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A major endothelial plasmalemmal sialoglycoprotein, gp60, is immunologically related to glycophorin

(receptors/capillary permeability/vasculitis/lectins/serum albumin)

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ABSTRACT **Glycophorins, the major sialoglycoproteins of red blood cells in many species, are generally considered to be specific to erythroid cells. Using polyclonal antibodies directed against mouse glycophorin (αgp), we have identified a glycoprotein antigenically related to glycophorin on the surface of bovine and rat cultured endothelial cells. Immunoblotting with αgp identified a single 60-kDa polypeptide on transfers of SDS/polyacrylamide gels of solubilized confluent endothelial monolayers. In addition, a 60-kDa polypeptide was immunoprecipitated by αgp from lysates of ^{125}I-labeled intact endothelial cells. Controls with preimmune serum were negative. This antibody interaction was inhibited by murine erythrocyte ghosts and purified glycophorins. Our past work identified several endothelial surface sialoglycoproteins including a 60-kDa glycoprotein (gp60) that (*i*) interacts with albumin, (*ii*) binds *Limax flavus*, *Ricinus communis*, and *Triticum vulgare* agglutinins but not other lectins, (*iii*) is sequentially precipitated from ^{125}I-labeled cell lysates by using *R. communis* agglutinin followed by *T. vulgare* agglutinin, and (*iv*) is sensitive to sialidase digestion. Immunoblotting of such precipitates with αgp demonstrates that lectins recognize the same glycoprotein, namely gp60. These results indicate that gp60, a major endothelial surface sialoglycoprotein, shares antigenic epitope(s) with glycophorin.**

The highly sialylated, polyanionic glycocalyx of the microvascular endothelium creates a significant permselective barrier that restricts intravascular flow and transvascular transport of the molecular and cellular constituents of the blood. The negative charge of most plasma macromolecules, of all circulating blood cells, and of the endothelial glycocalyx ensures significant electrorepulsion between these elements, thereby limiting nonspecific contact between the vascular wall and both the blood cells and the plasma proteins (for details, see ref. 1). Little is known about the glycoproteins that form the endothelial glycocalyx. Recently, we have identified on the endothelial surface a group of sialoglycoproteins (gp140, gp120, gp100, gp60, and gp47). Some of these proteins may be involved in a number of important vascular phenomena, including receptor-mediated transcytosis, cellular diapedesis, hemostasis, and blood-borne metastasis (1). The specific binding of albumin to the endothelial surface (2–5) via one of these sialoglycoproteins, gp60 (6), as well as two other albumin-binding proteins (7, 8), apparently increases capillary wall permselectivity (9) by increasing both charge and volume exclusion within the endothelial glycocalyx (10–12).

In contrast to endothelial sialoglycoproteins, the main sialoglycoproteins of erythrocytes, collectively known as glycophorins, have been investigated extensively (for review, see refs. 13 and 14) and have served as a benchmark for the study of transmembrane sialoglycoproteins. They have been isolated from various species and shown to contain up to 70% (wt/wt) carbohydrate, present primarily as O-linked oligosaccharides. The function of glycophorins has not been established. Since they are heavily sialylated and usually contain up to 70% of the sialic acid of the cell, one of their functions could be to restrict low-affinity, nonspecific interactions at the cell surface. Indeed, sialic acid residues on the cell surface appear to prevent nonspecific hemagglutination, erythrocyte removal from the circulation by the spleen, and erythrocyte binding to the vascular endothelium (13–15). The glycosylated ectodomains of the glycophorins form blood-group antigens, bind lectins, and act as receptors for bacteria and viruses (13, 14). The development of autoantibodies to their ectodomains may result in autoimmune hemolytic anemia (16). Their endodomains interact with the infrastructure of the plasmalemma via a high-affinity linkage protein, band 4.1 (17, 18), which ultimately may modulate changes in red-cell rigidity and deformability upon interactions at the cell surface (18, 19).

Murine glycophorins, gp2 and gp3, like their human counterparts, glycophorins A and B, are major sialoglycoproteins of the erythrocyte membrane (13, 14) and, until recently, have been considered to be erythroid cell-specific. Based on indirect evidence, the presence of glycophorin-like molecules in a variety of nonerythroid cells has been suggested for the glycophorins A, B, and C (20–26). In this study, we investigate possible structural similarities between endothelial sialoglycoproteins and glycophorins by using a polyclonal antiserum directed against murine glycophorins. Preliminary results of this work have been published in abstract form (27).

