The Transmembrane Proteins in the Plasma Membrane of Normal Human Erythrocytes. Evaluation Employing Lactoperoxidase and Proteases†

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ABSTRACT: The molecular architecture of the human erythrocyte membrane has been probed using lactoperoxidase-catalyzed iodination in conjunction with Pronase hydrolysis. Resealed, hemoglobin-free ghosts were labeled at the cytoplasmic surface and the external membrane surface was subsequently digested with Pronase. Changes in size of the components labeled at the cytoplasmic surface were readily detected by sodium dodecyl sulfate gel electrophoresis. The protein 3 molecular weight class labeled at the cytoplasmic surface was extensively hydrolyzed at the external surface to produce a major 65000 molecular weight fragment and a minor 45000 molecular weight fragment. When resealed membranes were labeled on the external surface the same 65000 molecular weight labeled component is produced. These results unequivocally demonstrate that the same polypeptides in the protein 3 molecular weight class that can be labeled by lactoperoxidase at the cytoplasmic membrane surface are digested by Pronase at the external surface and are, therefore, transmembrane components. Where it is possible to label one surface of a membrane with lactoperoxidase and reseal the membrane this procedure represents an alternate method for establishing transmembrane configuration of membrane proteins.

Studies of the normal human erythrocyte membrane have provided the basis for much of the information on the arrangement of membrane proteins and lipids. The use of nonpenetrating membrane probes has clearly established that the proteins in the erythrocyte membrane are asymmetrically organized and that the outside of the membrane contains relatively few proteins while the inner face of the membrane contains most of the membrane protein (for recent reviews, see Wallach, 1972; Juliano, 1973; Zwaal et al., 1973; Singer, 1974; Steck, 1974). This evidence has further suggested that all of the glycoproteins which exist on the outer surface of the membrane may have transmembrane orientations (Morrison et al., 1974; Mueller and Morrison, 1974).

Several approaches have been used to demonstrate the presence of transmembrane components in the human erythrocyte membrane. These include: comparison of peptide digestion products of proteins isolated from intact red cells and open stroma preparations that had been labeled with nonpenetrating reagents (Bretscher, 1971a,b; Segrest et al., 1973); proteolytic digestion of inside-out and right-side-out vesicles (Steck, 1972); and lactoperoxidase-catalyzed iodination of the exterior and interior surface of sealed ghosts (Reichstein and Blostein, 1973; Boxer et al., 1974; Morrison et al., 1974; Mueller and Morrison, 1974; Shin and Carraway, 1974).

The results presented in this paper illustrate another approach for detecting transmembrane components in membranes. The procedure employs Pronase digestion at the *external* surface of sealed erythrocyte ghosts that had been previously labeled at the *internal* membrane surface with the lactoperoxidase iodination technique.

Materials and Methods

Lactoperoxidase was isolated as described by Morrison and Hultquist (1963). The nuclides ^{125}I and ^{131}I were obtained from Schwarz/Mann and New England Nuclear, respectively. Catalase (2× crystallized), glucose oxidase (type VI), Tos-Arg-OMe,[1] and $PhCH_2SO_2F$ were purchased from Sigma Chemical Co. Pronase, B grade, was a product of Calbiochem. Dextran T250 was a product of Pharmacia Fine Chemicals. All other reagents were of reagent grade or better.

Membrane Preparation. Packed red cells, obtained from bank blood not more than 2 weeks old, were washed three times with cold 0.9% saline and one time with cold isotonic phosphate buffer (pH 7.4), care being taken to remove the "buffy coat". Hemoglobin-free, lysed stroma were prepared by hemolysis and washing in cold 20 mosm (hypotonic) phosphate buffer (pH 7.4).

Resealing. Resealed ghosts were prepared by diluting packed, hemoglobin-free stroma 1:5 with resealing medium containing hypotonic phosphate buffer (pH 7.4), 0.18 *M* NaCl, and 1.2 m*M* $CaCl_2$. The ghosts were then incubated at 37° for 45 min with gentle shaking. Ghosts to be subsequently labeled at the cytoplasmic membrane surface were resealed in the presence of 0.15 μM lactoperoxidase and 10–15 munits/ml of glucose oxidase, whereas ghosts to be labeled at the external surface were resealed in the presence of 10–15 munits/ml of glucose oxidase. Following the sealing period, the membranes were sedimented at 20000*g* for 10–15 min and washed four times with approximately 20 volumes of cold resealing medium (hypotonic phosphate buffer, 0.15 *M* NaCl–1 m*M* $CaCl_2$ (pH 7.4)).

