ANIONIC SITES OF HUMAN ERYTHROCYTE MEMBRANES

II. Antispectrin-Induced Transmembrane Aggregation of the Binding Sites for Positively Charged Colloidal Particles

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ABSTRACT

The effects of affinity-purified antispectrin γ-globulins on the topographic distribution of anionic residues on human erythrocytes membranes was investigated using colloidal iron hydroxide labeling of mounted, fixed, ghost membranes. Antispectrin γ-globulins were sequestered inside ghosts by hemolysis and the ghosts were incubated for 30 min at 37°C and then fixed with glutaraldehyde. The topographic distribution of colloidal iron hydroxide clusters on ghosts incubated with low (<0.05 mg/ml) or high (>5–10 mg/ml concentrations of sequestered antispectrin was dispersed, but the distribution at intermediate concentrations (0.1–5 mg/ml) was highly aggregated. The aggregation of colloidal iron hydroxide binding sites was time and temperature dependent and required the sequestering of cross-linking antibodies (antispectrin Fab could not substitute for γ-globulin antibodies) inside the ghosts. Prior glutaraldehyde fixation or fixation at the time of hemolysis in antispectrin solutions prevented the antispectrin-induced colloidal iron site aggregation. The antispectrin reacted exclusively at the inner ghost membrane surface and the colloidal iron hydroxide bound to *N*-acetylneuraminic acid residues on the outer membrane surface which are overwhelming on the sialoglycoprotein glycophorin. These results were interpreted as evidence for a structural transmembrane linkage between the inner surface peripheral protein spectrin and the integral membrane component glycophorin.

INTRODUCTION

The human erythrocyte membrane is composed of lipids, proteins and glycoproteins, some of which are known to be arranged in a highly asymmetric fashion (Bretscher, 1971 *a*, and 1972; Marchesi and Palade, 1967; Nicolson et al., 1971; Phillips and Morrison, 1971; Perrone and Blostein, 1973). Evidence from controlled glycosidase (Eylar et al., 1962) and protease (Steck et al., 1971; Steck, 1972; Winzler et al., 1967) digestion, chemical labeling (Steck, 1972), and electron microscope studies (Nicolson and Singer, 1971, and footnote 1.) have shown that most (if not all) of the mem-

[1] G. L. Nicolson and S. J. Singer. 1974. Distribution and asymmetry of mammalian cell surface saccharides utilizing ferritin-conjugated plant agglutinins as specific saccharide stains. *J. Cell Biol.* In press.

brane glycoprotein oligosaccharides are expressed at the outer surface of the membrane. Some of these molecules (but not their saccharide residues) appear to be expressed also at the inner surface from chemical-labeling studies (Bretscher, 1971 *a* and 1971 *b*; Segrest et al., 1973) and proteolytic digestion experiments using inside-out ghost vesicles (Steck et al., 1971; Steck, 1972). These findings suggest that certain membrane components or glycoproteins traverse the membrane (Bretscher, 1971 *a*; Marchesi et al., 1972; Segrest et al., 1973; Pinto da Silva et al., 1973). Other membrane components such as the protein spectrin (Marchesi and Steers, 1968; Marchesi et al., 1969), also called tektin A (Clarke, 1971; Mazia and Ruby, 1968), are exclusively located at the inner surface of the membrane (Nicolson et al., 1971; Nicolson and Singer, 1971) and appear to be peripheral membrane proteins (Singer and Nicolson, 1972).

In the previous article of this series, techniques for topographic localization of *N*-acetylneuraminic acid (NANA)[2] residues with colloidal iron hydroxide (CIH) were used to investigate the effects of trypsin, phospholipase C, and pH on the surface organization of the human erythrocyte ghost (Nicolson, 1973). It was found that incubating ghosts at pH 5.5 in low osmolarity buffers resulted in aggregation of the CIH binding sites (Nicolson, 1973) and the sites that bind a high isoelectric point derivative of ferritin (Pinto da Silva et al., 1973) in an entirely reversible manner similar to the pH-reversible aggregation of intramembranous or membrane-intercalated particles revealed by freeze-cleavage techniques (Pinto da Silva, 1972). From the properties of isolated spectrin, it was proposed that this protein might aggregate at the inner surface at pH 5.5 and cause a concomitant aggregation of sialoglycoproteins which traverse the membrane and express their NANA residues on the ghost exterior (Nicolson, 1973). Here we report on the aggregation of spectrin at the inner membrane surface by affinity-purified antibodies against purified spectrin and its transmembrane effects on the distribution of CIH binding NANA sites on the exterior membrane surface.

[2] *The following abbreviations are used in this paper:* CIH, colloidal iron hydroxide; NANA, *N*-acetylneuraminic acid; 300 PB, 0.05 M sodium phosphate-0.6% sodium chloride buffer, pH 7.5, 300 mosmol; 15 PB, 300 PB diluted 1:20, pH 7.5; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

MATERIALS AND METHODS

Glacial acetic acid, ferric chloride, calcium chloride, sodium acetate, sodium chloride, and sodium phosphates were obtained as reagent grade materials from Mallinckrodt Chemical Works, St. Louis, Mo. Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) was obtained from Sigma Chemical Co., St. Louis, Mo. Glutaraldehyde was obtained from Union Carbide Corp., New York, as a 50% solution and was fractionally distilled before use. Osmium tetroxide and Epon 812 were obtained from Electron Microscopy Sciences, Fort Washington, Pa.

