APPEARANCE AND DISTRIBUTION OF SURFACE PROTEINS OF THE HUMAN ERYTHROCYTE MEMBRANE

An Electron Microscope and Immunochemical Labeling Study

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ABSTRACT

We have used freeze-etching, before and after immunoferritin labeling, to visualize spectrin molecules and other surface proteins of the human erythrocyte membrane. After intramembrane particle aggregation was induced, spectrin molecules, identified by labeling with ferritin-conjugated antispectrin, were clustered on the cytoplasmic surface of the membrane in patches directly underlying the particle clusters. This labeling pattern confirms the involvement of spectrin in such particle aggregates, as previously inferred from indirect evidence. Ferritin-conjugated antihapten molecules, directed against external and cytoplasmic surface proteins of the erythrocyte membrane which had been covalently labeled nonspecifically with the hapten p-diazoniumphenyl-β-D-lactoside, were similarly found in direct association with such intramembrane particle aggregates. This indicates that when spectrin and the intramembrane particles are aggregated, all the major proteins of the erythrocyte membrane are constrained to coaggregate with them. Although giving no direct information concerning the freedom of translational movement of proteins in the unperturbed erythrocyte membrane, these experiments suggest that a close dynamic association may exist between the integral and peripheral protein components of the membrane, such that immobilization of one component can restrict the lateral mobility of others.

KEY WORDS: erythrocyte membrane, surface proteins, immunochemical labeling, freeze-etch electron microscopy, spectrin

Although the original formulation of the fluid mosaic model of membrane organization (39) did not stress the existence of interactions between membrane proteins, it has subsequently become evident that protein-protein associations play an important role in controlling the mobility and functional characteristics of cell surface components (26, 38). The existence of such associations is particularly evident in the erythrocyte membrane. For example, Nicolson and Painter (28) have demonstrated that perturbation of spectrin molecules on the cytoplasmic surface of the mem-
brane causes a redistribution of cationic sites on the membrane's external surface, and work in our laboratory (9, 10, 54) has shown that the associative properties of spectrin may provide a good model system to investigate how peripheral-integral membrane protein interactions can modulate cell surface organization.

Using freeze-fracture techniques, we have shown that the intramembrane particles of the erythrocyte membrane are normally dispersed in the plane of the membrane. Pretreatments, which remove most of the spectrin and actin molecules from the cytoplasmic surface of the membrane, allow subsequent intramembrane particle aggregation to be induced by simple manipulations of ionic strength or pH, or by the addition of polyvalent cations (9). Because the degree to which the particles are aggregated closely parallels the degree to which spectrin can be precipitated from aqueous solution by similar treatments, we have suggested (10) that it is the microprecipitation of residual spectrin molecules into small patches on the cytoplasmic surface of pretreated erythrocyte membranes which causes the observed particle aggregation. This implies either a specific association between spectrin and components of the intramembrane particles, or the nonspecific entrapment of the particles in the spectrin precipitate. In this paper, we describe experiments which extend our understanding of these phenomena in two ways.

First, to confirm our hypothesis that spectrin aggregates must underlie the intramembrane particle clusters, we have determined the locations of spectrin molecules on the cytoplasmic surface of the erythrocyte membrane by freeze-etching after labeling with affinity-purified ferritin-conjugated rabbit anti-human spectrin antibody.

Second, we have tried to examine whether spectrin and intramembrane particle aggregation involves the concomitant aggregation of all the other major erythrocyte membrane proteins or whether some of these proteins remain freely floating in the membrane's lipid bilayer. This question was of interest as other studies (17, 48) have shown that in the lymphocyte plasma membrane certain surface proteins (including immunoglobulins and glycoproteins bearing lectin receptors) behave quite independently of the intramembrane particles, whereas in the erythrocyte membrane specific receptors for phytohemagglutinin and concanavalin A are associated with the underlying intramembrane particles, coaggregating when these particles are aggregated by trypsin (46) or pH treatment (35). Rather than using specific labels to determine whether certain receptors or antigens are associated with the intramembrane particles, we employed a nonspecific protein-modifying hapten (51), and asked whether any substantial number of proteins exposed on the extracellular or the protoplasmic surface of the erythrocyte membrane were not associated with the particles.

MATERIALS AND METHODS

Preparation of Antibodies and their Ferritin Conjugates

Antibodies against spectrin were produced in rabbits by multiple-site injections of 200 μg per rabbit of chromatographically purified spectrin in isotonic saline emulsified with Freund's complete adjuvant. The procedures used to obtain purified spectrin free of erythrocyte actin and proteolytic cleavage products will be described in detail elsewhere. Initial intradermal and footpad injections were followed by intramuscular booster injections at 3-wk intervals. Bleedings (40 ml per rabbit, at 1- to 3-wk intervals) were started 12 days after the first boost, and the antisera were titered by precipitin tests. High-titer antisera were pooled, and the γ-globulins were precipitated by addition of an equal volume of 80% ammonium sulfate.

