Signal Transduction by Glycophorin A: Role of Extracellular and Cytoplasmic Domains in a Modulatable Process

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Abstract. Binding of ligands to the extracellular region of the erythrocyte transmembrane protein glycophorin A induces a decrease in membrane deformability. Since the property of membrane deformability is regulated by the skeletal proteins on the cytoplasmic side of the membrane, this suggests that ligand binding may initiate a transmembrane signal. To further study this process, we examined which domains of the extracellular region of glycophorin are involved in signal transduction and whether the cytoplasmic domain of the molecule is necessary for transmitting the signal. Using the ektacytometer, we compared the effect on deformability of four monoclonal antibodies that detect different epitopes on glycophorin A. We found that 9A3 (which recognized the amino terminus of glycophorin) caused a 5.8-fold increase in rigidity, R-10 and 10F7 (which recognized epitopes in the mid-region of the extracellular domain) caused a 10.8-fold increase in rigidity and B14 (which binds to glycophorin close to the membrane) caused a 18-fold increase in rigidity. Further, a direct relationship was observed between the degree of antibody-induced rigidity and the amount of glycophorin A that became associated with the skeletal proteins in a Triton shell assay. In Miltenberger V erythrocytes, which contain a hybrid sialoglycoprotein with no cytoplasmic domain, antibody binding did not induce an increase in rigidity. These results imply that glycophorin A is capable of a modulatable form of transmembrane signaling that is determined by the extracellular domain to which the ligand binds, and the cytoplasmic domain of glycophorin A is crucial for this process.

RECEPTOR binding initiates numerous and varied biological responses in many cell systems. The molecular processes by which ligand binding generates transmembrane signaling through receptor molecules appear to vary. One well-described mechanism entails ligand-induced changes in the associations of integral and skeletal membrane proteins. In platelets, ligand binding and stimulation of platelets causes an increased association of membrane-spanning glycoproteins IIb and IIIa with the actin cytoskeleton (17, 28, 29). In neutrophils, a similar lectin-induced association of glycoproteins and skeletal components occurs which may modulate cell motility (34). While the basic mechanism of ligand-induced protein–protein association has been established as a transmembrane signaling process, important details remain to be defined. For example, the effect of ligand binding to different extracellular domains has not been studied nor has the role of the cytoplasmic domain. This study attempts to address these issues using the erythrocyte as a model system.

The bases for the present study are our earlier findings that transmembrane signaling can indeed be induced through changes in glycoprotein–membrane interactions in the red blood cell. We found that membrane deformability was markedly decreased in erythrocytes that were pretreated with ligands specific for the transmembrane protein, glycophorin A (also known as sialoglycoprotein α; 4), but remained normal in the presence of ligands specific for other cell surface components such as band 3 and blood group antigens A and B (10). The decrease in membrane deformability appeared to depend upon a transmembrane process, and not upon a rigidity-inducing lattice on the outside surface of the cell because a monovalent Fab of an anti glycophorin IgG produced decreased deformability (10). Since the erythrocyte skeletal proteins play an important role in determining membrane deformability (9, 12, 16, 25), we looked for a ligand-induced change in the associations of glycophorin with skeletal proteins. In Triton X-100–insoluble residues, we found that the partitioning of glycophorin A with the skeletal proteins increased after binding of ligands specific for glycophorin A (10). These results implied that a form of transmem-

brane signaling occurs in the erythrocyte that is mediated by glycophorin A, and results in changes in the cellular property of membrane deformability.

To further study the mechanism by which ligand binding to glycophorin A can modulate membrane deformability, we asked what domain(s) along the extracellular portion of the molecule was involved in the process, and whether the cytoplasmic peptide plays a role in signal transduction. Using monoclonal antibodies that detect different epitopes on the extracellular region of glycophorin A, we found that every antibody tested induced membrane rigidity, but the degree of rigidity varied: the closer the binding epitope was to the membrane, the greater the degree of rigidity induced. Moreover, there was a direct relationship between the degree of rigidity and the amount of glycophorin A that was associated with the skeletal proteins. To determine whether the cytoplasmic domain has a role in these ligand-induced changes in membrane deformability, we studied homozygous Miltenberger V erythrocytes, which contain a hybrid sialoglycoprotein (α-δ; 6, 37) composed of glycophorins A and B (also known as sialoglycoprotein δ; 4) but with no cytoplasmic domain (22). The Miltenberger V cells exhibited little change in membrane deformability after ligand binding, implying that the cytoplasmic region of glycophorin A is essential for signal transduction.

