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Proteolytic and chemical dissection of the human erythrocyte glucose transporter

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Treatment of the purified, reconstituted, human erythrocyte glucose transporter with trypsin lowered its affinity for cytochalasin B more than 2-fold, and produced two large, membrane-bound fragments. The smaller fragment (apparent M_r 18000) ran as a sharp band on sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis. When the transporter was photoaffinity labelled with [4-^{3}H]cytochalasin B before tryptic digestion, this fragment became radiolabelled and so probably comprises a part of the cytochalasin B binding site, which is known to lie on the cytoplasmic face of the erythrocyte membrane. In contrast, the larger fragment was not radiolabelled, and ran as a diffuse band on electrophoresis (apparent M_r 23000–42000). It could be converted to a sharper band (apparent M_r 23000) by treatment with endo-β-galactosidase from *Bacteroides fragilis* and so probably contains one or more sites at which an oligosaccharide of the poly(*N*-acetyl-lactosamine) type is attached. Since the transporter bears oligosaccharides only on its extracellular domain, whereas trypsin is known to cleave the protein only at the cytoplasmic surface, this fragment must span the membrane. Cleavage of the intact, endo-β-galactosidase-treated, photoaffinity-labelled protein at its cysteine residues with 2-nitro-5-thiocyanobenzoic acid yielded a prominent, unlabelled fragment of apparent M_r 38000 and several smaller fragments which stained less intensely on SDS/polyacrylamide gels. Radioactivity was found predominantly in a fragment of apparent M_r 15500. Therefore it appears that the site(s) labelled by [4-^{3}H]cytochalasin B lies within the *N*-terminal or *C*-terminal third of the intact polypeptide chain.

Intensive efforts have been made by a number of groups over the past few years to identify the protein(s) responsible for catalysing the passage of glucose across the human erythrocyte membrane. Although some groups have suggested that the transporter is a protein of M_r 100000 (Shelton & Langdon, 1983), much evidence has now been accumulated in favour of a protein of apparent M_r 55000 as the native transporter (for references, see Shanahan, 1982; Carter-Su *et al.*, 1982). This integral membrane protein has been isolated, and has been shown to catalyse the uptake of D-glucose into phospholipid vesicles with kinetics similar to those shown by the intact erythrocyte (Kasahara & Hinkle, 1977; Baldwin *et al.*, 1981; Wheeler & Hinkle, 1981). It also binds cytochalasin B, a potent reversible inhibitor of glucose transport, with the same affinity and sensitivity to inhibition by D-glucose as the transport system in the intact erythrocyte (Baldwin *et al.*, 1979). Further evidence for the identity of this protein as the transporter has been provided by the recently developed technique of photoaffinity labelling with [4-^{3}H]cytochalasin B. Irradiation of freshly prepared erythrocytes or their membranes with high-intensity u.v. light in the presence of [4-^{3}H]-cytochalasin B results in the covalent labelling of a membrane component that runs as a broad band of apparent M_r 45000–60000 on SDS/polyacrylamide gels (Shanahan, 1982; Carter-Su *et al.*, 1982). This pattern of labelling closely resembles the distribution of Coomassie Blue-staining material in SDS/polyacrylamide gels of the purified transporter. In addition, the labelling of this component is inhibited by D-glucose and by other substrates of

Abbreviations used: SDS, sodium dodecyl sulphate, NTCB, 2-nitro-5-thiocyanobenzoic acid.

the transport system, but not by L-glucose or by other non-transported sugars.

The isolated transporter is a glycoprotein and bears oligosaccharides of the poly(*N*-acetyllactosamine) type on that part of the protein exposed on the extracellular surface of the membrane (Sogin & Hinkle, 1978; Gorga *et al.*, 1979). Heterogeneity of glycosylation causes the protein to run as a broad band corresponding to an apparent M_r of 43000–74000 on SDS/polyacrylamide-gel electrophoresis. However, much of the carbohydrate can be removed by treatment with endo-β-galactosidase from *Escherichia freundii*, which specifically cleaves internal β-galactosidic linkages of oligosaccharides of the poly(*N*-acetyllactosamine) series (Fukuda & Matsumura, 1976), and the protein then runs as a sharper band, with an apparent M_r of 46000, on electrophoresis (Gorga *et al.*, 1979; Baldwin *et al.*, 1982). The polypeptide spans the membrane, and contains sites sensitive to tryptic cleavage only in its cytoplasmic domain (Baldwin *et al.*, 1980). At present nothing more is known about the topography of the transport protein in the erythrocyte membrane. We have recently developed a procedure for the purification of the transporter that gives a higher yield of protein and a greater specific activity for cytochalasin B binding than previously published methods (Baldwin *et al.*, 1982). In the present paper we use this preparation in the identification of the sites of tryptic cleavage, cytochalasin B labelling and carbohydrate attachment, in order to elucidate partially the topography of the transporter in the erythrocyte membrane.

