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PHOTOLABELING OF GLUCOSE-SENSITIVE CYTOCHALASIN B BINDING PROTEINS IN ERYTHROCYTE, FIBROBLAST AND ADIPOCYTE MEMBRANES

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SUMMARY (^{3}H)Cytochalasin B has been photoincorporated into membrane fractions of the human erythrocyte, Rous sarcoma virus-transformed chicken embryo fibroblast and rat adipocyte. Identification of D-glucose sensitive cytochalasin B binding sites was achieved by photolyzing membranes with radioligand in the presence of 0.5-0.7M D- or L-glucose. In the erythrocyte the major labeled bands on SDS-polyacrylamide gels were at 55,000 and 46,000 daltons. In the virus-transformed fibroblasts a major labeled band was at 55,000 daltons, and in adipocyte microsomal membranes, peaks at 50,000 and 45,000 daltons were observed. Binding characteristics of these polypeptides suggest that they are the putative glucose transport proteins in these three cell types.

INTRODUCTION

The movement of D-glucose across the plasma membrane of a variety of mammalian cells is mediated by a facilitated diffusion transport system (1). In recent years membrane polypeptides constituting this transport system in the human erythrocyte have been identified and isolated (2-4). In this cell the transport system appears to consist of heterogeneously glycosylated polypeptides that run as a broad band on SDS gel electrophoretograms, with a range of molecular weights of 46,000-72,000 (2-4). Unequivocal identification of the glucose transporter in other mammalian cells, however, has not yet been achieved.

Cytochalasin B is known to be a potent competitive inhibitor of glucose transport in the erythrocyte as well as in fibroblasts and adipocytes (5-7). One of us has recently developed a method for photoincorporating (^{3}H)cytochalasin B into the glucose transporter of the human erythrocyte (8). In this report we demonstrate that

Abbreviations: CB, cytochalasin B; CE, cytochalasin E; SDS-PAGE, sodium dodecyl sulfate gel electrophoresis.

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this technique labels similar polypeptides in membrane fractions derived from the human erythrocyte, chicken embryo fibroblast and the rat adipocyte.

MATERIALS AND METHODS

(^{3}H)Cytochalasin B was purchased from Amersham Radiochemicals. Sodium lauryl sulfate was obtained from Pierce Chemical Co. and electrophoresis reagents from BioRad. All other reagents were from Sigma Chemical Co. Erythrocytes were prepared from outdated human blood obtained from the American Red Cross, Madison, WI.

Erythrocyte membranes were prepared by the method of Steck and Kant (9). Low density microsomes were prepared from insulin-free cells of epididymal fat from Sprague-Dawley rats according to the method of Karnieli et al. (10). The cell membrane particulate fraction was obtained from Rous sarcoma virus-transformed chick embryo fibroblasts as described elsewhere (6). These latter two preparations contained 70 and 30-40 pmol of D-glucose inhibitable CB binding sites per mg protein, respectively (10,6).

Membrane fractions (0.5 mg protein/ml) were suspended in cold 5 mM Na^+-phosphate buffer, pH 6.0, in 15 ml Corex centrifuge tubes. Membranes were then preincubated in the presence of 10^{-4}M cytochalasin E and 0.5M D- or L-glucose for 15 min in an ice water bath. (^{3}H) cytochalasin B was then added to a final concentration of 1-5 x 10^{-7}M. Both CB and CE were dissolved in ethanolic stock solutions such that the final ethanol concentration never exceeded 1%. Following addition of CB the membrane suspension was allowed to incubate for 15 min in an ice bath prior to photolysis.

Membrane suspensions were photolyzed in an ice bath for 20-30s with a 1 kW Pyrex glass-jacketed high pressure mercury lamp at a distance of 10 cm from the lamp (11). Following photolysis, erythrocyte membranes were pelleted under conditions used in the membrane isolation (9). Adipocyte and fibroblast membranes were diluted up to 10 ml with Na-phosphate buffer and centrifuged at 168,000 x g_{max} for 2 h in a Beckman L-8 ultracentrifuge using a Ti 40 rotor. The membrane pellets were solubilized in Laemmli sample buffer (12) without heating.

SDS-PAGE was performed according to the method of Laemmli (12) on 7% acrylamide slab gels. Following electrophoresis gels were fixed, stained, destained and scanned at 633 nm with an LKB laser densitometer. Gels were sliced with a Hoeffer vibrating gel slicer and 1.5 mm slices were placed in 20 ml scintillation vials with 10 ml of scintillation fluid consisting of 5% Protosol and 95% Econofluor (New England Nuclear). Gels were swollen overnight at 45°C and analyzed by liquid scintillation counting.