The antispectrin antibodies (20-40 μg/mg of precipitated washed γ-globulins, estimated by quantitative precipitin assays) were then purified by affinity chromatography (28). Purified spectrin (1 mg/ml in 0.1 M NaHCO₃ buffer, pH 9.0) was coupled to cyanogen bromide-activated Sepharose 4B (1.2 mg of spectrin/ml of swollen Sepharose; efficiency of coupling 94%), and, after thorough washing, 24 ml of the conjugate was packed in a 12-cm column and equilibrated with isotonic phosphate-buffered saline (PBS). The precipitated γ-globulins

1 D. M. Shotton and D. Branton. Chemical, biophysical, and electron microscope studies on the molecular structure of human erythrocyte spectrin. Manuscript in preparation.

Abbreviations used in this paper: DTT, dithiothreitol; lac, p-diazoniumphenyl-β-D-lactoside or its covalent reaction product (e.g. lac-ghosts); PB, sodium phosphate buffer, pH 7.6 (The phosphate buffers are designated according to their ideal milliosmolar value. Thus, 20 mosM sodium phosphate buffer is designated as 20 PB, 310 mosM sodium phosphate buffer is designated as 310 PB, 1 osM sodium phosphate buffer is designated 1,000 PB, etc. The pH of all the buffers is pH 7.6 unless specified otherwise in the text); PBS, isotonic phosphate-buffered saline (145 mM NaCl-20 PB, pH 7.6); SDS, sodium dodecyl sulfate.
were dissolved to a concentration of 30 mg/ml by exhaustive dialysis against ice-cold PBS, incubated twice on ice with washed human erythrocytes for 30 min in PBS to absorb nonspecific antibodies directed against external components of the erythrocyte membrane, and then passed through the spectrin-Sepharose affinity column. The column absorption and subsequent washing and elutions were all performed at room temperature, using buffers containing 0.5 mM sodium azide. After the column was thoroughly washed with PBS until the eluate absorbance at 280 nm was <0.05, the bound antispectrin was eluted as a sharp peak with 2 M NaCl-0.5 M glycine-HCl, pH 2.8. Surprisingly, but reproducibly, a second sharp peak of antispectrin was eluted when the column was reequilibrated with PBS. At antibody concentrations of ~1 mg/ml, 75% of the total protein in both eluted peaks could be precipitated by the addition of purified spectrin in PBS, with equivalence as determined by quantitative precipitation assays at antibody/antigen ratios of ~3.5:1 (wt/wt). These peaks were therefore pooled and the antispectrin was stored at 4°C either in solution in PBS-0.5 mM sodium azide for immediate use, or as a precipitate after dialysis against 50% ammonium sulphate in PBS-0.5 mM sodium azide.

Glutaraldehyde (Ladd Research Industries, Burlington, Vt., electron microscopy grade) was used to coupling the purified antispectrin to ferritin (Polysciences, Inc., Warrington, Pa., e.m. grade), employing the improved method (procedure II) of Kishida et al. (19). To determine the degree of ferritin conjugation, the agarose column eluate fractions were negatively stained with uranyl acetate, a solution of unconjugated ferritin molecules in PBS being similarly stained as a control (see Results). The peak containing low molecular weight ferritin antispectrin conjugates was dialyzed against PBS, sterilized by passage through a 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass.), and stored at 4°C in 0.5 mM sodium azide for use in the labeling experiments.

High-titer rabbit anti-p-diazoniumphenyl-β-D-lactoside (anti-lac) antisera were isolated from rabbits hyperimmunized by standard procedures against a lac-keyhole limpet hemocyanin conjugate (7). From the pooled antisera, anti-lac antibodies were purified by Sepharose affinity chromatography, as described by Wofsy and Burr (52). These antibodies were conjugated with fluorescein by use of the procedures of Goldman (14). The fraction having 1-3 fluorescein groups per antibody molecule was used for subsequent ferritin conjugation. Ferritin was coupled to the purified fluorescent anti-lac with glutaraldehyde by the procedure of Nicholson and Singer (29). After chromatographic separation from free ferritin, the ferritin-conjugated, fluorescein-labeled anti-lac was stored at 4°C in the presence of 10 mM sodium azide until used for the labeling studies described below. The antibody activity of these conjugates was monitored by testing the ability of the preparation to agglutinate and to cause intense ring-fluorescence of lac-modified erythrocytes and lac-ghosts.

**Preparation of Inside-Out Vesicles and Resealed Ghosts**

Inside-out vesicles (42) were prepared from fresh human erythrocyte ghosts (8, 9) by 1:40 dilution with 0.5 mM sodium phosphate buffer (PB), pH 8.2, and by incubation for 24-48 h at 0°C without agitation. To preserve large inside-out vesicles, no further manipulations were employed; the vesicles were neither sheared by passage through a hypodermic needle nor fractioned on a dextran gradient. Resealed ghosts were prepared by resuspending freshly prepared leaky ghosts (8) in PBS and incubating overnight without agitation at 0°C (28).

**Preparation of p-Diazoniumphenyl-β-D-Lactoside and lac-Modified Membranes**

The diazonium salt of the hapten phenyl-β-D-lactoside was prepared by a modification of the method of Truffa-Bachi and Wofsy (47). 10 ml of 10 mM p-aminophenyl-β-D-lactoside in 30 mM HCl was mixed with an equal volume of ice-cold 10 mM sodium nitrite. The mixture was incubated on ice for 20 min, and the resulting 5 mM acidic solution of p-diazoniumphenyl-β-D-lactoside (lac) was either used immediately or stored frozen in 2-ml aliquots for subsequent use.

