Band 3–Glycophorin A Association in Erythrocyte Membranes Demonstrated by Combining Protein Diffusion Measurements with Antibody-Induced Cross-Linking†

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ABSTRACT: A new approach to the study of molecular protein interactions in biological membranes is presented. The technique is based on measuring the rotation of a membrane protein in the presence and absence of specific antibodies directed toward a purported complex partner. As a first illustration of the method, the putative association of band 3 with glycophorin A in the human erythrocyte membrane was investigated. The rotational diffusion of band 3 was strongly reduced following cross-linking of glycophorin A with divalent antibodies. However, little or no effect on band 3 rotation was produced by monovalent antiglycophorin A Fab fragments, antispectrin, or nonspecific antibodies, ruling out major effects on band 3 mobility due to steric hindrance, unspecific antibody adsorption, or transmembrane interactions involving spectrin. It is concluded that immobilization of band 3 by antiglycophorin A antibodies is directly caused by cross-linking of a preexisting band 3–glycophorin A complex in the human erythrocyte membrane.

Band 3 and glycophorin A are the two major membrane-spanning proteins of the human erythrocyte membrane. Band 3 is a glycoprotein of molecular weight ~90 000 which comprises ~25% of the total membrane protein and is responsible for anion transport [for a review, see Cabantchik et al. (1978)]. Glycophorin A [nomenclature according to Furthmayr (1977)] is the major sialoglycoprotein; two-thirds of its mass consists of carbohydrates including most of the sialic acid of the erythrocyte membrane. It carries some blood group antigens and receptors for plant lectins and viruses [for a review, see Marchesi et al. (1976)]. The possible association of band 3 with glycophorin A in the erythrocyte membrane has been the subject of a longstanding debate (Guidotti, 1972; Pinto da Silva & Nicolson, 1974; Yu & Steck, 1975; Shotton et al., 1978). Whereas electron microscopic experiments have suggested that band 3 and glycophorin A might form a noncovalent complex in the membrane (Pinto da Silva & Nicolson, 1974), no stable association among these proteins has been demonstrated directly (Yu & Steck, 1975). The scarcity of information relevant to the interaction between two of the most extensively studied membrane proteins convincingly demonstrates the need for new techniques to investigate the molecular arrangement of proteins in biological membranes. Here we introduce a new approach to this problem by combining protein rotation measurements with the binding of specific antibodies.

The rotational mobility of band 3 has recently been measured by observing flash-induced transient dichroism (Cherry et al., 1976; Nigg & Cherry, 1979a) and phosphorescence polarization (Austin et al., 1979) of the protein-bound triplet probe eosin. Protein rotation is sensitive to protein–protein interactions, and hence these measurements provide a powerful method of investigating the arrangement of proteins in the membrane. Thus, it was previously shown that the presence of the peripheral proteins spectrin and actin has no observable effect on band 3 rotation (Cherry et al., 1976), indicating that the bulk of spectrin–actin is not directly linked to band 3. More recently, the dimeric association of band 3 has been demonstrated by measuring band 3 rotation before and after formation of covalent dimers by chemical cross-linking (Nigg & Cherry, 1979b).

Here we further extend these studies to investigate the existence of the putative association of band 3 with glycophorin A in the erythrocyte membrane. The method is based on measuring the rotation of band 3 in the presence and absence of specific antiglycophorin A antibodies. If band 3 and glycophorin A form a complex, then it would be expected that cross-linking of glycophorin A with antibodies would result in immobilization of band 3 (Figure 1A). No such effect would be expected if band 3 and glycophorin A are independently existing species (Figure 1B).

Experimental Procedures

Rotational Diffusion Measurements. The flash photolysis technique used to measure band 3 rotation has previously been described in detail (Cherry et al., 1976; Nigg & Cherry, 1979a; Cherry, 1978). Briefly, band 3 was selectively labeled by incubating intact erythrocytes with the triplet probe eosin-5-maleimide (Nigg & Cherry, 1979a). The rotational diffusion of band 3 was then measured in ghosts by observing the transient dichroism of ground-state depletion signals arising from excitation of the probe by a linearly polarized light pulse. Excitation was at 540 nm by a flash of 1–2-μs duration, and absorbance changes were recorded at 520 nm. The transient dichroism measurements were analyzed by calculating the absorption anisotropy $r(t)$ given by

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

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are, respectively, the absorbance changes at time t after the flash for light polarized parallel and perpendicular with respect to the polarization of the exciting flash. All results reported here were obtained by averaging 32 signals with a Datalab DL 102A signal averager. Data analysis was accomplished by a Hewlett-Packard HP 9825A desk top computer interfaced to the signal averager.