Materials and Methods

Reagents

Dextran was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); anti-N and anti-M from Ortho Diagnostic Systems, Inc. (Westwood, MA).

The monoclonal antibodies used in these studies were all of the IgG class and have been described in detail elsewhere (5, 7, 8, 11). In brief, 9A3 recognizes an epitope involving the first NH_2-terminal amino acid and is specific for blood group antigen M; R-10 and 10 F7 bind to the 39 amino acid trypsin-sensitive NH_2-terminal peptide of M or N erythrocytes (5, 8, 11); B-14 recognizes an epitope between residues 56 and 67, close to the membrane (31); and BRIC-10 binds to the NH_2 terminus of glycophorin C (sialoglycoprotein β; 7).

Deformability Measurements

After obtaining informed consent, blood from normal volunteers and from an individual with Miltenberger V blood type was drawn into acid-citrate-dextrose and the erythrocytes were collected and washed three times in 5 mM Tris, 140 mM NaCl, pH 7.4. The cells were then resuspended to 0.5% hematocrit in 3 ml of dextran (40,000 mol wt, 25 g/100 ml in 10 mM phosphate buffer, pH 7.4) and examined by ektacytometry, a laser diffraction method previously described (26). Briefly, suspended cells are exposed to an increasing shear stress (0–400 dyn/cm^2) and the change in their laser diffraction pattern from a circle to an ellipse is monitored. Photometric measurement of light intensities at two points in the diffraction image produces a signal, designated the deformability index, which quantitates cell ellipticity. By an automatic image analysis system, the deformability index is recorded as a continuous function of applied shear stress. The curve thus produced is a measure of the ability of the cells to deform under defined fluid stresses.

To evaluate the effect of antibodies on deformability, washed erythrocytes were resuspended to 0.5% hematocrit in dextran solution containing monoclonal antibodies (5–360 μg/ml). The suspensions were incubated for 30 min at room temperature and then examined in the ektacytometer.

Hemagglutination Assay

Equal volumes of anti-M, anti-N, or monoclonal antibodies and a 4% saline suspension of washed red cells were incubated at room temperature for 1 h. The cell suspensions were then centrifuged at 1,000 rpm for 10 s and examined macroscopically for hemagglutination.

Triton X-100 Extraction of Erythrocytes

Erythrocytes were extracted with Triton X-100 using a method modified from that first described by Sheetz (33). In brief, freshly drawn erythrocytes were washed twice in 140 mM NaCl, 10 mM sodium phosphate, pH 7.4. 1.1 ml of washed cells (hematocrit 70%) was then lysed in Triton X-100 (100 mg/10^{10} cells) in 140 mM KCl, 20 mM Hepes, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.05 mM $CaCl_2$, 2 mM reduced glutathione, and 1 mM DFP. This lysate was layered on a linear sucrose density gradient (10–60%) and centrifuged for 1 h at 22,000 rpm in an ultracentrifuge (model L3-50; Beckman Instruments, Inc., Fullerton, CA). The single visible band on the gradient was collected and resuspended in 140 mM KCl, 20 mM Hepes, 1 mM EDTA, 2 mM reduced glutathione, and 0.03 mM phenylmethylsulfonyl fluoride (PMSF; pH 7.4) and centrifuged at 22,000 rpm for 30 min. The resulting pellet was solubilized and analyzed by electrophoresis as described below.

To evaluate Triton extractability after ligand binding, 1.1 ml of washed cells (hematocrit 70%) was resuspended in 58.2 ml in dextran (40,000 mol wt, 25 g/100 ml in 10 mM phosphate buffer, pH 7.4) containing 7.5–180.0 μg/ml monoclonal antibody. The cells were incubated for 30 min at room temperature, then pelleted at 2,000 rpm for 5 min in a Sorvall RC-2B (DuPont Instruments, Sorvall Biomedical Division, Newton, CT). These pretreated erythrocytes were then processed as described above.