Human Erythrocyte Ghosts

Blood (B^+) was collected in heparinized vacuum tubes (Becton-Dickinson and Company, Rutherford, N. J.) and washed several times in 0.9% NaCl. The white coat was aspirated after each centrifugation. The erythrocyte ghosts were prepared at 4°C by a modification of the method of Dodge et al. (1963), except that sodium phosphate buffer, pH 7.5, 15 mosmol (15 PB) was used as the hemolyzing buffer (Nicolson, 1973). The lysed cells were washed three to five times in hemolyzing buffer by centrifugation at 15,000 *g* for 10–15 min and finally resuspended in 0.05 M sodium phosphate-0.6% sodium chloride buffer, pH 7.5, 300 mosmol (300 PB), and allowed to reseal overnight at 4°C. At each step the erythrocyte ghosts were examined by phase microscopy using a modified Vanox-Olympus Universal microscope fitted with Zeiss optics.

Human Spectrin

Human spectrin was extracted from erythrocyte ghosts by a slight modification of previously described procedures (Reynolds and Trayer, 1971). The ghosts were washed by centrifugation with 10 vol of cold distilled water (titrated to pH 7.5 with dilute NH_4OH) containing 5×10^{-4} M NaN_3. The pelleted ghosts were resuspended in cold NaN_3-water at a concentration of about 1 mg membrane protein/ml and dialyzed against the same solvent for 10 h in the cold. The suspension was finally centrifuged for 90 min at 100,000 *g* and the supernate carefully removed. After concentration by vacuum dialysis, the supernate was applied to a Sephadex G-200 column (2.5 cm × 90 cm; Pharmacia Fine Chemicals, Piscataway, N. J.) and eluted with 20 mM Tris-HCl (pH 7.5). Spectrin was judged to be pure by electrophoresis on 5.6% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fairbanks et al., 1971).

Purification of Antispectrin

New Zealand white rabbits were given 5-mg purified human spectrin each in Freund's complete adjuvant both in the footpads and subcutaneously. 2 mg were given in adjuvant after 1 mo and the animals were bled 10 days later. The serum was pooled from five animals and a titre of 0.5 mg/ml was obtained as judged by quantitative precipitin analysis.

Antispectrin was purified from sera by affinity chromatography. The antigen was coupled to CN-Br-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.1 *M* sodium phosphate buffer (pH 7) using the method of Cuatrecasas (1969). 20 ml of washed Sepharose-coupled spectrin (2 mg spectrin/ml packed by Sepharose) was added to 40–50 ml of clarified antisera and stirred slowly at 37°C for 30 min. The slurry was then packed in a column and washed with 300 PB until the effluent had an A_{280} of less than 0.05. The antibody was eluted as a single peak from the column at room temperature with 0.1 *M* acetic acid and immediately pooled and titrated to pH 5.5 with 1 M sodium acetate. The solution was dialyzed overnight against cold 300 PB and finally centrifuged at 100,000 *g* for 30 min to remove aggregated and precipitated protein. The final yield of antibody was 70–80% and the preparation was >85% precipitable with human spectrin. The protein was stored under 50% $(NH_4)_2SO_4$ at 4°C. Antispectrin Fab fragments were made and purified by the procedures of Porter (1959).

Labeling Procedures

Antispectrin γ-globulin molecules were sequestered inside resealed ghosts by hypotonic lysis (Seeman, 1967). To a loose pellet of ghosts (approximately 0.05 ml) in 300 PB, 0.5 ml of antispectrin (in 15 PB) was added and the ghosts were allowed to reseal for 1–2 min (Seeman, 1967). At that time they were either fixed in 1.5% glutaraldehyde (60 min, 20°C, same buffer) or incubated for various times at 0°, 20°, or 37°C. Control samples contained antispectrin Fab, absorbed normal γ-globulin, or buffer alone. After the incubation the ghosts were washed twice in 15 PB and fixed in 1.5% glutaraldehyde for 60 min at room temperature and washed again. The glutaraldehyde-fixed ghosts were floated on an air-water (or 15 PB) interface, mounted on collodion-coated (carbon-strengthened) electron microscope grids (Nicolson and Singer, 1971), and labeled with CIH as described previously (Nicolson, 1972 *c* and 1973). Ferritin-conjugated antispectrin was coupled and purified as described by Nicolson et al. (1971).

To determine the specificity and confirm the exterior localization of the CIH label on mounted ghosts, the following experiments were performed. First, human red blood cell ghosts were treated with *Vibrio cholerae* neuraminidase (1–10 U/10^7 ghosts) (Calbiochem, San Diego, Calif.) in 0.01 M Tris-HCl-0.01 M calcium chloride buffer, pH 6, for 30 min at 37°C. Under these conditions approximately 85–90% of the NANA is cleaved from the membrane (Eylar et al., 1962). After the neuraminidase treatment, ghosts were washed, fixed, and labeled as described above. Second, resealed ghosts in 300 PB were first lysed on an air-water (or 15 PB) interface, mounted on support films, and then fixed with 1.5% glutaraldehyde for 30–60 min at room temperature. Some of these ghosts lysed in a manner such that their inner membrane surfaces could be identified through holes in the membranes formed at lysis (see inset to Fig. 11) (Nicolson and Singer, 1971). The mounted ghosts were labeled with CIH to determine if their inner surfaces were capable of binding CIH. Samples were air-dried and observed in a Hitachi model HU-12 electron microscope.

