Loss of Rotational Mobility of Band 3 Proteins in Human Erythrocyte Membranes Induced by Antibodies to Glycophorin A

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ABSTRACT The effect of antibodies to glycophorin A on the rotational diffusion of band 3 in human erythrocyte membranes was investigated by transient dichroism. Three antibodies that recognize different epitopes on the exofacial domain of glycophorin A all strongly reduce the rotational mobility of band 3. The effect is at most only weakly dependent on the distance of the epitope from the membrane surface. The degree of immobilization obtained with two of the antibodies, BRIC14 and R18, is very similar to that produced by antibodies to band 3 itself. Similar results were obtained with membranes stripped of skeletal proteins. Fab fragments and an antibody to glycophorin C had no effect on band 3 rotational mobility. These results rule out a mechanism whereby band 3 rotational immobilization results from enhanced interactions with the membrane skeleton that are mediated by a conformational change in glycophorin A. Rather, they strongly indicate that the antibodies to glycophorin A cross-link existing band 3-glycophorin A complexes that have lifetimes that are long compared with the millisecond time scale of the transient dichroism measurements.

INTRODUCTION

Glycophorin A and band 3 are major integral proteins of the human erythrocyte membrane. Band 3 contains a large membrane-spanning domain thought to consist of up to 14 transmembrane α-helices and a 42-kDa cytoplasmic domain that is a site of attachment of the membrane skeleton via ankyrin (Reithmeier, 1993). Glycophorin A consists of a glycosylated extracellular domain, a single transmembrane α-helix and a cytoplasmic COOH-terminal domain (Tomita et al., 1978). Glycophorin A is probably not normally an attachment site for the erythrocyte skeleton (Pinder et al., 1992).

Measurements of the rotational diffusion of band 3 have provided considerable information on protein-protein interactions in the erythrocyte membrane (Cherry, 1992). Transient dichroism and phosphorescence anisotropy measurements with eosin-labeled band 3 reveal the presence of populations of band 3 with different correlation times (Nigg and Cherry, 1980; Tilley et al., 1990; Matayoshi and Jovin, 1991). The interpretation of these measurements is complex, not least because each population contributes two components to the anisotropy decay (Nigg and Cherry, 1979; Matayoshi and Jovin, 1991). Although the different populations may in part correspond to different oligomeric states of band 3, it is clear that a significant fraction of band 3 has a highly restricted motion that probably involves interactions with ankyrin and/or band 4.1 (Nigg and Cherry, 1980). The precise details of these interactions remain, however, to be elucidated (Clague et al., 1989).

Received for publication 22 August 1994 and in final form 2 February 1995.
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0006-3495/95/05/1881/07 $2.00

Evidence that band 3 and glycophorin A form a complex in the erythrocyte membrane was originally obtained from rotational diffusion measurements (Nigg et al., 1980). When polyclonal antibodies to glycophorin A are bound to the membrane, most of band 3 becomes rotationally immobile. This effect is not observed when Fab fragments rather than intact antibodies are used. These experiments are most simply interpreted as indicating the existence of band 3-glycophorin A complexes.

Subsequently, lateral diffusion measurements have provided a somewhat different picture. Glycophorin A and band 3 diffuse laterally at slightly different rates in normal cells and at greatly different rates in certain erythrocytes containing mutant band 3 molecules (Golan, 1989; Jarolim et al., 1994; Mohandes et al., 1992; Schofield et al., 1992). This would not be expected if band 3 and glycophorin A formed long-lived complexes. Additional evidence favoring complexes, however, was obtained from the finding that the Wr^b antigen appears to involve both band 3 and glycophorin A (Telen and Chasis, 1990). Very recently, Bruce et al. (1995) have identified the mutation $Glu_{658} \rightarrow Lys$ in band 3 from a Wr(a+b–) individual and proposed that anti-Wr^b antibodies stabilize an interaction between Glu_{658} of band 3 and Arg_{61} of glycophorin A. In addition, it is found that band 3 is expressed more efficiently at the surface of *Xenopus* oocytes when glycophorin A is coexpressed (Groves and Tanner, 1992).