SDS-PAGE

Samples for electrophoresis were solubilized in 0.5 M Tris-HCl (pH 6.8), 1.25% SDS, and 0.38 M dithiothreitol (DTT), heated to 100°C for 2 min, and analyzed in the discontinuous system described by Laemmli (19) on slab gels composed of a separating gel of 10% acrylamide and a stacking gel of 3% acrylamide. After electrophoresis, the gels were fixed and stained for carbohydrate with periodic acid–Schiff reagent (7). Quantitation of periodic acid–Schiff reagent-positive bands was obtained by gel scanning at 550 nm using a spectrophotometer (model DU-8; Beckman Instruments, Inc.).

Quantitation of Spectrin

The amount of spectrin in the Triton-insoluble residues was determined as previously described (1). The washed Triton shells were dissolved in SDS and subjected to electrophoresis through 3.5–17.0% polyacrylamide slab gels using the Fairbanks buffer system (13). The slabs were then stained with Coomassie Brilliant Blue R250 and destained until the background was colorless. The bands corresponding to spectrin were excised, the dye was eluted in 25% (vol/vol) pyridine in water, and the absorbance of the spectrin eluate was measured at 605 nm (15).

Results

Effect of Glycophorin A-specific Antibodies on Membrane Deformability

To study which domains along the extracellular portion of glycophorin A play a role in modulating membrane deformability, we used monoclonal antibodies as probes. The binding sites for these antibodies are depicted in Fig. 1. This figure is a schematic diagram of glycophorin A as it traverses the lipid bilayer with its amino terminus on the cell surface and its carboxy terminus within the cytoplasm (18, 21, 32, 36). The antibody, with an epitope most distal to the lipid bilayer was 9A3. This antibody has specificity for the blood group antigen M and recognizes an epitope that involves amino acid 1 but not amino acid 5 (8). R-10 and 10F7 bind closer to the lipid bilayer than 9A3 but do not bind to erythrocytes pretreated with trypsin (5, 8, 11). B14 detects an epitope between residues 56 and 67, a region of the sialoglycoprotein closely adjacent to the bilayer (31).

The effect on deformability of antibody binding was measured by ektacytometry. Initially, dose response curves were done with each antibody to determine the concentration at which the maximal rigidity occurred. The effect of increas-

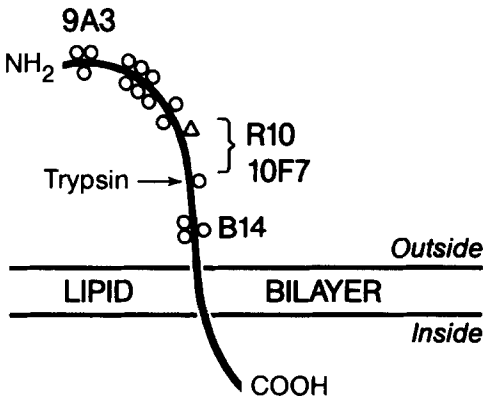

Figure 1. Schematic diagram of glycophorin A with the antibody binding sites. The peptide backbone with its 15 O-linked (○) and 1 N-linked (△) tetrasaccharides is shown traversing the lipid bilayer. The blood group antigens M and N are determined by variations within the first five amino acids in the amino terminal end of the molecule. The monoclonal antibody 9A3 has anti-M specificity and binds to the amino terminus. Antibodies R-10 and 10F7 bind in the mid-region of the extracellular portion of glycophorin A distal to the trypsin cleavage site. B14 detects an epitope closely adjacent to the lipid bilayer, between residues 56 and 67.

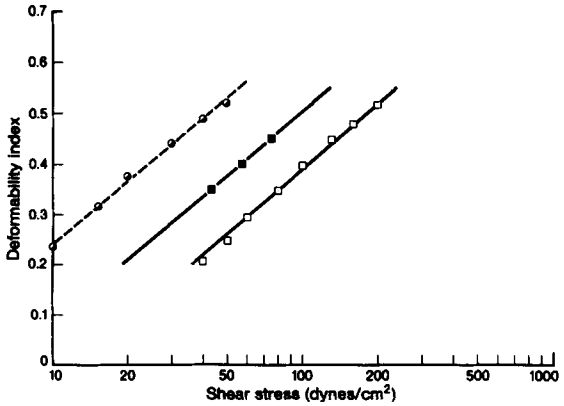

Figure 3. Effect of 9A3 on the deformability of MM, MN, and NN erythrocytes. Antibody-treated NN erythrocytes (●), which lack the antigenic determinant for 9A3, and erythrocytes without antibody (control RBC, ○) had normal deformability in response to low shear stress. MN cells (■) and MM cells (□) with bound 9A3 required greater amounts of shear stress to produce the same degree of deformation as cells without bound antibody. MN cells with 9A3 bound were 2.5 times more rigid than normal, and MM cells with bound 9A3 were 4.5 times more rigid.