RESULTS

Characterization of Antispectrin

For the subsequent studies it was necessary to rule out the possibility that contaminating antibodies against membrane components other than spectrin were present in the purified antibody preparation. Several different criteria were used for purity and specificity: (*a*) Purified antibody did not agglutinate intact red blood cells. In addition, ferritin anti-spectrin conjugates did not bind to the surface of intact cells in agreement with the findings Nicolson et al. (1971). (*b*) Purified antibody was more than 85% precipitable with pure antigen in agreement with the findings concerning other affinity-purified antibody preparations (Cuatrecasas, 1969; Painter et al., 1973). (*c*) One band was obtained in double diffusion experiments. (*d*) Unfixed ghosts which were spread on an air-water surface, picked up on thin support films, and stained with ferritin antispectrin as described previously (Nicolson and Singer, 1971) were labeled only on surfaces identified as *inner* in agreement with previous findings (Nicolson and Singer, 1971). (*e*) Purified antibodies incubated with SDS-solubilized ghost membrane components separated by Sepharose 4B chromatography in SDS[3] fixed complement only in fractions containing spectrin (R. G. Painter and S. J. Singer, unpublished results).

[3] Antibody reactivity assayed by complement fixation to purified spectrin denatured with 2% SDS is completely recovered by exhaustive dialysis against neutral buffers.

Antispectrin Effects on CIH Topography

In the previous article of this series it was shown that ghosts incubated in low ionic strength buffers at neutral pH did not change their morphology when fixed in glutaraldehyde (Nicolson, 1973). The glutaraldehyde-fixed ghosts were carefully floated onto buffer surfaces and mounted on coated grids for CIH labeling. Since the ghosts were mounted flat by this technique, large areas (up to one-half) of the ghost surface can be visualized (see inset to Fig. 1) and the topographic distribution of CIH sites can be obtained. Human ghosts fixed at pH 7.5 and labeled with CIH show particles distributed randomly across the entire membrane surface in higher than average densities in discrete regions (Fig. 1) (Nicolson, 1972 *c* and 1973; Weiss et al., 1972).

To test the effects of antispectrin on the topography of CIH sites on the membrane outer surface, antibodies were sequestered inside the ghosts by hypotonic lysis. If ghosts are (*a*) hemolyzed in buffers containing low concentrations of antispectrin (<0.05 mg/ml), (*b*) incubated for 30 min at 37°C and washed, (*c*) fixed with glutaraldehyde, and (*d*) mounted and labeled with CIH, the distribution of CIH is similar to that on ghosts that have been directly fixed and labeled (Fig. 2). However, if higher concentrations of antispectrin are used (0.5–5 mg/ml),[4] the CIH sites are aggregated[5] on most of the cells examined (Figs. 3 and 4). At high concentrations of antispectrin (>5–10 mg/ml) the CIH sites are again dispersed on the membrane (Figs. 5 and 6). The effects of antispectrin on the distribution of CIH requires that the antibodies be sequestered inside the ghosts. In experiments where this was not performed no effect of antispectrin on CIH topography is obtained. If antispectrin Fab molecules are substituted for γ-globulin antibodies, the CIH sites are not aggregated at concentrations (0.25 mg/ml) that cause significant rearrangements in CIH sites by γ-globulin (Fig. 7). If antispectrin γ-globulin is sequestered inside ghosts and incubated at 0°C, the CIH sites are not aggregated (Fig. 8). Glutaraldehyde fixation of the ghosts before hemolysis in antispectrin solutions (Fig. 9) or a short time thereafter (Fig. 10) does not change the CIH distributions compared to untreated controls. These data are summarized in Table I.

Specificity of CIH Labeling

As found previously, neuraminidase dramatically reduced CIH labeling (Nicolson, 1972 *c* and 1973). Ghosts treated with 1 U neuraminidase/10^7 ghosts for 60 min at 37°C bound approximately 10% as many CIH particles as untreated ghosts. In a test of localization of CIH binding sites to the outer ghost surface using previous procedures (Nicolson and Singer, 1971), CIH was exclusively and specifically bound on surfaces identified as *outer*. Few CIH particles were associated with surfaces identified as inner membrane surface (Fig. 11).

DISCUSSION

On untreated glutaraldehyde-fixed human erythrocyte ghosts, CIH particles are bound in higher than average density clusters which are randomly dispersed across the surface (Nicolson, 1973; Weiss et al., 1972). At the pH where CIH staining is performed, most anionic surface groups are uncharged except for some NANA residues (Gasic et al., 1968; Weiss et al., 1972; Weiss and Zeigel, 1972), sulfate (Gasic et al., 1968; Weiss et al., 1972), and ribonucleic acid or phosphate residues (Weiss et al., 1972; Weiss and Mayhew, 1969). On the human erythrocyte 85–90% of the CIH labeling can be blocked by neuraminidase (Nicolson, 1973; Weiss et al., 1972) which removes approximately 90% of the NANA residues (Eylar et al., 1972; Weiss et al., 1972); thus, the CIH label in this system is overwhelmingly bound to the carboxyl groups of NANA (Weiss et al., 1972). Of the components isolated from the human erythrocyte ghost, one, the sialoglycoprotein or glycophorin, is known to contain approximately 60–80% of the total membrane NANA (V. T. Marchesi, personal communication). Glycophorin

[4] This is equivalent to approximately 5×10^8 ghosts. The total spectrin/ghost present can be calculated from the known protein content/ghost (5.7×10^{-10} mg/ghost; Dodge et al., 1963; Weed et al., 1969) and the known percentage of spectrin (25% of the total protein weight; Steck et al., 1971) or ~71 μg spectrin per 0.05 ml packed ghosts.