Preparation of Antisera. Antisera to the major red cell membrane proteins were raised in rabbits. Sialoglycoproteins, band 3, and spectrin (bands 1 and 2) were extracted by published procedures (Marchesi & Andrews, 1971; Kahlen-

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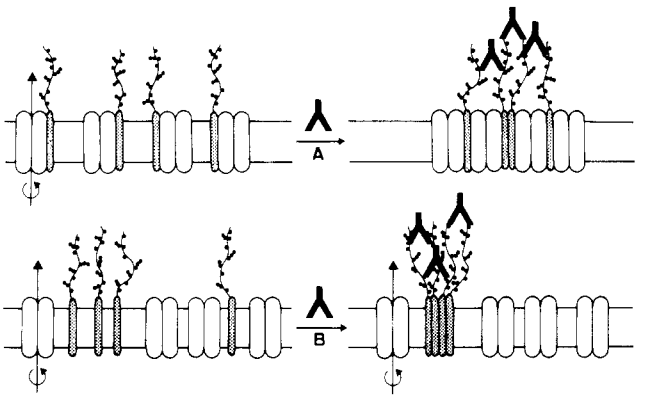

FIGURE 1: Schematic model illustrating the effects of antiglycophorin A antibodies on band 3 rotational mobility. (A) Band 3 dimers and glycophorin A (shaded) form complexes which are aggregated by antiglycophorin A. Aggregation reduces the rotational mobility of band 3. (B) Band 3 dimers and glycophorin A are independently existing species. Band 3 rotation is unaffected by antibody-induced cross-linking of glycophorin A. For clarity, other proteins, such as spectrin–actin, are omitted. Some possible interactions are discussed in the text. Band 3 proteins are shown as dimers, although higher aggregates may also exist in the membrane (Nigg & Cherry, 1979a). Although antibodies are depicted as interacting exclusively with antigens on the outer surface of the membrane, interactions with antigenic sites on the inner surface undoubtedly occur as well (see Figure 2A, III).

berg, 1976; Marchesi, 1974) and further purified by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis and subsequent elution of single bands. Sialoglycoproteins were labeled with ^{125}I by the chloramine-T method (McConahey & Dixon, 1966) prior to electrophoresis. The proteins were resolved on 10% $NaDodSO_4$–polyacrylamide gels in a discontinuous buffer system (Laemmli, 1970). Individual bands were cut from fixed and dried gels by using autoradiography for detection of the sialoglycoprotein bands PAS-2 and PAS-4 [nomenclature according to Fairbanks et al. (1971)] and Coomassie blue staining for detection of band 3 and spectrin. [The band PAS-1, the dimer of glycophorin A (Marchesi et al., 1976), was not used for immunizations to avoid contamination by band 3 due to the proximity of the two bands in the gel.] Elution of the protein bands was performed by soaking dried gel pieces for 24 h in 0.2 M ammonium bicarbonate, pH 8.5, containing 1% $NaDodSO_4$. After mechanical homogenization, the suspension was centrifuged and washed several times. The supernatants were collected, dialyzed against deionized water, and lyophilized. Antisera were prepared by repeated intramuscular injections of these $NaDodSO_4$–polyacrylamide gel eluted polypeptides together with complete Freund's adjuvant. Immune and normal IgG and their Fab fragments were prepared from antisera and preimmune sera by published procedures (Kekwick, 1940; Putnam et al., 1962; Porter, 1959). The complexity of the PAS bands observed in $NaDodSO_4$–polyacrylamide gels (Furthmayr, 1977) raises some problems in antibody nomenclature. Hereafter, we refer to antibodies raised by injection of either PAS-2 or PAS-4 as antiglycophorin A. Identical results were obtained irrespective of whether PAS-2 or PAS-4 was used for immunization.

Characterization of Antibody Specificity. All antisera were tested for the presence of antibodies directed to cell surface antigens. This was performed by complement-mediated hemolytic testing using ^{51}Cr-labeled target cells as described previously (Bron & Gallagher, 1974).

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff base stain; IgG, immunoglobulin G; Fab, antigen binding fragment; NET, NaCl–EDTA–Tris; NP-40, Nonidet P-40.

The specificity of each antiserum was tested by several immunochemical techniques, namely, by indirect immunoprecipitation of ghosts prepared from surface-labeled erythrocytes, by two-dimensional immunoelectrophoresis, and by a direct immunochemical identification of proteins in $NaDodSO_4$–polyacrylamide gels overlaid with antiserum-containing agarose layers.

Prior to indirect immunoprecipitation, erythrocytes were surface labeled either by a glucose oxidase–lactoperoxidase-catalyzed radioiodination method (Hubbard & Cohn, 1975) or by tritiation according to Gahmberg (1976) after pretreatment of red cells with neuraminidase. A total of 250×10^6 labeled cells were solubilized in 1 mL of NET lysis buffer (Kessler, 1976) containing 0.5% of the nonionic detergent NP-40 and 1 mM phenylmethanesulfonyl fluoride. Free iodine was eliminated by dialysis against lysis buffer adjusted to 0.05% in NP-40 and 5 mM KI. For indirect immunoprecipitation, 150–200 μL of various antisera diluted 1:10 in NET buffer containing 0.05% NP-40 was added to aliquots of 150 μL of lysed cells. After 2 h of incubation at 37 °C and overnight at 4 °C, the immune complexes were precipitated with 200 μL of a 10% suspension of *Staphylococcus aureus*. After 15 min of incubation at 4 °C and several washings in NET buffer containing 0.05% NP-40, 5 mM KI, and 1 mg/mL ovalbumin, the complexes were eluted from the bacterial adsorbant by treatment with 100 μL of 0.05 M Tris-HCl buffer containing 4% $NaDodSO_4$ and 6 M urea at pH 8.4 at 100 °C for 3 min. Reduced and alkylated samples were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