Experimental

Materials

[4-^{3}H]Cytochalasin B and 2-nitro-5-[*cyano*-^{14}C]-thiocyanobenzoic acid were obtained from New England Nuclear (Southampton, Hants., U.K.). NTCB was purchased from Fluka (via Fluorochem, Glossop, Derbys., U.K.). Diphenylcarbamyl chloride-treated trypsin and molecular weight standards for SDS/polyacrylamide-gel electrophoresis were obtained from Sigma (Poole, Dorset, U.K.). Boehringer Corp. (London) (Lewes, East Sussex, U.K.) supplied bovine lung aprotinin. CNBr-cleaved myoglobin came from Pharmacia Fine Chemicals (G.B.) (Hounslow, Middx., U.K.). Spectra/Por 3 dialysis tubing was purchased from Raven Scientific (Haverhill, Suffolk, U.K.). SDS was from Pierce & Warriner (U.K.) (Chester, Cheshire, U.K.). All other reagents and solvents were bought from Sigma or from BDH (Poole, Dorset, U.K.) and were the highest grades available. Out-dated human blood was provided by the blood bank of the Royal Free Hospital.

Preparation of the glucose transporter

The glucose transporter was prepared from out-dated human blood by the method of Baldwin *et al.* (1982) with the following modifications. The detergent extract of protein-depleted membranes was made 25mM in NaCl before being subjected to DEAE-cellulose chromatography, and 25mM-NaCl was also included in the column buffer. Reconstitution of the transporter was achieved by dialysis at 4°C against 50mM-sodium phosphate buffer, pH 7.4, containing 100mM-NaCl and 1mM-EDTA. These modifications increased the yield of the transporter by about 40%, but in all other respects the properties of the purified protein were identical with those previously described.

Photoaffinity labelling

Photoffinity labelling of the transporter with [4-^{3}H]cytochalasin B was carried out by a modification of the method used by Shanahan (1982) for labelling erythrocyte membranes. Samples of the purified transporter at a protein concentration of 160–180μg/ml in 50mM-sodium phosphate, pH 7.4, containing 100mM-NaCl, 1mM-EDTA, and either 500mM-D- or L-glucose were incubated with 10^{-6}M-[4-^{3}H]cytochalasin B (14.7Ci/mmol) for 10min on ice in quartz cuvettes of 1cm path length. The cytochalasin was added as a solution in ethanol, such that the final concentration of ethanol was 1% (v/v). The samples were then exposed to high-intensity u.v. light from a 100W lamp (Mineralight model R-52, Ultraviolet Products Inc., San Gabriel, CA, U.S.A.) at a distance of 10cm for 10min unless otherwise noted. They were then dialysed exhaustively at 4°C against a buffer appropriate to the subsequent experiments, in order to remove noncovalently bound cytochalasin B.

Tryptic digestion

Tryptic digestion was carried out at 25°C in 50mM-sodium phosphate, pH 7.4, containing 100mM-NaCl and 1mM-EDTA; the concentration of the transporter was 300μg/ml and of the trypsin, 3μg/ml, unless otherwise noted. In order to ensure that digestion had reached completion, fresh trypsin (3μg/ml) was added after 2h and again after 4h during 6h digestion experiments. At various times, the digestion was terminated by addition of bovine lung aprotinin to give a final concentration by weight equal to twice that of the trypsin. If the digestion was to be followed by endo-β-galactosidase treatment, the trypsin was inactivated by the addition of 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') to a concentration of 1mM.

Cleavage with NTCB

Before cleavage with the NTCB the transporter (0.5–1 mg/ml) was incubated at 37°C under N_2 for 15 min with 0.8 mM-dithiothreitol in 42 mM-Tris/acetate, pH 8, containing 0.8 mM-EDTA and 1.7% (w/v) SDS. NTCB was then added to a concentration of 10 mM, and the incubation was continued in the dark for a further 15 min. One-half of the incubation volume of 0.1 M-sodium borate, pH 9.3, was then added and the pH adjusted to 9.3 by addition of NaOH. The mixture was incubated for 24 h at 37°C in the dark before terminating the reaction by the addition of 265 mM-2-mercaptoethanol. Samples of the cleaved transporter were either prepared directly for SDS/polyacrylamide-gel electrophoresis, or were first dialysed against 1 mM-Tris/HCl, pH 6.8, containing 0.1% SDS, with the use of Spectra/Por 3 dialysis tubing, in order to remove excess reagent. Cleavage of the transporter with 2-nitro-5-[*cyano*-^{14}C]thiocyanobenzoic acid (sp. radioactivity 8.8 mCi-mmol) was carried out in the same manner, except that the concentrations of dithiothreitol and of radiolabelled NTCB were halved.

Endoglycosidase digestion

Endo-β-galactosidase was isolated from *Bacteroides fragilis* as previously described (Scudder *et al.*, 1983). Digestion of the glucose transporter (0.4–1 mg/ml) was carried out at 37°C for 18 h in 50 mM-sodium phosphate, pH 6.0, containing 1 mM-dithiothreitol and 1 mM-EDTA, with an enzyme concentration of 0.56 units/ml.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out either by the procedure of Laemmli (1970) using 10%, 12% or 15% acrylamide gels, or by the procedure of Hashimoto *et al.* (1983) using 10–18% linear gradient acrylamide gels containing 7 M-urea. Proteins of known M_r used as markers were bovine serum albumin (M_r 66000), ovalbumin (M_r 45000) rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), bovine erythrocyte carbonic anhydrase (M_r 29000), soya bean trypsin inhibitor (M_r 20100), equine cytochrome *c* (M_r 12400) and bovine lung aprotinin (M_r 6000). Markers of low M_r were provided by CNBr cleavage of equine myoglobin (M_r values 17200, 14632, 8235, 6383 and 2556). Polypeptides were stained with Coomassie Blue (Page Blue 83 from BDH) and their relative amounts were estimated from spectrophotometric scans. Fluorographic detection of radioactive bands on gels was carried out by using sodium salicylate in the procedure of Chamberlain (1979). A linear relationship was found between the concentration of radioactivity on the gel and the extent of film darkening (results not shown). A more quantitative estimate of the distribution of radioactivity in gels was obtained by solubilization of gel slices according to the method of Goodman & Matzura (1971), followed by liquid-scintillation counting.