[5] The appearance of less CIH on ghosts with aggregated CIH sites may be due to a CIH particle-packing problem. The CIH particles are quite large (50–100 Å) and may on the average cover more NANA sites per particle on the antispectrin-treated ghosts. In fact, the CIH-labeling method is probably not revealing the total number of NANA sites in either the aggregated or the dispersed states (Weiss and Ziegel, 1972).

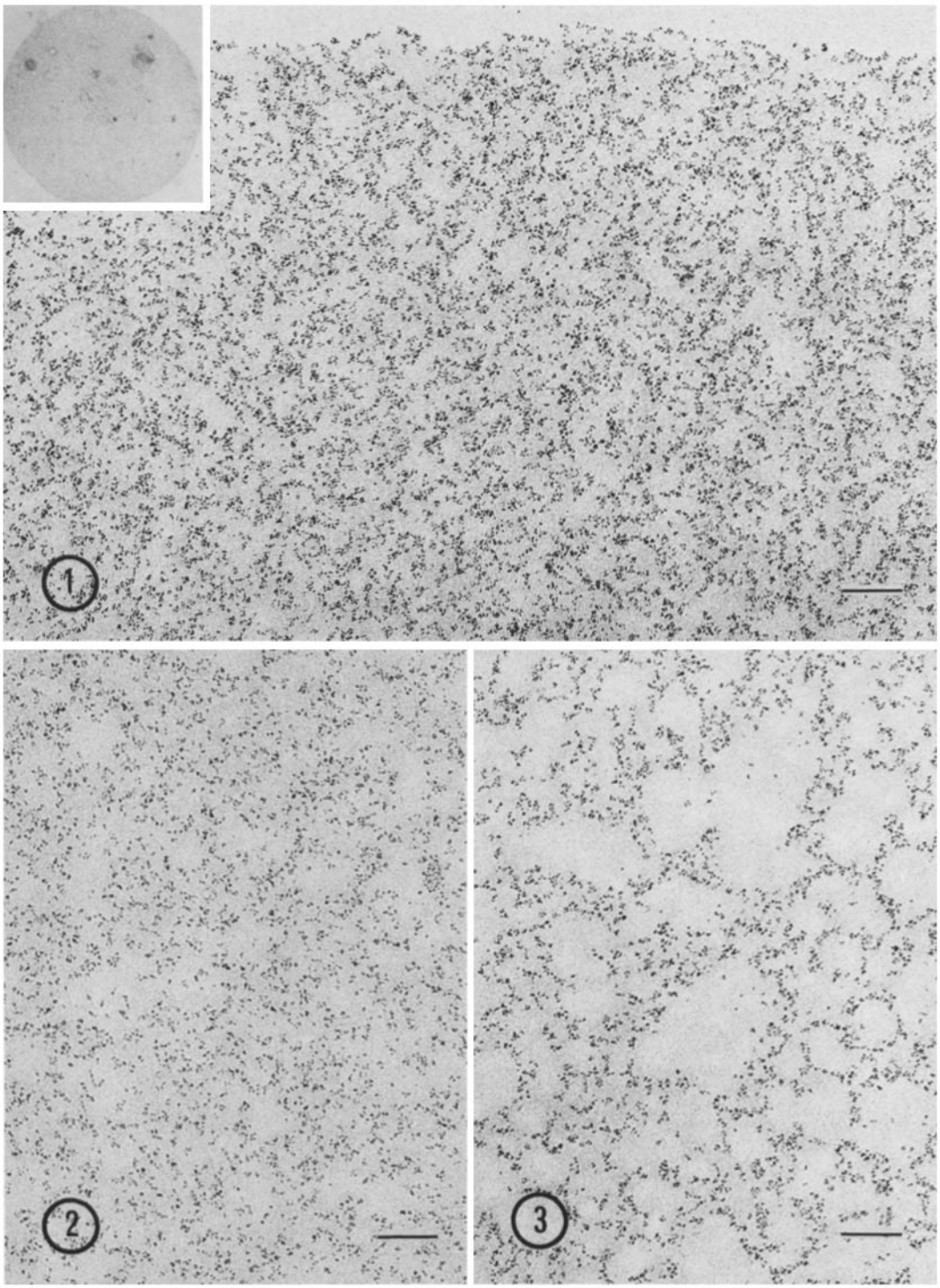

FIGURE 1 Topographic distribution of CIH on the surface of a glutaraldehyde-fixed human erythrocyte ghost. Ghosts were incubated for 30 min at 37°C in low osmolarity buffer, pH 7.5, and then washed twice. The ghosts were fixed at pH 7.5 with glutaraldehyde. After fixation the ghosts were mounted on carbon collodion films (low magnification *inset*) and stained with CIH. Bar equals 0.1 μm. × 84,000. *Inset* × 2,000.

FIGURE 2 The legend is the same as in Fig. 1 except that the incubation mixture contained 0.05 mg/ml purified antispectrin. The distribution of CIH is dispersed. Bar equals 0.1 μm. × 84,000.

FIGURE 3 The legend is the same as in Fig. 2 except that the antispectrin concentration was 0.25 mg/ml. The distribution of CIH is aggregated. Bar equals 0.1 μm. × 84,000.

