PEPTIDE HETEROGENEITY IN THE BAND 3 PROTEIN OF RABBIT ERYTHROCYTE PLASMA MEMBRANES

E. R. Vimr and J. R. Carter, Jr., M.D.*

Department of Medicine
Case Western Reserve University
Cleveland, Ohio 44106

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SUMMARY: We have examined the band 3 protein(s) of rabbit erythrocyte membranes by a combination of differential extraction and surface labeling methods. Only one major peptide was labeled when intact red cells were exposed to $^{125}I^-$ and lactoperoxidase; this coincided with band 3. When intact cells were exposed to galactose oxidase followed by [^{3}H]borohydride, numerous surface glycoproteins were labeled, one of which clearly coincided with band 3. Differential extraction with lithium diiodosalicylate revealed one major band 3 glycoprotein which contained both the $^{125}I^-$ and ^{3}H surface labels and three peptides which were unlabeled; these three peptides are apparently not exposed at the cell surface.

The protein composition of mature red cell membranes, as demonstrated by polyacrylamide gel electrophoresis, is similar in all mammalian species so far examined, with 50-60 per cent by weight composed of the two "spectrin" molecules and band 3 (1, 2). Glycoprotein composition, as assessed by periodic acid-Schiff staining of polyacrylamide gels, is much more variable (2); rabbit red cell membranes, with which we have been working, show no major glycoprotein by this technique. However, Gahmberg (3) has shown, with specific labeling of galactosyl residues with [^{3}H]borohydride and galactose oxidase, that many more glycoproteins are present in human erythrocyte membranes than can be observed with periodic acid Schiff staining.

Considerable interest has focused on band 3 due to its quantitative importance and because it is a glycosylated peptide which spans the full width of the erythrocyte membrane (4, 5). Several specific functions have now been associated with this glycopeptide (6-8). There has been disagreement as to whether this

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diffuse band represents a single peptide species with variable glycosylation (9) or several co-migrating species (10). We report here experiments indicating that band 3 in rabbit erythrocyte membranes is composed of at least one glycosylated, surface-exposed peptide and three nonglycosylated peptides of closely similar molecular weight which are apparently not exposed at the cell surface.

Materials & Methods

Materials: Lithium diiodosalicylate was obtained from Eastman Kodak Co. Galactose oxidase and lactoperoxidase were from Sigma Chemical Co. Carrier-free $^{125}I^-$ (11-17mCi/μg) and sodium [^{3}H]borohydride (10Ci/mol) were from Amersham/Searle. All other reagents were reagent grade.

Preparation and Labeling of Red Cells: Rabbit blood was collected from an ear vein into a heparinized beaker. Red cells were washed three times in NKM-PO_4[1] buffer. For galactose labeling, washed packed cells were diluted in 5 volumes of NKM-PO_4 buffer and treated essentially as described by Gahmberg (3). To 0.5ml of cell suspension at 37° was added 12.75 units of galactose oxidase; the cells were incubated for 1 hour with intermittent gentle shaking, then washed twice with NKM-PO_4 buffer and resuspended in 0.5ml of the same buffer at 37°. Reduction of galactose residues was performed for 30 minutes with the addition of 1mCi of [^{3}H]borohydride. The reaction was stopped by the addition of 10 volumes of ice cold NKM-PO_4 buffer and the cells washed three times. Only terminal galactose residues are labeled with this method. Controls incubated without galactose oxidase incorporated only 1-2% as much ^{3}H as cells exposed to the enzyme.

Intact erythrocytes suspended in NKM-PO_4 buffer were labeled on the external surface with $^{125}I^-$ in the presence of peroxidase and H_2O_2 exactly as described by Morrison (11).

Preparation and Gel Analysis of Membranes: After labeling intact cells, red cell "ghost" membranes were prepared as described by Cabantchik and Rothstein (8). Membrane proteins were fractionated in sodium dodecyl sulfate and dithiothreitol on 5.6% polyacrylamide gels as described by Fairbanks et al (1), stained with Coomassie Blue, and scanned at 580 nm with an Isco model 659 gel scanner. The gels were then sliced at 1 or 2 mm intervals in a Mickle Gel-Slicer. ^{125}I in each slice was determined directly in a well-type gamma counter. To determine ^{3}H incorporation, slices were dissolved in H_2O_2 and radioactivity determined in a toluene-based fluor in a liquid scintillation counter.

