Vol. 122, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1984 Pages 218-224

CHEMICAL IDENTITY OF THE GLUCOSE TRANSPORTER WITH THE [^{3}H]CYTOCHALASIN B-PHOTOLABELLED COMPONENT OF HUMAN ERYTHROCYTE MEMBRANES. EQUAL SENSITIVITY TO TRYPSIN AND ENDOGLYCOSIDASE F

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Received May 21, 1984

SUMMARY The protein photolabelled by [^{3}H]cytochalasin B and band 4.5, which contains the human erythrocyte hexose transporter, were compared by electrophoretically monitoring the effect of digestion with endoglycosidase F and trypsin. Band 4.5 was found to consist of two minor components, Mr 58,000 and 52,000, and one main component, Mr 60,000-50,000. Deglycosylation by endoglycosidase F converted both the [^{3}H]-labelled species and the main polypeptide of band 4.5 from a mixture of polypeptides of Mr 50,000-60,000 to a sharp component of Mr 46,000. Tryptic cleavage of the photolabelled protein produced a [^{3}H]-labelled peptide of 19,000 daltons, which corresponded to an analogous tryptic fragment of the main component of band 4.5. Endoglycosidase F treatment of trypsin-treated samples had no effect on the 19,000 dalton fragment or the labelled 19,000 component, indicating that both species lack the carbonydrate moiety of the parent protein. This parallel chemical behaviour indicates that the photolabelled polypeptide is representative of the main constituent of band 4.5. Photolabelling may be used with confidence to quantitate glucose transporters in other cells.

The glucose transport activity of human red blood cell membranes has been recovered in a purified glyco-polypeptide of Mr 50,000-60,000 designated as "band 4.5" (1,2). The strategy used to purify this protein (selective exclusion from diethyl aminoethyl cellulose) cannot be used to purify the glucose transporters of other cell types, primarily due to the large diversity of other polypeptides composing those membranes, as well as to the relative scarcity of the transporter in other cells compared to the human erythrocyte.

A distinct property of the human red cell glucose transporter is the reversible, high-affinity binding of the mold metabolite cytochalasin B (Kd = 0.1 μM) (3). This binding inhibits hexose transport (K_i = 0.1 μM), and D-glucose competes stereospecifically with cytochalasin B at this high affinity site (see 4). [^{3}H]Cytochalasin B binds to band 4.5 at a molar ratio of 0.7 (15), suggesting that a large proportion of the mass of band 4.5 constitutes the glucose transporter.

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0006-291X/84 $1.50

A procedure for covalently photolabelling the glucose transporter of red blood cells with [^{3}H]cytochalasin B has recently been described (5,6). This reaction is thought to result from photo-activation of aromatic residues in the transporter itself (7). This procedure, in combination with polyacrylamide gel electrophoresis (PAGE), enables one to identify the glucose transporter without the need of prior protein purification.

Polypeptides have been covalently photolabelled with [^{3}H]cytochalasin B in membranes from cells as diverse as chick fibroblasts (8), rat adipocytes (9), rat skeletal muscle (10) and human placenta (11). Identification of these polypeptides with the glucose transporter has been based upon D-glucose protection of photolabelling. However, when compared with the maximum (D-glucose-sensitive) reversible binding of cytochalasin B, the yield of photolabelling appears to be less than 2% in most cases. Attempts to achieve higher yields of photolabelling have not been entirely successful (see 7,10) in part due to competition of this reaction with photo-inactivation of the protein, evidenced by a decrease in glucose transport capacity (12) and in cytochalasin B binding of the remaining unlabelled transporters (7). Due to the low yield of photolabelling, a distinct possibility remains that [^{3}H]cytochalasin B labels covalently a D-glucose-sensitive protein different from the transporter, which co-migrates in PAGE with the broad band 4.5 and constitutes some 5% of the protein in that region. For thisthis reason, it would be desirable to determine the composition of band 4.5 and compare chemical properties of its constituents with those of the photolabelled component.