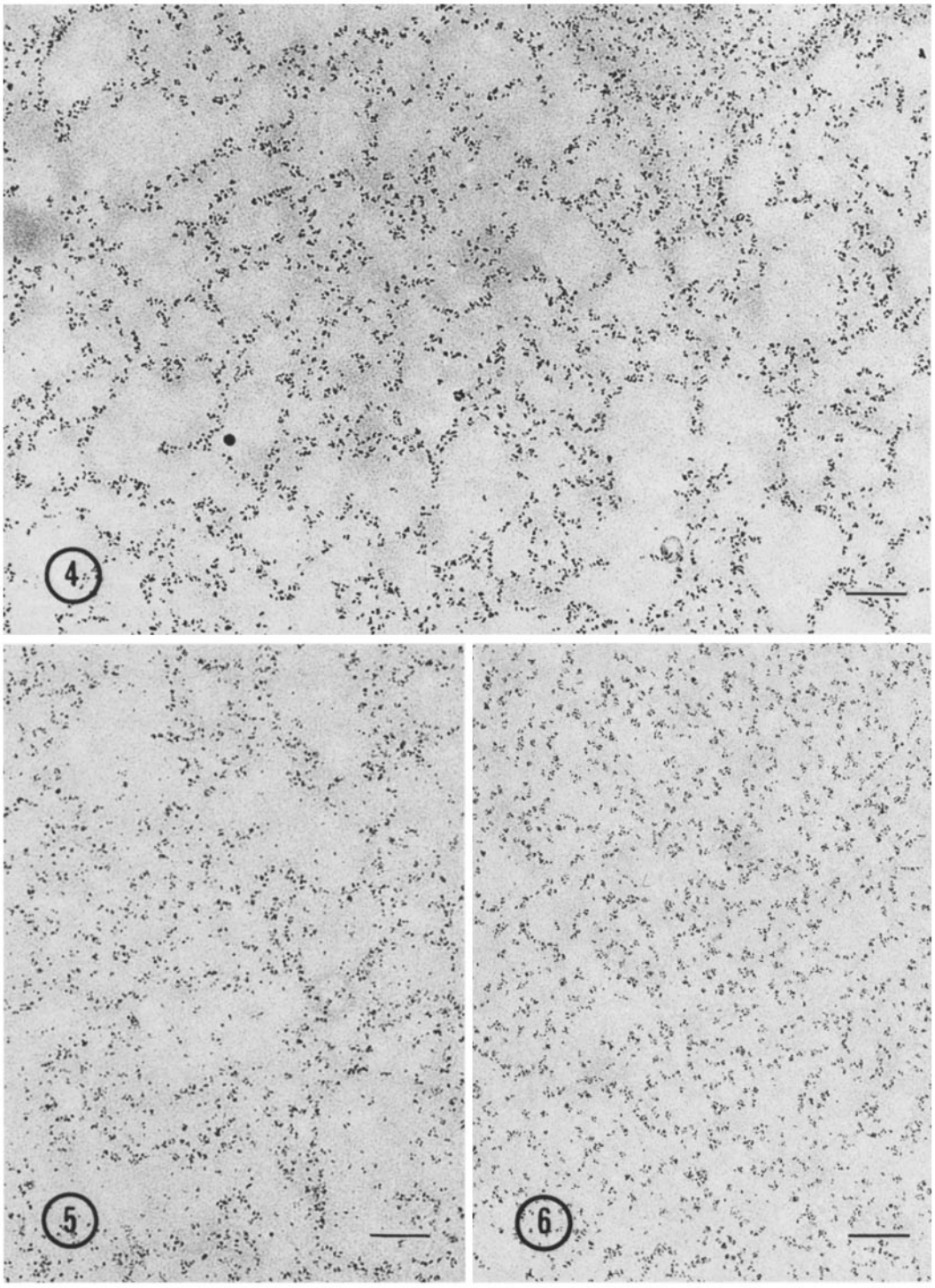

FIGURE 4 The legend is the same as in Fig. 3 except that the antispectrin concentration was 1 mg/ml. Bar equals 0.1 μm. × 84,000.

FIGURE 5 The legend is the same as in Fig. 2 except that the concentration of antispectrin is 5 mg/ml. The distribution of CIH is partly aggregated on this surface. Bar equals 0.1 μm. × 84,000.

FIGURE 6 The legend is the same as in Fig. 2 except that the concentration of antispectrin is 10 mg/ml. The distribution of CIH is dispersed. Bar equals 0.1 μm. × 84,000.