Ghosts whose extracellular surface membrane proteins had been lac modified (hereafter referred to as lac(ES)-ghosts) were prepared by lac modification of fresh erythrocytes washed four times at 4°C in 30 vol of 40 mM glucose-120 mM NaCl-20 PB, pH 7.6 (25). 2 ml of ice-cold 5 mM lac was premixed on ice with 1 ml of 1,000 PB, pH 7.6. 2 ml of the packed washed erythrocytes were added, and the mixture was incubated overnight at 4°C with gentle tumbling. The lac-modified erythrocytes were then washed three times with 50 mM glucose-120 mM NaCl-20 PB and once with 310 PB. A very small amount of lysis occurred during the overnight incubation but presented no subsequent problems because the ghosts formed were removed with the supernate after the low-speed centrifugations which followed these washes. Ghosts were then prepared from these lac-modified erythrocytes by the method of Dodge et al. (8) as previously described (9). Erythrocytes which had been incubated in parallel, with the omission of lac, were similarly lysed to yield unmodified fresh ghosts for use in control experiments.

Large vesicles whose protoplasmic surface membrane proteins were lac-modified (hereafter referred to as lac(PS)-vesicles, to distinguish them from the lac(ES)-ghosts prepared from lac-modified intact erythrocytes)

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3 Nomenclature of Branton et al. (4).
were prepared by lac modification of fresh ghosts. 2 ml of packed fresh ghosts in 20 PB was lac modified by a procedure identical to that detailed above for intact erythrocytes, except that 20 PB, pH 7.6, was substituted for the 1,000 PB buffer in the reaction mixture. The pH of the final mixture varied between pH 5.0 and 5.5 in different preparations, and no further pH adjustment was made. After overnight reaction, the ghosts had formed large lac-modified vesicles. These were gently washed three times with 20 PB before antibody staining.

**Pretreatments and Induction of Particle Aggregations**

To allow subsequent particle aggregation, normal and lac(ES)-ghosts were pretreated in some experiments by incubation in 20 PB, pH 9.0, for 10 h at 37°C, or in 20 PB, pH 7.6, for 16 h at 30°C, after the addition of 0.5 mM sodium azide (9). Care was taken to avoid mechanical fragmentation of the fragile pretreated vesicles. Intramembrane particles in the pretreated membranes were aggregated before antibody labeling by one of the procedures described in our previous papers (9, 10). Most commonly, incubations in 310 PB at pH 7.6 or in PBS at pH 5.0 for 30–120 min on ice were employed. Details are given in the figure legends.

**Immunodiffusion and Immunoprecipitation**

The specificity of antibody preparations was checked by double immunodiffusion in agar (30). For photography, the washed agar gels were air-dried and the white precipitin lines stained by brief (2–5 min) immersion in a 0.025% solution of Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, Calif.) in 10% acetic acid-25% isopropanol. For quantitative precipitin assays or for analysis of the protein composition of immunoprecipitates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, antibody and antigen were incubated together in PBS overnight at 4°C. Insoluble membrane proteins to be used for immunodiffusion or immunoprecipitation were first solubilized by incubation for 30 min at 37°C in 4 mM sodium phosphate buffer, pH 8.0, containing 0.5% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.). In some cases the membrane preparations were first depleted of essentially all their spectrin (bands 1 and 2) and actin (band 5) by incubation at 37°C for 30 min in 0.1 mM EDTA-0.05 mM dithiothreitol (DTT) titrated to pH 9.5 with dilute NH₄OH. In either case, the small amount of insoluble material remaining after this treatment was found to have substantially the same protein composition as the Triton solution.

The resulting immunoprecipitates were washed three times with ice-cold PBS (containing 0.5% Triton X-100 where appropriate) and pelleted each time by centrifugation at 16,000 rpm for 10 min at 4°C. The washed precipitates were then either dissolved in 0.1 N NaOH to allow the protein absorbance at 280 nm to be measured or, alternatively, dissolved in 1% SDS-10% sucrose-3 mM DTT-1 mM EDTA-10 mM Tris-HCl, pH 8.0, by incubating for 30 min at 37°C for subsequent protein analysis on polyacrylamide gels. In cases where the antigens were membrane proteins solubilized by Triton extraction, 0.5% Triton X-100 was included in the PBS throughout the immunoprecipitation and subsequent washings, and the washed precipitates were analyzed immediately by SDS-polyacrylamide gel electrophoresis. Storage of the protein-Triton-SDS mixture resulted in a deterioration of the gel patterns obtained.

**Immunochemical Labeling of Ghost Preparations with Ferritin-Conjugated Antibodies**

To investigate the ferritin-conjugated, antispectrin labeling of spectrin aggregates on the protoplasmic surface of erythrocyte membranes, aliquots of the antibody conjugate were incubated in PBS with preparations of inside-out vesicles, parallel incubations with resealed ghosts serving as controls for nonspecific adsorption of the ferritin conjugate. In addition, vesicles and ghosts were incubated with PBS containing free ferritin, rather than ferritin-conjugated antispectrin, to act as controls both for the effective removal of unbound ferritins during the subsequent washes and for the effects of the incubation conditions themselves, in the absence of antibody, on the morphology and particle distribution of the membranes.