Recently, endoglycosidase F has been used to remove the carbohydrate from the glycoprotein band 4.5 and this resulted in conversion of the broad band Mr 50,000-60,000 observed in PAGE to a sharp Mr 46,000 band (13). In addition, trypsin has been shown to cleave purified, reconstituted glucose transporter into two fragments of average Mr 30,000 and 20,000 (14).

In this study we compare the sensitivity of the [^{3}H]cytochalasin B photolabelled component with that of the main constituent of band 4.5 to endoglycosidase F and to trypsin. The results show a parallel behaviour of both components under the action of these enzymes.

MATERIALS AND METHODS

[^{3}H]cytochalasin B (17 Ci/mmol) was purchased from Amersham. Endoglycosidase F was obtained from New England Nuclear. Trypsin (TPCK treated) from bovine pancreas, n-octylglucoside and EP-475 were obtained from Sigma.

Purification of band 4.5 in reconstituted lipid vesicles from human erythrocyte membranes was performed essentially according to Baldwin et al. (15). Briefly, protein-depleted membranes were extracted with 34 mM octylglucoside and the extract was applied to a DEAE-cellulose column, from which only band 4.5 and erythrocyte membrane lipids were eluted. Protein was determined by the procedure of Lowry et al. (16).

Photolabelling of band 4.5 with [^{3}H]cytochalasin B was performed essentially as described before (7,14). Briefly, exposure of 0.5 mg protein/ml to 1 μM

[^{3}H]cytochalasin B was performed in 50 mM Tris-HCl pH 7.4, 100 mM NaCl, and 1 mM EDTA containing 500 mM D- or L-glucose, and irradiated at 280 nm in an Aminco Bowman fluorometer equipped with a xenon arc lamp, for 1 h at room temperature. Removal of free label occurred during subsequent PAGE.

Reaction with endoglycosidase F was carried out essentially as described (13), using the proportions of band 4.5 and enzyme described in each case, in 0.1 M sodium phosphate pH 6.1 containing 50 mM EDTA, 75 mM β-mercaptoethanol, 0.5% triton X-100 and 0.05% sodium dodecylsulfate, and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM), EP-475 (5 μM) and pepstatin A (0.1 μg/ml). After 16 h with agitation at room temperature samples were subjected to PAGE.

Trypsin treatment was essentially as described (14) exposing 0.5 mg protein of reconstituted band 4.5 to 5 μg of trypsin in 1 ml of 50 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA, pH 7.4 for 90 min at room temperature. The reaction was stopped by addition of 0.1 mM phenylmethylsulfonyl fluoride, prior to reaction with endoglycosidase F or to PAGE.

PAGE according to Laemmli (17) was carried out as described in (7,15) using Pierce lauryl sulfate and 12% polyacrylamide. Protein bands were visualized by staining with Coomassie blue or by the silver staining procedure of Wray et al. (18). Gel slices (2 mm) were digested in 0.75 ml of 30% H_2O_2, and radioactivity was determined by liquid scintillation counting with an efficiency of 0.22.

RESULTS

Fig. 1A shows the effect of endoglycosidase F on the Coomassie-blue stained polypeptide(s) of band 4.5. As had been observed by Lienhard et al. (13) with a non-commercial preparation of the enzyme, removal of the oligosaccharide of band 4.5 by increasing amounts of commercially obtained endoglycosidase F (lanes 3-5) resulted in a progressive decrease of the characteristic diffuse 50,000-60,000 dalton band and an increase in a sharp 46,000 band. Complete conversion was achieved with 1 unit enzyme per 20 μg protein of band 4.5. The glycosidase itself could not be detected by the stain in this gel system (lane 6). Although Coomassie-blue staining did not reveal it, silver staining showed band 4.5 to be composed of at least three entities (Fig. 1B, gel +): A broadly migrating component of Mr 50,000-60,000 which is converted to a sharp, 46,000 dalton one, and two minor but distinct sharp components of Mr 58,000 and 52,000. The latter two become apparent only after deglycosilation of the main component and its concomitant reduction to a Mr of 46,000 by endoglycosidase F.