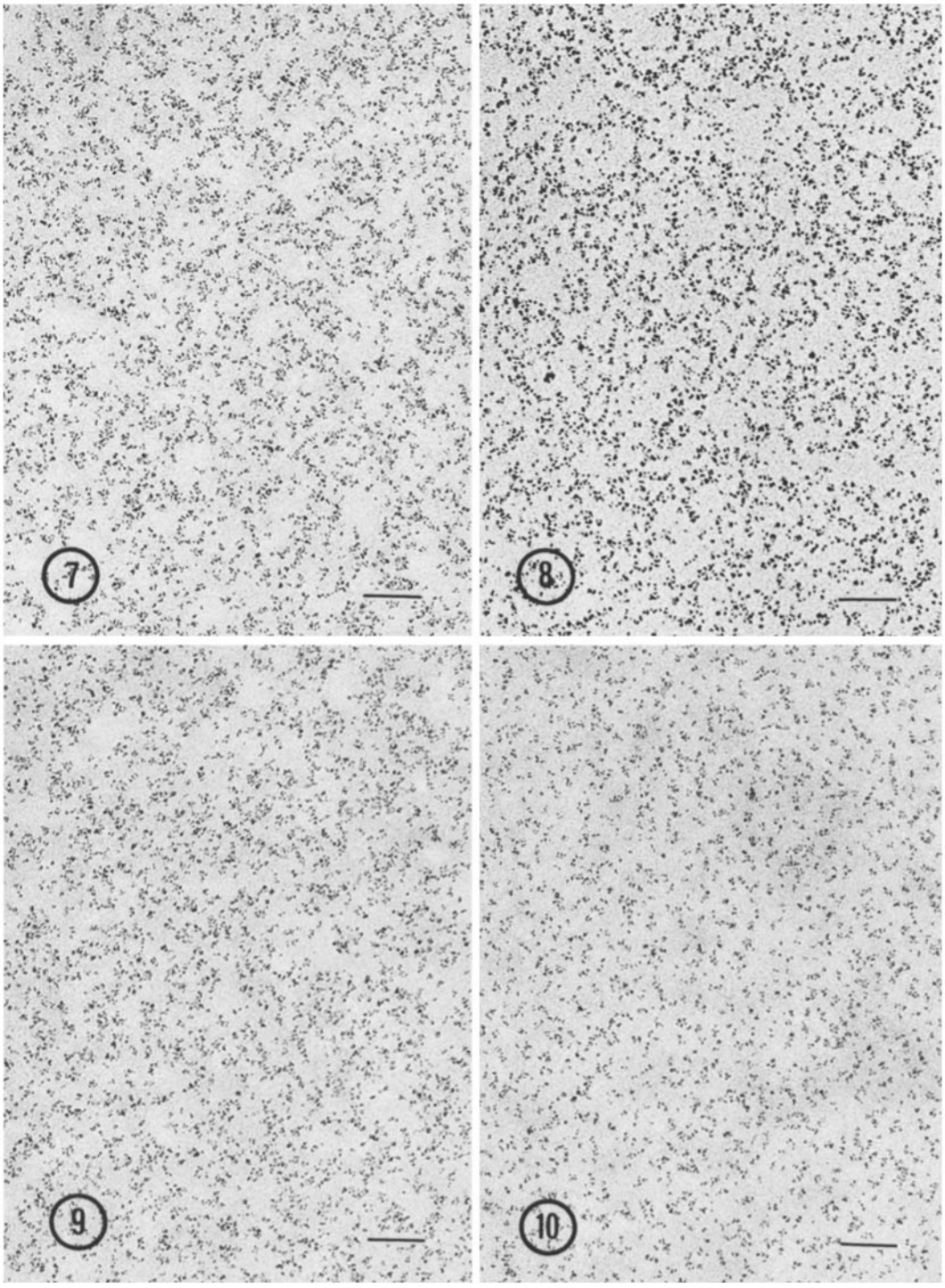

FIGURE 7 Fab control. The legend is the same as in Fig. 3 except that antispectrin Fab was substituted for antispectrin γ-globulins. The distribution of CIH is dispersed. Bar equals 0.1 μm. × 84,000.

FIGURE 8 Temperature control. The legend is the same as in Fig. 3 except that the incubation temperature was 0°C instead of 37°C. Bar equals 0.1 μm. × 84,000.

FIGURE 9 Fixation control. The legend is the same as in Fig. 3 except that the ghosts were immediately fixed with glutaraldehyde before the antispectrin incubation. Bar equals 0.1 μm. × 84,000.

FIGURE 10 Incubation time control. The legend is the same as in Fig. 3 except that the incubation time was 1 min instead of 30 min. Bar equals 0.1 μm. × 84,000.

TABLE I

Effects of Antispectrin on Human Erythrocyte CIH Site Topography

Antibody concentration	Incubation time	Incubation temperature	Topography of CIH clusters‡
(*mg/ml*)	(*min*)*	(°C)	
0	30	0	Dispersed
0	30	20	Dispersed
0	30	37	Dispersed
0.05	30	37	Dispersed
0.10	30	37	Dispersed/aggregated§
0.25	30	37	Aggregated
0.25	0‖	37	Dispersed
0.25	0.5	37	Dispersed
0.25	30	0	Dispersed
0.25	30	20	Aggregated
0.25 Fab	30	37	Dispersed
0.5	30	37	Aggregated
0.5 Fab	30	37	Dispersed
1	30	37	Aggregated
5	30	37	Dispersed/aggregated§
10	30	37	Dispersed

* At the end of the incubation period the ghosts were fixed in 1.5% glutaraldehyde for 60 min at room temperature.

‡ Topographic distribution of bound CIH clusters. Dispersed, the clusters cover the entire membrane surface; aggregated, the clusters are definitely aggregated into "superclusters" and do cover the entire surface.

§ A majority of the ghost membranes had dispersed distributions of CIH clusters while the remainder had definite aggregation of CIH clusters into superclusters.

‖ Ghosts were fixed with glutaraldehyde before incubation with antispectrin.

has recently been isolated and characterized as an asymmetric glycoprotein which is 60% carbohydrate and 40% protein in the form of a single polypeptide chain with its oligosaccharide chains attached near the *N*-terminal end (Marchesi et al., 1972; Segrest et al., 1973). Several of these oligosaccharide chains contain NANA residues at terminal positions (there are approximately 30 NANA residues per glycophorin molecule); this could explain why CIH binds to the erythrocyte ghost in certain regions with higher than average densities or clusters containing several CIH particles (Nicolson, 1973; Weiss et al., 1972). Glycophorin can be chemically labeled from both the inside and outside of the human erythrocyte ghost by reagents that have low penetrability in the membrane hydrophobic regions, leading to the proposal that this component traverses the membrane (Bretscher, 1971 *a* and *b*; Segrest et al., 1973). Also this component has been found to be associated with the membrane-intercalated particles found by freeze-cleavage (Marchesi et al., 1972; Pinto da Silva et al., 1971; Tillack et al., 1972; Pinto da Silva et al., 1973).