ing concentrations of 9A3 on the deformability of MM erythrocytes is shown in Fig. 2. In this figure, the deformability index is plotted as a function of the logarithm of the shear stress. It can be seen that the cells treated with increasing concentrations of 9A3 required greater amounts of shear stress to produce the same degree of deformation as cells without antibody. Since the lines are parallel, one can calculate that cells treated with 5 μg/ml of 9A3 required 1.7-fold greater shear stress than cells without antibody to reach equivalent deformation at all points along the curve, indicating that these cells were 1.7 times more rigid than normal. Erythrocytes treated with 10 μg/ml were 2.3 times more rigid; those treated with 20 μg/ml were 3.7 times more rigid; and those treated with 40–160 μg/ml were 4.5 times more rigid. Thus, increasing concentrations of 9A3 caused a dose-dependent increase in rigidity, and at 40 μg/ml the maximal effect was observed. Subsequent experiments were then performed using that concentration.

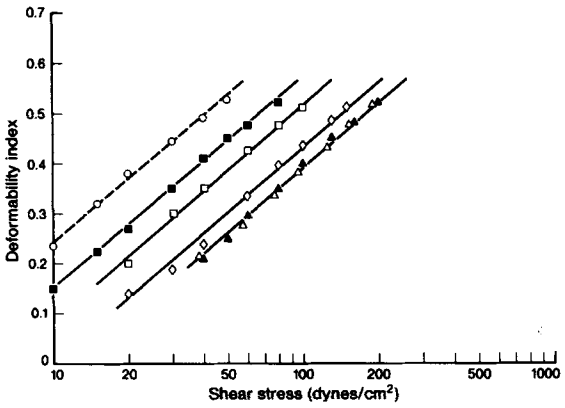

Figure 2. Effect of 9A3 on deformability of MM erythrocytes. The deformability of cells without antibody (control RBC, ○) was normal in response to low shear stress. Cells treated with increasing concentrations of 9A3 required greater amounts of shear stress to produce the same degree of deformation as cells without antibody. The lines are parallel; thus cells treated with 5 μg/ml of 9A3 (■) required 1.7-fold greater shear stress than cells without antibody to reach equivalent deformation at all points along the curve, indicating that these cells were 1.7 times more rigid than normal. 10 μg/ml of 9A3 (□) induced a relative rigidity of 2.3; 20 μg/ml (◇) induced a relative rigidity of 3.7; and 40 (▲) and 160 (△) μg/ml induced a relative rigidity of 4.5. The maximal effect was observed at 40 μg/ml. The deformability index is plotted as a function of the logarithm of the shear stress.

The effect of 9A3 on the deformability of MM, MN, and NN erythrocytes is shown in Fig. 3. NN erythrocytes, which lack the antigenic determinant for 9A3, were unaffected by incubation with this antibody. MN and MM cells pretreated with 9A3 required greater amounts of shear stress to produce the same degree of deformation as cells without antibody. MN cells with 9A3 bound were 2.5 times more rigid than normal, while MM cells with 9A3 bound were 4.5 times more rigid than untreated erythrocytes.

Dose-response curves similar to those performed with 9A3 were obtained with R-10, 10F7, and B14. Like 9A3, increasing concentrations of R-10, 10F7, and B14 caused dose-dependent increases in rigidity in the following range of antibody concentrations: 10F7, 0–360 μg/ml; R10, 0–240 μg/ml, and B14, 0–200 μg/ml. The maximal effect on rigidity was observed at a 10F7 concentration of 90 μg/ml, an R-10 concentration of 40 μg/ml, and a B14 concentration of 50 μg/ml. Subsequent experiments using these antibodies were then performed at concentrations that induced maximum rigidity. The maximum effect of R-10 binding on deformability is illustrated in Fig. 4. Unlike 9A3, this antibody reacts with glycophorin A irrespective of its MN type. Antibody binding increased the rigidity of MM, NN, and MN erythrocytes 10-fold.