For two-dimensional immunoelectrophoresis, 50 μg of membrane proteins was resolved on 10% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) in the first dimension and electrophoresed in the second dimension into a layer of agarose containing 10% of specific antibody.

Agarose immunoreplicates were prepared as follows. Red cell ghosts were iodinated by the chloramine-T method (McConahey & Dixon, 1966) and resolved on 5–13% $NaDodSO_4$–polyacrylamide gels in a discontinuous buffer system (Laemmli, 1970). Gels were layered down onto agarose plates containing 10–20% antisera or control normal rabbit serum and left overnight at room temperature in a moisture chamber. Agarose plates were then thoroughly washed for 24 h in saline and dried. Autoradiography was carried out with Kodirex X-ray film.

Incubation of Ghosts with Antibodies. Ghosts were prepared from eosinmaleimide-labeled erythrocytes as described previously (Nigg & Cherry, 1979a). One hundred microliters of packed ghosts (containing ~4 mg of protein per mL) was incubated with variable amounts of antibodies or antisera in a total volume of 1.2 mL of 5 mM phosphate buffer, pH 7.4, for 1 h at 4 °C. Prior to flash photolysis experiments, ghosts were washed 3 times in the same buffer at 4 °C. The samples were then flushed with argon (Cherry, 1978), and flash photolysis measurements were made at 37 °C after a 10-min equilibration.

Results

Specificity of Antisera. The specificity of each antiserum was demonstrated by several immunochemical methods. Results of indirect immunoprecipitation of ghosts prepared from surface-labeled erythrocytes are shown in Figure 2. Antiglycophorin A specifically recognized the monomer of glycophorin A (PAS-2) and its aggregated form (PAS-1). The same specificity was also observed when using an antiserum against a peptide derived from the C-terminal portion of glycophorin A (Cotmore et al., 1977) provided by H. Furth-