It is well established that antibodies to glycophorin A increase membrane rigidity in erythrocytes (Chasis et al., 1985; Chasis et al., 1988; Clough et al., 1995; Pasvol et al., 1989). The magnitude of this effect decreases with the distance of the epitope from the membrane surface (Chasis et al., 1988; Pasvol et al., 1989). An effect on membrane rigidity is still observed when Fab fragments rather than intact antibodies are bound (Chasis et al., 1985; Pasvol et al., 1989). Additional interest in the effects of anti-glycophorin A on membrane structure arises from the finding that they decrease

invasion of erythrocytes by malarial parasites (Clough et al., 1995; Hadley et al., 1986; Pasvol et al., 1989).

In view of the above results and the availability of antibodies with well defined epitopes on glycophorin A, we considered it worthwhile to perform additional experiments on the effect of antibody binding on band 3 rotational diffusion. In particular, we wished to investigate whether different results are obtained with antibodies that bind to glycophorin A at different distances from the membrane surface. As the membrane skeleton has been implicated in the effects of antibodies on membrane rigidity and malarial invasion, we have performed experiments on both normal ghosts and on ghosts stripped of skeletal proteins.

During the course of this work, two groups have reported that antibodies to glycophorin A decrease the lateral mobility of both glycophorin A and band 3 (Clough et al., 1995; Knowles et al., 1994). Knowles et al. (1994) found that these effects are not observed in Miltenberger V cells containing a mutant form of glycophorin A that lacks the cytoplasmic domain. They thus conclude that antibody binding at the exoplasmic surface results in changes in membrane-skeleton interaction mediated by the cytoplasmic domain of glycophorin A.

MATERIALS AND METHODS

Antibodies and Fab fragments

The murine monoclonal antibodies to glycophorin A used in this study were R1.3, R18, and BRIC 14 (B14). The following two antibodies directed against extracellular epitopes on band 3 were also employed: BRIC 6 (B6), a murine monoclonal antibody, and BRAC 17 (B17), a rat monoclonal antibody. In addition, experiments were performed with BRIC 10 (B10), a murine monoclonal antibody to an extracellular epitope on glycophorin C. The above antibodies and their Fab fragments were generously supplied by Dr. David J. Anstee (International Blood Group Reference Laboratory, Bristol, UK). They were prepared and characterized as previously described (Gardner et al., 1989; Smythe et al., 1994, 1995).

Preparation of ghosts for rotational diffusion measurements

Band 3 was labeled with eosin-5-maleimide from Molecular Probes (Eugene, OR) in intact cells from which ghosts were prepared as previously described (Nigg and Cherry, 1979). For studies with ghosts depleted of skeletal proteins, band 6 was first removed by washing twice with phosphate-buffered saline (pH 7.5). Removal of skeletal proteins was achieved by incubation with 1 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 12, on ice for 10 min (Tilley et al., 1993). These membranes are hereafter referred to as stripped ghosts.

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard after solubilizing ghosts in 1% sodium dodecyl sulfate, whereas the eosin concentration was determined by measuring absorbance at 531 nm. The eosin/band 3 molar ratio was calculated assuming that band 3 accounted for 25% of the total membrane protein and taking the extinction coefficient for bound eosin to be 83000 M^{-1} cm^{-1} (Cherry et al., 1976). The ratio was found to be approximately 0.9:1 in all samples.

For transient dichroism measurements, 200 μl of packed ghosts containing ~5 mg/ml protein were incubated with variable amounts of antibodies or Fab fragments in a total volume of 0.5 ml of 5 mM phosphate buffer, pH 7.5 (5PB), for 1 hour at 4°C. The samples were then washed three times in 5PB at 4°C. A similar procedure was employed with stripped ghosts except that, after antibody binding, ghosts were resuspended in 5PB containing 66% glycerol to obviate complications caused by vesicle tumbling.