The relative rigidity of MM erythrocytes exposed to 9A3, R-10, 10F7, and B14 is depicted in Fig. 5. Control cells with no bound antibody have normal deformability and a relative

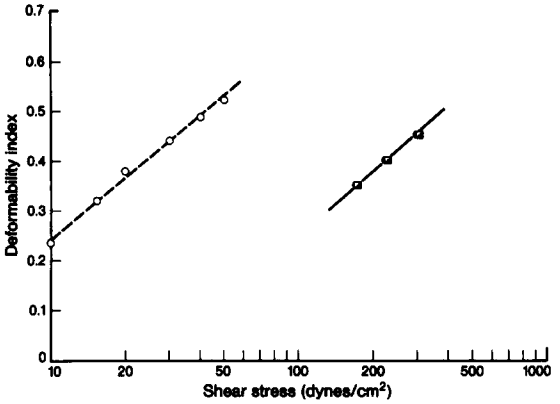

Figure 4. Effect of R-10 on the deformability of MM, MN, and NN erythrocytes. R-10 binding increased the rigidity of NN (●), MN (■), and MM (□) erythrocytes 10-fold. ○, control RBC.

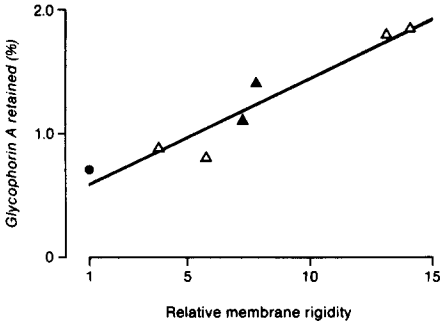

Figure 6. Ligand-induced association of glycophorin A with the skeletal proteins and relative membrane rigidity. The greater the degree of ligand-induced membrane rigidity, the greater the percentage of glycophorin A retained with the skeletal proteins. ●, erythrocytes with no antibody; △, erythrocytes with various concentrations of R-10; ▲, erythrocytes with various concentrations of 9A3.

rigidity of 1. Binding of 9A3 produces cells with a relative rigidity of 5.8 ± 1.5; 10F7 cells with a relative rigidity of 10.8 ± 1.4; R-10 cells with a relative rigidity of 10.8 ± 2.1; and B14 cells with a relative rigidity of 18.2 ± 2.7.

From these observations we can conclude that ligand binding to three different regions of the extracellular peptide of glycophorin A induces increased membrane rigidity. Interestingly, binding of antibodies to different regions induces different degrees of change and the pattern observed is that the closer to the membrane the ligand binds the greater the change in rigidity. Moreover, two antibodies, 10F7 and R-10, which have epitopes in close proximity to one another, change membrane rigidity to the same degree suggesting that the region of ligand binding determines the degree of the resulting change in the membrane property of deformability.

Relationship between Membrane Rigidity and the Association of Glycophorin A with the Skeletal Proteins

We next investigated whether there was a quantitative relationship between the degree of ligand-induced membrane rigidity and ligand-induced association of glycophorin A with the skeletal proteins. For these studies, MM erythrocytes were incubated with various concentrations of the monoclonal antibodies 9A3 and R10. Aliquots from each incubation mixture were assayed by ektacytometry to measure membrane rigidity. Then Triton-insoluble residues were prepared from the liganded erythrocytes. The amount of glycophorin A associated with the skeletal proteins in the Triton lysates was quantitated by densitometry of SDS polyacrylamide gels stained with periodic acid–Schiff reagent. To compare different preparations with one another, the spectrin content of each preparation was determined as described in the Materials and Methods section and then the amount of glycophorin A retained in the membrane skeleton of the Triton lysate was normalized with the spectrin content. The percentage of glycophorin A retained in the Triton lysate was expressed as a ratio of the glycophorin A retained to the total glycophorin A content of intact membranes. As illustrated in Fig. 6, the greater the degree of ligand-induced membrane rigidity, the greater the percentage of glycophorin A retained with the skeletal proteins. These results imply that there is a direct relationship between membrane rigidity and enhanced association of glycophorin A with the skeletal proteins.