The membrane component spectrin is quite unlike glycophorin in its properties and membrane location. Spectrin has been characterized as a fibrous protein which associates in solution into rod-shaped polymers (Marchesi et al., 1969). Spectrin is categorically a peripheral membrane component because of its easy removal from the membrane with chelating agents (Marchesi et al., 1969; Marchesi and Steers, 1968) or distilled water (Clarke, 1971; Mazia and Ruby, 1968) without disrupting the bilayer integrity of the membrane. It has been exclusively localized at the inner surface by ferritin antibody (Nicolson et al., 1971; Nicolson and Singer, 1971) and chemical techniques (Bretscher, 1971 *b*; Phillips and Morrison, 1971; Steck, 1972).

The results here indicate that the distribution of NANA (present predominantly on glycophorin molecules at the membrane exterior) is influenced by the distribution of spectrin and possibly other

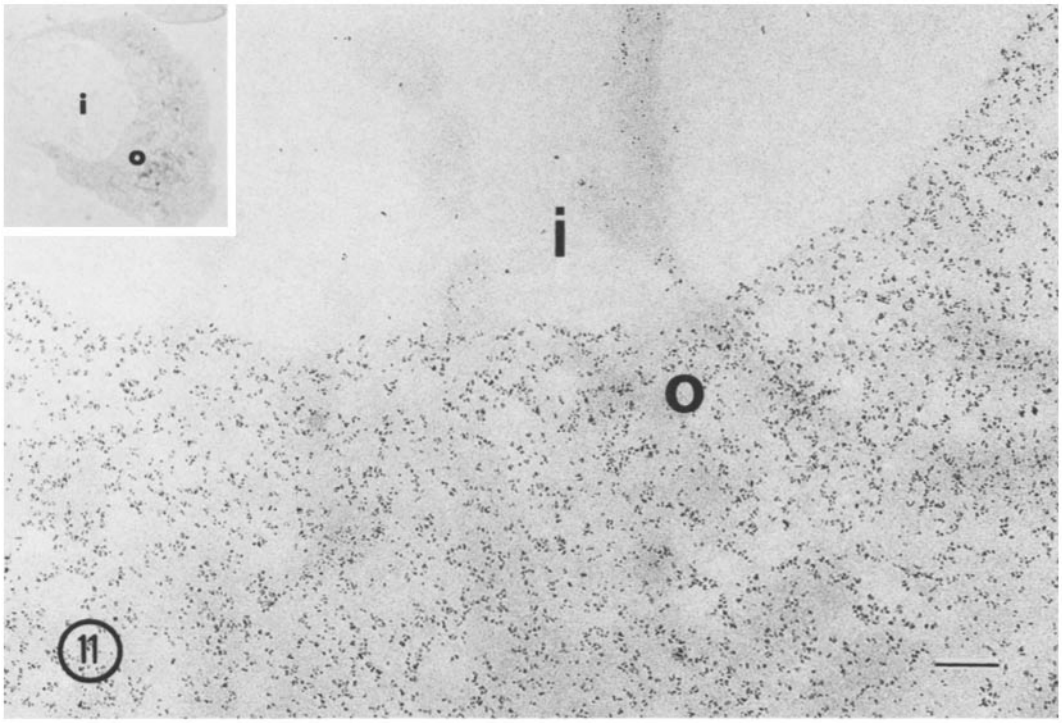

FIGURE 11 Human erythrocyte ghosts were lysed at an air-water interface and mounted on support films (see low magnification *inset*). Occasionally the upper membrane folds back revealing the inner surface (*i*) distinct from the outer surface (*o*). CIH binds to the outer surface. Bar equals 0.1 μm. × 84,000. *Inset* magnification, × 2,000.

components at the inner membrane surface (transmembrane control). Aggregation of CIH sites by antispectrin was found to be dependent on antibody concentration, accessibility of antibody to the inner ghost surface, time of incubation, temperature, and antibody valency. At low and very high concentrations of antispectrin no change in the distribution of CIH sites was found, but at intermediate antibody concentrations the CIH sites were extensively aggregated on most of the ghost surfaces examined. The fact that not every ghost surface showed aggregation of CIH sites was expected from prior hemolysis experiments where ferritin-conjugated antispectrin was sequestered inside human ghosts (Nicolson et al., 1971). There it was found that not all ghosts resealed at high osmolarity can be hemolyzed to allow entry of large molecules such as antibodies or ferritin conjugates (Nicolson et al., 1971). The presumed aggregation of membrane-bound spectrin at intermediate concentrations, but not at high or low antibody concentrations, is similar to precipitation of soluble antigens by antibodies. At low concentrations of antibody compared to antigen (similar to antigen excess) no cross-linking occurs and no precipitation occurs, at intermediate antibody concentrations (near equivalence) precipitation occurs, but at high antibody concentration (similar to antibody excess) precipitation does not occur. The observed antispectrin aggregation effects are dependent on hemolyzing the ghosts in the presence of antispectrin. Allowing the ghosts to reseal before addition of antispectrin prevents entry of ferritin antispectrin and prevents antispectrin-induced surface aggregation. The time and temperature dependency of the change in CIH site topography is also consistent with known aggregation times of membrane components (Edidin and Weiss, 1972; Karnovsky et al., 1972; Kourilsky et al., 1972; Taylor et al., 1971). Finally, the requirement for specific γ-globulin antibodies (antispectrin Fab or antidinitrophenyl γ-globulins had no effect) demonstrates that antibody binding itself is not sufficient for the aggregation effect.