Other procedures

Protein was measured by the procedure of Lowry *et al.* (1951) except that 0.5% (w/v) SDS was included in order to solubilize membranous samples. Routine measurements of cytochalasin B binding to the purified transporter were made by equilibrium dialysis using 4×10^{-8} M-[4-^{3}H]cytochalasin B as previously described (Zoccoli *et al.*, 1978). Non-specific binding of cytochalasin to lipids in the preparation accounted for less than 1% of the total binding, and so no correction was made for this. The ratio of bound to free cytochalasin under these conditions is approximately equal to the concentration of cytochalasin B binding sites divided by the dissociation constant for cytochalasin B, and is referred to as the cytochalasin B binding activity (Zoccoli *et al.*, 1978). For more accurate determination of the concentration of sites and for measurement of the dissociation constant, cytochalasin B binding was measured in duplicate over a range of concentrations and the data treated by the method of Scatchard, as previously described (Baldwin *et al.*, 1982).

Results

Photoaffinity labelling

When a mixture of the purified glucose transporter and [4-^{3}H]cytochalasin B was exposed to high-intensity u.v. light, some of the cytochalasin apparently became covalently linked to the protein; the radioactivity could not be removed by exhaustive dialysis nor by electrophoresis on SDS/polyacrylamide gels followed by the normal staining and destaining procedures. Fig. 1 (open bars) shows the result of electrophoresis of a sample that had been exposed to u.v. light for 5 min in the presence of [4-^{3}H]cytochalasin B and 500 mM-L-glucose. The distribution of radioactivity corresponded closely to the pattern of Coomassie Blue staining, except for the presence of some radiolabelled material with a mobility greater than the tracking dye. Approx. 73% of the radioactivity on the gel was associated with the broad band of transporter monomer (apparent M_r 55000) and another 14% was found in the region containing the dimer (slices 2 and 3) and larger aggregates (slice 1) of the transporter. The amount of radioactivity associated with the monomer band was equivalent to 3% of the cytochalasin B present in the original mixture. In the absence of u.v. light,

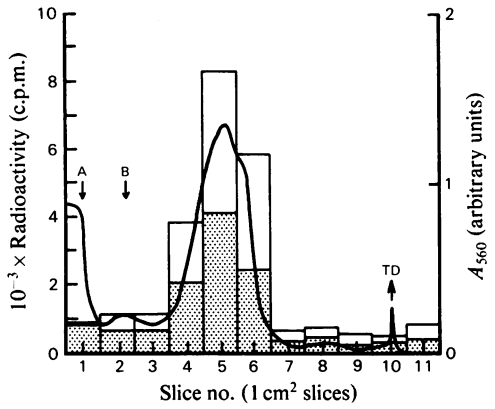

Fig. 1. *Photoaffinity labelling of the glucose transporter with [4-^{3}H]cytochalasin B in the presence of* D- *or* L-*glucose*
The glucose transporter (160 μg/ml) was labelled by irradiation with u.v. light for 5 min in the presence of 1×10^{-6} M-[4-^{3}H]cytochalasin B as described in the Experimental section. Irradiation was carried out either in the presence of 500 mM-L-glucose (open bars) or 500 mM-D-glucose (shaded bars). After electrophoresis on SDS/polyacrylamide gels containing 10% acrylamide, the gel tracks were cut into 1 cm squares and their radioactivity was determined by liquid-scintillation counting as described in the Experimental section. A spectrophotometric scan at 560 nm of the Coomassie Blue-stained gel track has been superimposed. TD, Tracking dye. A and B indicate the positions of highly aggregated and of dimeric transporter molecules respectively.