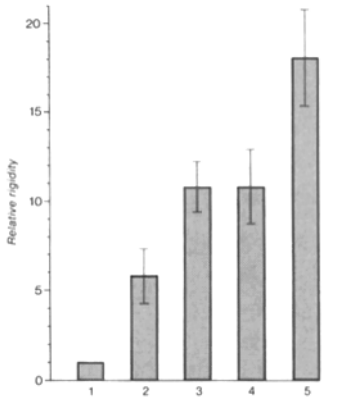

Figure 5. The maximum relative rigidity of MM erythrocytes induced by 9A3, R-10, 10F7, and B14. Antibody binding to different regions of glycophorin A induces different degrees of increase in membrane rigidity. Control erythrocytes with no bound antibody (lane *1*) have normal deformability and a relative rigidity of 1. Binding of 9A3 (lane *2*) produces cells with a relative rigidity of 5.8 ± 1.5 (n = 5); 10F7 (lane *3*) cells with a relative rigidity of 10.8 ± 1.4 (n = 10); R-10 (lane *4*) cells with a relative rigidity of 10.8 ± 2.1 (n = 17); and B14 (lane *5*) cells with a relative rigidity of 18.2 ± 2.7 (n = 6).

Study of the Role of the Cytoplasmic Domain of Glycophorin A

To study whether the cytoplasmic domain of glycophorin A participated in the transmembrane signaling process, we used Miltenberger V erythrocytes. A periodic acid–Schiff reagent stain of an SDS polyacrylamide gel of erythrocyte membranes prepared from the homozygous Miltenberger V individual (F.M.) is shown in Fig. 7. In individuals homozygous for this phenotype, the red cells lack normal glycophorin A and B and contain instead a hybrid sialoglycoprotein comprised of the extracellular portion of glycophorin A, the intramembranous portion of glycophorin B, and no cytoplasmic residues. Fig. 8 shows the effect of 10F7 and R-10 on the deformability of both normal and Miltenberger V erythrocytes. The deformability of unliganded Miltenberger V cells was normal. The rigidity of normal cells after antibody binding was markedly increased. Cells incubated with R-10 were 13.1 times as rigid as control and those incubated with 10F7 were 13.9 times as rigid. In contrast, the rigidity of Milten-

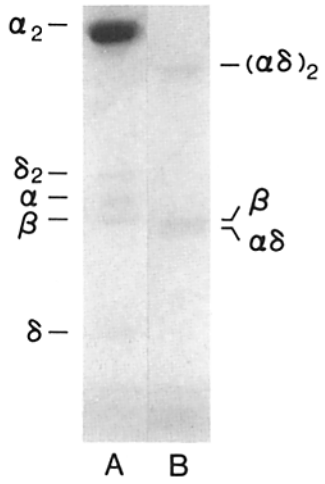

Figure 7. SDS polyacrylamide gel of membranes prepared from normal (lane *A*) and Miltenberger V (lane *B*) erythrocytes stained with PAS. Miltenberger V erythrocyte membranes have no glycophorin A (sialoglycoprotein α) or glycophorin B (sialoglycoprotein δ) but do have a hybrid sialoglycoprotein composed of glycophorins A and B (sialoglycoprotein α-δ).

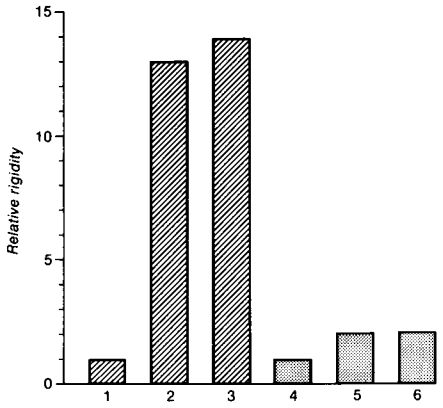

Figure 8. Effect of R-10 and 10F7 on the relative rigidity of normal erythrocytes and Miltenberger V erythrocytes. The deformability of nonliganded normal cells (lane *1*) and Miltenberger V cells (lane *4*) was normal with a relative rigidity of 1. The relative rigidity of normal cells after R10 (lane *2*) and 10F7 (lane *3*) binding was 13.1 and 13.9, respectively. The rigidity of Miltenberger V erythrocytes after R-10 (lane *5*) and 10F7 (lane *6*) binding was only minimally increased. These results imply that marked increases in antibody-induced rigidity require the presence of the cytoplasmic domain of glycophorin A.

berger V cells with both 10F7 and R10 was only minimally increased.