Iodination. The packed, resealed ghosts, which occupy approximately 40–50% of the volume of packed, lysed stroma, were resuspended with resealing medium to the original volume of packed stroma.

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[1] Abbreviations used are: Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; PAS, periodic acid–Schiff; LP, lactoperoxidase.

External iodination of resealed ghosts with glucose oxidase sealed inside was accomplished by incubation for 1 hr at 25° in the presence of 0.20–0.25 mCi of carrier-free ^{125}I or ^{131}I per ml, 1.6 m*M* glucose, and 0.075 μM lactoperoxidase. Uptake of glucose into the sealed ghosts results in the enzymatic production of hydrogen peroxide inside the vesicles. Since the membrane is freely permeable to peroxide, iodination by *external* lactoperoxidase is readily effected.

Internal iodination of resealed ghosts which had both lactoperoxidase and glucose oxidase sealed inside was carried out by incubation at 25° for 1 hr in the presence of 0.20–0.25 mCi of carrier-free ^{125}I or ^{131}I per ml, 1.6 m*M* glucose, and 8–10 μM catalase. The presence of residual external lactoperoxidase during internal iodination could lead to spurious results. Thus during internal labeling of resealed ghosts, external catalase was added to inhibit iodination by any residual external lactoperoxidase that was not removed by the washing procedures.

Following iodination, the labeled vesicles were washed one time with 60 μM KI in resealing medium and one time with resealing medium. The sealed ghosts were then separated from stroma which failed to reseal by centrifugation on discontinuous dextran 250 gradients (2.5–10% w/v) at 70000*g* for 10–15 hr. This is a modification of a procedure described by Steck et al. (1971). The sealed ghosts were removed from the top of the gradient, diluted with resealing medium, sedimented at 20000*g* for 15 min, and washed 2–3 times with resealing medium.

Proteolytic Digestion. Proteolytic digestion was carried out on 10% suspensions of labeled, resealed ghosts which had been isolated from the dextran gradients. The ghosts were treated at 37° with 10μg/ml of Pronase for 2 hr. Pronase digestion was inhibited by the addition of 5 μl/ml of $PhCH_2SO_2F$ (6.8 mg/ml in methanol; final concentration 0.2 m*M*). Following addition of the inhibitor, incubation was conducted for an additional 15 min at 37°, and the suspension was centrifuged at 20000*g* for 15 min. The sealed membranes were washed three additional times with approximately 100 volumes of cold resealing medium containing 0.2 m*M* $PhCH_2SO_2F$. The resealed ghosts were then lysed in cold hypotonic phosphate buffer containing 1.6 m*M* Tos-Arg-OMe and 0.2 m*M* $PhCH_2SO_2F$ and centrifuged to isolate the membranes. The packed membranes (200–500 μl) were diluted with 200 μl of hot gel sample buffer containing 0.01 *M* phosphate buffer (pH 7.4), 2% dodecyl sulfate, 2% mercaptoethanol, and 8 *M* urea, and immediately boiled for 3 min.

Dodecyl Sulfate Gel Electrophoresis. Dodecyl sulfate gel electrophoresis was performed on 7.5% polyacrylamide gels containing 0.1% dodecyl sulfate in 0.1 *M* phosphate buffer (pH 7.2–7.4). Membrane samples were prepared by boiling for 3 min in at least an equal volume of sample buffer containing 0.01 *M* phosphate buffer (pH 7.4), 2% dodecyl sulfate, 2% mercaptoethanol, and 8 *M* urea. The distribution of radioactivity was determined by cutting the gels into 2-mm slices and counting in a Nuclear Chicago γ spectrometer. Molecular weight markers employed were β-galactosidase (mol wt 130000), bovine serum albumin (mol wt 68000), ovalbumin (mol wt 43000), carbonic anhydrase (mol wt 29000), and myoglobin (mol wt 17200). Dansylated bovine serum albumin and carbonic anhydrase were frequently employed as internal fluorescent standards in gels to be cut and counted (Talbot and Yphantis, 1971). Gels were stained for protein with Coomassie Brilliant Blue using a modification of the method of Weber and Osborn (1969). Glycoproteins were visualized using the periodic acid–Schiff (PAS) procedure (Zacharias et al., 1969).