Rotational diffusion measurements

The transient dichroism apparatus used to measure rotational motion was similar to that described in detail elsewhere (Cherry, 1978). Excitation was by a Nd-YAG laser with the frequency-doubled emission at 532 nm. The pulse width was approximately 15 ns and the repetition rate 10 Hz. Transient absorbance changes at time t after the flash arising from ground-state depletion were simultaneously recorded at 515 nm for light polarized parallel ($A_\parallel(t)$) and perpendicular ($A_\perp(t)$) with respect to the polarization of the exciting flash. Up to 512 signals were averaged in a Datalab DL 102A signal averager. Data were analyzed and plotted by calculating the absorption anisotropy, $r(t)$, defined by

$$r(t) = \frac{A_\parallel(t) - A_\perp(t)}{A_\parallel(t) + 2A_\perp(t)} \quad (1)$$

The anisotropy decay curves were fitted in the absence of antibody by the double exponential equation

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_3 \quad (2)$$

by a nonlinear least squares analysis. After antibody binding, however, the inclusion of the second exponential term was usually not justified by the data and for consistency all such data were fitted by a single exponential decay plus a constant term. The constant term r_3 results from a combination of the effect of anisotropic motion and the contribution of a population of band 3 that is essentially immobile over the 2-ms duration of the measurement. Data analysis and interpretation are further discussed by Nigg and Cherry (1979) and by Matayoshi and Jovin (1991).

All samples were flushed with argon before measurement to obviate quenching of the eosin triplet state by oxygen. The eosin concentration was typically 1–2 μM and all experiments were performed at 37°C.

RESULTS

Fig. 1 shows anisotropy decay curves for eosin-5-maleimide-labeled band 3 in ghosts incubated with varying amounts of R18 and B17. These and all subsequent anisotropy decays are presented as semilogarithmic plots. In both cases, a dose-dependent reduction in rotational mobility of band 3 is observed. No additional change in rotational mobility occurs as the amount of antibody added is increased beyond 200 μg (added to 1.1 mg of ghost protein). Calculations made on the basis of the number of sites and affinity constants for these antibodies (Gardner et al. 1989; Smythe et al., 1995) indicate that the binding sites are close to saturation at 200 μg of antibody. This amount of immunoglobulin (IgGh) was normally used in subsequent experiments.

Fig. 2 compares the effects of the antibodies to glycophorin A, B14, and R1.3, on band 3 rotational mobility. Both of these antibodies immobilize band 3, although the effect is somewhat less complete with R1.3. B14 has a similar affinity constant to R18, but that of R1.3 is markedly lower (Gardner et al., 1989). The experiment with R1.3 was performed at twice the normal concentration of antibody at which it is calculated that the binding sites are 72% saturated.

The antibody to band 3, B6, also immobilizes band 3, but B10, an antibody directed against glycophorin C, has no effect even when used at double the normal concentration (Fig.3).

The effect of Fab fragments on band 3 rotational mobility was tested for the two antibodies to glycophorin A that bind closest to the membrane surface. As shown in Fig. 4, neither B14-Fab nor R18-Fab has any detectable effect on the anisot-

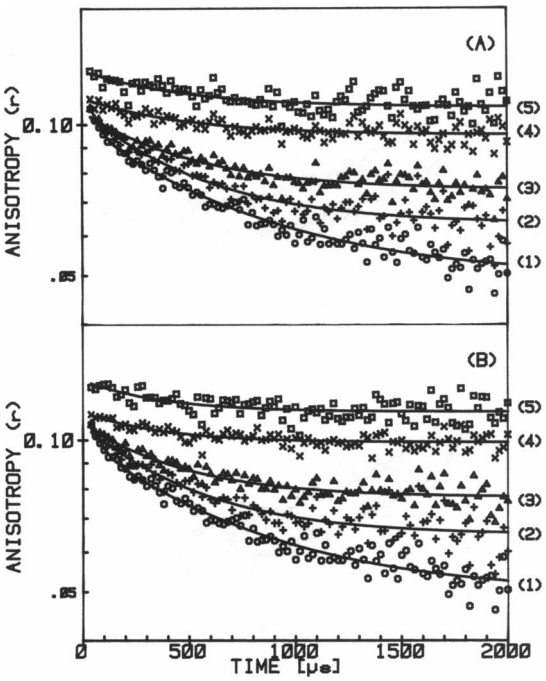

FIGURE 1 Effects of R18 and B17 on band 3 rotational mobility in ghosts. Samples contained 1.1 mg of membrane protein in 0.5 ml of 5PB, and measurements were at 37°C. (*A*) (1), control; (2), 50 μg R18; (3), 100 μg R18; (4), 200 μg R18; (5), 400 μg R18. (*B*) (1), control; (2), 50 μg RB17; (3), 100 μg B17; (4), 200 μg B17; (5), 400 μg B17. Note that in both panels, curve 5 has been displaced upwards for clarity; otherwise it superimposes on curve 4.