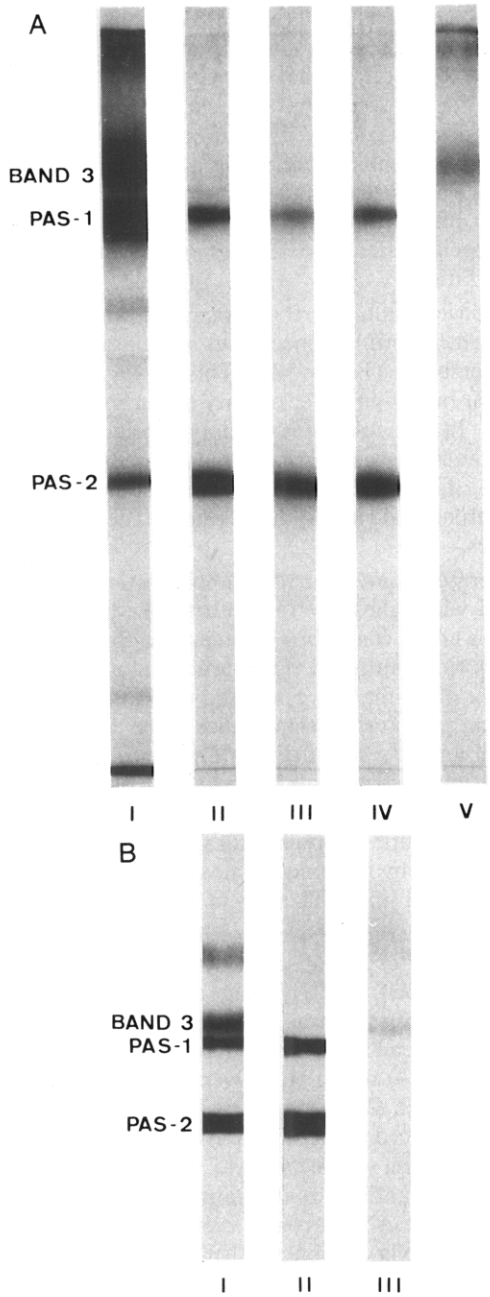

FIGURE 2: (A) Autoradiography of polyacrylamide slab gels of immunoprecipitate of ghosts from surface-iodinated red cells. Erythrocytes were surface labeled by a glucose oxidase–lactoperoxidase-catalyzed radioiodination method (Hubbard & Cohn, 1975). Indirect immunoprecipitation was performed as described under Experimental Procedures. Electrophoresis was carried out on 10% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970), and autoradiography was performed on fixed and dried gels with Kodirex X-ray film. (I) NP-40 lysed surface-iodinated red cells; (II) immunoprecipitate with antiserum to glycophorin A; (III) immunoprecipitate with antiserum to glycophorin A absorbed on intact red cells; (IV) immunoprecipitate with antiserum to C-terminal portion of glycophorin A; (V) immunoprecipitate with antiserum to band 3 (early bleeding). (B) Fluorography of polyacrylamide slab gels of indirect immunoprecipitate of ghosts from red cells labeled with tritiated sodium borohydride. Surface labeling was performed according to Gahmberg (1976) after pretreatment of red cells with neuraminidase. Indirect immunoprecipitation was performed as described under Experimental Procedures. The samples were analyzed on 5–13% gradient $NaDodSO_4$–polyacrylamide gels in a discontinuous buffer system (Laemmli, 1970). Fluorography using RP-X OMAT film was performed with fixed gels processed according to Bonner & Laskey (1974). (I) Immunoprecipitate with antiserum to band 3 (hyperimmune) containing antibodies to glycophorin A; (II) immunoprecipitate with antiserum to glycophorin A; (III) immunoprecipitate with antiserum to band 3 (early bleeding).

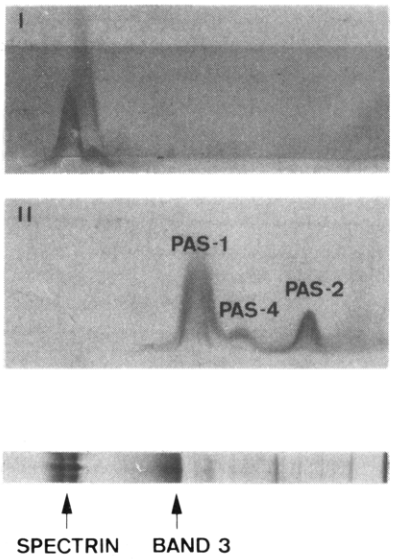

FIGURE 3: Two-dimensional immunoelectrophoresis of erythrocyte ghosts. (I) Antiserum to spectrin; (II) antiserum to glycophorin A. The lower gel shows Coomassie blue staining of the first dimension in order to locate the bands.

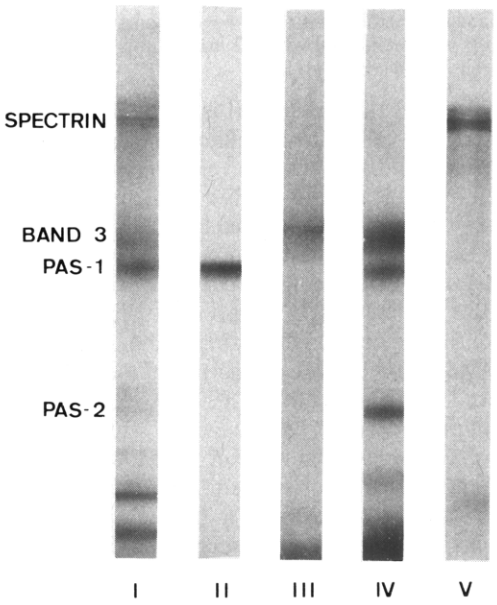

FIGURE 4: Autoradiography of agarose immunoreplicates from iodinated red cell ghosts. Agarose immunoreplicates were prepared as described under Experimental Procedures. (I) Iodinated erythrocyte ghosts showing pattern of iodination; (II) immunoreplicate with antiserum to glycophorin A; (III) immunoreplicate with antiserum to band 3 (early bleeding); (IV) immunoreplicate with antiserum to band 3 (hyperimmune) containing antibodies to glycophorin A; (V) immunoreplicate with antiserum to spectrin.

mayr. Specific antisera against band 3 were only obtained from early bleedings whereas prolonged immunization elicited the production of contaminating antibodies to glycophorin A.

Results of two-dimensional immunoelectrophoresis are shown in Figure 3. Antibodies to spectrin reacted exclusively with bands 1 and 2, whereas antiglycophorin A reacted with PAS-1, PAS-2, and PAS-4. As a final demonstration of the specificity of the antisera, a direct immunochemical identification of spectrin, band 3, and glycophorin A was performed on membrane proteins separated by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). This technique avoids the nonspecific coprecipitation of spectrin with band 3 sometimes observed in indirect immunoprecipitation. The results shown in Figure 4 clearly confirm the specificity of the

