, CA. NBTS/BRIC-10 reacts with an epitope on the extracellular domain of glycophorin C and 10F7 detects an epitope on the extracellular domain of glycophorin A. The 600-base pair (bp) cDNA clone for glycophorin C was a gift from J.P. Cartron of the Centre Nationale de Transfusion Sanguine, Paris, France. Protein 4.1 was purified from normal erythrocytes by the method of Tyler et al, dialyzed against hypotonic sodium phosphate buffer (pH 7.4, 40 mMol/kg), and used within 4 days. All other reagents were reagent grade chemicals from standard sources.

Immunochromatographic assays. The methods for preparation of erythrocyte membranes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and periodic acid Schiff staining, have been described previously. Pyridine elution of Coomassie blue-stained bands was performed as described by Agre et al. Immunoblotting was performed as described by Merry et al except 5% (wt/vol) low-fat milk in phosphate-buffered saline at pH 7.4 was used as the blocking reagent.

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**Flow cytometry.** The methods for dimethylsuberimidate (DMS) fixation of erythrocytes (to eliminate hemagglutination after treatment with MoAbs), preparation of the erythrocytes, and flow cytometry have been previously described in full. Briefly, DMS-fixed erythrocytes were mixed with an optimum concentration of primary antibody, then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Cappel Laboratories, Malvern, PA). Cell-associated fluorescence measurements were made using a dual-beam cytometer. Fluorescein was excited at 488 nm and the fluorescent signal was measured through a 514-nm narrow-band emission filter. Fluorescence measurements were standardized by comparing results to 1.8 μm fluorescent microspheres (Polysciences, Warrington, PA).

**Protein 4.1 reconstitution experiments.** To incorporate purified protein 4.1 into the protein 4.1-deficient membranes, we modified the technique of exchange hemolysis described by Clark and Shohet. In brief, protein 4.1-deficient erythrocytes were first washed with the isotonic sodium phosphate buffer (pH 7.4, 290 mOsm/kg) and then lysed in 40 vol of ice-cold hypotonic sodium phosphate buffer (pH 7.4, 40 mOsm/kg). The membranes were collected by centrifugation at 39,000 × g for 2 minutes at 0°C. Four-tenths milliliter of purified protein 4.1 solution at a concentration of 960 μg/mL (0.4 mL of hypotonic phosphate buffer as a control) was added to 0.4 mL of packed membranes. The mixture was gently stirred at 0°C for 20 minutes. After this incubation in the cold, 0.1 mL of a mixture of KCl, MgCl₂, and dithiothreitol (DTT) was added to the membrane suspension to give a final concentration of 100 mmol KCl, 1 mmol/L MgCl₂, and 1 mmol/L DTT. The membranes were then incubated at 37°C for 60 minutes to allow them to reseal. Incorporation of protein 4.1 into the protein 4.1-deficient membranes was confirmed by SDS-PAGE analysis of the reconstituted membranes, as well as by functionally demonstrating the restoration of normal membrane mechanical stability to the initially unstable membranes. The glycoprotein C content of membranes and Triton X-100-insoluble residues prepared from native protein 4.1-deficient membranes, protein 4.1 reconstituted membranes, and normal membranes was determined by Western blot analysis using MoAb BRIC-10.

**Southern blot analysis of genomic DNA.** Genomic DNA was extracted from peripheral-blood leukocytes, digested with restriction endonucleases, and subjected to electrophoresis in horizontal slab gels of 0.8% agarose. The DNA was transferred to nitrocellulose filters and hybridized to radiolabeled cDNAs derived from a 2.5-kilobase (kb) protein 4.1 clone and a 600-bp glycophorin C clone.

**Northern blot analysis of messenger RNA (mRNA).** mRNA was isolated from reticulocytes obtained from normal and protein 4.1-deficient individuals as previously described. Northern blot analysis was performed by electrophoresis of glyoxal-denatured RNA on 1% agarose gels, followed by transfer to nitrocellulose filters and hybridization to nick-translated cDNA probes for protein 4.1 and glycophorin C.