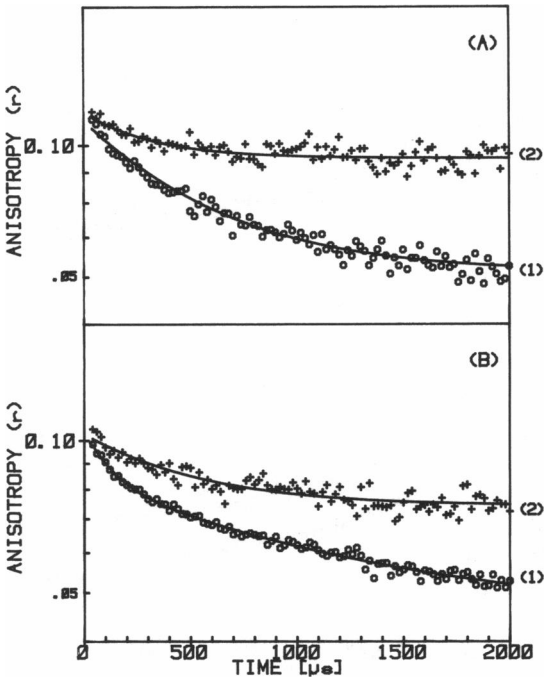

FIGURE 2 Effects of B14 and R1.3 on band 3 rotational mobility in ghosts. Samples contained 1.1 mg of membrane protein in 0.5 ml of 5PB; measurements were at 37°C. (*A*) (1), control; (2), 200 μg B14. (*B*) (1), control; (2), 400 μg R1.3.

ropy decay curves. The Fab fragments of the two antibodies to band 3 used in this study also failed to influence band 3 rotational mobility (data not shown). Fab fragments have lower affinities than their parent antibodies (Gardner et al., 1989). In the worst case, R18-Fab has an affinity constant of $2.5 \times 10^6\ M^{-1}$, giving 75% saturation of binding sites under the conditions used to measure the anisotropy decay. Because the number of sites for Fab is generally approximately twice that for the parent IgG, however, the number of occupied sites is actually greater in the experiments with Fab than in those with IgG.

Additional experiments were performed with ghosts stripped of skeletal proteins. Both antibodies to glycophorin A and to band 3 were effective in reducing band 3 mobility in this system (Figs. 5 and 6). As expected from the transient dichroism measurements with normal ghosts, Fab fragments of these antibodies were without effect in stripped ghosts (data not shown).

It is not possible to make an absolute quantitation of the immobilization produced by the different antibodies as the residual anisotropy r_3 depends on the time over which data are collected. Also, after antibody binding, it is possible to resolve only a single correlation time that nevertheless is likely to represent an average for different populations. To provide a numerical comparison of the effects of different antibodies, we calculate a parameter *I*%, defined by

$$I\% = 100(r_3 - r_{3(\text{control})})/(100 - r_{3(\text{control})}) \qquad (3)$$

where r_3 and $r_{3(\text{control})}$ are the values of r_3 (expressed as a percentage of the initial anisotropy) obtained in the presence and absence of antibody. *I*% thus varies between 0% when the antibody has no effect and 100% when band 3 is totally immobilized by the antibody on the 2-ms time scale of the experiment. Values of *I*% are collected together in Table 1.

DISCUSSION

We have examined the effects of three monoclonal antibodies to glycophorin A on the rotational mobility of band 3 in the human erythrocyte membrane. B14 binds to the Wr^b blood group antigen, which involves both band 3 and glycophorin A (Telen and Chasis, 1990; Bruce et al., 1995). The epitope on glycophorin A is between residues 61 and 70 (Bruce et al., 1994), close to the transmembrane segment (residues 73–92). The epitope for R18 is on the trypsin-resistant portion of glycophorin A between residues 40 and 56, whereas R1.3 recognizes an epitope close to the NH_2 terminus within residues 1–26 (Pasvol et al., 1989). These three antibodies thus bind to the exofacial region of glycophorin A at different distances from the point at which the peptide chain enters the lipid bilayer. All three antibodies produce an immobilization of band 3 in ghosts (Figs. 1 and 2). The immobilization parameter, *I*%, is close to 75% for both R18 and B14 as well as for the anti-band 3 antibodies B6 and B17 (Table 1). A lower value of *I*% (52%) was obtained for R1.3 even when added at higher concentration. This may reflect the rather low affinity of this antibody.