Similarly, to investigate the distribution of lac-modified proteins on the outer surface of erythrocyte membranes, aliquots of ferritin-conjugated fluorescein-labeled anti-lac in PBS were incubated with preparations of lac(ES)-ghosts and pretreated lac(ES)-ghosts, with fresh and pretreated unmodified ghosts serving as specificity controls. To investigate the distribution of hapten molecules on the cytoplasmic surface of lac(PS)-vesicles, aliquots of ferritin-conjugated fluorescein-labeled anti-lac in PBS were similarly incubated with preparations of lac(PS)-vesicles, using hapten-free inside-out vesicles and resealed ghosts as controls.

The following general experimental protocol was found convenient: 20–50-μl samples of a 50% suspension of an experimental or control membrane preparation were added to 20 μl of a 2–5 mg/ml solution of ferritin-conjugated antibody or free ferritin in PBS. After incubation on ice for 1–2 h, a small sample was taken for phase-contrast light microscopy to observe any agglutination which had occurred. The membranes were then washed twice with PBS, centrifuged at 5,000 rpm for 10 min at 4°C, and then resuspended in approximately their own volume of PBS, any agglutinated clumps being partially dispersed by tapping the tube.
some of the antispectrin labeling experiments, the pH of the PBS was lowered to pH 5.0 to maintain a high degree of particle aggregation. The degree of labeling with ferritin-conjugated fluorescein-labeled anti-α was determined by fluorescence microscopy. Samples were then frozen for freeze-etch electron microscopy.

**Freeze-Etch Electron Microscopy**

Concentrated 1-μl samples of ghost suspensions were frozen onto copper specimen supports by immersion into freezing Freon-22 (chlorodifluoromethane), cooled in liquid nitrogen, and subsequently freeze-fractured and etched for 60 or 70 s at −100°C in a Balzers freeze-etch device (Balzers AG, Balzers, Lichtenstein) by standard techniques (12). Replicas were cast, using Pt-C and C anodes heated by electron bombardment from tungsten cathodes (24).

In spite of the relatively high salt content of PBS, the salt-containing eutectic-like phase which this buffer formed on freezing obscured the freeze-etched membrane surfaces only in localized areas. Thus, when ghosts and vesicles were labeled, washed and frozen in PBS, favorable replica areas were found in which etching exposed membrane surfaces that had been in contact with pure ice. These membrane surfaces were unobscured by the eutectic-like phase, appearing as though they had been frozen in water or a low ionic strength buffer. In some experiments the labeled membranes were given a final wash in ice-cold 20 PB before freezing, a procedure which did not dissociate the previously bound ferritin-antibody conjugates. Details for each micrograph are given in the figure legends.

**RESULTS**

**Affinity-Purified Antispectrin**

The absorbed serum from spectrin-immunized rabbits and the affinity-purified antispectrin prepared from it gave single, sharp, confluent precipitin lines when tested by double immunodiffusion against purified spectrin (Fig. 1a, b). Similarly, purified spectrin, EDTA extracts of ghosts (containing spectrin and actin), and Triton X-100 extracts of ghosts (containing all the major erythrocyte membrane proteins) gave single, sharp, confluent precipitin lines when tested against antispectrin, while Triton X-100 extracts of spectrin-free vesicles showed no bands (Fig. 1c, d). Analysis of such immunoprecipitates by SDS-polyacrylamide gel electrophoresis (Fig. 2) showed that the only major polypeptide bands present, in addition to those expected from the antispectrin preparation itself (shown in control gel 5), were those of spectrin (gels 6 and 7). Traces of other high molecular weight bands, almost certainly spectrin proteolysis products, were also visible in these gels, as were two minor bands running in the region of bands 4.1 and 4.2. The bands in the 4.1 and 4.2 region were not unexpected as it has been observed that a proportion of these polypeptides remains associated with spectrin when erythrocyte membranes are dissolved in Triton X-100 (43) or Triton X-114 (Sheetz, personal communication). Although the band 5 region was obscured by the monomeric IgG heavy-chain band from the antispectrin, bands 3 and 6 were clearly absent from these immunoprecipitates. When antispectrin was incubated with a Triton X-100 solution containing all the erythrocyte membrane proteins except spectrin and actin (gel 4), no polypeptides precipitated, demonstrating the specificity of the affinity-purified antispectrin.

**Ferritin-Conjugated Antispectrin**

Although we prepared ferritin antispectrin conjugates by using procedure II of Kishida et al. (19) without modification, two aspects of our results are noteworthy. First, though our column fractionation of the ferritin-conjugated antispectrin gave an elution profile (Fig. 3) very similar to that reported by Kishida et al. (19) cf. Fig. 3 of their paper), the second peak of eluted ferritin protein contained active low molecular weight ferritin antibody conjugates, not the third and largest peak, as implied in their paper. No active ferritin antispectrin conjugates were detected in peak 3 when binding to inside-out vesicles was assayed.