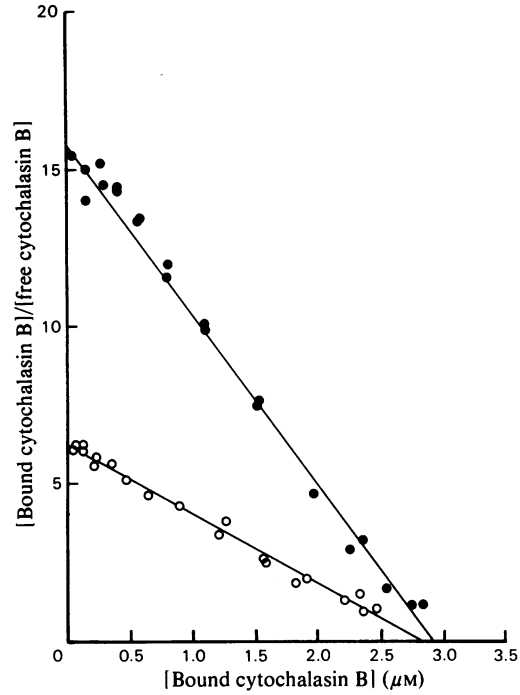

Fig. 2. *Scatchard plot analysis of cytochalasin B binding to the transporter before and after tryptic digestion*
Freshly prepared glucose transporter (187 μg/ml) was digested at 25°C for 2 h with trypsin (3 μg/ml). A second addition of trypsin was then made, bringing the total concentration to 6 μg/ml, and digestion was continued for 22 h. The digestion was halted by the addition of bovine lung aprotinin (12 μg/ml). A control sample was treated identically, except for the omission of trypsin. Cytochalasin B binding was measured by equilibrium dialysis over the concentration range from 0.5×10^{-7} M to 75.5×10^{-7} M and the data were subjected to Scatchard analysis. (●) Control sample; (○) trypsin-treated sample. The lines are the best fits according to linear least squares analysis.

only 0.12% of the original radioactivity remained associated with the monomer band on SDS/polyacrylamide-gel electrophoresis. The incorporation of radioactivity into the transporter upon illumination was essentially the same in the absence of L-glucose as in its presence at a concentration of 500 mM (results not shown). However, substitution of the L-glucose by 500 mM-D-glucose reduced the incorporation of radioactivity into the monomer band by 52% (Fig. 1, shaded bars).

The effects of longer periods of exposure to u.v. light were also investigated. It was found that when the protein was exposed for 10 min the incorporation of radiolabel into the transporter monomer increased 2-fold. The pattern of labelling was very similar to that resulting from 5 min illumination (cf. Fig. 5) and showed an identical sensitivity to inhibition by D-glucose. Occasionally a slight aggregation of the transporter polypeptide and signs of its partial degradation were visible on SDS/polyacrylamide gels of such samples. Exposure of the transporter to u.v. light for longer periods (e.g. 20 min) resulted in a marked increase in the amount of degradation with only a small further increase in the incorporation of radioactivity (results not shown). Therefore 10 min was adopted as the optimal period for illumination and this period of exposure was used for experiments in which labelling was followed by cleavage of the protein. Exposure of the transporter to u.v. light in the absence of cytochalasin B even for this short time resulted in the loss of 80% of the cytochalasin B binding activity.

Tryptic digestion

Prolonged (24 h) digestion of freshly prepared glucose transporter (187 μg/ml) with trypsin (3 μg/ml added initially and again after 2 h) resulted in the loss of 60% of the cytochalasin B binding activity. Scatchard analysis of the binding showed that there was no change in the number of

binding sites in the preparation, but that their affinity for cytochalasin B was decreased: the K_s for cytochalasin B binding to the unproteolysed transporter was 1.8×10^{-7} M, whereas that for binding to the trypsin-treated preparation was 4.5×10^{-7} M (Fig. 2). The linearity of the Scatchard plot indicated that very few of the higher affinity sites remained. All the other experiments to be described were carried out using transporter that had been stored frozen at −70°C. Tryptic digestion of this material led to a slightly smaller loss of activity (45–50%) and to the loss of about 75% of the transporter band on SDS/polyacrylamide gels (Fig. 3). The addition of fresh trypsin after 2h and 4h of digestion led to little further loss of activity. The presence of a small remainder of undigested transporter was reflected in curvature of the Scatchard plots for cytochalasin binding to this material (results not shown).

The products of tryptic digestion of the glucose transporter were analysed by SDS/polyacrylamide-gel electrophoresis of samples taken at various times during the digestion (Fig. 4*a*). Prolonged digestion of the transporter gave rise to two large fragments, both of which remained associated

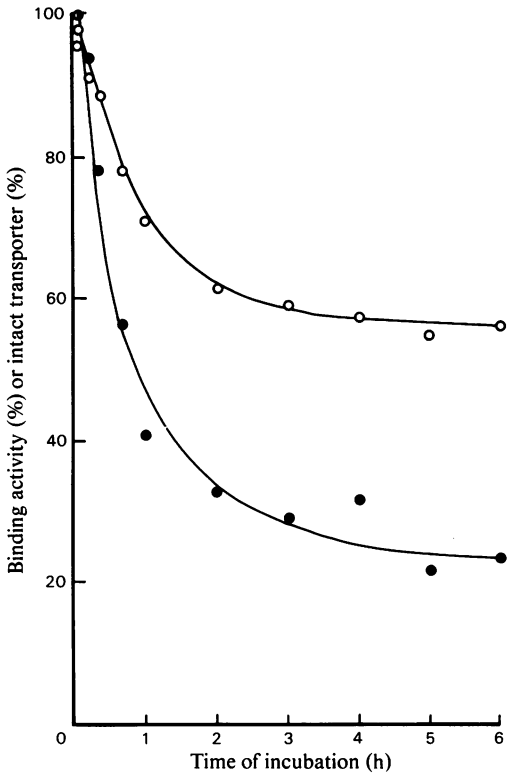

Fig. 3. *Effect of tryptic digestion on the glucose transporter*
Tryptic digestion of the glucose transporter (300 μg/ml) was carried out at 25°C with an initial trypsin concentration of 3 μg/ml. Fresh trypsin was added at the same concentration after 2h and again after 4h. At the time points indicated, samples were removed and added to bovine lung aprotinin in order to stop the digestion. They were then either prepared for electrophoresis on SDS/polyacrylamide gels containing 12% acrylamide, or assayed for cytochalasin B binding activity as described in the Experimental section. (○) Percentage of initial cytochalasin B binding activity remaining; (●) percentage of intact transporter polypeptide remaining, estimated by spectrophotometric scanning of the Coomassie Blue-stained gel.