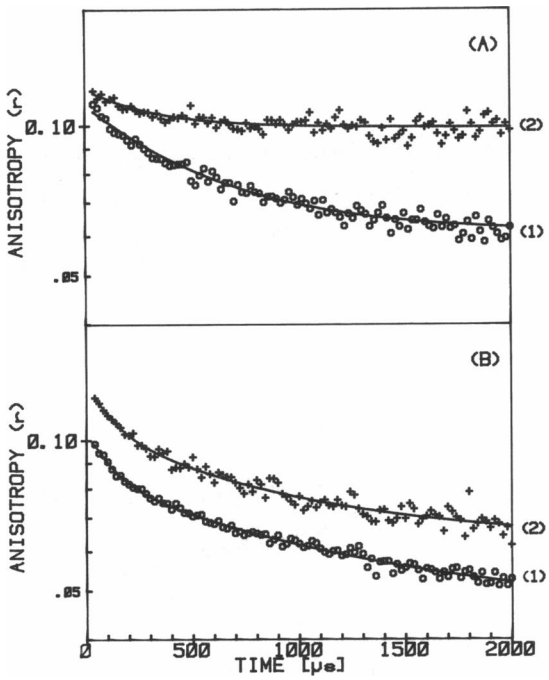

FIGURE 3 Effects of B6 and B10 on band 3 rotational mobility in ghosts. Samples contained 1.1 mg of membrane protein in 0.5 ml of 5PB; measurements were at 37°C. (*A*) (1), control; (2), 200 μg B6. (*B*) (1), control; (2), 400 μg B10. Note that curve 2 has been displaced upwards for clarity; otherwise it superimposes on curve 1.

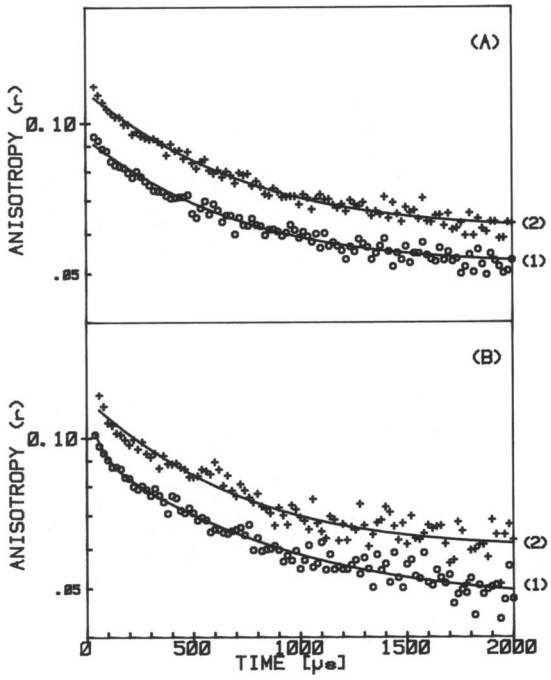

FIGURE 4 Effects of Fab fragments of B14 and R18 on band 3 rotational mobility in ghosts. Samples contained 1.1 mg of membrane protein in 0.5 ml of 5PB; measurements were at 37°C. (*A*) (1), control; (2), 130 μg B14-Fab. (*B*) (1), control; (2), 130 μg R18-Fab. The upper curves in each panel are displaced for clarity; otherwise they superimpose on the control.

It has previously been shown that binding Fab fragments of antibodies to glycophorin A to red cells increases membrane rigidity, with the greatest effect occurring for epitopes close to the surface of the lipid bilayer (Chasis et al., 1985; Pasvol et al., 1989). We therefore tested the effects of Fab fragments of R18 and B14 on band 3 rotational mobility. In contrast to the intact IgG, the Fab fragments had no effect (Fig. 4). A similar result was obtained with Fab fragments of antibodies to band 3. Although Fab fragments have lower affinity than their parent IgG, they recognize a greater number of binding sites, probably because of steric factors (Gardner et al., 1989). In all cases, we calculate that the number of sites occupied by Fab in the above experiments was greater than the number of sites occupied by IgG when maximum immobilization of band 3 occurred. These experiments thus demonstrate that loss of rotational mobility of band 3 requires cross-linking of glycophorin A (or band 3).