In light of these data, it was important to establish that R-10 and 10F7 bound to Miltenberger V erythrocytes to the same extent as to normal cells. Antibody binding was evaluated using two techniques. The first, a standard hemagglutination assay, showed that 10F7 and R-10 of 1:100 dilution did indeed bind to Miltenberger V cells to the same degree as they did to control cells. These results were confirmed using a second, independent method in which the relative effectiveness of Miltenberger V and normal cells in binding R-10 was determined by assaying the ability of supernatants collected from erythrocyte and antibody incubations to induce rigidity in normal cells. In this assay, 4.9×10^6 normal cells and 9.8×10^6 Miltenberger V cells were separately incubated in aliquots of R-10 (50 μg/ml). The number of Miltenberger V cells used was twice that of control cells, since the number of copies per cell of the hybrid sialoglycoprotein is about one-half that of glycophorin A in normal cells (6, 23, 37). This estimation of the number of hybrid molecules per cell is based upon the findings that the sialic acid content of these particular Miltenberger V erythrocytes is 53% of normal, and that there are 100,000 molecules in a heterozygous cell (23, 37). After incubation, the erythrocytes were centrifuged and the ability of the supernatants to induce rigidity in normal cells evaluated by ektacytometry. Both the supernatant from normal and Miltenberger V cells failed to induce rigidity in normal cells implying that Miltenberger V cells bound R-10 as effectively as normal cells. This set of experimental data clearly shows that the cytoplasmic domain of glycophorin A is crucial to antibody-induced rigidity.

Effect of Glycophorin C-specific Antibody on Membrane Deformability

To determine whether ligand binding to another transmembrane sialoglycoprotein with a cytoplasmic domain had an effect on membrane deformability, we measured deformability after incubation of erythrocytes with BRIC-10, a monoclonal antibody specific for glycophorin C (sialoglycoprotein β; 7). We found that antibody binding to glycophorin C induced no change in membrane deformability (data not shown).

Discussion

Our results show that binding of ligands with specificity for different regions of glycophorin A induces an increase in membrane rigidity and that the magnitude of this increase is related to the site of ligand binding. This differential effect on rigidity is not the result of differences in the amount of each antibody bound, since it has previously been shown that the number of binding sites per cell and the binding affinities are very similar for the antibodies used in this study (20, 24). We conclude from this evidence that more than one extracellular domain of the sialoglycoprotein is capable of altering membrane mechanical properties after ligand binding and that the extent of alteration is modulatable by the domain specificity of the ligand. The similarity in the degrees of rigidity induced by 10F7 and R-10, two antibodies with epitopes which are in close proximity to one another, supports this conclusion.

Based on our earlier studies and on the results presented in this study, we conclude that the ligand-induced alteration in membrane rigidity is via a transmembrane signaling process. We previously observed that binding monovalent Fab fragments of R-10 caused membrane rigidity (10). These results implied that the increase in membrane rigidity depended upon a transmembrane event and not upon a rigidity-inducing lattice on the outside surface of the cell. The hypothesis that ligand-induced rigidity is a transmembrane process is further strengthened by the current data on Miltenberger V erythrocytes. The inability of these cells to become rigid after antibody binding demonstrates that the cytoplasmic domain of glycophorin A must be present for ligand-induced membrane rigidity to occur.

How can we explain the differential response to ligand binding? One possible model that we propose is that the binding of ligands to glycophorin A induces a conformational change in the polypeptide that results in a change in

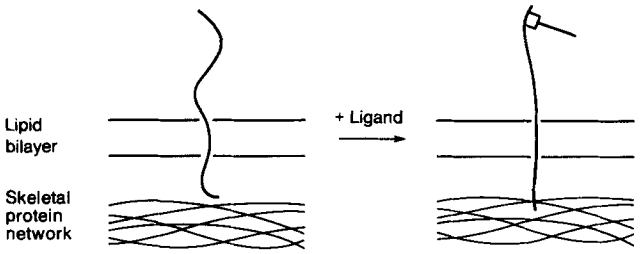

Figure 9. Model of effect of ligand binding on glycophorin A. In the nonliganded state, the cytoplasmic domain of glycophorin A is free floating and not associated with the skeletal protein network. Ligand binding induces a conformational change which causes the cytoplasmic domain of glycophorin to come into close contact with the skeletal protein lattice resulting either in entanglement in the lattice or in a specific protein–protein interaction with a component of the lattice. Both of these occurrences would cause increased membrane rigidity. Individual protein components of the skeletal network have not been delineated, since the exact nature of the interaction of liganded glycophorin A with any one specific component is unclear at the present time.