Results & Discussion

When rabbit erythrocyte membrane glycoproteins were labeled with tritiated borohydride and analyzed by polyacrylamide gel (Fig. 1), a number of radioactive peaks were observed; the majority of these were at a molecular weight < 90,000 and did not coincide with specific Coomassie Blue-stained peptides. The single

1) The abbreviations used are: NKM-PO_4 buffer: 115mM NaCl, 3.75mM KCl, 3.75mM $MgCl_2$, 30mM phosphate buffer pH 7.5; LIS, lithium diiodosalicylate.

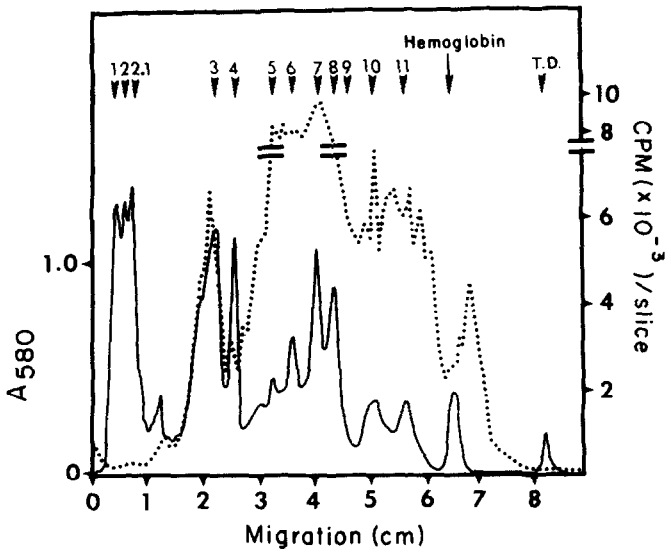

Fig. 1 Peptide and [^{3}H]galactose profiles of rabbit erythrocyte membrane proteins labeled with sodium [^{3}H]borohydride after oxidation with galactose oxidase. Protein band numbering is according to Koch et al (12). Solid line, profile of Coomassie Blue stained peptides (A_{580}); Dotted line, tritium /1 mm gel slice.

Table I. Extraction of rabbit erythrocyte membranes with lithium diiodosalicylate.[a]

LIS Concentration (mM)	% Protein Extracted	% [^{3}H]Galactose Extracted
0	10	12
10	19	8
30	30	8
50	38	11
100	65	35
200	67	41

[a]Extractions were performed as described by Furthmayer et al (13). Each extraction with the indicated concentration of LIS was performed using 1mg of membrane protein containing 3.7×10^6 tritium cpm.

radioactive peak migrating at a molecular weight of 100,000 clearly corresponded to band 3 (Fig. 1).

When labeled membranes were extracted with increasing concentrations of LIS, the amount of protein extracted increased steadily with each increment in LIS (Table I). In contrast, a significant increase in [^{3}H]galactose extracted was not observed until 100mM LIS was reached (Table I).

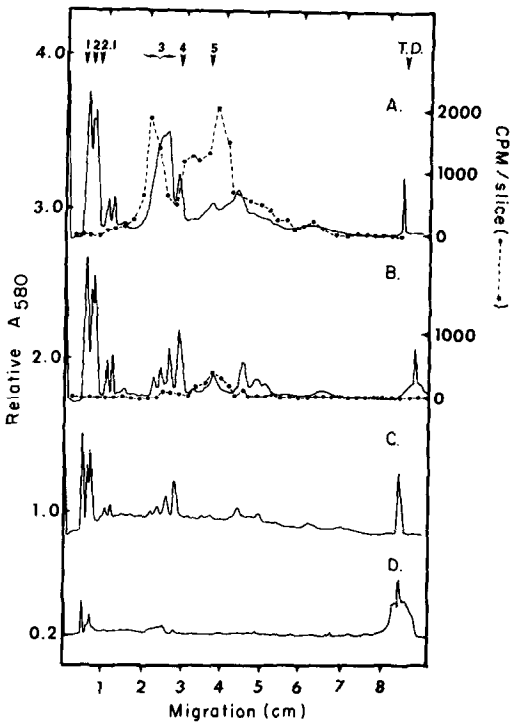

Fig. 2 Identification of erythrocyte membrane proteins extracted by LIS and their extent of glycosylation. From the experiment described in Table I, equal volumes (50µl) of control, 10, 50 and 100mM LIS extracts were fractionated, stained and scanned on polyacrylamide gels as described in Methods (solid line). In addition, the radioactive profiles of the 100mM and 50mM extracts were determined by counting 2 mm gel slices (broken line). A, 100mM LIS extract; B, 50mM; C, 10mM; D, 0mM.