METHODS

Cell Cultures. Microvascular endothelial cells from rat epididymal fat pads [rat fat capillary (RFC) cells] were isolated, grown, and plated to achieve confluent monolayers as described (1). Bovine endothelial cells isolated from the pulmonary artery (BPA), pulmonary vein (BPV), and microvessels of lung tissue (BLMV) were generously provided by P. DelVecchio and were grown on plastic dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The endothelial origin of the cell monolayers was checked periodically (5). Murine erythroleukemia (MEL) cells were grown and induced to differentiate as described (28).

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Abbreviations: αgp, anti-glycophorin; BPA, bovine pulmonary artery; BPV, bovine pulmonary vein; BLMV, bovine lung microvessel; RFC, rat fat capillary; MEL, murine erythroleukemia; RCA, *Ricinus communis* agglutinin; WGA, *Triticum vulgare* (wheat germ) agglutinin.

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Immunoprecipitation of Radioiodinated Cell Surface Polypeptides. As described previously (1), confluent monolayers of endothelial cells were radioiodinated by the lactoperoxidase technique and processed for immunoaffinity chromatography and SDS/PAGE. MEL cells (10^7 per sample) were washed several times in phosphate-buffered saline (PBS), pelleted, and then resuspended in 1.0 ml of PBS containing 75 μg of lactoperoxidase and 50 ng (1.0 mCi; 37 MBq) of $Na^{125}I$ (Amersham). Initially and at four successive 3-min intervals, 10 μl of 0.35 mM H_2O_2 in double-distilled water was added to the cell suspension and mixed by mild circular agitation (5 sec). After 15 min, the reaction was stopped by first pelleting the cells, then aspirating the reaction mixture, and finally washing the cells with three 1-ml portions of PBS (1 min per wash). The cells were lysed and processed for immunoaffinity chromatography and SDS/PAGE as described (28).

Radioiodination of IgG Fraction. IgG, purified from anti-glycophorin (αgp) serum by using protein A-Sepharose beads as per manufacturer's instructions (Pharmacia), was radioiodinated in 0.5 ml of PBS (0.5 mg/ml) with 0.2 mg of iodogen (1,4,5,6-tetrachloro-3α,6α-diphenylglycouril) and 1.5 mCi of $Na^{125}I$ stock (75 ng) as described (5). Free ^{125}I was removed by using a 10-ml Bio-Gel P-6 desalting column (Bio-Rad). The specific activity was $\approx$2.5 mCi/mg and the purity of the labeled protein was verified by SDS/PAGE.

Immunoblotting of Total Cell Lysate. Confluent monolayers of endothelial cells were washed, lysed, and processed for preparative SDS/PAGE (1). The separated proteins were electrophoretically transferred onto either nitrocellulose or Immobilon filters (1). Several 3- to 5-mm-wide strips cut from the filters were (*i*) quenched for 1 hr with PBS containing 0.05% Nonidet P-40 and 1% gelatin (PBS-NG), (*ii*) incubated for 1 hr with rabbit serum in PBS-NG (1:500 to 1:5000 dilution, (*iii*) washed three times with PBS (10 min per wash), (*iv*) incubated for 1 hr with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma; 0.6 μg/ml) in PBS-NG, and (*v*) washed three times with PBS (10 min per wash). The strips were reacted with alkaline phosphatase substrates as described (1).

Immunoblotting of Lectin-Affinity-Purified Glycoproteins. Confluent RFC monolayers were washed and lysed (1) using 3 ml of cold PBS containing 5% (vol/vol) Triton X-100 and 1% (wt/vol) SDS. Cell lysate (100 μl) was mixed with biotinylated *Ricinus communis* (RCA) or *Triticum vulgare* (WGA) agglutinin (E-Y Laboratories or Sigma; 200 μg in 400 μl of 0.68 mM $CaCl_2$ in PBS) and incubated at 4°C for 1 hr on a Nutator (Adams Chemical, Round Lake, IL). This lectin/cell lysate mixture was then added to 200 μl of avidin- or streptavidin-agarose beads (previously washed with PBS containing 1% Triton X-100 and 0.2% SDS at 4°C) and incubated with agitation on a Nutator for 45 min at 4°C. The beads were washed and processed for preparative SDS/PAGE followed by electrotransfer of the separated glycoproteins onto nitrocellulose filters; the latter were immunoblotted as described above.