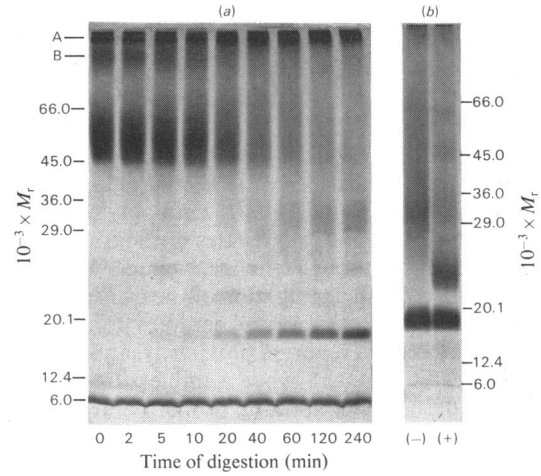

Fig. 4. *Separation of the tryptic fragments of the transporter by electrophoresis and the effect of treatment with endo-β-galactosidase*
(*a*) Electrophoretic analysis of the patterns of fragmentation produced by tryptic digestion of the transporter for the times indicated. Digestion was performed as described in the legend to Fig. 3. (*b*) The effect of endo-β-galactosidase treatment on the electrophoretic behaviour of the tryptic fragments. Glucose transporter (300 μg/ml) was digested with trypsin (3 μg/ml) for 2h at 25°C, and then a second addition of trypsin was made, equal in amount to the first, and digestion continued for a further 22h. After inactivation of the trypsin by addition of 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK') to a final concentration of 1 mM, samples were treated with (+) and without (−) endo-β-galactosidase as described in the Experimental section. Electrophoresis was performed as described in the legend to Fig. 3 and the gels were stained with Coomassie Blue. The following marker proteins of known M_r were used: bovine serum albumin (66000); ovalbumin (45000); glyceraldehyde-3-phosphate dehydrogenase (36000); carbonic anhydrase (29000); soya bean trypsin inhibitor (20100); cytochrome *c* (12400) and aprotinin (6000). A and B indicate the positions of tetrameric or more highly aggregated transporter molecules, and of dimeric transporter molecules respectively.

with the membrane (being recovered in the pellet following centrifugation at 100000*g* for 1h). The larger fragment ran as a diffuse, poorly staining band corresponding to an apparent M_r of 23000–42000 on SDS/polyacrylamide-gel electrophoresis. The smaller fragment, of apparent M_r 18000, ran as a sharp band. At earlier times of digestion, small amounts of a polypeptide of apparent M_r 23000 were also visible on the gels (Fig. 4*a*). The intensity of this band decreased as that of the M_r 18000 polypeptide increased, and so it probably represents a precursor of the latter. In addition, within the broad band of the fragment with apparent M_r 23000–42000, a sharper band corresponding to a species of apparent M_r 33000 could be seen to increase in amount in parallel with the M_r 18000 fragment during the course of the digestion. We believe that this comprises a dimeric form of the M_r 18000 fragment, both because of its electrophoretic mobility and because it can be photoaffinity labelled with [4-^{3}H]cytochalasin B (see below). The bands seen just below the top and at the top of the gel in Fig. 4(*a*) probably represent tetramers and higher aggregates of the transporter respectively, although the M_r values were not determined. Such aggregation of the transporter in SDS during preparation of samples for electrophoresis has been reported previously, and is found in increased amount when samples are electrophoresed on gels containing a high percentage of acrylamide (Baldwin *et al.*, 1982).

In order to ascertain whether the M_r 23000–42000 band represented a heterogeneously glycosylated fragment, samples of the digest were treated with endo-β-galactosidase from *B. fragilis*. Preliminary experiments had shown that the effect of this enzyme on the transporter was identical to that of endo-β-galactosidase from *E. freundii*: the broad band corresponding to an apparent M_r of 55000 was converted to a sharper band corresponding to an apparent M_r of 46000 on SDS/polyacrylamide gels (Baldwin *et al.*, 1982). The two enzymes are known to have almost identical specificities (Scudder *et al.*, 1984). Digestion of the transporter appeared to be equally effective in the presence or absence of a concentration of octylglucoside (1%, w/v) that completely clarified the suspension. However, incubation of the transporter at 37°C in the presence of octylglucoside caused irreversible aggregation of some of the protein, and so endoglycosidase digestions were routinely performed in the absence of detergent. Endoglycosidase treatment of the trypsin-digested transporter had no effect on the electrophoretic mobility of the M_r 18000 fragment. However, the broad band corresponding to the fragment of apparent M_r 23000–42000 largely disappeared, to be replaced by a sharper band with an electrophoretic mobility corresponding to an apparent M_r of 23000 (Fig. 4*b*).