Polyacrylamide gel analysis of the LIS extracts revealed a striking difference in the band 3 region (Fig. 2). The 50mM LIS extract, separated and stained with Coomassie Blue, showed three small but discrete peaks in the band 3 region (Fig. 2B), whereas the 100mM extract (Fig. 2A) showed the typical broad, diffuse band observed in intact membranes (Fig. 1). When these gels were sliced and the presence of [^{3}H]galactose determined, a large peak coinciding with the trailing edge of band 3 was observed in the 100mM LIS extract (Fig. 2A); virtually none was seen coinciding with the three peaks in the 50mM extract (Fig. 2B).

Externally oriented membrane proteins can be labeled with ^{125}I in the presence of lactoperoxidase and H_2O_2; this technique has been used to show that

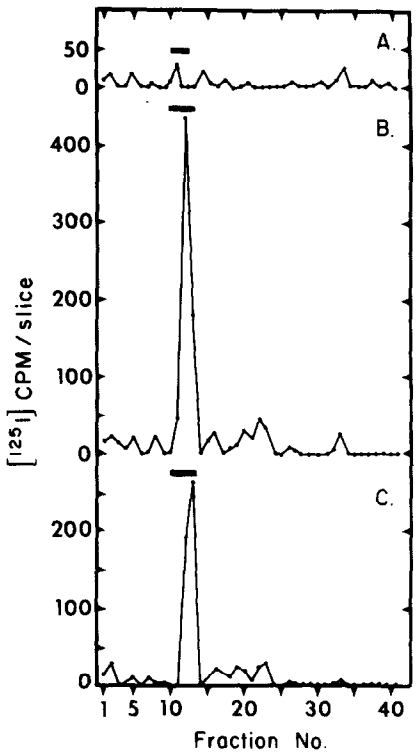

Fig. 3 Polyacrylamide gel fractionation of ^{125}I-labeled membrane proteins. A, 50mM LIS extract; B, 100mM LIS extract; C, unextracted membranes. The horizontal bars indicate the region of each gel containing stained protein of band 3. Approximately 25μg of protein was fractionated on each gel.

band 3 is in fact exposed on the outer surface of the cell (5). We combined this technique with differential extraction of rabbit erythrocyte membranes with LIS to examine further the possibility of heterogeneity in band 3 (Fig. 3). Electrophoresis of intact labeled membranes (Fig. 3C) indicated that only one major peptide, band 3, was labeled; this is in contrast to human membranes, where glycophorin is also labeled by this technique, and coincides with the absence of a major PAS-stainable band in rabbit membranes. The 50mM LIS extract (Fig. 3A) containing the three discrete peaks within the band 3 region showed essentially no radioactivity. On the other hand, the 100mM LIS extract (Fig. 3B) contained the sharp peak of ^{125}I-labeled peptide observed in the intact membranes.

It is thus apparent that "band 3" in rabbit erythrocyte membranes is composed of at least 4 peptide species. One of these is glycosylated, spans the cell membrane, is extracted with 100mM LIS and appears to represent the majority (∿ 75%) of the protein in band 3; whether this represents one or more peptide species is not evident from the present studies. In addition there are three smaller, discrete peaks which contain no terminal galactosyl residues and do not appear to be exposed to the outer cell surface; these peaks are not clearly defined when intact membranes are examined because of preponderance of the glycoprotein. At the present time, there is no data to indicate whether these smaller peptides have any association with the band 3 glycoprotein within the membrane or simply co-migrate with it by virtue of similar molecular weights. In the case of anion transport, the studies of Cabantchik and Rothstein (14) would suggest that transport is associated with the glycoprotein itself. Of the other functions assigned to band 3, it would be logical to assume that water and glucose transport might also be associated with the transmembrane glycoprotein.

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