Glucose Transport Carrier in Human Erythrocyte Membranes

DINITROPHENYLATION OF A MEMBRANE COMPONENT MODIFIED BY D-GLUCOSE*

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CHAN Y. JUNG AND LINDA M. CARLSON

From the Nuclear Medicine Service, Veterans Administration Hospital, and Department of Biophysical Sciences, State University of New York at Buffalo, Buffalo, New York 14215

SUMMARY

The effect of D-glucose on dinitrophenylation of membrane proteins of human erythrocyte ghosts by 1-fluoro-2,4-dinitrobenzene was studied in the absence and in the presence of D-glucose. A double isotopic, differential labeling technique followed by gel electrophoresis of extracts in the presence of sodium dodecyl sulfate revealed the presence of a polypeptide, or polypeptides, in human erythrocyte membranes, the reactivity of which to 1-fluoro-2,4-dinitrobenzene was significantly enhanced in the presence of D-glucose. Molecular weights of the peptides were estimated to be approximately 180,000. The effective differential labeling of the peptides required specific conditions identical with the ones which maximize the differential between inactivation of the carrier by 1-fluoro-2,4-dinitrobenzene and the dinitrophenylation of bulk membrane (Jung, C. Y. (1974) J. Biol. Chem. 249, 3568). A differential labeling was also demonstrated as a single peak by LH-20 column chromatography of chloroform-methanol-soluble membrane components. The peak contained both protein and phospholipids, indicating its proteolipid-like nature. A possible relationship of the differentially labeled polypeptides to the glucose carrier is discussed.

A major difficulty in isolating membrane carriers is that it is impossible to test the isolated carrier for the transport function once it has been removed from the membrane. This difficulty can be avoided if the carrier is labeled prior to its isolation. FDNB, a protein reagent, is a potent, irreversible inhibitor of carrier-mediated glucose transport across human erythrocyte membranes (1). Inactivation by this reagent is characteristically modified in the presence of substrates (2-6). Attempts to use this fact in labeling the glucose carrier selectively have been made with little success (2, 7). Recent work in our laboratory (8) indicates that, under certain conditions, D-glucose significantly enhances the reaction of the carrier to FDNB, but not the concomitant bulk membrane dinitrophenylation. Conditions have been found in which this differential labeling can be optimized. The present study, using hemoglobin-free, carrier-active ghosts (9) and adopting a double isotope, differential labeling technique (10), demonstrated a sharp region on sodium dodecyl sulfate gel electrophoresis of the membrane proteins, which was dinitrophenylated much more readily in the presence of D-glucose. Such a differential labeling was also demonstrated on LH-20 column chromatography of chloroform-methanol-soluble membrane components.

EXPERIMENTAL PROCEDURE

Materials—d-[14C]Glucose, [14C]FDNB, and [3H]FDNB were obtained from Amersham/Searle, Arlington Heights, Ill. D-Glucose, n-mannitol, and FDNB were purchased from K & K Laboratories, Plainview, N. Y. Fresh human blood was obtained from healthy donors by venipuncture. Glucose carrier-active, resuled erythrocyte ghosts practically free of hemoglobin were used. The method of preparation and chemical and functional properties of the ghosts are described elsewhere (9, 11).

Single and Double Isotope Labeling of Ghosts with FDNB—Single isotope labeling of ghosts with FDNB was carried out as described previously (8). For double isotope, differential labeling of ghosts with FDNB, the general principle of Fox and Kennedy (10) was adopted. The ghost preparation was divided into two equal parts and each was allowed to react with FDNB in an identical manner, except that one included a tracer amount of [14C]-FDNB in the presence of 50 mM D-glucose, and the other a tracer amount of [3H]FDNB in the absence of D-glucose. In some experiments, the [14C]FDNB and [3H]FDNB were interchanged. Typically, the reaction mixture contained 5 mg of ghost protein and 18 &mu;mol of FDNB, with 40 &mu;Ci of [14C]FDNB or 80 &mu;Ci of [3H]FDNB in a total of 4 ml of 1:10 isotonic balanced salt solution (Na+, 12.5 mM; K+, 0.5 mM; Ca++, 0.38 mM; Mg++, 0.25 mM; all as chlorides) buffered at pH 8.0 with 10 mM Tris-HCl. At the end of the reaction, the two parts were washed with prechilled buffer to free the unreacted FDNB, and pooled. The pooled membrane pellets were solubilized with sodium dodecyl sulfate for gel electrophoresis or were extracted with a chloroform-methanol (2:1, v/v) mixture. Parallel to the double isotope labeling experiments, inactivation of the transport function was measured by following the isotopic equilibrium exchange flux of D-glucose (12).