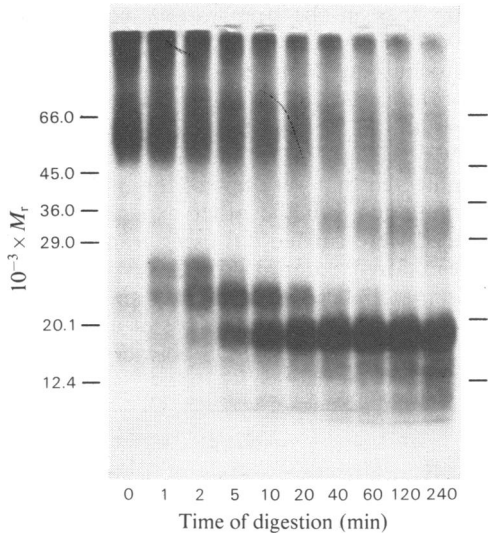

Fig. 5. *Tryptic digestion of glucose transporter photoaffinity labelled with [4-^{3}H]cytochalasin B*

Glucose transporter was photoaffinity labelled with [4-^{3}H]cytochalasin B in the presence of 500mM-L-glucose as described in the Experimental section; the period of illumination with u.v. light was 10min. Tryptic digestion was then carried out and samples taken at the times indicated for electrophoresis, as described for Fig. 3. The fluorograph shown was prepared as described in the Experimental section. The positions of marker proteins of known M_r, described in the legend to Fig. 4, are indicated.

Tryptic digestion of transporter that had been photoaffinity labelled with cytochalasin B gave rise to the same pattern of Coomassie Blue-staining bands on SDS/polyacrylamide gels as did the unlabelled protein. Fluorography revealed that after prolonged digestion of the protein, the radiolabel was associated predominantly with the fragment of apparent M_r 18000 and with its putative dimer of M_r 33000 (Fig. 5). Apart from the region occupied by this dimer, the broad band comprising the fragment of apparent M_r 23000–42000 was not labelled. During the very early part of the digestion, before the M_r 18000 band became prominent, two other labelled bands were also seen. The first to appear was a fragment of apparent M_r 25500 which had completely disappeared by 10min. A second fragment of M_r 23000 reached a peak intensity by about 5min and had largely disappeared by 40min. It is likely that both these fragments represented precursors to the M_r 18000 fragment, which increased in amount as they disappeared, although the kinetics of this process have not been investigated in detail.

NTCB cleavage

Treatment of the intact transporter with NTCB in SDS usually resulted in the fragmentation of about 50% of the polypeptides in the preparation, although the extent of cleavage was rather variable. On SDS/polyacrylamide gels, a broad band of Coomassie Blue-staining material of slightly higher mobility than the intact transporter appeared, but was poorly resolved from the latter (Fig. 6*a*). A clearer picture was seen when endo-β-galactosidase-treated transporter was cleaved with NTCB. Here a prominent fragment of apparent M_r 38000 ran as a fairly sharp band, well resolved from the intact transporter (apparent M_r 46000) (Fig. 6*a*). In both cases, a number of lower-M_r fragments were also produced. These had apparent M_r values of 31000, 19000, 15500 and 8000 (Fig. 6*a*).

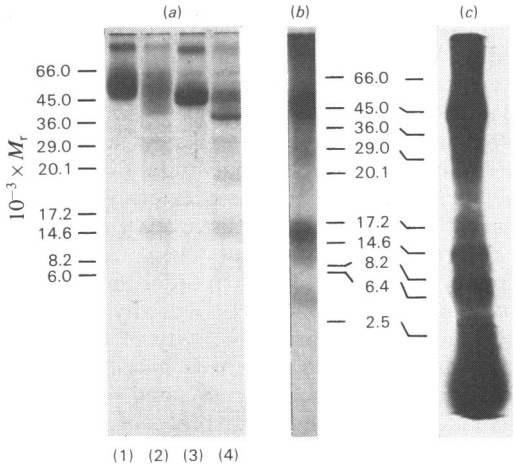

Fig. 6. *Cleavage of unlabelled, and of photoaffinity labelled, glucose transporter with NTCB*

Photoaffinity labelling, endo-β-galactosidase treatment, cleavage with NTCB and fluorography were carried out as described in the Experimental section. Samples were electrophoresed on SDS/polyacrylamide gels containing a gradient of 10–18% acrylamide. The positions of marker proteins of known M_r, described in the legend to Fig. 4, are indicated. In addition, myoglobin and its CNBr fragments were used as markers, as described in the Experimental section. (*a*) Coomassie Blue-stained gel; lane (1), untreated transporter; lane (2), untreated transporter after NTCB cleavage; lane (3), endo-β-galactosidase-treated transporter; lane (4), endo-β-galactosidase-treated transporter after NTCB cleavage. (*b*) A fluorograph of photoaffinity labelled, endo-β-galactosidase-treated and NTCB-cleaved glucose transporter after electrophoresis. (*c*) A fluorograph of endo-β-galactosidase-treated transporter that had been cleaved with 2-nitro-5-[*cyano*-^{14}C]thiocyanobenzoic acid and then subjected to electrophoresis.