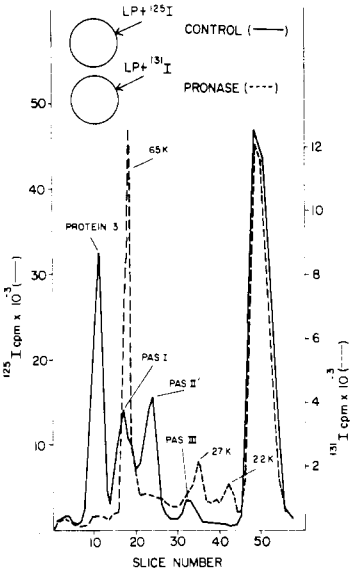

FIGURE 1: The effect of Pronase digestion on the distribution of iodine label in polypeptides isolated from resealed ghosts labeled externally with the lactoperoxidase iodination technique. Control resealed ghosts were labeled externally with ^{125}I (—). Resealed ghosts labeled externally with ^{131}I were subsequently digested with 10 μg/ml of Pronase (- - -). The solubilized membrane samples were coelectrophoresed on a 7.5% dodecyl sulfate acrylamide gel.

Results

The effects of Pronase treatment on resealed ghosts labeled at the *external* surface are illustrated in Figure 1. The control membranes were labeled with ^{125}I and the Pronase treated resealed ghosts were labeled with ^{131}I. In agreement with previous results, external labeling with lactoperoxidase results in the iodination of protein 3, three sialoglycoproteins designated PAS I, II′, and III, as well as a low molecular weight component (Morrison et al., 1974; Mueller and Morrison, 1974). Slicing and counting a gel previously stained for protein demonstrates that the radioactive peak designated protein 3 corresponds with the Coomassie stained peak designated protein 3 by Steck et al. (1971). The mobilities of the components designated PAS I, II′, and III are all increased following treatment with neuraminidase, demonstrating that they are sialoglycoproteins. In addition, these components can be isolated and purified using the procedure of Hamaguchi and Cleve (1972), and the radioactive peaks correspond with PAS staining components (Mueller and Morrison, 1974).

Pronase digestion results in extensive hydrolysis of protein 3 and the appearance of a heavily labeled component which migrates with an apparent molecular weight of 65000. Analysis of gels stained for protein also reveals the loss of staining material in the protein 3 region and the appearance of a fragment of 65000 molecular weight. Pronase also causes extensive degradation of PAS I and II′ and the appearance of two smaller labeled fragments of approximately 27000 and 22000 molecular weight. The loss of the labeled PAS I component is not readily discerned in this figure since the 65000 molecular weight fragment migrates in the same region of the gel. However, analysis by PAS staining of the gel reveals the loss of the PAS I band. In addition, no labeled component is found in the PAS I region following electrophoresis of the glycoproteins isolated from

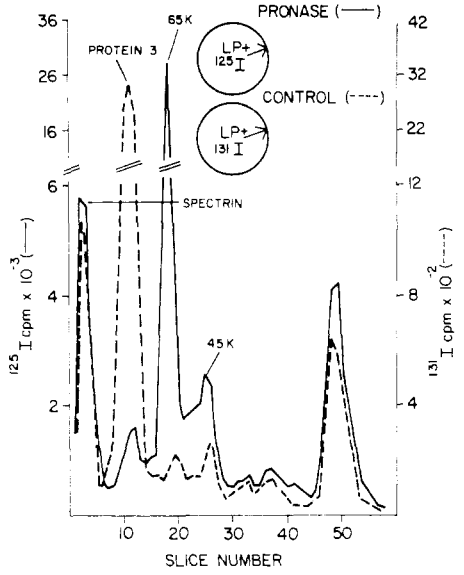

FIGURE 2: The effect of Pronase digestion on the distribution of iodine label in the polypeptides isolated from resealed ghosts labeled internally with the lactoperoxidase technique. Control resealed ghosts were labeled internally with ^{131}I (- - -). Resealed ghosts labeled with ^{125}I were subsequently treated with 10 μg/ml of Pronase (—). The solubilized membrane samples were coelectrophoresed on a 7.5% dodecyl sulfate acrylamide gel.