Sodium Dodecyl Sulfate Gel Electrophoresis—Electrophoresis using 5% polyacrylamide gels containing 1% sodium dodecyl sulfate was carried out by a method essentially similar to that of Fairbanks et al. (13). FDNB-treated ghosts were solubilized for sodium dodecyl sulfate gel electrophoresis as follows: 3 ml of 50% sucrose, 0.073 ml of 1 M Tris-HCl (pH 8.0), 0.088 ml of 0.2 mM EDTA (pH 8.0), 0.75 ml of 0.4 mM dithiothreitol, and 0.14 ml of 0.5% pyronin Y (0.5 mg/ml) were added to 2 ml of a ghost suspension containing approximately 10 mg of membrane protein and were mixed for 10 minutes. Then 1.5 ml of 50% sucrose, 0.073 ml of 1 M Tris-HCl (pH 8.0), 0.088 ml of 0.2 mM EDTA (pH 8.0), 0.75 ml of 0.4 mM dithiothreitol, and 0.14 ml of 0.5% pyronin Y (0.5 mg/ml) were added and the mixture was heated to 60°C for 10 minutes. After cooling, 25 ml of 5% sodium dodecyl sulfate was added, followed by 1% sodium dodecyl sulfate.
to 27° for 20 min. An aliquot of the above mixture, containing 75 to 150 μg of membrane proteins, was applied to each column (0.5 cm diameter, 7.0 cm length). Pyronin migrated 6.0 cm within 60 min with the voltage gradient at 9 volts/cm and the current at 8 mA/tube. Four gels were run for each experiment; two were stained with Coomassie blue and the others were sliced into 30 to 36 equal sections without staining. The stained gels were scanned at 350 nm with a Gilford spectrophotometer equipped with a model 2410 linear transport accessory. The molecular weights of the polypeptide bands were estimated from their relative mobilities (see Figs. 1 and 2). The gel slices were counted for their ¹⁴C and ³H radioactivities in a liquid scintillation counter (Mark II liquid scintillation systems, Nuclear-Chicago Corp.). A standard two-channel counting procedure for dual-labeled samples was adopted. The ratio of ¹⁴C:³H (or vice versa) was calculated for each gel slice with a standard quenching correction. In some experiments, scans or both the Coomassie blue-stained polypeptide bands and the radioactivities were done on a single gel after longitudinal slicing into two equal parts.

**LH-20 Column Chromatography—**Membrane pellets equivalent to approximately 10 mg of protein were mixed in an Erlenmeyer flask with 100 ml of chloroform-methanol (2:1, v/v) and extracted for 30 min at room temperature, with occasional shaking. The extract was filtered into a separatory funnel through glass wool. The residue was extracted twice more in a similar manner. NaCl (75 ml of a 0.75% aqueous solution) was added to 300 ml of combined extract, mixed gently, and stored overnight at 4°. The lower phase was recovered and dried in vacuo, then redissolved in 5 ml of Folch’s lower phase solvent mixture (14). A Sephadex LH 20 column (2.5 X 27 cm) was prepared and equilibrated in chloroform for 24 hours prior to the run. The column was eluted successively with 50 ml of chloroform; 10 ml of chloroform-methanol 15:1; 20 ml each of chloroform-methanol 10:1, 6:1, and 4:1; and 75 ml of chloroform-methanol 2:1 (15). Fractions (2 to 3 ml) were collected at a flow rate of 0.85 to 1.0 ml/min. Finally, the column was eluted three times with 40 ml of methanol. Each fraction was assayed for ¹⁴C and ³H activities. Lowry proteins (16) and lipid phosphorus (17) were also measured for each fraction. Some pooled fractions were separated by silica gel thin layer chromatography with chloroform-methanol-acetic acid-water (50:25:10.7:4) (18).