**PCR amplification of glycoprotein mRNA from reticulocytes.** One microgram of human reticulocyte total RNA was transcribed into single-stranded DNA at 37°C for 120 minutes in a 50-μL reaction containing: 40 mmol/L KCl, 50 mmol/L Tris-HCl (pH 8.3), 0.8 mmol/L MgCl₂, 0.5 mmol/L dNTPs, 50 pmol random primer (hexamer; Pharmacia LKB Biotechnology Inc, Piscataway, NJ), 200 U of M-MLV reverse transcriptase (Bethesda Research Labs, Gaithersburg, MD), and 10 U of RNASin (Promega Biotec, Madison, WI). Ten milliliters of this cDNA reaction was diluted to 100 μL in Taq polymerase buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 1.5 mmol/L MgCl₂, 0.1% gelatin) supplemented with 50 pmol each of the two glycoprotein C-specific oligonucleotide primers (see below), additional dNTPs to a final concentration of 0.2 mmol/L and 5 μL of Taq polymerase (Cetus, Emeryville, CA). Forty cycles of amplification were performed using an automated Perkin Elmer-Cetus thermal cycler under the following conditions: denaturation, 30 seconds at 94°C; renaturation, 30 seconds at 60°C; extension 1 minute 45 seconds at 72°C. Amplified in this reaction was a 395-bp product extending from –8 to +387 in the glycoprotein C cDNA, encompassing the entire coding region. DNA fragments were analyzed by 5% PAGE. The oligonucleotides used in this report are as follows: 5'-GCCAGGAATGTTGTCGAGA-3' (sense strand); and 5'-TCAAATAAAGTACTCCTTCT-3' (antisense).

**RESULTS**

**Sialoglycoprotein content of erythrocytes.** Glycoprotein A and C content of normal and protein 4.1-deficient erythrocytes was quantified by flow cytometry. Representative histograms of cell-associated fluorescence using monoclonal anti-glycoprotein C for normal and protein 4.1-deficient erythrocytes are shown in Fig 1. The membrane content of glycoprotein C in erythrocytes totally deficient in protein 4.1 was only 9% of normal, while that in erythrocytes partially deficient in protein 4.1 (50% of normal protein 4.1 levels) was 44% of normal. The data on cell-associated fluorescence using MoAbs against both glycoprotein A and glycophorin C for various erythrocyte samples are summarized in Table 1. In contrast to glycoprotein C, the membrane content of glycoprotein A in erythrocytes either totally or partially deficient in protein 4.1 was within normal limits. Further confirmation for flow cytometric data on the membrane content of various sialoglycoproteins was obtained by comparing the staining intensity of bands corresponding to various glycoporphins on PAS-stained SDS-PAGE gels of different membrane preparations. The staining intensity of bands corresponding to glycoprotein C was undetectable in membranes totally deficient in protein 4.1, while it was moder-
GLYCOPHORIN C AND PROTEIN 4.1 IN RBCs

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<th>Table 1. Flow Cytometric Analysis of Glycophorin C and Glycophorin A Content of Various Erythrocyte Samples</th>
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<td>Cell-associated fluorescence with anti-glycophorin C</td>
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Results expressed as percentage of fluorescence of standard fluorescent microspheres.

Abbreviation: NT, not tested.

ately reduced in membranes partially deficient in protein 4.1. In contrast, the PAS staining intensity of bands corresponding to glycophorin A in protein 4.1-deficient membranes was indistinguishable from that seen in normal membranes (data not shown). These data imply that the membrane content of glycophorin C but not that of glycophorin A is decreased in protein 4.1-deficient erythrocytes, and further suggest that protein 4.1 may play a role in regulating the membrane content of glycophorin C.

Membrane content of glycophorins A and C of erythrocytes from individuals who are either homozygous or obligate heterozygotes for glycophorin C deficiency was also quantitated by flow cytometry, and those data are summarized in Table 1. No cell-associated fluorescence using monoclonal anti-glycophorin C could be detected in erythrocytes from individuals with homozygous glycophorin C deficiency, implying complete absence of this sialoglycoprotein in these membranes. Surprisingly, the membrane content of glycophorin C in erythrocytes from obligate heterozygotes was found to be normal. These flow cytometric data on membrane content of glycophorin C were further confirmed by the observed staining intensities of bands corresponding to this sialoglycoprotein on PAS-stained SDS-PAGE gels. These findings are in marked contrast to glycophorin A deficiency in which the membrane content of glycophorin A in the obligate heterozygotes, as is to be expected, is 50% of normal.10 Quantitation of the protein 4.1 content of membranes in erythrocytes from individuals with homozygous and heterozygous glycophorin C deficiency by SDS-PAGE analysis showed that all of these membranes have normal amounts of this skeletal protein. Our finding of normal glycophorin C content in membranes of erythrocytes from individuals who are obligate heterozygotes for glycophorin C deficiency suggests that this sialoglycoprotein may be synthesized in excess by erythroid precursor cells, and the amount of protein either assembled or retained with the erythrocyte membrane is regulated by protein 4.1.