To investigate whether the membrane skeleton is involved in the effects of the antibodies on band 3 rotational mobility, we performed experiments on ghosts stripped of peripheral proteins. We found that the results were qualitatively similar to those obtained with normal ghosts. The antibodies to glycophorin A, R18 and B14, strongly immobilized band 3 (Fig. 5) as did antibodies to band 3 (Fig. 6). Fab fragments of these antibodies had no effect (data not shown). These results thus demonstrate that loss of band 3 rotational mobility upon antibody binding to glycophorin A (or band 3) does not require the presence of the membrane skeleton.

In additional control experiments, an antibody to glycophorin C was tested. There is no reason to suppose an association between band 3 and glycophorin C, and the same antibody (B10) was also used in control experiments by Knowles et al. (1994). Fig. 3 shows that B10 has no effect on band 3 rotational mobility in ghosts, even when added at double the normal concentration.

Taken together, the experiments described here provide rather conclusive evidence that antibodies to glycophorin A reduce band 3 rotational mobility by cross-linking band 3-glycophorin A complexes. This conclusion agrees with that reached previously by Nigg et al. (1980) using polyclonal antibodies. None of the anti-glycophorin A antibodies totally immobilize band 3, but neither do anti-band 3 antibodies. This could be because, close to saturation, not all binding results in intercomplex cross-linking. The relationship between antibody binding and immobilization is in any case likely to be complex as band 3 and probably glycophorin exist in oligomeric states in the membrane (Reithmeier, 1993; Welsh et al., 1985). R1.3 produces significantly less immobilization than the other antibodies. Although this could be because of lower affinity, it is also possible that, as R1.3 binds close to the NH_2 terminus of glycophorin A, flexibility in the peptide chain might permit some restricted rotational motion, even in cross-linked complexes.

The simplest mechanism whereby cross-linking causes immobilization is that aggregates are formed of sufficient size to be immobile or very slowly rotating on the 2-ms time scale of the measurement. This is probably what happens in stripped ghosts. Values of *I%* are, however, noticeably higher in normal ghosts than in stripped ghosts. In normal ghosts there are significant populations of immobile and

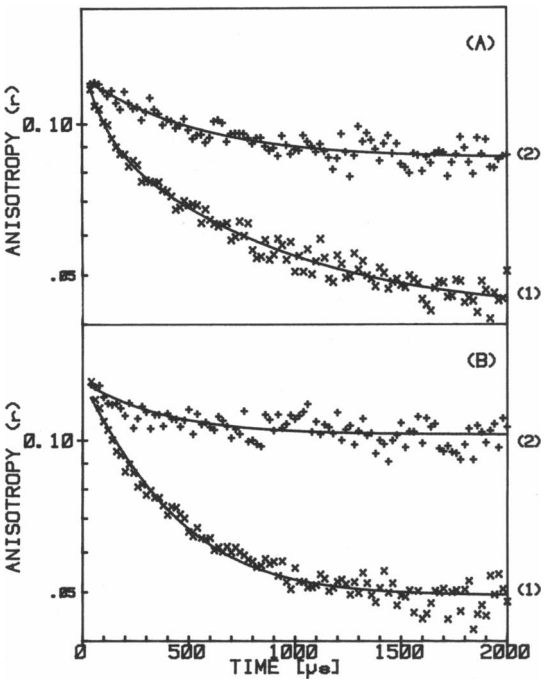

FIGURE 5 Effects of B14 and R18 on band 3 rotational mobility in stripped ghosts. Samples contained 0.5 mg of membrane protein in 0.5 ml of 5PB; measurements were at 37°C. (*A*) (1), control; (2), 200 μg R18. (*B*) (1), control; (2), 200 μg B14.