RESULTS

(^{3}H)Cytochalasin B binds to human erythrocyte membranes with several apparent classes of high affinity sites (5). The dissociation constant (K_D) for the glucose-displacable sites associated with glucose transport has been reported between 10^{-7} - 10^{-8}M (5,13). Figure 1A shows the photoincorporation of (^{3}H)cytochalasin B into erythrocyte membrane polypeptides separated by SDS PAGE.

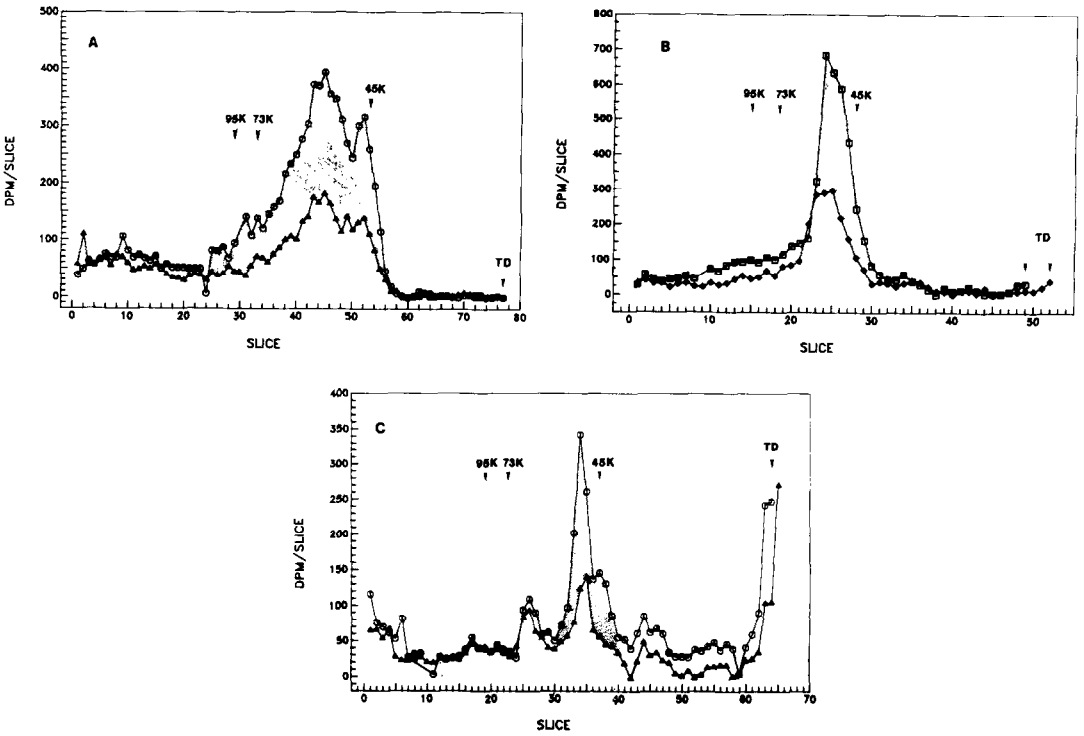

Figure 1. Distribution of radioactivity on SDS-PAGE of membrane polypeptides photolabeled with (^{3}H)cytochalasin B. (A) Photolabeling pattern of human erythrocyte ghost membranes: ○ , membranes labeled in the presence of 0.5 μM (^{3}H)CB, 500 mM L-glucose and 100 μM CE. ▲ , membranes labeled as above, but with 500 mM D-glucose present. The shaded area represents the labeling difference between D- and L-glucose. Photolysis time was 30 s; 90 μg of protein of each sample were separated on a 1.5 mm thick 7% acrylamide slab gel. (B) Photolabeling pattern of membranes derived from Rous sarcoma virus-transformed cultured chicken embryo fibroblasts: □ , labeling in the presence of 0.1 μM ^{3}H(CB) 500 mM L-glucose and 100 μM CE. ◆ , labeling as above but with 500 mM D-glucose. Photolysis time was 20 s; 100 μg of protein was applied to the gels. (C) Photolabeling pattern of the light microsomal fraction from rat adipocytes: ○ ,labeling in the presence of 0.5 μM (^{3}H)CB, 100 μM CE and 700 mM L-glucose. ▲ , conditions as above, but with 700 mM D-glucose. Photolysis time was 30 s; 250 μg membrane protein was applied to the gel. Arrows on all figures indicate positions of migration of molecular weight markers, TD = tracking dye.