Second, a substantial proportion of the small, active conjugates obtained by using this improved method contained more than a single ferritin molecule. We examined the degree of conjugation in the eluted fractions from the agarose column by negative staining, the negatively stained ferritin molecules appearing as clear circles with central electron-dense cores. Representative fields from the different preparations, having fairly low and approximately equal densities of ferritin molecules, were counted to determine the proportions of solitary, paired, trimeric, and oligomeric ferritin molecules present. The results are shown in Table 1. The third peak (Fig. 3) was largely unconjugated ferritin, lacking active ferritin antispectrin conjugates. It appeared virtually identical to a buffered control solution of unconjugated ferritin negatively stained at an equal ferritin density, the apparent association of individual ferritin molecules into dimers, trimers, etc. in both these samples being due to random nonco-
Photographs of spectrin-antispectrin precipitin lines obtained by Ouchterlony double immunodiffusion in agar. (a) Test of antispectrin sera: center well, 2.5 μg of purified spectrin; well 1, 6 μg of affinity-purified antispectrin (rabbit 1); well 2, 8 μl of 1:30 diluted absorbed serum (rabbit 1); well 3, 8 μl of undiluted serum (rabbit 2); well 4, 8 μl of 1:6 diluted serum (rabbit 2); well 5, 8 μl of undiluted serum (rabbit 3); well 6, 8 μl of 1:6 diluted serum (rabbit 3). (b) Test of affinity-purified antispectrin preparations from rabbit 1: center well, 8 μg of purified spectrin; well 1, 14 μg of affinity-purified antispectrin (preparation 1); well 2, 12 μg of affinity-purified antispectrin (preparation 2); well 3, 10 μl of 1:30 diluted absorbed serum (rabbit 1); well 4, 12 μg of affinity-purified antispectrin (preparation 3); well 5, 6 μg of affinity-purified antispectrin (preparation 2); well 6, 10 μg of affinity-purified antispectrin (preparation 4). (c) Test of purified spectrin at different concentrations: center well, 12 μg of affinity-purified antispectrin; wells 1–6, 0, 1, 2, 4, 8, and 12 μg of purified spectrin, respectively. (d) Immunoprecipitation of spectrin from an EDTA extract of spectrin and actin, and from Triton-solubilized mixtures of erythrocyte membrane proteins. Center well, 12 μg of affinity-purified antispectrin; well 1, 0.5% Triton X-100 buffer alone (control); well 2, 7 μg of purified spectrin; well 3, 10 μg of EDTA extract of spectrin and actin; well 4, as well 3, plus 0.5% Triton X-100; well 5, 16 μl of a 0.5% Triton X-100 solution of whole ghosts; well 6, 16 μl of a 0.5% Triton X-100 solution of EDTA-extracted ghosts (lacking spectrin and actin).

valent side-by-side absorption of individual ferritin molecules on the electron microscope grids. A significantly higher degree of association was observed in peak 2, indicating that 57% of the ferritin molecules in peak 2 were covalently associated as dimers or higher oligomers, probably via antispectrin molecules to which they had been independently cross-linked. Peak 1 contained highly aggregated material and was discarded with peak 3, while the active low molecular weight ferritin antispectrin conjugates from peak 2 were used for the labeling experiments. The occurrence of ferritin oligomers, both in these preparations of ferritin-conjugated antispectrin and in the more conventional preparations of ferritin-conjugated fluorescein-labeled anti-lac, explains the appearance of small ferritin clusters seen on the etched surfaces of membranes labeled with these preparations in situations where the antigens themselves were dispersed.

**Lac(ES)-Ghosts and Lac(PS)-Vesicles**

Four tests were employed to ensure that the lac(ES)-ghosts and lac(PS)-vesicles, prepared by
FIGURE 2 SDS-polyacrylamide gels of erythrocyte membrane protein preparations, and the antispectrin immunoprecipitates obtained from them. The procedures used were essentially those of Fairbanks et al. (11) with the minor modifications described by Steck and Yu (43). Hb, residual hemoglobin; Ap, the pancreatic protease inhibitor aprotinin (Sigma Chemical Co., St. Louis, Mo.) added to the samples used for gels 3-5 to prevent proteolysis. Gel 1, 7.5 μl of packed fresh ghosts; gel 2, 50 μl of a 0.5% Triton X-100 solution of whole ghosts; gel 3, 10 μl of an EDTA extract of spectrin and actin; gel 4, 50 μl of a 0.5% Triton X-100 solution of EDTA-extracted ghosts (lacking spectrin and actin); gel 5, 30 μl of the antispectrin immunoprecipitate obtained from the 0.5% Triton X-100 solution of whole ghosts (gel 2); gel 6, 20 μl of the antispectrin immunoprecipitate obtained from the EDTA-extract of spectrin and actin (gel 3). No immunoprecipitate was obtained when the mixture of antispectrin and the 0.5% Triton X-100 solution of EDTA-extracted ghosts (lacking spectrin and actin — gel 4) was reacted in parallel with the sample giving gel 6.

reaction of the diazonium reagent with intact erythrocytes and fresh ghosts, respectively, had been satisfactorily modified. These were agglutinability, freeze-etch electron microscopy, fluorescence labeling, and protein analysis by SDS-polyacrylamide gel electrophoresis.

Intact lac-modified erythrocytes, lac(ES)-ghosts, and lac(PS)-vesicles could all be induced to agglutinate by the addition of anti-lac antibodies or their ferritin fluorescent conjugates. Unmodified membranes treated identically were unaffected by the addition of anti-lac.