FIG. 1. Sodium dodecyl sulfate gel electrophoresis of erythrocyte ghosts allowed to react with FDNB in a single isotope labeling experiment. The reaction of mixture (4 ml) contained 0.6 mg of membrane protein (100 ghosts) and 16 μmol of FDNB, with 40 μCi of [¹⁴C]FDNB in the standard buffer solution (pH 8.0). No sugar was included in the reaction mixture. Treatment was carried out for 60 min at 24°. Washed pellets were solubilized, and approximately 100 μg of protein were applied to sodium dodecyl sulfate acrylamide gel for electrophoresis. Separate gels were used for the absorbance scan (continuous curve) and for the radioactivity scan (discrete lines). Electrophoretic mobilities were expressed relative to the mobility of pyronin Y as a tracking dye.

**RESULTS**

The isolated membranes used in this study were hemoglobin-free, resealed, human erythrocyte ghosts with intact glucose carrier activity. This activity was inactivated by FDNB, as described previously (9). Concomitant with this inactivation, the major membrane polypeptides reacted with FDNB, as revealed on sodium dodecyl sulfate gel electrophoresis (Fig. 1). The extent of the reaction varied, however, with different polypeptides. Polypeptides of relative mobilities—0.42, 0.48, 0.53 (Band V (13)), 0.57 (Band VI (13)), 0.65, and 0.73—reacted less than those of 0.08, 0.10 (Spectrin, or Bands I and II, respectively (13)), 0.28 (Band III (13)), and 0.33 (Band IV (13)). The identical pattern was reproduced regardless of whether tritium or ¹⁴C labeled FDNB was used. The presence of n-glucose at a 50 mM concentration did not change this pattern appreciably, with one important exception: the double isotope labeling technique, applied under certain experimental conditions, revealed that some specific membrane polypeptides were detectably more dinitrophenylated in the presence than in the absence of the sugar (Fig. 2). The differential labeling occurred as a relatively sharp peak in terms of the ¹⁴C:³H ratio on sodium dodecyl sulfate gel electrophoresis, with an estimated molecular weight of approximately 180,000. The differentially labeled peak did not

**Fig. 2.** Sodium dodecyl sulfate gel electrophoresis of erythrocyte ghosts allowed to react with FDNB in a double isotope, differential labeling experiment. Two aliquots of 5.2 mg of membrane protein equivalent of ghosts were allowed to react separately with 4 mM FDNB at 21°, pH 8.0, one with [¹⁴C]FDNB and the other with [³H]FDNB. n-Glucose (50 mM) was included with [³H]FDNB in the reaction mixture. Protein (150 μg) from the treated mixture was applied per gel. Separate gels were used for the absorbance scan and for the radioactivity counting. Upper panel, absorbance at 550 nm is shown as a continuous curve. The radioactivity of each slice is shown as a discrete line in percentage of the total radioactivities of the gel. These were calculated from actual counting data for both [¹⁴C]FDNB and [³H]FDNB and their counting efficiencies and specific activities. Differences (greater than 10%) between the results of the two isotopic forms of FDNB are shown as solid blocks, signifying the reaction of [¹⁴C]FDNB in excess of [³H]FDNB. Lower panel, the ¹⁴C:³H ratio in the ordinate signifies the molar ratio of [¹⁴C]FDNB:[³H]FDNB. Slice 0 represents a blank mixture of the two isotopes in the absence of ghost.
The adoption of a particular set of conditions used in this experiment, such as the FDNB concentration (4 mM) and temperature (21\(^\circ\)) rather than 37\(^\circ\), appeared to be critical for effective differential labeling. The differential labeling was not obvious when carried out with 2 mM as opposed to 4 mM FDNB, or at 37\(^\circ\) as opposed to room temperature (Fig. 3). The labeling was not demonstrable if n-mannitol, instead of n-glucose, was used (Fig. 3). It appears that the optimal conditions for differential labeling are those which maximize the differential between carrier inactivation by FDNB and membrane bulk dinitrophenylation (8). An equally significant differential labeling was demonstrated with chloroform-methanol-soluble membrane components on Sephadex LH-20 column chromatography (Fig. 4). The differentially labeled peak was eluted as a peak containing both protein and lipid phosphorus. Silica gel thin layer chromatography of this peak revealed that the phospholipids were mainly (more than 80\%) phosphatidic acid. Both phosphatidylethanolamine and phosphatidylserine were present in small amounts.