Band 4.5 was photolabelled with [^{3}H]cytochalasin as indicated under Methods. Fig. 1B (solid trace) shows the distribution of radioactivity from covalently bound [^{3}H]cytochalasin B photo-incorporated onto band 4.5. The diffuse radioactive peak extends along all of the region occupied by band 4.5. Fig. 1B (broken trace) shows that treatment of [^{3}H]cytochalasin B-labelled band 4.5 with endoglycosidase F results in conversion of 73% of the radioactively labelled material into a sharp peak, corresponding to a Mr of 46,000. In parallel, most of the silver-stained diffuse region comprising Mr 50,000-60,000 was converted into a sharp 46,000 dalton band (gel +).

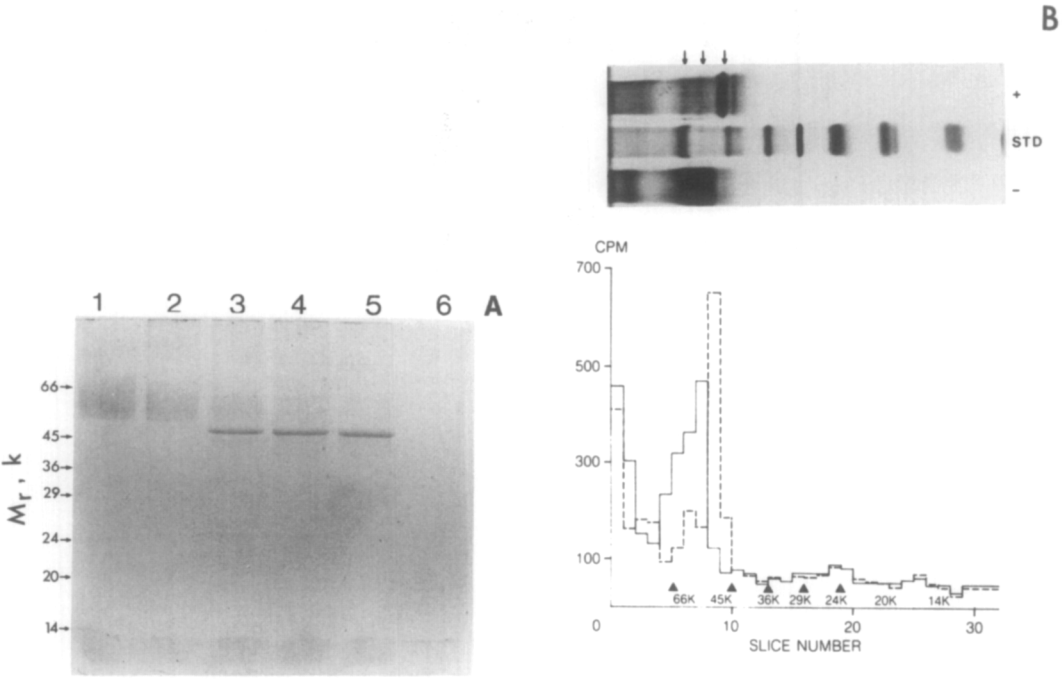

Fig. 1A Effect of endoglycosidase F on band 4.5. Purified band 4.5 was exposed to endoglycosidase F as described under methods and resolved by PAGE in 12% polyacrylamide stained with Coomassie blue. Lane 1: Purified band 4.5 (20 µg). Lane 2: band 4.5 (20 µg) incubated for 18 h in the reaction buffer without endoglycosidase F. Lanes 3, 4 and 5: Band 4.5 (20 µg) reacted with 0.2, 0.4 or 1 units of endoglycosidase F, respectively. Lane 6: Endoglycosidase F (0.4 units). The scale at the left shows the calibration of molecular weights.