The lac(ES)-ghosts appeared white and possessed the normal biconcave shape typical of healthy fresh ghosts. When examined by freeze-etch electron microscopy, both their fracture faces and etched surfaces were indistinguishable in appearance from those of fresh unmodified ghosts. After labeling with ferritin-conjugated fluorescein-labeled anti-lac (described in detail below), ferritin molecules were confined to the extracellular surfaces of the membranes, as expected. The labeled ghosts themselves exhibited intense, uniform yellow-green ring fluorescence against a dark background when examined by fluorescence microscopy, and unhaptenized control ghosts were dark or showed extremely weak fluorescence, indicating insignificant levels of nonspecific absorption or entrapment of the antibodies. After SDS-polyacrylamide gel electrophoresis, the staining pattern of the membrane proteins of lac(ES)-ghosts was the same as that of unmodified control ghosts (Fig. 4a). Unlike Berg (3) and Bender et al. (1), who used 3 mM p-diazoniumphenyl sulfonic acid at 23°C, we observed no significant destruction of membrane proteins after diazo-coupling at 4°C.

The lac(PS)-vesicles were also white, the diazo-coupling reaction conditions producing a mixed population of large (1-6 μm) right-side-out, inside-out, and multilamellar vesicles whose relative proportions, revealed by freeze-etch electron microscopy, varied among preparations. The fracture faces and etched surfaces of these vesicles appeared identical to those of unmodified inside-out and right-side-out vesicles. After incubation with ferritin-conjugated fluorescein-labeled anti-lac, the density of ferritin molecules on the etched protoplasmic surfaces of the inside-out vesicles was found to be more than 20 times greater than that observed on the etched extracellular surfaces of the right-side-out vesicles, hence the description
Ferritin Monomers, Dimers, and Higher Oligomers in Antibody Conjugate Fractions

| Monomeric ferritin preparation | Antibody conjugate fractions | Corrected values for Peak 2 | Peak 1 | Peak 2* | Peak 3* |
|--------------------------------|-------------------------------|-----------------------------|--------|--------|--------|
| Monomers                       |                               |                             | %      | %      | %      |
| 70                             | Not determined                | 30 (± 6)                    | 43     |
| Dimers§                         |                               | 42 (± 13)                   | 33     |
| Higher oligomers§               |                               | 28 (± 7)                    | 24     |

* Figures given are mean values (±SD) for two fields from two different conjugate preparations, each containing ~200 ferritin molecules. These are observed values, which have not been corrected to account for fortuitous juxtaposition of ferritin monomers on the electron microscope grid. Because all the preparations were stained at approximately equal ferritin densities, such corrections could be made by assuming that the proportions of true monomers appearing as dimers and higher oligomers were the same in each preparation. In the preparation of monomeric ferritin the proportions were 70:21:9. Those in peak 3 were almost identical, indicating that peak 3 contained very few true dimers or oligomers. In peak 2, the percentage of monomers appearing as dimers and oligomers can be calculated from the percentage of observed monomers in this preparation (30% as monomers, hence 9% appearing as dimers and 4% appearing as oligomers, that is, 30:9:4 = 70:21:9).

‡ The corrected percentages of monomers, dimers, and oligomers in conjugate peak 2 was obtained by correcting the observed values for the percentages of monomers appearing as dimers and oligomers; true monomers, 30% + 9% + 4% = 43%; true dimers, 42% - 9% = 33%; true oligomers, 28% - 4% = 24%. These values are approximate, but they clearly illustrate that a substantial proportion of true dimers and oligomers are present in conjugate peak 2.

§ For explanation, see text.

Figure 3 Elution profile of the conjugates of ferritin with antiserum on 6% agarose A-5m (200-400 mesh, Bio-Rad Laboratories) in 0.1 M Tris-HCl, pH 7.6, at 4°C. Column volume 200 ml; flow rate 4 ml/h. Peaks 1-3 are described in text. The small A₃₅₀ peak eluting after peak 3 probably represents unconjugated antibody.

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The Appearance and Distribution of the Proteins on the External Surface of Erythrocyte Ghosts before and after Immunochemical Labeling

The etched extracellular surface of the lac(ES)-ghosts (Fig. 5a) could be labeled by binding of the ferritin-conjugated antibodies to hapten-modified proteins exposed on the surface (Fig. 5b). The degree of particle aggregation in these specimens was only moderate and did not change appreciably on binding the antibody conjugates, whose distribution closely resembled that of the intramembrane particles themselves. The ferritin molecules appeared to be slightly more aggregated than the intramembrane particles, but this was in part an optical illusion due to their greater size and in part a reflection of the oligomeric state of a proportion of the ferritin conjugates (discussed previously). A few isolated ferritin molecules were visible on the etched surface, their numbers corresponding with the rare appearances of isolated intramembrane particles on the fracture faces of these preparations. The binding of the ferritin conjugate was totally inhibited when 10 mM D-lactose was included in the incubation medium (Fig. 5c), demonstrating that binding was due to specific antibody-hapten recognition.

When a higher degree of particle aggregation was first induced in the membranes of lac(ES)-ghosts, the distribution of surface components and bound ferritin conjugates could be more closely correlated with that of the intramembrane particles (Figs. 6, 7). Fig. 6 shows the situation in the absence of ferritin anti-lac conjugate labeling. The exposed extracellular surface shows a texturing which was visible only at very low shadow angles and occurred in limited areas which corresponded clearly and precisely with the distribution of the underlying intramembrane particles. These textured areas are presumably caused by the extracellular portions of membrane proteins projecting beyond the extracellular surface of the phospholipid bilayer (32, 34). Such features were equally apparent in normal (non-lac) ghosts in which particle aggregation had been similarly induced, the lac modification itself having brought about no observable change in the freeze-etch appearance of the membranes.