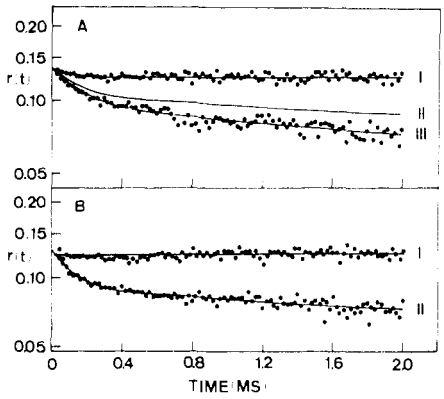

FIGURE 5: Time dependence of the absorption anisotropy for erythrocyte membranes following binding of antiglycophorin A antibodies. (A) Ghosts were incubated with the indicated amounts of antibodies (expressed per milligram of membrane protein) as described under Experimental Procedures. Flash-induced transient dichroism of the eosin probe was measured, and $r(t)$ was calculated as described previously (Nigg & Cherry, 1979a; Cherry, 1978). The solid lines were obtained by fitting the data as described previously, assuming a residual anisotropy of 25% (Nigg & Cherry, 1979a). For greater clarity experimental points have been omitted from curve II and curves were slightly adjusted to the same initial anisotropy r_0. (I) 12 mg of IgG from antiglycophorin A antiserum; (II) 12 mg of IgG from nonimmunized control rabbits; (III) no addition. (B) As in (A) except that ghosts were incubated with 0.3 mL of antiglycophorin A serum (I) or preimmune serum (II).

three types of reagents used. It is particularly important to emphasize that all of the immunochemical tests performed strongly indicate that antisera to glycophorin A are devoid of antibodies to band 3. It is further of interest to note that, as judged by complement-mediated hemolytic testing, only antibodies to glycophorin A were cytolytic, whereas antibodies to spectrin and band 3 failed to detect any surface antigens. This latter finding confirms earlier studies indicating that rabbit antibodies prepared against band 3 react only with antigens within the cytoplasmic portion of the protein (England & Steck, 1978; Fukuda et al., 1978).

Immobilization of Band 3 by Antiglycophorin A. Figure 5A shows the time dependence of the absorption anisotropy at 37 °C after incubation of eosin-labeled erythrocyte membranes with antiglycophorin A antibodies, together with the curves obtained when no or nonspecific antibodies are present in the incubation medium. It is clear that antiglycophorin A antibodies have a striking effect on band 3 mobility. In the absence of antibodies (Figure 5A, III) the absorption anisotropy clearly decays with time. As discussed in detail previously (Nigg & Cherry, 1979a), this decay is due to rotational motion of band 3 proteins about an axis perpendicular to the plane of the membrane. In the presence of antiglycophorin A antibodies (Figure 5A, I), the absorption anisotropy is almost constant with time, indicating a virtually complete immobilization of band 3 on the time scale of the experiment. It should be noted that rotational relaxation times of intrinsic membrane proteins depend on the square of their cross sectional diameter in the plane of the membrane (Saffman & Delbrück, 1975). Thus, microaggregation is sufficient to account for the observed immobilization, and it is not necessary to invoke patching or capping phenomena as described in lymphoid cells (Taylor et al., 1971). Such microaggregations may take place within the interstices of the spectrin–actin network underlying the erythrocyte membrane.

Similar measurements were made by using control IgG obtained from nonimmunized rabbits in order to test for possible effects of unspecific adsorption. In some cases, a slight immobilization of band 3 was produced by these control IgG (Figure 5A, II). Although the effect was considerably weaker than that observed with antiglycophorin A, it was clearly important to ascertain that unspecific effects were not responsible for the result shown in curve I of Figure 5A. We therefore directly compared the effects of antiglycophorin A antiserum and preimmune serum from the same rabbit (Figure 5B). It is evident that immobilization of band 3 is produced only after immunization of the rabbit, hence demonstrating that unspecific effects are not responsible for the observed immobilization.

The above results are those expected when band 3 and glycophorin A form a noncovalent association in the erythrocyte membrane (Figure 1A). This conclusion, however, is clearly dependent on the specificity of the antiglycophorin A reagents. In particular, it is crucial to exclude effects due to contamination of the antiglycophorin A reagents by antibodies to band 3. The immunochemical tests described above indeed provide strong evidence for the absence of such contaminating antibodies.

Furthermore, only a partial immobilization of band 3 was produced when ghosts were incubated with an antiband 3 antiserum under conditions analogous to those used for antiglycophorin A antisera. This result is most likely due to a lower titer of antiband 3 compared with antiglycophorin A. For present purposes, the significance of the experiment is that even with an antiserum deliberately raised against band 3, complete immobilization of band 3 was not achieved. It therefore appears safe to conclude that any contamination of our antiglycophorin A preparations by antiband 3 below the level detectable by immunochemical tests could not account for the observed immobilization of band 3.

In further experiments, we were able to confirm our results by using two different antisera which have both been shown to be highly specific for glycophorin A. (1) An antiserum against glycophorin A was extensively absorbed by C. G. Gahmberg on membranes derived from erythrocytes of the rare phenotype En(a−) which lack glycophorin A (Gahmberg et al., 1976; Tanner & Anstee, 1976; Dahr et al., 1976). This antiserum produced a clear loss of rotational mobility of band 3. Immobilization was incomplete, probably due to a rather low titer of the antiserum after absorption on En(a−) cells. Antiglycophorin A is partially removed by this treatment because these cells contain another sialoglycoprotein (band PAS-3) which apparently is related to glycophorin A in structure and antigenicity. (2) A complete immobilization of band 3 was observed by using the antiserum against the C terminal of glycophorin A (Cotmore et al., 1977) which was provided by H. Furthmayr. Taken together, these results leave no doubt that immobilization of band 3 is really due to antiglycophorin A antibodies.