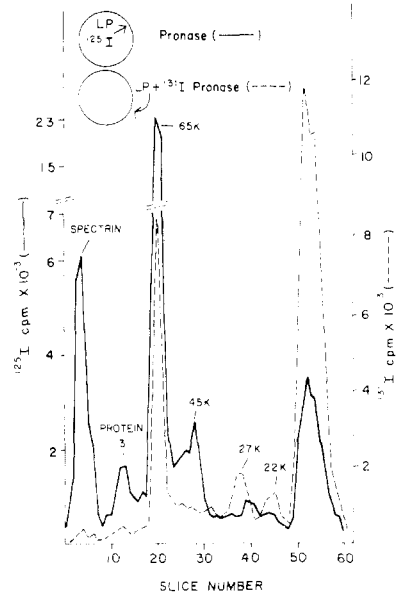

FIGURE 3: The effect of Pronase digestion on the distribution of iodine label in polypeptides isolated from resealed ghosts labeled either externally or internally using the lactoperoxidase iodination technique. Resealed ghosts, which had been labeled internally with ^{125}I (—) or externally with ^{131}I (- - -), were digested with 10 μg/ml of Pronase. The solubilized membrane samples were then coelectrophoresed on a 7.5% dodecyl sulfate acrylamide gel.

Pronase-treated membranes. Pronase treatment under the conditions employed liberated approximately 15% of the membrane associated radioactivity.

In an attempt to identify more conclusively which proteins of the erythrocyte membrane are truly "transmembrane" in nature, we have labeled resealed ghosts from the inside with lactoperoxidase. Following dextran gradient centrifugation to remove any unsealed membranes, the resealed ghosts were digested at the external surface with Pronase. The results are illustrated in Figure 2. Untreated resealed ghosts were labeled internally with ^{131}I, while Pronase treated resealed ghosts were labeled on the inside with ^{125}I. Most of the internal label is incorporated into two components. The most prominent of these migrates in the region of protein 3. A high molecular weight class of proteins, which on Coomassie-stained gels corresponds with the spectrin components, is also labeled at the cytoplasmic membrane surface. Almost all of the other components of the membrane are also labeled, but not as extensively. Pronase digestion at the external surface results in extensive hydrolysis of the labeled components migrating in the protein 3 region of the gel, with the appearance of a fragment of 65000 molecular weight and a minor fragment of 45000 molecular weight. Under the conditions employed, protein 3 is not totally digested. Whether this represents incomplete digestion, or the presence of other peptides which are more resistant to Pronase hydrolysis, is not known. Little hydrolysis of "spectrin" is observed, suggesting that the bulk of the ghosts remain sealed. The labeled spectrin components are not resistant to proteolysis since digestion of lysed membranes leads to extensive digestion (see also Bender et al., 1971; Carraway et al., 1971; Speth et al., 1972; Steck, 1972; Carter et al., 1973).

Figure 3 compares the effects of Pronase digestion at the external surface of membranes that had been labeled at *both* the external and internal surfaces. It can be seen that the 65000 molecular weight digestion products from both samples comigrate. Thus most of the iodine incorporated into protein 3 at both membrane surfaces comigrates both before and after Pronase digestion, suggesting that the same component is labeled at both membrane surfaces. This indicates that most of the material in the protein 3 region of the gel may consist of a single component, or a group of very similar proteins which span the membrane.