Fig. 1B Effect of endoglycosidase F on the [^{3}H]cytochalasin B-labelled component. Band 4.5 was photolabelled with [^{3}H]cytochalasin B as described in Methods, and 5 µg were incubated without (gel - and solid trace) or with (gel + and broken trace) 0.3 units endoglycosidase F. The samples were resolved by PAGE (2 µg protein/lane). The silver-stained gels and the distribution of radioactivity are displayed. Molecular weight standards (STD) are shown in the middle gel and indicated in the lower panel trace (Dalton Mark VII-L, Sigma: α-lactalbumin, 14,300; trypsin-inhibitor 20,100; trypsinogen, 24,000; carbonic anhydrase, 29,000; glyceraldehyde-3-P-dehydrogenase, 36,000; egg albumin, 45,000; bovine serum albumin, 66,000).

Fig. 2 (upper panel) shows the effect of trypsin on the silver-stained components of band 4.5. The protease produced two major fragments detectable by PAGE: a diffuse band at 30,000-26,000 daltons (arrow, gel -) and a sharper band of 19,000 daltons, essentially as observed before (14). The minor components of band 4.5 of Mr 58,000 and 52,000 were not affected by trypsinization. Upon exposure of trypsinized band 4.5 to endoglycosidase F the carbohydrate-bearing 30,000-26,000 dalton fragment, which was visible as a diffuse band (arrow, gel -), was converted by endoglycosidase F to a family of discrete bands ranging in molecular weight from 20,000-23,000 daltons (arrow, gel +). On the other hand the glucosidase did not alter either the molecular size or the width of the 19,000

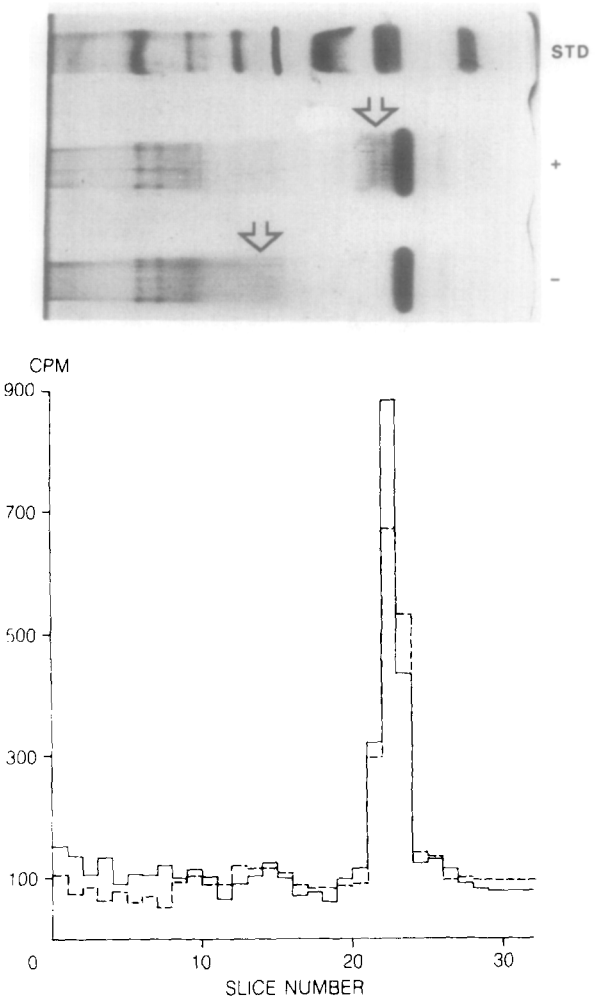

Fig. 2 Effect of trypsin on photolabelled band 4.5 treated with endoglycosidase F. Photolabelled band 4.5 was exposed to trypsin as described under Methods, followed by 18 h incubation in the absence (gel - and solid line) or presence (gel + and broken line) of endoglycosidase F (conditions as described in Fig. 1B). The samples were resolved by PAGE (2 µg protein/lane). The silver-stained gels and the distribution of radioactivity are displayed. Molecular weight standards (as in Fig. 1B) are shown in the top gel.

dalton tryptic component (gel +). This is in keeping with the observation that this fragment does not bear any of the carbohydrate of the parent glycoprotein (14). The 58,000 and 52,000 dalton minor components were not affected by treatment with the glycosidase (gel +).