In order for membrane-bound spectrin to be aggregated, it is necessary to presume that spectrin is capable of lateral movement on the inner surface of washed ghosts at 37°C. Fortunately, there is now ample evidence for a fluid structure of biological membranes (Fluid Mosaic Membrane Model [Singer and Nicolson, 1972]) where lateral diffusion of membrane components is expected. Several investigators have shown that γ-globulin antibodies cause aggregation of external surface receptors (Edidin and Weiss, 1972; Karnovsky et al., 1972; Kourilsky et al., 1972; Taylor et al., 1971; Unanue et al., 1972).

The validity of the conclusions drawn from experiments presented here on the direct or indirect interaction of spectrin with glycophorin at the inner membrane surface depends on (*a*) the purity of antispectrin, (*b*) the precise location and structure of spectrin and glycophorin, and (*c*) the reliability of the CIH labeling techniques used to monitor outer surface NANA present on glycophorin. The purity of affinity-purified γ-globulins made against highly purified spectrin was established by (*a*) quantitative reaction against purified spectrin, (*b*) reactivity with solubilized ghost proteins identified as spectrin, but not with other components, and (*c*) labeling of ferritin-conjugated antispectrin exclusively to the inner surface of human erythrocyte ghosts (Nicolson et al., 1971; Nicolson and Singer, 1971; R. G. Painter, unpublished data). The locations of spectrin and glycophorin have been well established using chemical labeling (Bretscher, 1971 *a* and 1971 *b*; Phillips and Morrison, 1971; Segrest et al., 1973), controlled proteolysis (Steck, 1972; Triplett and Carraway, 1972), and electron microscope labeling techniques (Nicolson, 1973; Nicolson and Singer, 1971). The reliability of the CIH labeling technique was established in the first paper of this series (Nicolson, 1973): (*a*) neuraminidase blocked CIH labeling to human erythrocyte ghosts, (*b*) changes in CIH distribution correlated with pH-induced reversible changes in the distribution of freeze-cleavage intramembranous particles (Pinto da Silva, 1972), (*c*) CIH distribution correlated with protease- and lipase-induced changes in the distribution of ferritin-conjugated lectins on human erythrocyte membranes (Nicolson, 1972 *b* and 1973), and (*d*) CIH labeled only the outer ghost surface (Fig. 11). Additionally, Ji (1973) has found that spectrin can be cross-linked to glycophorin and other glycoproteins by the bifunctional reagent dimethyl adipinsidate dihydrochloride, indicating close association in the membrane.

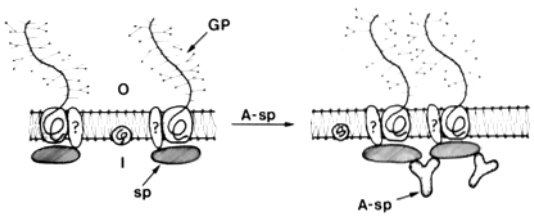

FIGURE 12 A proposal to explain the effects of antispectrin on the distribution of surface NANA residues. The membrane model is based on the proposal of Singer and Nicolson (1972); the glycoprotein component glycophorin (*GP*) on the proposals of Morawiecki (1964), Marchesi et al. (1972), Tillack et al. (1972), and Winzler (1970); the location of spectrin (*sp*) on the results of Marchesi et al. (1969), Jacob et al. (1971), Nicolson and Singer (1971), and Nicolson et al. (1971). Antispectrin (*A-sp*) is sequestered inside the ghost where it can bind to spectrin and cause aggregation of spectrin molecules at the inner (*I*) surface and also NANA resides on glycophorin at the outer (*O*) surface. The interaction between spectrin and glycophorin may be direct or it may occur through another unidentified component (?) that is present in a macromolecular complex as suggested by Bretscher (1971 *a*) and Guidotti (1972).

The findings here suggesting that internal surface peripheral proteins may exert direct or indirect control on the external topography of some membrane components (the converse may also be true) (Fig. 12) has far reaching consequences on the expression of a cell's exterior.[6] Structural associations such as the one found here may provide a linkage from the cell cytoplasm to the plasma membrane outer surface which could be

[6] By direct control we mean that direct noncovalent interaction between spectrin and glycophorin occurs at the interior surface of the membrane such that distributional perturbation of one type of molecule results in perturbation of the other type. By indirect control we mean that one or more other molecule(s) are involved in a structural linkage between spectrin and glycophorin (Fig. 12). Guidotti (1972) has suggested that glycophorin is part of a macromolecular complex containing another glycoprotein (90,000–100,000 daltons molecular weight) and that this complex is the membrane-intercalated particle revealed by freeze-fracture. If this is correct, spectrin should control the distribution and mobility of the human erythrocyte membrane-intercalated particle as suggested in a previous paper (Nicolson, 1973).

used to control the distribution of certain recognition or receptor sites and provide a matrix for the attachment of contractile proteins involved in cell motility, endocytosis, etc. Additionally, peripheral proteins like spectrin may contribute to the resistance to deformation and sheer of the erythrocyte (Jacob et al., 1971) by providing an internal lattice of fibrous peripheral proteins attached to integral membrane components firmly bound and intercalated into the hydrophobic regions of the membrane.

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