Discussion

Work from several laboratories (Bretscher, 1971a; Steck, 1972; Reichstein and Blostein, 1973; Boxer et al., 1974; Mueller and Morrison, 1974; Shin and Carraway, 1974) has suggested the transmembrane disposition of protein 3 in the human erythrocyte. Bretscher (1971a) employed chemical labeling of both intact red cells and lysed stroma followed by dodecyl sulfate gel electrophoresis of the labeled peptides. Peptide mapping of the protein 3 region of the gel revealed that additional sites were labeled in the open stroma preparation that were not labeled in the intact cell. Bretscher (1971a) proposed that these new sites were actually on the internal surface of the membrane, since gross alteration of the membrane during lysis, exposing cryptic sites on the external surface, would not be expected to occur. However, several recent reports (Zwaal et al., 1973; Morrison et al., 1974; Mueller and Morrison, 1974; Staros et al., 1974) indicate that such alterations of the external membrane surface may in fact occur.

Other workers have attempted to show the transmembrane orientation of protein 3 using lactoperoxidase-catalyzed iodination of both the cytoplasmic and external membrane surfaces of resealed ghosts. The studies resulted in iodine incorporation into protein 3 at both membrane surfaces (Reichstein and Blostein, 1973; Boxer et al., 1974; Mueller and Morrison, 1974; Shin and Carraway, 1974). However, several reports (Bretscher, 1971a; Phillips and Morrison, 1971; Knüfermann et al., 1973; Bhakdi et al., 1974; Findlay, 1974; Steck, 1974; Anselstetter and Horstmann, 1975; Knüfermann et al., 1975; Roses et al., 1975)

have suggested that the protein 3 region in dodecyl sulfate gels is heterogeneous. In addition, the red cell Na^+-K^+-ATPase (Avruch and Fairbanks, 1972; Williams, 1972; Knauf et al., 1974), acetylcholinesterase (Bellhorn et al., 1970), the glucose transport protein (Taverna and Langdon, 1973; Lin and Spudich, 1974), and the anion transporter (Cabantchik and Rothstein, 1972, 1974a,b; Ho and Guidotti, 1975) have all been ascribed to proteins migrating in this region of the gel. This raises the possibility that the above mentioned probes may not be acting on the same component at each membrane surface, but on different components migrating together on the gels. The possibility also exists that the same protein may not exist in a transmembrane orientation, but may exist as a discrete entity on each side of the membrane.

Steck (1972) has presented convincing evidence for the transmembrane nature of band 3 polypeptides utilizing proteolytic digestion of sealed inside-out and right-side-out vesicles. Digestion at either membrane surface resulted in the loss of the bulk of the Coomassie staining material in the protein 3 region, indicating that at least a portion of the band 3 peptides are transmembrane components.

The results presented in this paper confirm and extend the previous studies from other laboratories suggesting that protein 3 is a transmembrane component. These results, as well as similar data reported by Reichstein and Blostein (1974), illustrate an alternative approach for convincingly demonstrating the presence of transmembrane components in the human erythrocyte. These data in regard to protein 3 are unequivocal, since the same protein molecule labeled at one surface of a sealed membrane was altered by proteolytic digestion at the opposite membrane surface.

A somewhat similar approach—labeling at one membrane surface in conjunction with proteolysis at the opposite surface—has also been utilized with *intact* erythrocytes. Whiteley and Berg (1974) labeled the proteins at the cytoplasmic surface with a "penetrating", amidinating reagent, following saturation of the external reactive sites with an unlabeled, "nonpenetrating" reagent. Subsequent digestion with Pronase at the external surface produced several labeled peptides with altered electrophoretic mobilities, among them protein 3. However, since this reagent might also label proteins within the membrane, the results may not distinguish a protein which spans the membrane from one which only extends into the bilayer.