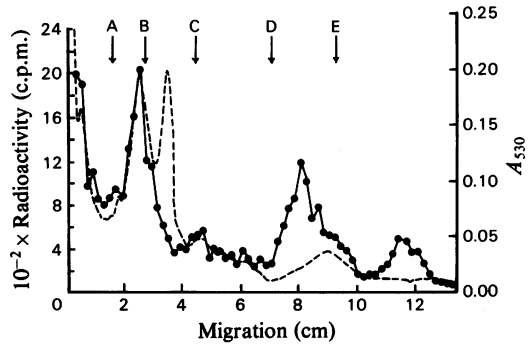

Fig. 7. *Identification of radiolabelled fragments produced by NTCB cleavage of endo-β-galactosidase-treated, photoaffinity labelled glucose transporter*

Glucose transporter was photoaffinity labelled with [4-^{3}H]cytochalasin B, then treated with endo-β-galactosidase and cleaved by NTCB as described in the Experimental section. A sample was electrophoresed on an SDS/polyacrylamide gel containing 15% acrylamide. The Coomassie Blue-stained gel was scanned at 530nm (----) and then cut into 2mm slices for determination of radioactivity (●) by liquid-scintillation counting as described in the Experimental section. Arrows indicate the positions of marker proteins, described in the legend to Fig. 4, that had the following M_r values: A, 66000; B, 45000; C, 29000; D, 20100; E, 12400

An identical pattern of fragments was produced by NTCB treatment of endo-β-galactosidase-treated transporter that had been photoaffinity labelled with [4-^{3}H]cytochalasin B. Fluorography showed that the radiolabel was associated predominantly with a fragment of apparent M_r 15500 and with the remaining intact transporter polypeptide (Fig. 6*b*). The prominent fragment of M_r 38000 appeared to be unlabelled; this finding was confirmed by slicing the gel into 2mm sections and determining their radioactivity by the method of Goodman & Matzura (1971) (Fig. 7). Treatment of the transporter with 2-nitro-5-[*cyano*-^{14}C]thiocyanobenzoic acid produced the same pattern of fragments as for the unlabelled reagent, although the amount of cleavage in this particular experiment was rather low. Fluorography showed that all the fragments were radiolabelled (Fig. 6*c*).

Discussion

The technique of photoaffinity labelling with [4-^{3}H]cytochalasin B has been used in several laboratories to identify glucose-sensitive, cytochalasin B-binding polypeptides in a variety of cell types (Shanahan, 1982; Carter-Su *et al.*, 1982; Shanahan *et al.*, 1982). Although the mechanism of the photoincorporation reaction is not yet known,

its characteristics strongly suggest that the polypeptides labelled are glucose transport proteins (Shanahan, 1983). In the present study we have confirmed this suggestion by demonstrating that the isolated glucose transporter can be photoaffinity labelled by using cytochalasin B. At the concentrations of transporter (3.9×10^{-6} M) and of cytochalasin B (1×10^{-6} M) used in our experiments, approx. 95% of the cytochalasin would have been bound noncovalently to the protein before u.v. irradiation. After exposure to u.v. light for 10 min, 6% of the total cytochalasin B had become covalently associated with the protein, correspondingly to approx. 6.3% of the cytochalasin formerly bound noncovalently. It is difficult to compare this value with the results described by other workers who have labelled erythrocyte membranes, because of the different concentrations of ligand and protein employed, but the efficiencies of labelling appear to be roughly equivalent (Carter-Su *et al.*, 1982; Shanahan, 1982). Other workers have also noted the aggregation of protein and loss of activity upon u.v. irradiation that we have described here (Shanahan, 1983). The specificity of the labelling reaction is demonstrated by its susceptibility to inhibition by the transported sugar, D-glucose. If the K_i for inhibition by D-glucose of cytochalasin B binding to the transporter is taken to be 21 mM (Zoccoli *et al.*, 1978), then the presence of 500 mM-D-glucose during photolabelling experiments would be expected to inhibit the noncovalent binding by approx. 55%. This value is very similar to the 52% inhibition of incorporation of radioactivity caused by the presence of glucose. Several lines of evidence suggest that cytochalasin B and glucose compete for binding to a site on the transport protein that faces the cytoplasm (Devés & Krupka, 1978; Baldwin *et al.*, 1980). Therefore the site(s) photoaffinity labelled by cytochalasin B is probably located in the cytoplasmic domain of the protein.

In a previous study it was suggested that the procedure we have used here for purification of the glucose transporter yields mainly unsealed membranous structures (Gorga & Lienhard, 1982). The consequent accessibility to the medium of both the cytoplasmic and extracellular domains of most of the transporter molecules in the preparation has been confirmed in the present study, and has greatly facilitated the use of enzymes for investigation of the protein structure. Approx. 75% of the polypeptides were degraded upon addition of trypsin. Since trypsin is known to cleave the transporter only when present at the cytoplasmic surface of the erythrocyte membrane (Baldwin *et al.*, 1980), these polypeptides must have had their cytoplasmic domains exposed to the medium. Similarly, the majority of the transporters must have had extracellular domains accessible to the medium, because solubilization of the membrane in detergent did not enhance the ability of endo-β-galactosidase to remove carbohydrate from the protein; the oligosaccharide chains are known to be exposed on the extracellular surface of the intact erythrocyte (Gorga *et al.*, 1979). The partial loss of accessibility to trypsin produced by freezing and thawing the preparation probably resulted from the production of sealed structures or aggregates.