The traces in the lower panel show the fate of the [^{3}H]cytochalasin B label after trypsinization before (solid line) or after (broken line) treatment with endoglycosidase F. Virtually all of the label originally associated with the parent, untrypsinized band 4.5 migrated as a single peak of Mr 19,000 and this pattern was not altered by treatment with endoglycosidase F.

DISCUSSION

The glucose transporter of red cells is associated with band 4.5 but the purity of the latter is still in question (13). Indeed, band 4.5 is known to contain the nucleoside transporter as well (19). [^{3}H]Cytochalasin B has been used to label band 4.5 and it is assumed that the label is on the glucose transporter polypeptide mainly on the basis of protection of photolabelling by D-glucose. However, in all cases protection has been only partial (~ 50%) (5,6). In an experiment parallel to that depicted in Fig. 1B, protection by 500 mM D-glucose was 56%. Complete protection of photolabelling cannot be expected since the reaction with [^{3}H]cytochalasin B is irreversible, while interaction with the sugar is reversible. Alternatively, incomplete protection could result from labelling of a polypeptide other than the glucose transporter. Since only a small percentage of the polypeptides in band 4.5 becomes covalently labelled with [^{3}H]cytochalasin B (0.4% in the experiments in Fig. 1B), and since band 4.5 is here shown to be heterogeneous (see arrows in gel +, Fig. 1B), any of its three constituents could be the photolabelled entity.

The results presented in this study suggest that the [^{3}H]cytochalasin B-labelled component of band 4.5, although corresponding to only a small percentage of the total mass of this protein, is representative of its main constituent. The evidence leading to this conclusion can be summarized as follows: (i) Removal of the carbohydrate of band 4.5 results in a parallel behaviour of the main polypeptide(s) (detected by Coomassie blue or silver staining) and of the photolabelled component, decreasing their apparent molecular weight and increasing their sharpness on PAGE. (ii) The covalent radioactive label comigrates quantitatively with one of two major tryptic fragments of band 4.5 of Mr 19,000. (iii) This 19,000 dalton radioactive peak is not affected by endoglycosidase F, and neither is an effect of the enzyme observed on the silver-staining pattern of this fragment.

These studies have also provided additional information concerning the carbohydrate-bearing fragment of the transporter. Since the molecular weight of the deglycosylated protein is 46,000 daltons, and that of the unglycosylated [^{3}H]cytochalasin B-labelled peptide is 19,000 daltons, the carbohydrate-bearing tryptic polypeptide fragment should have a molecular weight of approximately 27,000 daltons. The series of somewhat smaller peptides observed with endoglycosidase F treated samples (arrows, gel +, Fig. 2) suggest that this fragment contains additional trypsin-sensitive sites that are cleaved at lower yields.

Given that the 70% of the mass of band 4.5 is the glucose transporter (15), the established identity of the [^{3}H]cytochalasin B-labelled component with the main constituent of band 4.5 can be of great importance in studies of regulation of glucose transport by hormones and other physiological stimuli in several cell types. Specifically this may be useful for the study of mechanisms of regulation

of glucose transport that may involve changes in the number of transport sites. Indeed, an increase in the number of transporters at the plasma membrane has been proposed to occur in insulin-treated fat cells (20) and diaphragm muscle (21) in studies of reversible binding of [^{3}H]cytochalasin B. The recently reported increase in the amount of [^{3}H]cytochalasin B photolabelling in membranes from insulin treated fat cells (22) may now be ascribed with greater confidence to a corresponding increase in the number of transporters in these membranes. The photolabelling method in conjunction with endoglycosidase F may provide the additional advantage of allowing the disclosure of possible differences in the molecular weights of the basal and insulin-stimulated transporters. Studies to this aim are currently underway in our laboratory.

ACKNOWLEDGEMENTS

We are indebted to Dr. G.E. Lienhard for making available to us information on the use of endoglycosidase F before its publication. This work was supported by the Medical Research Council of Canada. AK is a MRC Scholar.

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