the spatial relationship of the cytoplasmic domain of glycophorin and the skeletal protein network. Precedence for ligand-induced polypeptide conformational change exists. Falke and Koshland have recently shown that transmembrane signaling by the aspartate receptor involves a global change in the structure of the protein (14). In our model, shown in Fig. 9, in the nonliganded state the conformation of glycophorin A is such that its cytoplasmic domain is free floating and is not associated with the skeletal protein network. This protein network, depicted in the model in simplified form, is actually a complex group of interacting skeletal proteins which includes spectrin, actin, adducin, tropomyosin, and proteins 4.1 and 4.9. The binding of a ligand to the extracellular domain of glycophorin induces a change in the conformation of the molecule which causes the cytoplasmic domain to come into close contact with this skeletal protein network. This, in turn, results in increased protein–protein associations leading to increased membrane rigidity. The differential response to various ligands can then be envisioned to result from differences in the molecular conformation of glycophorin induced by these ligands which in turn might determine the number of glycophorin molecules that come into close contact with the skeletal protein network. We suggest that the greater the extent of conformational change, the higher the likelihood of glycophorin molecules becoming associated with skeletal proteins and the larger the increase in membrane rigidity. In support of this model are our results showing the direct relationship between increased membrane rigidity and increased association of glycophorin A with skeletal proteins in the Triton shells. It is interesting to note that there were only small increases in the amount of glycophorin A associated with the skeletal proteins despite marked increases in rigidity. There are at least two possible explanations for these results. First, it may be that a small number of copies of glycophorin becoming associated with the membrane skeleton is indeed sufficient to account for the observed increases in membrane rigidity. There is some precedent for small increases in the number of protein–protein associations within the membrane skeleton leading to increased rigidity. Previous investigators have observed a three- to four-fold increase in membrane rigidity associated with only a 3.7% increase in spectrin–hemoglobin cross-linking after treating erythrocyte membranes with hydrogen peroxide (35). Alternatively, it may be that only high affinity associations between glycophorin and the membrane skeleton can be detected by the technique we applied, and that lower affinity associations are also occurring which contribute to the increased rigidity. At this time, we cannot distinguish between these possibilities.

It is also unclear at the present time whether the increased association of glycophorin A with the skeletal protein lattice is due to specific binding to a component of the lattice, or alternatively, whether it is due to physical entanglement of the cytoplasmic domain within the lattice. Anderson and Lovrien have previously shown that the cytoplasmic domain of glycophorin A can bind to protein 4.1, and further characterization of this binding by Anderson and Marchesi suggests that it is regulated by the phosphoinositides (2, 3). However, studies of En(a-) erythrocytes, which are deficient in glycophorin A, showed that these cells had normal membrane deformability and membrane stability (30). Since the membrane properties of deformability and stability are regulated by the skeletal protein network, the observation that glycophorin A–deficient cells have normal membrane properties suggests that glycophorin A does not associate with skeletal proteins in the normal physiological state, but does so only after ligand binding. These findings further support our proposed model in which the cytoplasmic domain of glycophorin A is free floating in the nonliganded state.

Our observation that ligand binding to glycophorin C causes no increase in membrane rigidity is consistent with the report that this sialoglycoprotein, in contrast to glycophorin A, is already linked to the membrane skeleton via an interaction with protein 4.1 (27). With an association of its cytoplasmic domain already in place, ligand binding would not be expected to induce new associations between this integral protein and the skeletal protein lattice.

The results presented here imply that glycophorin A is capable of a novel form of signal transduction that is modulated by the domain specificity of the ligand. Furthermore, the data allow us to establish unequivocally that the cytoplasmic domain of glycophorin A is crucial for this signaling process. Glycophorin A may hence serve as a model of modulatable transmembrane communication. It will be of interest to determine whether this modulatable process is unique to glycophorin A or whether it is observed in other signaling proteins.

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