It could be argued that binding of antiglycophorin A antibodies to the membrane might produce immobilization of band 3 by some sort of steric hindrance. It appears to us very unlikely that this would occur in rotational diffusion measurements, although it might present a problem in experiments involving the observation of lateral diffusion. However, even in the latter case spatial independence between different protein populations in lymphoid cell membranes has been demonstrated by observing the lateral redistribution of fluorescent-labeled antibodies or lectins [for a review, see de Petris (1977)]. Additional evidence against an involvement of steric hindrance in our experiments has been obtained from studies with Fab fragments prepared from antiglycophorin A IgG. The effect on band 3 mobility of monovalent Fab fragments was found to be within the range of unspecific effects (Figure 6). This

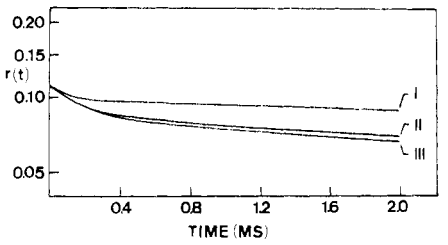

FIGURE 6: Effect of antiglycophorin A Fab fragments on the time dependence of absorption anisotropy for erythrocyte membranes. Experiments were carried out as described in the legend to Figure 5A. (I) 8 mg of IgG from antiglycophorin A serum; (II) 6 mg of Fab prepared from antiglycophorin A IgG; (III) 8 mg of IgG from nonimmunized control rabbits. The immobilization produced by antiglycophorin A IgG is less complete than in Figure 5A due to the lower concentration of antibodies used. For clarity experimental points are omitted; the scatter is similar to that in Figure 5. The solid lines were obtained by fitting the data as described previously, assuming a residual anisotropy of 25% (Nigg & Cherry, 1979a).

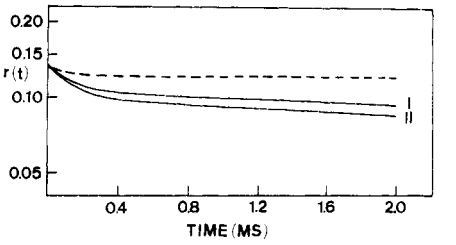

FIGURE 7: Effect of antispectrin antibodies on the time dependence of the absorption anisotropy for erythrocyte membranes. Experiments were carried out as described in the legend to Figure 5A. (I) 12 mg of IgG from antispectrin serum; (II) 12 mg of IgG from nonimmunized control rabbits. The dashed line is curve I from Figure 5A for comparison. For clarity experimental points are omitted; the scatter is similar to that in Figure 5A. The solid lines were obtained by fitting the data as described previously, assuming a residual anisotropy of 25% (Nigg & Cherry, 1979a).

experiment strongly indicates that indeed multivalent antibodies are required to immobilize band 3. However, because Fab and IgG molecules considerably differ in size and because the binding capacity of the two reagents used could conceivably differ, a contribution of steric effects to the immobilization of band 3 cannot be totally ruled out.

Absence of Transmembrane Effects. In principle, one alternative explanation of our data should be considered. It is conceivable that the observed immobilization of band 3 by antiglycophorin A antibodies might be mediated by a transmembrane effect involving the peripheral proteins spectrin and actin. In fact, many studies have shown that both band 3 (Bretscher, 1973; Steck, 1974; Morrison et al., 1974; Shin & Carraway, 1974) and glycophorin A (Bretscher, 1971; Tomita & Marchesi, 1975; Cotmore et al., 1977) span the membrane and are accessible from both sides. On the basis of both rotational (Cherry et al., 1976) and lateral diffusion measurements (Fowler & Branton, 1977), it appears unlikely that the bulk of spectrin is directly attached to band 3; moreover, recent biochemical data indicate that neither band 3 nor glycophorin A constitutes direct high-affinity spectrin binding sites (Luna et al., 1979; Yu & Goodman, 1979; Bennett & Stenbuck, 1979a). Nevertheless, it could be argued that cross-linking glycophorin A might lead to a rearrangement of the spectrin–actin network at the cytoplasmic surface of the membrane. This rearrangement might then in turn result in an immobilization of band 3. Some support for this chain of events may be derived from earlier studies providing evidence for transmembrane effects across the erythrocyte membrane (Ji & Nicolson, 1974; Nicolson & Painter, 1973).