The polypeptide region of M_r 40,000-70,000 is extensively labeled by (^{3}H)cytochalasin B. Peaks of D-glucose-sensitive labeling occur at 46,000 daltons and 53,000 daltons but several additional unresolved shoulders are also evident. Estimates from the literature for the number of glucose-displaceable cytochalasin B binding sites for erythrocyte membranes are around 300-400 pmol bound/mg membrane protein (12). In the experiment depicted in figure 1A the total photoincorporation of cytochalasin B in the zone 4.5 region was 2.0 pmol/mg membrane protein. 53% of this binding was masked when D-glucose was substituted for L-glucose during the incubation.

Figure 1B presents the results of a similar experiment performed with a cell membrane fraction from virus-transformed chicken embryo fibroblasts. The molecular weight markers are based upon the relative positions of unlabeled erythrocyte membrane proteins run simultaneously in an adjacent gel. It is evident that D-glucose-inhibitable photoincorporation of (^{3}H)CB occurred in fibroblast polypeptides of similar mobility to those in the erythrocyte. With the fibroblasts the major peak, which is centered at 55,000 daltons, is a sharper band than that for the erythrocyte. In the experiment shown here only a shoulder is evident at 45,000 daltons, but in other experiments we have observed a second distinct peak of label at 45,000 daltons.[1] The total amount of photoincorporation in the 40-60,000 dalton polypeptides was 0.16 pmoles/mg protein; 0.5 M D-glucose reduced this amount by 52%.

Similar experiments were also performed with the low density microsomal membranes from rat adipocytes, the membrane fraction from this cell type that possesses the highest concentration of glucose transporters (10). The results are indicated in Figure 1C. Polypeptides with mobilities corresponding to 45,000 and 50,000 daltons were labeled in a D-glucose-inhibitable fashion. A small peak of D-glucose-insensitive labeling at 69,000 daltons was also seen. The total amount of incorporation into the two main peaks was 0.21 pmoles CB per mg protein; this amount was reduced 57% by D-glucose.

Since CB is known to bind to high-affinity sites on F-actin (14) and since one of the photolabeled polypeptides has a molecular weight similar to that of actin (43,000), it might be concluded that this labeled polypeptide is membrane bound actin. In addition, in experiments to be reported elsewhere, one of us has shown that actin can be photolabeled with (^{3}H)CB.[1] However, it is unlikely that the 45,000 dalton labeled polypeptide is actin, since actin photolabeling is totally blocked by CE, but not inhibited by D-glucose.[1]

DISCUSSION

The results presented here show that the membranes of transformed fibroblasts and adipocytes contain CB-labled polypeptides that are similar to the glucose transporter in human erythrocytes. In all three cell types, the major targets of

[1]Shanahan, M.F. unpublished observation.

photolabeling are two polypeptides. One of apparent molecular weight 50-55,000, and a second polypeptide of apparent molecular weight 45-46,000 which is labeled to a lesser degree. In each case, D-glucose inhibits the labeling relative to that observed with L-glucose. Furthermore, the efficiency of labeling for experiments presented here is in the range of 0.3 to 0.5%. It thus seems likely that one or both of the labeled polypeptides is the glucose transporter(s) of transformed fibroblasts and adipocytes. Very recently, Pessin et al (15) have described similar results for the photoaffinity labeling of untransformed fibroblasts with CB.

Antibodies against the purified glucose transporter from human erythrocytes are available, and recently these have been used to identify the glucose transporter in transformed chicken embryo fibroblasts and rat adipocytes (16-18). The results of these antibody studies are in approximate agreement with the results presented in this report. Polypeptides that ran as a broad band centered at 41,000 and 45,000 upon SDS PAGE were immunologically identified as the glucose transporter in fibroblasts and adipocytes, respectively. We feel that the differences in identification of the glucose transporter between the previous reports (16,18) and this communication are likely a result of using different methods of detection and electrophoresis and not due to an actual difference in the polypeptides being labeled. In regard to this latter point, it has previously been shown that the mobility of the erythrocyte transporter in SDS-PAGE varies substantially with the conditions of electrophoresis (4).

The basis for the heterogeneity in the polypeptides susceptible to D-glucose-inhibitable labeling is not known with certainty. The glucose transporter from erythrocytes has been shown to be heterogenously glycosylated (19), and thus the labeled polypeptides may consist of a single polypeptide with various carbohydrate moieties attached.

The results described herein indicate that the glucose transporter in a variety of cell types can be identified through photoaffinity labeling with CB. This method should prove useful for purification of the transporter and for investigations of the regulation of glucose transport.

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