RESULTS

In this study, we examine the possibility that some of the endothelial plasmalemmal sialoglycoproteins that we have recently identified both *in situ* and in culture (1) are structurally related to mouse glycophorins. Polyclonal antiserum raised against purified mouse glycophorin gp3 (29), αgp, which specifically recognizes both glycophorins from mouse erythrocytes (29), erythroblasts (29), and MEL cells (28) was used to probe lysates of endothelial cells cultured from various sources (rat and bovine) for the presence of glycophorin-like molecules.

Immunoblotting with αgp Serum. Proteins of solubilized confluent endothelial monolayers were separated by SDS/PAGE and electrotransferred onto filters. Endothelial cells derived from the microvasculature of the rat epididymal fat pad (RFC) and bovine lung (BLMV) and from large vessels such as bovine pulmonary artery (BPA) and vein (BPV) were used. Immunoblotting with αgp specifically identified a single 60-kDa polypeptide in each cell line tested (Fig. 1). Controls with preimmune and nonimmune serum were negative. Since each confluent endothelial cell monolayer was processed under identical conditions and the same lysate volume was loaded onto each gel lane, the stronger signal observed for the endothelial cells derived from microvessels (RFC and BLMV) rather than large vessels (BPA and BPV) suggests that microvascular endothelium expresses more of this protein per unit surface area than other endothelia. Furthermore, microvascular cells appear to express more gp60 on a per cell basis than other endothelia, based on the observation that BPA and BLMV cells have similar cell surface densities, while BPV cells are much less spread out and have a greater cell surface density (data not shown).

Immunoprecipitation of Radioiodinated Plasmalemmal Proteins with αgp Serum. Lactoperoxidase-catalyzed radioiodination of intact cells, followed by cell lysis and lysate processing through SDS/PAGE and autoradiography, demonstrated the presence of several cell surface proteins. Immunoprecipitation of this radiolabeled cell lysate with αgp serum specifically revealed a single radiolabeled polypeptide with an apparent mass of 60 kDa, whereas the preimmune serum failed to recognize any plasmalemmal proteins (Fig. 2). Similar results were obtained with the three bovine endothelial cell lines (data not shown). As a positive control, radioiodinated MEL cells were also subjected to immunoprecipitation with αgp and only the glycophorins gp3, gp2, and gp1 (which is an aggregate of gp2 and especially of gp3) were recognized by the serum.

Competition of αgp Antibody Binding to gp60. Although αgp was shown to be specific for murine glycophorins (28, 29), it is possible that contaminating antibodies unrelated to the glycophorins accounted for the observed immune recognition of the 60-kDa endothelial protein. However, this seems unlikely since (*i*) the preimmune serum did not recognize the 60-kDa protein by immunoprecipitation or immunoblotting and (*ii*) the binding of αgp to gp60 was inhibited by glyco-

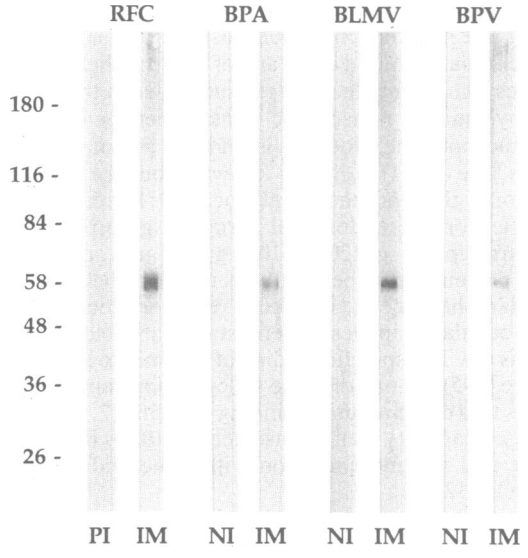

FIG. 1. Immunoblotting of endothelial cell lysates with rabbit serum raised against murine glycophorins. Proteins from the indicated confluent endothelial monolayers were solubilized, separated by SDS/PAGE, electrophoretically transferred onto filters, and then immunoblotted with either preimmune (PI), nonimmune (NI), or immune (IM) αgp serum. Molecular mass markers (kDa) are at left.