To test this possibility, we exposed leaky ghosts directly to antispectrin antibodies. When compared to the same amount of antiglycophorin A IgG, these antibodies produced at most a slight reduction of band 3 mobility (Figure 7). Such an effect may be explained in terms of an increased compartmentalization of the membrane following cross-linking of spectrin by antibodies (Nicolson & Painter, 1973). An increase in local protein concentration could then lead to a slightly increased aggregation of band 3. We have shown previously that band 3 proteins may in fact exist in different states of aggregation in the membrane (Nigg & Cherry, 1979a). In a further experiment, membranes from which the bulk of spectrin and actin had been extracted (Fairbanks et al., 1971) were incubated with antiglycophorin A antibodies. We found that antiglycophorin A IgG was equally effective in immobilizing band 3 in these depleted membranes as in normal ghosts. The above experiments thus strongly argue against a role of spectrin in the immobilization of band 3 following binding of antiglycophorin A antibodies. We conclude that the immobilization of band 3 by antiglycophorin A antibodies is directly caused by cross-linking of a preexisting band 3–glycophorin A complex (Figure 1A) and does not involve transmembrane effects.

Discussion

In this report, we present direct evidence for the existence of a band 3–glycophorin A complex in the human erythrocyte membrane. This evidence is derived from the observation that band 3 rotation is strongly reduced following binding of divalent antiglycophorin A antibodies but not by monovalent Fab fragments, antispectrin, or nonspecific antibodies.

Other interactions involving band 3 have previously been investigated by rotational diffusion measurements (Cherry et al., 1976; Nigg & Cherry, 1979b). The following arrangement of band 3 in the human erythrocyte membrane can be deduced from these studies in conjunction with the present experiments. Band 3 proteins exist in the membrane as stable dimers (Nigg & Cherry, 1979b) which possibly may undergo temperature-dependent self-aggregation (Nigg & Cherry, 1979a). These dimers form a complex with the major sialoglycoprotein, glycophorin A. The band 3–glycophorin A complexes apparently protrude into the interstices of a spectrin–actin network underlying the cytoplasmic surface of the erythrocyte membrane so that their long-range lateral diffusion is hindered (Elgsaeter & Branton, 1974; Fowler & Bennett, 1978). However, the rotational mobility of the band 3–glycophorin A complexes is not dependent on the presence or absence of the bulk of spectrin and actin (Cherry et al., 1976), indicating that in the erythrocyte ghost, no direct physical linkage exists between most of these peripheral membrane proteins and band 3. The possibility that a proportion of band 3 may be immobilized by interaction with ankyrin (band 2.1), as recently suggested (Bennett & Stenbuck, 1979b), remains to be further investigated.

It is commonly believed that band 3 and/or glycophorin A are responsible for the appearance of intramembranous particles revealed by freeze–fracture electron microscopy of the erythrocyte membrane (Pinto da Silva et al., 1971; Tillack et al., 1972; Bretscher, 1973; Pinto da Silva & Nicolson, 1974; Grant & McConnell, 1974; Yu & Branton, 1976; Bächi et al., 1977; Gahmberg et al., 1978; Weinstein et al., 1978), although the exact composition of these particles remains in doubt. The present experiments demonstrate that immobilization of band 3 by antiglycophorin A is close to quantitative. This implies that at most a minor fraction of the mobile band 3 is unassociated with glycophorin A. According to latest calculations

of the amount of glycophorin A protein in erythrocyte membranes, there are $\sim 1.0 \times 10^6$ copies of glycophorin A per cell (Gahmberg et al., 1979). Since there are also $\sim 1 \times 10^6$ band 3 monomers and $\sim 5 \times 10^5$ intramembranous particles per cell (Steck, 1974), this might appear to imply that each intramembranous particle contains one band 3 dimer associated with two copies of glycophorin A. However, it is also conceivable that only one glycophorin A molecule is associated with each band 3 dimer, in which case half of the glycophorin A protein in the erythrocyte membrane could be unassociated with band 3. Moreover, a previous analysis of the rotational diffusion of band 3 indicated that aggregates of band 3 dimers may be present in the membrane (Nigg & Cherry, 1979a). Since each aggregate need contain only one glycophorin A in order to be immobilized by antibodies, the fraction of free glycophorin A could be even higher than 50%. It may be this free glycophorin A which selectively segregates into small vesicles when erythrocyte ghosts are treated with Triton X-100 after preincubation at low pH (Lutz et al., 1979). The present experiments therefore do not settle the question of the stoichiometry of the intramembranous particles, and it remains important to establish the chemical composition and homogeneity of these particles.

In conclusion, we emphasize that rotational diffusion measurements provide a powerful technique for investigating protein–protein interactions in membranes. In particular, the present experiments demonstrate how the combination of mobility measurements with antibody-induced cross-linking may be used as a general approach to the study of molecular interactions in biological membranes. As a further example, the mode of interaction between two key components of the electron transport chain in microsomes, cytochrome P-450 and NADPH-cytochrome P-450 reductase, is an unresolved question at the present time (Yang, 1977; Peterson et al., 1976). Measurements of rotational diffusion of cytochrome P-450 have recently been reported (Richter et al., 1979), and investigation of its association with the reductase can therefore be pursued by methods analogous to those described here.