Several workers (Bender et al., 1971; Bretscher, 1971a; Phillips and Morrison, 1971; Hubbard and Cohn, 1972; Steck, 1972; Triplett and Carraway, 1972; Boxer et al., 1974; Cabantchik and Rothstein, 1974b; Reichstein and Blostein, 1974) have reported the digestion of protein 3 to a 60000–73000 molecular weight fragment. Steck (1972) and Cabantchik and Rothstein (1974b) have observed the appearance of both a major 65000 and a minor 35000–45000 molecular weight peptide following digestion of intact red cells with chymotrypsin or Pronase. We have also observed two fragments of 65000 and 45000 molecular weights following Pronase digestion, both of which are labeled at the cytoplasmic membrane surface by lactoperoxidase. Several interpretations of these data are plausible. One explanation is that the 65000 and 45000 molecular weight fragments are each derived from a separate peptide migrating in the protein 3 region of the gel. Alternatively, they may arise from the same peptide. However, if the two fragments do indeed arise from the same component, then two alternate explanations are feasible. The smaller fragment may arise from further digestion of the larger fragment. However, digestion with higher concentrations of Pronase leads to no apparent increase in the smaller fragment at the expense of the larger component. Alternatively, the original peptide may form a loop in the membrane with two distinct and discontinuous regions of the peptide exposed at the cytoplasmic surface and separated by a segment of the peptide chain that is exposed at the external surface and contains the Pronase sensitive site. This type of conformation would be compatible with the "S-shaped" structure recently proposed by Jenkins and Tanner (1975). In addition our data would suggest that the minor fragment produced by Pronase digestion *also* extends through the bilayer and is accessible at the cytoplasmic membrane surface. Further work is in progress to clarify this.

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The Isolation and Structure of the Core Oligosaccharide Sequences of IgM†

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ABSTRACT: Methods are presented for separating the three IgM heavy chain sialoglycopeptides associated with asparagines 170, 332, and 395. The core glycopeptide units containing the disaccharide fucosyl-*N*-acetylglucosamine were obtained through the use of an endo-β-*N*-acetylglucosaminidase from *Diplococcus pneumoniae,* following exoglycosidase treatment of the sialoglycopeptides. In addition to the core glycopeptides, high yields of a tetrasaccharide, $(Man)_3GlcNAc$, were obtained. The fucose in the core disaccharide is glycosidically linked to the 6-O position of the *N*-acetylglucosamine residue in Asn-GlcNAc. This core unit is resistant to glycosyl asparaginase, but becomes susceptible to hydrolysis on removal of the fucosyl residue by a purified hen oviduct α-L-fucosidase. The core sequence of the immunoglobulin M sialoglycopeptides appears to be similar to that of most other asparagine-linked oligosaccharides in consisting of a basic unit composed of β-D-Man(1→4)β-D-GlcNAc(1→4)β-D-GlcNAc(1→4), but with L-fucose linked α-(1→6) to the proximal GlcNAc. The two nonreducing terminal ends of $(Man)_3GlcNAc$ are linked to β-D-Man by α-(1→3) and α-(1→6) bonds, respectively.

Following our demonstration (Tarentino et al., 1970; Sukeno et al., 1971) that the "core" glycopeptide region of ribonuclease B consists of β-D-Man(1→4)β-D-GlcNAc(1→4)β-D-GlcNAc-Asn,[1] studies on glycopeptides derived from several glycoproteins have revealed this sequence to be common to both neutral and acidic oligosaccharides linked to asparagine (Kabasawa and Hirs, 1972; Lee and Socca, 1972; Sugahara et al., 1972; Sukeno et al., 1972; Tarentino et al., 1972, 1973; Baenziger et al., 1974; Baenziger and Kornfeld, 1974; Spik et al., 1975). Pertinent to these findings is the association of one or the other type of oligosaccharide with some glycoproteins (ovalbumin, deoxyribonuclease A, ribonuclease B, and IgG) and of both types with others (thyroglobulin and IgM).

It was shown recently by us (Tarentino et al., 1974) that an endoglycosidase from *Streptomyces griseus* is capable of releasing neutral oligosaccharides from glycoproteins or glycopeptides by hydrolyzing the di-*N*-acetylchitobiosyl residue in the core region. This enzyme, unfortunately, cannot release acidic oligosaccharides from glycoproteins or

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[1] Abbreviations used are: Asn-GlcNAc, 2-acetamido-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; GlcNAc, 2-acetamido-2-deoxy-β-D-glucosamine; GlcN, 2-amino-2-deoxy-β-D-glucosamine; Gal, galactose; Fuc, fucose; Man, mannose; PGA, pyroglutamate; Me_3Si, trimethylsilyl.