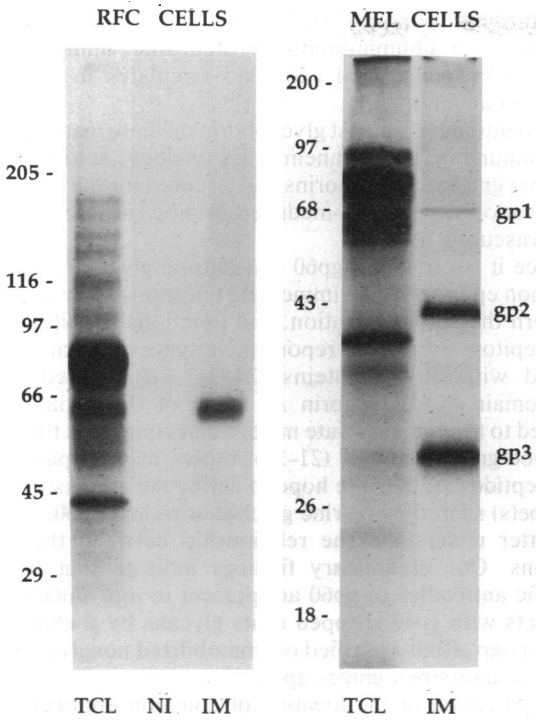

FIG. 2. Radioiodinated surface proteins of RFC and MEL cells immunoprecipitated with αgp serum, separated by SDS/PAGE, and visualized by autoradiography. ^{125}I-labeled lysates from confluent RFC monolayers and from MEL cells grown in suspension were subjected to immunoprecipitation (as in refs. 1 and 28), with the serum indicated for each lane (labeled as in Fig. 1). TCL, total cell lysate.

phorin. The latter was demonstrated in several ways. First, immunoblotting was performed after incubating αgp serum with various concentrations of lyophilized ghosts prepared from murine erythrocytes as described (30). Binding to the 60-kDa protein was significantly inhibited at protein concentrations greater than 0.1 mg/ml (Fig. 3). Second, since ghost preparations contain a heterogeneous mixture of lipids and proteins that at higher concentrations potentially could interfere with antibody binding, purified murine glycophorins were used as a more specific inhibitor of αgp antibody binding (Fig. 4). To increase the sensitivity of our detection system, we purified and then radioiodinated an IgG fraction from the αgp serum. By immunoblotting, this radiolabeled IgG recognized a 60-kDa protein at protein concentrations as low as 50 ng/ml (data not shown). This binding was diminished significantly in the presence of added glycophorin (≈70% inhibition at protein concentrations of 10 μg/ml). A control using ovalbumin at 1 mg/ml showed less than a 10% decrease in

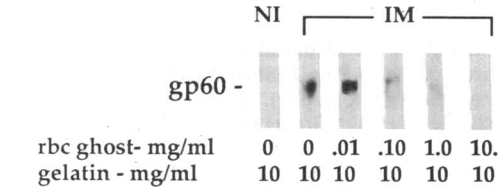

FIG. 3. Inhibition/competition with erythrocyte membranes of αgp binding to gp60 on immunoblots. To determine the specificity of αgp binding to gp60, αgp serum was preincubated for 1 hr with the indicated final concentrations of lyophilized membranes from ghosts of murine erythrocytes (rbc ghosts) before immunoblotting of the transferred proteins from a BLMV cell lysate. The serum (NI, nonimmune; IM, immune) was diluted 1:1500 in the presence of 1.0% gelatin and the antibody binding was detected using goat anti-rabbit IgG conjugated to alkaline phosphatase.