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Incorporation of Glycosidically Linked Sialic Acid from Radiolabeled Free Sialic Acid and Cytidine Monophosphate–Sialic Acid by Intact Hamster Fibroblasts: A Reexamination†

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ABSTRACT: When incubated with cytidine monophosphate (CMP)–[^{14}C]sialic acid or [^{14}C]sialic acid, intact hamster fibroblasts incorporate labeled sialic acid into glycosidic linkage to cellular acceptors. A comparative study under strictly identical conditions shows that the two substrates behave differently in the following ways: (a) incorporation of sialic acid into glycosidic linkage from CMP–sialic acid is immediate and shows no lag, whereas a distinct lag is seen with the free sugar; (b) intact cells pretreated with neuraminidase incorporate 4–8 times more sialic acid from CMP–sialic acid (as compared to untreated control), but no difference in incorporation is seen by using free sialic acid with or without neuraminidase pretreatment of the cells; (c) when cells are prelabeled with CMP–sialic acid, greater than 50% of acceptor-bound sialic acid is released by neuraminidase; however, only a negligible fraction is released from labeled cells preincubated with free sialic acid; (d) CMP, a competitive inhibitor of sialyltransferase activity and which does not penetrate intact cells, inhibits sialic acid incorporation from CMP–sialic acid but not from the free sugar. Additionally, by employment of a gentle extraction procedure, a significant amount of sialyltransferase which exhibits some unique properties is extracted from intact cells. We conclude from these data that, at least in hamster fibroblasts, acceptors on the cell surface are readily glycosylated by direct transfer of the sialic acid moiety from CMP–sialic acid and that the loosely attached cell surface sialyltransferase may play a role in catalyzing the transfer reaction.

A variety of mammalian glycoproteins and glycolipids contain sialic acids at the terminal nonreducing end of the oligosaccharide chains (Kornfeld & Kornfeld, 1976; Fishman & Brady, 1976). The enzyme sialyltransferase (EC 2.4.99.1) catalyzes the incorporation of sialic acid onto these macromolecules by transferring the sialic acid moiety from the nucleotide sugar CMP–sialic acid[1] (Roseman, 1970). Most sialyltransferases are membrane-bound enzymes, the major cellular site being the Golgi apparatus (Roseman, 1970; Fleischer, 1977); other cellular organelles such as mitochondria (Bosmann, 1971), synaptosomes (Den et al., 1975), and rough endoplasmic reticulum (Bernacki, 1975; Shier & Trotter, 1976; Jarnefelt, 1976) also appear to contain significant amounts of enzyme activity. Several recent studies have shown that incubation of intact cells with radiolabeled CMP–sialic acid results in the incorporation of radioactivity into acid-precipitable macromolecular components. The following lines of evidence indicate that a major fraction of the cell-associated radioactivity can be accounted for by the labeling of the external acceptors on the cell surface: treatment of intact labeled cells with exogenously added neuraminidase released a large fraction of the acceptor-bound sialic acid (Datta, 1974; Bernacki, 1974; Patt & Grimes, 1974; Porter & Bernacki, 1975; Painter & White, 1976; Cervén, 1977); intact cells pretreated with neuraminidase incorporated three- to eightfold more sialic acid as compared to untreated cells (Datta, 1974; Bernacki, 1974; Porter & Bernacki, 1975; Cervén, 1977); exposure of intact cells to galactose oxidase to modify terminal galactose residues before incubation with CMP–sialic acid diminished sialic acid incorporation (Cervén, 1977); autoradiography of labeled cells revealed that greater than 80% of the radioactivity was associated with the plasma membrane (Porter & Bernacki, 1975; Cervén, 1977). Additional experimental data that are consistent with the above notion show the following: (a) addition of a 100–1000-fold excess of nonradioactive free sialic acid in the incubation fluid containing labeled CMP–[^{14}C]sialic acid did not reduce the incorporation of labeled sialic acid into acid-precipitable material (Datta, 1974; Porter & Bernacki, 1975; Painter & White, 1976; Cervén, 1977), although very high concentrations (e.g., 20 mM) of unlabeled sialic acid abolished incorporation of radioactivity from CMP–sialic acid (Hirschberg et al., 1976; Deppert & Walter, 1978) presumably because of contaminating inhibitor or due to the toxic effect of sialic acid itself [see Deppert & Walter (1978)]; (b) the initial rate of uptake of radioactivity from CMP–sialic acid into the *acid-soluble* fraction was ~25% of that found incorporated into the *acid-precipitable* fraction (Datta, 1974; Painter & White, 1976); (c) inhibitors that block transport of sugars, amino acids, and nucleosides did not influence the

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[1] Abbreviations used: CMP–sialic acid, cytidine monophosphate–sialic acid; Con A, concanavalin A; CMP, cytidine monophosphate; UDP, uridine diphosphate; α-methyl mannoside, methyl α-D-mannopyranoside.

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