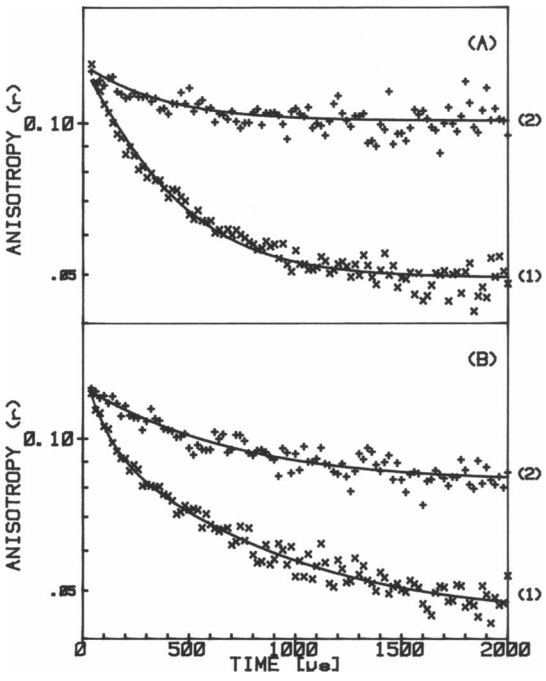

FIGURE 6 Effects of B6 and B17 on band 3 rotational mobility in stripped ghosts. Samples contained 0.5 mg of membrane protein in 0.5 ml of 5PB; measurements were at 37°C. (*A*) (1), control; (2), 200 μg B6. (*B*) (1), control; (2), 200 μg B17.

slowly rotating band 3 (Nigg and Cherry, 1980). Thus, loss of mobility may occur when freely rotating complexes are cross-linked to these existing species. Larger scale aggregation is likely to be inhibited by restrictions to lateral motion imposed by the membrane skeleton. There is evidence from electron microscopy that polyvalent ligands do not induce large scale aggregation of integral membrane proteins in ghosts (Triche et al., 1975). For this reason, it is unlikely that band 3 becomes immobilized by simple entrapment between cross-linked glycophorin A molecules, as suggested by Matayoshi and Jovin (1991).

TABLE 1 Immobilization of band 3 by antibodies and Fab fragments

Antibody	Amount (μg)	Membrane	*I*%
B14	200	Normal ghosts	74%
R18	200	Normal ghosts	75%
R1.3	400	Normal ghosts	52%
B6	200	Normal ghosts	74%
B17	200	Normal ghosts	79%
B14-Fab	130	Normal ghosts	1.6%
R18-Fab	130	Normal ghosts	−1.6%
B6-Fab	130	Normal ghosts	0%
B17-Fab	130	Normal ghosts	−1.6%
B10	200	Normal ghosts	−1.7%
B14	200	Stripped ghosts	65%
R18	200	Stripped ghosts	54%
B6	200	Stripped ghosts	63%
B17	200	Stripped ghosts	49%

I% was calculated according to Eq. 3. Errors in *I*% are typically ±3. Experimental details are given in the figure legends. The anti-glycophorin A antibodies bind to epitopes located between residues 61 and 70 (B14), 40 and 51 (R18), and 1 and 26 (R1.3). B6 and B17 are antibodies to band 3, whereas B10 is an antibody to glycophorin C.

Although the present studies show that antibodies to glycophorin A cross-link glycophorin A-band 3 complexes, it is conceivable that these complexes exist only in the presence of the bound antibody. This possibility is made more plausible by the evidence that antibody binding induces a conformational change in glycophorin A that is transmitted to the cytoplasmic domain (Chasis et al., 1988; Pasvol et al., 1989; Knowles et al., 1994). In this model, Fab fragments would also be expected to promote association with band 3. We find, however, no effect of Fab fragments on band 3 rotational diffusion. This rules out antibody-induced complex formation unless it is supposed that the complex is insufficiently different in size to the uncomplexed band 3 to appreciably change the anisotropy decay curves. An additional argument is made on the basis that the conformational change that alters membrane rigidity is strongly dependent on the site of antibody binding to glycophorin A (Chasis et al., 1988; Pasvol et al., 1989). We find no difference in the ability of R18 and B14 to immobilize band 3, although R18 has a much smaller effect than B14 on membrane rigidity. In addition, R1.3 immobilizes a significant proportion of band 3 but has little or no effect on membrane rigidity (Pasvol et al., 1989). Finally Nigg et al. (1980) reported that antibodies directed against the COOH-terminal domain of glycophorin A also immobilize band 3. It seems highly unlikely that all of these different antibodies can promote complex formation with band 3. Rather, the evidence strongly indicates that complexes preexist in the erythrocyte membrane.