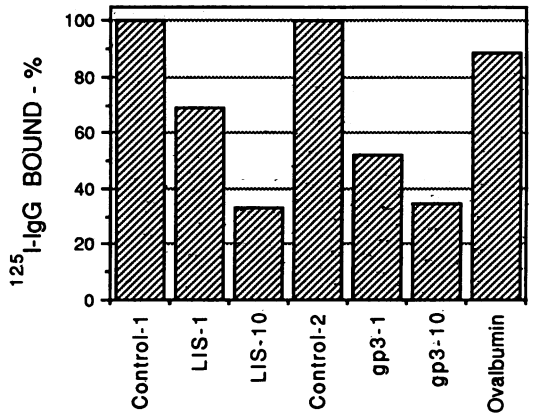

FIG. 4. Inhibition/competition with purified glycophorins of αgp binding to gp60 on immunoblots. A radioiodinated IgG fraction of the αgp serum was used to assess binding of αgp IgG to gp60 in the presence or absence of purified murine glycophorins. ^{125}I-labeled IgG (200 ng/ml) was first incubated for 1 hr with either a lithium diiodosalicylate (LIS) extract of murine erythrocyte ghosts (30) or murine glycophorin gp3 [prepared by elution from polyacrylamide gel slices and concentration in a Centricon-3 microconcentrator (Amicon)] and then incubated with filters in the presence of 1% gelatin. LIS-1 and LIS-10 indicate that the LIS extract was used at 1 and 10 μg/ml, respectively. gp3-1 and gp3-10 indicate that gp3 was used at an estimated concentration of 1 and 10 μg/ml, respectively. The binding was quantitated by γ counting of the radioactive signal in the region of the strip where gp60 was located. The results are expressed relative to the controls of each set of experiments. Ovalbumin at 1 mg/ml was used as another control. The amounts given for gp3 are maximal values, based on 100% recovery of glycophorin, calculated from the specific radioactivity of gp3 [assuming six methionine residues as indicated by its amino acid sequence (31)] isolated from MEL cells metabolically labeled with [^{35}S]methionine under defined isotope dilution conditions.

signal. Hence, the 60-kDa protein interacts specifically with the subset of antibodies directed against glycophorin and, therefore, has one or more epitopes in common with murine glycophorin.

Immunoblotting of Lectin-Purified Glycoproteins with αgp Serum. Previous work (1, 3) identified a 60-kDa, albumin-binding sialoglycoprotein (gp60) on the surface of RFC cells that binds *Limax flavus* agglutinin, RCA, and WGA but not 14 other lectins including succinylated WGA, concanavalin A, or *Glycine max*, *Ulex europaeus*, or *Bandeiraea simplicifolia* I agglutinins. Furthermore, gp60 was sequentially precipitated from ^{125}I-labeled cell lysates with RCA followed by WGA (1). In order to verify that αgp serum recognizes gp60 (and not just another protein of the same apparent mass), endothelial cell glycoproteins were isolated from RFC cell lysates by lectin-affinity chromatography using RCA and WGA. These glycoproteins were separated by SDS/PAGE and electrotransferred to nitrocellulose filters. Immunoblotting with αgp identified specifically in both cases a single band at 60 kDa that corresponded precisely with the detected radiolabeled gp60 (Fig. 5). It appears that the lectins and αgp recognize the same radiolabeled surface glycoprotein, namely gp60.

DISCUSSION

As shown in this paper, bovine and rat cultured endothelial cells have a 60-kDa protein on their surface that is immunologically related to murine glycophorins. Antibodies to glycophorin recognize by immunoprecipitation and immunoblotting a 60-kDa endothelial sialoglycoprotein in lysates of radioiodinated intact cells. Antibody binding is inhibited by murine erythrocyte ghosts and by purified glycophorins,

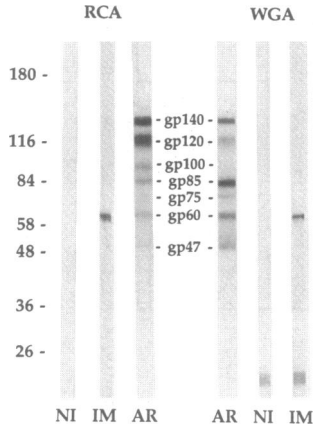

FIG. 5. Immunoblotting of lectin-purified glycoproteins from RFC cell lysates with αgp serum. Endothelial cell glycoproteins were isolated from RFC cell lysates by lectin affinity chromatography using RCA and WGA. The isolated glycoproteins were resolved by SDS/PAGE, electrotransferred to nitrocellulose filters, and then immunoblotted with either nonimmune (NI) or immune (IM) αgp serum. In this case, 50 μl of radioiodinated cell lysate was added to the 1 ml of unlabeled cell lysate before processing for SDS/PAGE. The AR lanes show the RCA- and WGA-precipitated glycoproteins detected by autoradiography of the filter.

indicating that this protein does indeed share one or more epitopes with murine glycophorins. Furthermore, this protein proved to be gp60, a major endothelial plasmalemmal sialoglycoprotein that we have recently identified (1) and shown to bind albumin (6). Like glycophorin, gp60 is sensitive to sialidase (neuraminidase) digestion and contains predominantly, if not solely, O-linked oligosaccharides (1, 13, 28, 29). Immunoblotting with αgp serum indicates that gp60 is expressed to a greater degree by microvascular endothelium than by endothelium of large arteries and veins. This finding correlates well with the observation that albumin binds more to microvascular than to other endothelia (32, 33).