Glycophorin C association with the membrane skeleton. Having established that protein 4.1 might play a role in regulating the membrane content of glycophorin C, we next examined whether an association exists between these two proteins in the erythrocyte membrane. To accomplish this, the glycophorin C content of intact membranes and membrane skeletons (prepared by Triton X-100 extraction) was examined by Western blot analysis using monoclonal anti-glycophorin C. As shown in Fig 2, glycophorin C is retained in membrane skeletons derived from normal erythrocyte membranes (compare lane a and a'). In contrast, while glycophorin C is present in membranes derived from erythrocytes totally deficient in protein 4.1, it is not retained in the membrane skeletons (compare lane b and b'). This finding suggests that protein 4.1 may be necessary for the association of glycophorin C with the membrane skeleton.

To establish unequivocally that protein 4.1 is indeed necessary for the association of glycophorin C with the normal membrane skeleton, we reconstituted membranes totally deficient in protein 4.1 with purified protein 4.1 and examined the glycophorin C content of membrane skeletons derived from native and reconstituted membranes. Whereas no glycophorin C was retained in membrane skeletons of protein 4.1-deficient erythrocytes (lane b'), this sialoglycoprotein was retained with the membrane skeleton after reconstitution of these erythrocyte membranes with purified protein 4.1 (lane c'). While these data clearly show that glycoprotein

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**Fig 2.** Western blot analysis using anti-glycophorin C antibody on membranes and Triton shells (membrane skeletons) from the protein 4.1-deficient red blood cells before and after reconstitution with purified protein 4.1. The first two lanes show the presence of glycophorin C in membranes (lane a) and Triton shells (lane a') from control red blood cells. The third lane shows glycophorin C to be present in totally protein 4.1-deficient erythrocyte membranes (lane b) but absent from Triton shells prepared from these membranes (lane b'). The last two lanes show the results of tests on membranes from the protein 4.1-deficient membrane that have been reconstituted with exogenous protein 4.1. Glycophorin C is present in both the membrane (lane c) and Triton shell preparations (lane c').
C is retained with membrane skeletons of protein 4.1-deficient cells after reconstitution, we were unable to accurately quantitate the fraction of total glycophorin C retained in these membranes. This inability was due in large part to the limited number of these rare cells available for analysis. However, this finding with reconstituted cells provides strong suggestive evidence that glycophorin C interacts with the erythrocyte membrane skeleton via protein 4.1.

Glycophorin C gene in protein 4.1-deficient cells. To rule out the possibility that reduced membrane content of glycophorin C in protein 4.1-deficient membranes is due to a defect in the gene coding for this sialoglycoprotein, we analyzed the glycophorin C gene in an individual with total 4.1 deficiency. Southern blot analysis, using two different restriction enzymes (Bgl II and EcoRI) did not show any differences between DNA from an individual with total protein 4.1 deficiency and normal DNA when probed with glycophorin C cDNA (data not shown). Similarly, Northern blot analysis showed that this DNA probe hybridized with a 1,200-bp band in mRNA extracted from reticulocytes totally deficient in protein 4.1, which was indistinguishable from that seen in normal reticulocyte mRNA (Fig 3, lanes a and a'). This is in marked contrast to hybridization with protein 4.1 cDNA, where major differences were noted in both Southern and Northern blot analyses. Glycophorin C mRNA from protein 4.1-deficient reticulocytes was additionally characterized by enzymatic amplification using polymerase chain reaction technique. RNA was reverse transcribed into cDNA, followed by amplification with glycophorin C-specific primers to produce a 395-bp DNA fragment encompassing the entire coding region of the mRNA (Fig 3). Because normal and protein 4.1-deficient reticulocytes yielded identical products (lanes b and b'), these data imply no major alteration in the coding region of glycophorin C gene in the protein 4.1-deficient cells.