The evidence that band 3 and glycophorin A are not associated in the erythrocyte membrane is largely on the basis

of observation of different lateral diffusion rates (measured by fluorescence recovery after photobleaching) for the two proteins under certain situations (Golan, 1989; Mohandes et al., 1992; Schofield et al., 1992). One possible explanation for the discrepancy is that there are two populations of glycophorin A, one associated with band 3 that produces the effects seen in the present study and the other independently mobile. The credibility of this suggestion depends critically on the numbers of glycophorin A and band 3 molecules present in the membrane and the stoichiometry of the complex. Extensive binding studies with Fab fragments indicate that there are of the order of 1×10^6 copies of glycophorin A per cell (Gardner et al., 1989), as is also the case for band 3 (Reithmeier, 1993). Thus, if the complex consisted of a tetramer of band 3 and a dimer of glycophorin A, then as much as 50% of glycophorin A could be in an uncomplexed state.

An alternative explanation of the different rates of lateral diffusion of band 3 and glycophorin A can be made on the basis of the fact that rotational and lateral diffusion measurements operate on completely different time scales. The results of the rotational diffusion measurements require that the band 3-glycophorin A complexes have lifetimes longer than a few milliseconds. Over the few minutes duration of a lateral diffusion measurement, however, complexes could dissociate and reassociate many times so that the components would exhibit independent rates of diffusion. The fact that band 3 and glycophorin can be independently extracted (Scheuring et al., 1986) suggests that they are not tightly associated in the membrane. As their concentrations in the membrane are high (effectively, approximately 5 mM), however, only a modest association constant is required to maintain a high equilibrium concentration of complexes.

Knowles et al. (1994) found that Fab fragments of antibodies to glycophorin A strongly inhibit glycophorin A lateral mobility. They interpreted this result as indicating increased association of glycophorin A with skeletal proteins. If band 3 and glycophorin A are complexed, it might be thought that, contrary to our observation, this attachment would influence band 3 rotational mobility. It has, however, previously been shown that attachment to the spectrin-actin network does not reduce band 3 rotation as measured by transient dichroism (Nigg and Cherry, 1980; Clague et al., 1989). This is probably because the network is too flexible to hinder rotational randomization over $\pm \pi/2$, which determines the time course of the anisotropy decay curves. Antibodies to glycophorin A also strongly reduce lateral diffusion of band 3 (Knowles et al., 1994, Clough et al., 1995). An effect on the lateral mobilities of both glycophorin A and band 3 would be expected if they form a complex.

It is possible that the effects of antibodies to glycophorin A on erythrocyte membrane rigidity involve more than a single mechanism. As Fab fragments can increase rigidity (Chasis et al., 1985; Knowles et al., 1994; Pasvol et al., 1989), it was supposed that cross-linking plays no role in this phenomenon. The data of Pasvol et al. (1989), however, clearly show that Fab fragments are considerably less effective in increasing membrane rigidity than are intact antibodies. We suggest, therefore, that cross-linking of glycophorin A-band 3 complexes enhances the effect of any increased interactions with the membrane skeleton. In studies with hereditary ovalocytes, which have a mutant band 3 and high membrane rigidity (Mohandes et al., 1992; Schofield et al., 1992), we found that band 3 forms linear aggregates and suggested that aggregation of membrane attachment sites can increase rigidity (Che et al., 1993). Tilley et al. (1993) also proposed that band 3 aggregation influences membrane rigidity in hereditary ovalocytes as well as in camelid red blood cells (McPherson et al., 1993).

In conclusion, the present studies show that antibodies to glycophorin A strongly reduce the rotational mobility of band 3. The effect is at most weakly dependent on the position of the epitope on the exofacial domain of glycophorin A. It is highly probable that the antibodies cross-link preexisting band 3-glycophorin A complexes that have lifetimes that are long compared with the millisecond time scale of the transient dichroism measurements. The effects of the antibodies on band 3 rotational diffusion appear to be largely distinct from the increase in membrane rigidity and the decreased lateral diffusion of glycophorin A and band 3 that are mediated by enhanced interactions with the membrane skeleton.

We are indebted to Dr. D. J. Anstee for supplying antibodies and to Brigitte Gardner for preparing Fab fragments. We are also grateful to Professor M. J. A. Tanner for stimulating the present study.

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