**DISCUSSION**

The double isotopic, differential labeling, as applied in the present study on a glucose carrier-active, hemoglobin-free ghost preparation, demonstrated the existence of a polypeptide or polypeptides in human erythrocyte membranes, the dinitrophenylation of which by FDNB was significantly enhanced in the presence of n-glucose. The peptides, with an estimated molecular weight of approximately 180,000, did not correspond with any of the major polypeptide peaks on sodium dodecyl sulfate gel electrophoresis of human erythrocyte membranes (13). This differential labeling appeared to require specific conditions, such as the use of n-glucose rather than mannitol, room temperature (21\(^\circ\)) rather than 37\(^\circ\), and 4 mM rather than 2 mM FDNB. As reported elsewhere (8), these are the conditions which maximize the differential between carrier inactivation and the bulk membrane dinitrophenylation. This close correlation would strongly suggest that the differential labeling is indeed related to an enhanced carrier inactivation. The differentially labeled dinitrophenylation of the peptides was estimated to be 2.2\% (an average of four independent runs with the standard deviation of ±0.8\%) of the total membrane protein dinitrophenylation, or approximately 4 \times 10^4 dinitrophenyl groups per ghost.

Assuming that two dinitrophenyl groups are involved for each peptide in this glucose-induced dinitrophenylation (8), this would represent reaction of 2 \times 10^4 peptide molecules. Since only 70\% of the total carriers formed a complex with glucose at the glucose concentration used (50 mM), the total number of the peptides would be 3 \times 10^4 per ghost if they are indeed the carrier. This is in reasonable accord with estimated numbers of the carrier per cell as reported in the literature, which are in the range of 0.7 to 3.3 \times 10^8 (19). The mass of the peptides, calculated from this number and the molecular weight of 18,000, is approximately 15\% of the total membrane protein, grossly exceeding a previous estimation (21).

Eady and Widdas (7) have attempted a differential labeling of the glucose carrier of human erythrocytes on the basis of the fact that the FDNB inactivation of this system is enhanced by 2-deoxy-n-glucose on one hand and is retarded by 4,6-O-ethylidene-n-glucose on the other. They have used intact cells and shown that dinitrophenylation of some membrane proteins was correspondingly affected by these sugars. The differential labeling was, however, not well localized in all experiments. The authors believe that the difficulty may have been avoided by the use of a hemoglobin-free ghost preparation. Discrepancies between this experiment and the present study may indeed be due to differences in the membrane preparations used. An attempt...
was recently made by Taverna and Langdon (20), who used n-glucosylisothiocyanate as an "active-site-directed" irreversible inhibitor in the affinity labeling of the glucose carrier. This study indicates that a specific incorporation of this sugar analog occurs at two polypeptide peaks on sodium dodecyl sulfate gel electrophoresis with molecular weights of approximately 75,000 and 100,000. The significance of the relation between these polypeptides and ours is not clear at this time.

The present study demonstrates a close parallelism between the reactivities to FDNB of membrane polypeptide (or polypeptides) of 180,000 and of the glucose carrier. Definite proof that the polypeptides are carriers is not provided in the present study, and further conclusions should await supportive evidence from independent approaches. Nevertheless, it is an interesting fact that the differential labeling of a single peak containing both proteins and phospholipids is also demonstrable by LH-20 column chromatography of chloroform-methanol extracted ghosts. This observation, as well as previous findings (21), would indicate that the glucose carrier of the human erythrocyte is proteolipid-like in nature.

REFERENCES

C Y Jung and L M Carlson


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