Scatchard plot analysis of cytochalasin B binding to the trypsin-treated transporter showed that the loss of binding activity resulted from a decreased affinity for the ligand. The fact that the cleaved transporter continues to bind cytochalasin B, albeit with a lower affinity, and the continued association of the protein fragments with the membrane, suggest that the tertiary structure of the protein may not be greatly altered by trypsin treatment. In a previous study, the loss of cytochalasin B binding activity upon trypsin treatment of the purified transporter was ascribed largely to a complete loss of some binding sites, although a decrease in binding affinity was also observed (Baldwin *et al.*, 1980). Several possible explanations can be envisaged to explain the rather different findings of the current study. Firstly, in the previous investigation, more extensive degradation of the transporter may have occurred, because the trypsin used had not been treated with diphenylcarbamyl chloride in order to inhibit any contaminating chymotryptic activity. Secondly, the much higher specific activity of the current preparation for cytochalasin B binding, and the lower nonspecific binding, obviated the need for computerized curve-fitting procedures in Scatchard analysis: such procedures had introduced some uncertainty into the earlier findings (Baldwin *et al.*, 1980).

Tryptic digestion of the transport protein produced a large fragment which ran as a broad band on SDS/polyacrylamide-gel electrophoresis. It is likely that this fragment bears one or more oligosaccharide chains, because treatment with endo-β-galactosidase resulted in a sharper band with increased electrophoretic mobility. Since it is glycosylated and is produced by tryptic cleavage of the cytoplasmic domain of the protein, then it follows that this fragment must cross the lipid bilayer at least once. Comparison of its apparent M_r of 23000 after endoglycosidase treatment with that of the intact protein (46000) indicates that a trypsin-sensitive site must lie close to the middle of the transporter polypeptide. The other half of the polypeptide is probably represented by the fragments of apparent M_r 25500 and 23000 seen on SDS/polyacrylamide gels after short times of digestion. These fragments ran as sharp bands

even in the absence of endoglycosidase treatment. They appeared to be precursors to the fragment of apparent M_r 18000 which accumulated after prolonged digestion. Since the electrophoretic mobility of the M_r 18000 fragment was unchanged by endoglycosidase treatment, it is not possible to state whether or not it is glycosylated. Therefore although it must be exposed at the cytoplasmic surface of the membrane, whether it spans the lipid bilayer remains unknown. The fragment presumably forms a part of the cytochalasin B binding site, because it becomes radiolabelled following exposure of the intact protein to u.v. light in the presence of [4-^{3}H]cytochalasin B.

The glucose transporter contains only five cysteine residues (Baldwin *et al.*, 1982) and so treatment of the protein with NTCB was employed to generate a small number of polypeptide fragments. Cleavage of the photoaffinity-labelled, endo-β-galactosidase-treated transporter with NTCB yielded a large unlabelled fragment of apparent M_r 38000 and several smaller fragments. If endoglycosidase treatment was omitted the sharp band of the M_r 38000 fragment was replaced by a broad band corresponding to an apparent M_r of 36000–48000 on SDS/polyacrylamide gels, and so it is likely that this fragment contains one or more sites of glycosylation in the transporter. The absence of label from the large fragment suggests that the site(s) of labelling lies fairly close to one end of the intact polypeptide. From a comparison of the apparent M_r values of the fragment (38000) and of the intact transporter (46000) the site(s) labelled by cytochalasin would be expected to be found on a fragment(s) of $M_r \leqslant 8000$. Instead, radioactivity was predominantly associated with a fragment of apparent M_r 15500. However, the behaviour of the transporter on SDS/polyacrylamide-gel electrophoresis is known to be anomalous, even after endoglycosidase treatment to remove the carbohydrate (Baldwin *et al.*, 1982), and so the estimated sizes of the fragments may be incorrect. In an attempt to identify the fragment that contained the *N*-terminus of the protein, the transporter was cleaved with radiolabelled NTCB. The mechanism of cleavage is such that all fragments except that derived from the *N*-terminus should become labelled (Jacobson *et al.*, 1973). However, although the extent of cleavage in this experiment was low, all the peptides appeared to be labelled.

In this paper we have described methods for identifying the sites of glycosylation, tryptic cleavage and photoaffinity labelling within the transporter polypeptide. Since the positions of these sites on either the extracellular or cytoplasmic side of the membrane are already known, a knowledge of their location within the protein should give an insight into the folding pattern of the polypeptide in the membrane and thus eventually perhaps into the mechanism of action of the transporter. By means of tryptic cleavage we have been able to identify two separate regions of the protein, one of which is glycosylated and spans the bilayer and another which bears the cytochalasin B binding site. Cleavage with NTCB has further shown that the latter site lies close to one end of the protein. Thus considerable information has already been obtained concerning the topography of the transporter. Further work is necessary to isolate the fragments and determine their order within the intact polypeptide.

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