For many years, it has been assumed that glycophorins are restricted to and, therefore, specific for erythroid cells (13, 14). Antisera raised against purified murine glycophorins have been shown to react strongly with erythroid cells but not with platelets, lymphocytes, macrophages, and granulocytes (34). Other observations suggest, however, that molecules immunologically related to human glycophorins A and B (21–26) and C (20) may be present in a variety of cells. For instance, antibodies that recognize the M and N blood-group epitopes [known to be located in the ectodomain of glycophorins A and B (35)] interact with various tissues, including renal endothelium (21–23). Antibodies against other epitopes on the ectodomain of glycophorin A were reported to react with the surfaces of a number of normal and tumor cell lines (24–26). So far, only a 95-kDa protein has been indirectly identified as a potential glycophorin analogue on the basis of similar amino acid and hexose content (35). This protein may be the same as a 95-kDa molecule immunoprecipitated with αgp antibodies from mouse thymus lysates (36).

Blood cells and vascular endothelium originally stem from mesenchymal hemangioblastic cell islands that contain early erythroblasts at their center and primitive endothelial cells at their periphery (for review, see ref. 37). Their common embryological origin may explain the presence of similar proteins on endothelium and on erythroid cells, but the time at which the two cell lineages diverge remains uncertain. gp60 and possibly other proteins may be remnants of a set of molecules expressed early before lineage divergence that were maintained through subsequent cell differentiation. For instance, endothelial cells express several proteins that are also present in platelets, such as the von Willebrand factor (38), thrombospondin (39), SPARC (40), and GMP140 (41). SPARC is an albumin-binding protein that, unlike gp60, is secreted by endothelial cells and circulates in the blood bound to albumin (40).

Autoantibodies against glycophorin mediate many forms of autoimmune hemolytic anemia. By analogy, autoantibodies to either gp60 or glycophorins may account for the occasional observation of immune-mediated hemolysis in association with vasculitis (16).

Since it is clear that gp60 and murine glycophorins have common epitope(s), the immediate questions to be answered concern the nature, location, and functional significance of these epitopes. Previous reports have suggested that epitopes shared with other proteins (24–26) are located in the ectodomain of glycophorin A. Some of them may be restricted to the carbohydrate moiety that comprises the M and N blood-group epitopes (21–23); others may be part of the polypeptide (35, 36). We hope to define the chemistry of the epitope(s) shared by murine glycophorins and gp60, in order to better understand the relationship between these two proteins. Our preliminary findings indicate that peptide-specific antibodies to gp60 are present in αgp since (*i*) αgp interacts with gp60 stripped of its glycans by β-elimination and (*ii*) αgp affinity-purified on immobilized nonglycosylated gp3 precursors recognizes gp60.

Identification of the location of common epitope(s) may yield valuable insights into potential common functions for these proteins. For instance, if the epitope(s) were located in the endodomains of both molecules, then gp60, like the glycophorins, might interact with cytoskeletal elements and thereby provide anchoring points in the plasmalemma for an underlying infrastructure that stabilizes mechanically and chemically the plasmalemma (or specific domains thereof). In fact, many cells share common peripheral membrane proteins (band 4.1, spectrin, actin) with erythrocytes (18). In the case of gp60 (and other endothelial surface proteins), such interactions could modulate junctional permeability through cytoskeletal rearrangements. Alternatively, if the common epitope(s) were located in the ectodomains, glycophorins might, like gp60, interact with albumin. Albumin is known to bind to erythrocytes (42) and to influence strongly their shape, rigidity, and deformability (43–45). Both erythrocytes and endothelium are exposed to high fluid shear conditions in the circulation so that albumin binding to their surface may be necessary for normal rheological behavior. Erythrocytes hemolyze with hydrodynamic shear stress to a lesser extent in the presence of albumin (43). The molecular mechanisms underlying these findings are presently unknown but they may involve ligand-induced interactions of integral membrane proteins with cytoskeletal elements (45), in a manner similar perhaps to the changes in red-cell plasticity elicited by the binding of antibodies and lectins to the ectodomain of glyclophorin A (18, 19).

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