Fig. 7 shows these membranes after labeling with ferritin anti-lac conjugates. The ferritin clusters on the etched surface always covered the...
textured areas and were contiguous to and continuous with the particle clusters on the fracture face at all points along the fracture-etch boundary. The patterns made by the ferritin and by the particle clusters were virtually indistinguishable (Fig. 7c, d), and no change in particle aggregation was induced by the binding of the ferritin conjugates (cf. Figs. 6, 7). We therefore conclude that all the major proteins accessible to hapten modification on the extracellular surface of the erythrocyte membrane are closely associated with the intramembrane particles. No proteins present in sufficient abundance to be detected by this technique were distributed independently of the aggregated particles. Thus, the smooth areas between the aggregated proteins (whose appearance resembled the freeze-etched surfaces of pure phospholipid bilayers) were virtually free of accessible protein and provided direct views of the lipid head groups of the extracellular leaflet exposed by etching.

The Appearance and Distribution of the Proteins on the Protoplasmic Surface of the Erythrocyte Membrane before and after Immunochemical Labeling

In the fresh erythrocyte ghost, the etched protoplasmic surface showed a roughened appearance (Fig. 8a) due to both the protrusions of the intramembrane particles and the peripheral proteins bound to this surface. The surface roughness was quite different in appearance and much more prominent than the fine texturing seen at low shadow angles on the membrane's extracellular surface (compare with Fig. 6). Figs. 8a, b, c, and 9 form a series showing membranes in which the intramembrane particles were progressively aggre-
Details within these aggregates were hard to discern, but they appeared to be formed by the association of proteins of a globular rather than of a fibrous nature, distinctly different from the clearly fibrous freeze-etch images of the F-actin molecules used by Tillack and Marchesi (45) to label the extracellular surface of erythrocytes. As with the complementary views of these membranes showing the protoplasmic fracture face and extracellular etched surface (Fig. 6), large smooth lipid areas were left between the aggregated proteins. Normal (non-lac) ghosts, inside-out vesicles, and lac(PS)-vesicles appeared the same as these lac(ES)-vesicles when examined at identical degrees of particle aggregation and shadow angle, confirming our conclusion from the chemical studies that the level of hapten modification was insufficient to cause any discernible structural alteration in the membrane.

After immunochemical labeling of inside-out vesicles with ferritin-conjugated antispectrin (Fig. 10) and of inside-out lac(PS)-vesicles with ferritin-conjugated fluoresceinated anti-lac (Fig. 11), the ferritin conjugates were found to be exclusively confined to the roughened areas of aggregated protoplasmic surface protein. No ferritin molecules were detected in those regions which were free of intramembrane particles, and none bound to control membranes incubated in parallel with the experimental samples.
FIGURE 7 (a) and (b) Freeze-etch electron micrographs (× 60,000) of lac(ES)-ghosts after immunochemical labeling. The ghosts were prepared as described in the legend to Fig. 6, then incubated with ferritin-conjugated fluorescein-labeled anti-lac in PBS for 2 h at 0°C and washed twice in PBS. (c) and (d), as (a) and (b), respectively, with boundaries drawn between smooth and textured areas. Bar, 5,000 Å.
FIGURE 8 Freeze-etch electron micrographs (× 60,000) illustrating the appearance of the etched protoplasmic surface of the erythrocyte membrane. (a), (b), and (c) show, respectively, the protoplasmic surface (PS) and extracellular face (EF) of fresh ghosts, of ghosts pretreated in 0.5 mM sodium azide-20 PB, pH 9.0, for 10 h at 37°C, and of inside-out vesicles prepared by incubating fresh ghosts in 0.5 mM Na₂HPO₄, pH 8.2, for 24 h at 0°C. For comparison, the corresponding protoplasmic faces are shown in (d), (e), and (f). Bar, 5,000 Å.

DISCUSSION

The Distribution of Proteins on the Erythrocyte Membrane Surfaces

A number of specific labels have been used in combination with freeze-etching to investigate the distribution of various components on the external surface of the erythrocyte membrane (20, 22, 23, 32, 34, 35, 46). These studies have shown that the distribution of the integral membrane proteins, glycophorin and band 3, as well as the distribution of blood group A antigens, receptors for influenza virus, soybean agglutinin, phytohemagglutinin, and concanavalin A, and unidentified negatively charged sites follows that of the intramembrane particles. These experiments
FIGURE 9  (a) Freeze-etch electron micrograph (× 60,000) of an inside-out vesicle, showing protoplasmic surface and extracellular face views. Membranes prepared as detailed in legend to Fig. 6. (b) as (a), with boundaries drawn to enclose the rough areas on the protoplasmic surface and the areas formerly occupied by particles on the extracellular face. Bar, 5,000 Å.
FIGURE 10 (a), (b), and (c) Freeze-etch electron micrographs (× 85,000, × 85,000, and × 60,000, respectively) of inside-out vesicles after ferritin antispectrin conjugate labeling. Inside-out vesicles, prepared as described in Materials and Methods, were suspended in PBS, pH 5.0, for 30 min at 0°C to aggregate the intramembrane particles, labeled by incubation with ferritin-conjugated antiserum in PBS, pH 5.0, for 2 h at 0°C, and washed first in PBS, pH 5.0, and then 20% PB, pH 5.0. (d), (e), and (f), as (a), (b), and (c), respectively, with boundaries drawn between smooth and textured areas. Bar, 5,000 Å.
Figure 11 (a), (b), and (c) Freeze-etch electron micrographs (× 60,000) of inside-out lac(PS)-vesicles after immunochemical labeling. The vesicles, prepared as described in Materials and Methods, were incubated with ferritin-conjugated fluorescein-labeled anti-lac in PBS for 2 h at 0°C and then washed in PBS. (d), (e), and (f), as (a), (b), and (c), respectively, with boundaries drawn between smooth and textured areas. Bar, 5,000 Å.
strongly suggest that glycophorin and band 3 are components of the intramembrane particles, but they offer no specific information concerning the location of either those minor proteins which are exposed on the extracellular membrane surface (40, 41), or the variety of major peripheral proteins residing on the protoplasmic membrane surface (41).