DISCUSSION

The data presented here enable us to suggest that glycophorin C associates with the erythrocyte membrane skeleton potentially through its interaction with protein 4.1 and that, through this interaction, protein 4.1 may regulate the membrane content of glycophorin C. This conclusion is based on the following set of observations. The reduced quantity of glycophorin C in protein 4.1-deficient erythrocytes, in the presence of an apparently normal glycophorin C gene, indicates that protein 4.1 may play a critical role in regulating the glycophorin C content of the erythrocyte membrane. Further evidence to support this theory is provided by our finding that individuals who inherited only one gene for the expression of glycophorin C have a normal membrane content of this protein and not the expected 50% reduction in membrane content of glycophorin C. The basis for the suggestion that regulation of membrane content of glycophorin C is possibly through interaction between this protein and protein 4.1 is our observation that, while glycophorin C is totally removed from protein 4.1-deficient erythrocytes after extraction of integral proteins, it is retained with membrane skeletons if the protein 4.1-deficient membranes are reconstituted with exogenous protein 4.1 before extraction of the integral proteins. This observation provides the most compelling evidence to date for an association between protein 4.1 and glycophorin C in the erythrocyte membrane.

We are aware that each set of observations we have outlined do not in and of themselves convincingly show that glycophorin C content of erythrocyte membrane is regulated by protein 4.1 through the interaction between these two proteins. For example, it can be argued that our studies have not unequivocally shown that the glycophorin C gene is completely normal in the individual with protein 4.1 deficiency. However, this is unlikely because in two other families with a partial deficiency of protein 4.1, we have documented similar decreases in membrane content of glycophorin C, and it would be highly surprising that a defect in the protein 4.1 gene coexists with a defective glycophorin C gene in these three separate families. Similarly, our data on reconstituted membranes, which suggests an interaction between glycophorin C and protein 4.1, may be questioned in terms of the quantitative aspects of this interaction. However, we believe that, while these data do not unequivocally document a direct interaction between glycophorin C and protein 4.1 in erythrocyte membrane, it is strongly suggestive. Taken as an ensemble, the data we have outlined strongly support our suggestion for a role for protein 4.1 in regulating membrane content of glycophorin C.

Previous studies have provided indirect evidence for an association between protein 4.1 and glycophorin C in the erythrocyte membrane. Our data confirm and extend these observations. In this regard, it is interesting to note that there has been much discussion as to whether it is glycophorin A or glycophorin C that is associated with protein 4.1 in the native erythrocyte membrane.
Chasis et al.\textsuperscript{35} suggest that glycophorin A does not normally interact with erythrocyte membrane skeleton, but such an interaction can be induced by binding of antibodies to the extracellular domain of this sialoglycoprotein. In addition, Mueller\textsuperscript{11} has shown that, compared with normal membranes, protein 4.1 is more readily extracted from glycophorin C-deficient erythrocyte membranes, while its extraction from glycophorin A-deficient membranes is no different than from normal membranes. Viewed in the context of our finding that the content of glycophorin C but not that of glycophorin A is decreased in protein 4.1-deficient erythrocytes, these data suggest that, in the native erythrocyte membranes, the dominant interaction is likely to be between protein 4.1 and glycophorin C.

The quantitative relationship between protein 4.1 and glycophorin C and the finding that glycophorin C is retained in protein 4.1-deficient membrane skeleton preparations reconstituted with exogenous protein 4.1 indicates that protein 4.1 is required for the retention of normal amounts of glycophorin C in the erythrocyte membrane. This suggests that in protein 4.1-deficient individuals, glycophorin C may be synthesized, processed, and transported to the membrane in the normal way, but in the absence of protein 4.1 only one tenth of the normal quantity is retained in the mature erythrocyte membrane. It is likely that the remaining glycophorin C molecules are lost during the maturation of reticulocytes into erythrocytes. There is precedence for such a mechanism, since other membrane proteins such as transferrin receptors and fibronectin receptors are lost during this maturation process.\textsuperscript{36-38}

In summary, the data presented here suggest that in the native erythrocyte membrane, there may be a functionally important interaction between protein 4.1 and glycophorin C, and that the erythrocyte membrane content of glycophorin C but not that of glycophorin A is regulated by protein 4.1. In addition to the previously well-documented linkage between band 3 and ankyrin, the association between glycophorin C and protein 4.1 may be an important component of the anchoring mechanism involved in linking the lipid bilayer to the membrane skeleton. The linkages appear to be functionally significant, since cells in which these associations are either absent or defective exhibit abnormal shape and decreased mechanical integrity of the membrane. Finally, recent identification of protein 4.1 and glycophorin C in nonerythroid cells raises the possibility that interaction between these proteins may play a functionally important role in various other cells as well.

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