In the ferritin anti-lac labeling studies presented here, we have employed a nonspecific protein-modifying hapten to test whether a substantial number of the proteins exposed on either membrane surface were not coaggregated with the intramembrane particles. Although we have not determined the degree of lac-labeling of the various membrane proteins, we have shown that in the human erythrocyte there are few if any proteins detectable by this ferritin-immunochemical labeling method whose distribution in the phospholipid bilayer is independent of the integral proteins comprising the aggregated particles. Rather, our results indicate that the majority of proteins on both surfaces of the erythrocyte membrane coaggregate with the intramembrane particles. This conclusion is strongly supported by our previous observations (10) that no proteins were swept into the lipid vesicles blebbing from the surfaces of normal and lac(ES)-ghosts treated with protamine. It is thus clear that the erythrocyte membrane is different from the plasma membrane of other cells, such as the lymphocyte, where mobile surface proteins may be patched and capped without altering the distribution of intramembrane particles (17, 48, 51).

Although experiments in which morphological labels and membrane perturbations (e.g. intramembrane particle aggregation) were used can only suggest the existence of protein-protein interactions in the native membrane, our results accord with cross-linking results demonstrating a close proximity between spectrin and many other membrane proteins (49), with binding studies showing specific interaction between integral and peripheral membrane proteins (2, 44, 53, 54), and with other immunochemical or lectin perturbation studies showing transmembrane effects on integral and peripheral proteins (16, 28). The nature of our treatments and the reversibility of particle aggregation (9, 33) make it unlikely that the spatial distribution of proteins that we have observed is the result of a general denaturation phenomenon. Rather, this distribution suggests that associations exist between all the integral and peripheral protein components of the erythrocyte membrane, the proteins exposed on both sides of the membrane interacting with one another in such a way that immobilization of one component restricts the lateral distribution of the others.

The Molecular Dimensions and Organization of Spectrin on the Cytoplasmic Surface of the Erythrocyte Membrane

Many observations (see reference 18 for review) show that spectrin molecules located on the protoplasmic surface of the erythrocyte membrane (27) play an important structural role in imparting strength and elasticity to the erythrocyte. Following the suggestions of Clarke (6) and Guidotti (15) that spectrin may be a long, fibrous myosin-like protein, Elgsaeter and Branton (9), and later Kirkpatrick (18), published almost identical erythrocyte membrane models in which the spectrin molecules were portrayed as long fibers. Our recent comparative studies of spectrin and myosin by low-angle shadowing indicate that these models may be misleading. Rather than being a myosin-like molecule with two globular heads and a 1,500-Å long supercoiled α-helical tail (21), the spectrin molecule appears to be more compact, 500 Å in length, and composed of three globular domains of approximately equal size. Although the protein aggregates exposed by etching on the cytoplasmic surface of the erythrocyte membrane (Figs. 8, 9) are composed of other proteins in addition to spectrin, their nonfibrous appearance agrees well with this observed shape of the spectrin molecule.

Although spectrin molecules are under study in a number of laboratories (18, 36, 37), few details are available regarding spectrin's self-association or interaction with other components of the membrane. Previous work from our laboratory (9, 10) suggested that a dense cytoplasmic meshwork of spectrin molecules, perhaps together with erythrocyte actin, can limit the translational freedom of the intramembrane particles in fresh ghosts, and led us to postulate that a two-dimensional microprecipitation of residual spectrin molecules on the protoplasmic surface of pretreated ghosts could cause the aggregation of the intramembrane particles. Thus, we predicted that aggregated spectrin molecules should undergo such particle aggregates. The ferritin antispectrin immunochemical labeling studies reported here confirm this to be the case. The existence of an association between spectrin and firmly bound protein com-
component of the membrane has also been inferred from recent recombinant studies using solubilized membrane components (54), and from binding assays using spectrin-depleted vesicles and purified [32P]spectrin (2).

However, one must recognize that none of these studies provides direct information concerning movement of intramembrane particles or their constituent proteins in the intact and unperturbed erythrocyte membrane. Indeed, rotational diffusion studies (5) suggest that the integral protein constituent proteins in the intact and unperturbed membrane components (54), and from binding assays using spectrin-depleted vesicles and purified [32P]spectrin (2).

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