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Characterisation of monoclonal antibodies which specifically recognise the human erythrocyte glucose transport protein

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Two monoclonal antibodies (mabs) of subclass IgG_1 have been raised against the human erythrocyte glucose transport protein. The mabs bound to the purified glucose transporter in both its membrane-bound and detergent-solubilised forms. However, they exhibited little or no binding to the detergent-solubilised nucleoside transport protein, which is present as a minor contaminant in the glucose transport protein preparation. Both mabs inhibited the binding of cytochalasin B to the glucose transport protein, reducing the affinity of this binding by >2-fold. Each mab labelled the transporter polypeptide on Western blots both before and after treatment of the protein with endoglycosidase F, indicating that the epitopes recognised were located on the protein moiety of the glycoprotein. However, the mabs did not bind to the large fragments produced by tryptic or chymotryptic digestion of the native protein, although both mabs were shown to bind to sites on the cytoplasmic surface of the erythrocyte membrane.

Key words: monoclonal antibodies/glucose transport protein/human erythrocytes

Introduction

Hexose uptake across the plasma membrane represents a primary site of metabolic regulation in many cell types. Transport systems that facilitate the uptake of D-glucose are found in the plasma membrane of most mammalian cells (Elbrink and Bihler, 1975). One of the most abundant, and certainly the best characterised, of these facilitated diffusion systems is that of the human erythrocyte membrane (Lienhard *et al.*, 1983). Although it has been suggested that the erythrocyte glucose transporter is a protein of mol. wt. 100 000 (Shelton and Langdon, 1983), most of the available evidence indicates that transport is catalysed by a protein that runs as a broad band of apparent mol. wt. 55 000 on SDS-polyacrylamide gel electrophoresis (for references, see Shanahan, 1982; Carter-Su *et al.*, 1982). This protein has been purified to near homogeneity, although the isolated preparation contains small amounts of contaminating proteins (Kasahara and Hinkle, 1977; Baldwin *et al.*, 1979, 1982). A major defined contaminant is the nucleoside transporter protein, which may comprise up to 3% of the total protein (Jarvis and Young, 1981). The nucleoside transport protein also runs as a broad band of apparent mol. wt. 45 000-65 000 on SDS-polyacrylamide gel electrophoresis (Wu *et al.*, 1983) and it has been suggested that the two transporters may be structurally similar (Young *et al.*, 1983). Highly purified preparations of the glucose transport protein catalyse the uptake of D-glucose into artificial lipid vesicles with kinetics similar to those seen in the intact erythrocyte (Kasahara and Hinkle, 1977; Baldwin *et al.*, 1981; Wheeler and Hinkle, 1981). These preparations also bind cytochalasin B (a potent reversible inhibitor of glucose transport) with high affinity and with a stoichiometry of ~0.8 inhibitor molecules per polypeptide chain (Baldwin *et al.*, 1982). The glucose transport protein runs as a broad band on SDS-polyacrylamide gels as a result of its heterogeneous glycosylation (Gorga *et al.*, 1979); deglycosylation of the protein by treatment with endoglycosidase F causes it to run as sharp band of apparent mol. wt. 46 000 (Lienhard *et al.*, 1984). The protein is known to span the membrane, with the attachment site(s) for oligosaccharide on its extracellular domain (Gorga *et al.*, 1979) and sites susceptible to tryptic cleavage on its cytoplasmic domain (Baldwin *et al.*, 1980). However, little more is known about its arrangement in the lipid bilayer at present.

Much less is known about the molecular basis of glucose transport in cells other than the human erythrocyte. Putative transporter polypeptides of mol. wt. similar to the erythrocyte transporter have been identified in a number of cell types by the procedure of photoaffinity labelling with cytochalasin B (Shanahan, 1982; Shanahan *et al.*, 1982; Carter-Su *et al.*, 1982). Polyclonal antibodies to the erythrocyte transporter have also been successfully employed to identify the glucose transport proteins of other cells, indicating that these are probably similar to the erythrocyte transporter (Salter *et al.*, 1982; Wheeler *et al.*, 1982; Lienhard *et al.*, 1982; Klip *et al.*, 1983). However, since a completely pure preparation of the transporter is not yet available for use as an immunogen, the possibility exists that some fraction of the antibodies raised are in fact directed against other proteins. Here we describe the production and rigorous characterisation of monoclonal antibodies (mabs) against a highly purified preparation of the human erythrocyte glucose transport protein. The mabs characterised here will be of great value in the further elucidation of structure/function relationships in the glucose transport protein; mabs have recently been employed for this purpose with several membrane proteins, e.g., rhodopsin and bacteriorhodopsin (Molday and Mackenzie, 1983; Ovchinnikov *et al.*, 1985). Our antibodies may also be an important tool in the characterisation of glucose transport proteins in cells other than the human erythrocyte.

Results

Characterisation of mabs by ELISA

Both mabs (GTPR 1 and 2) were shown by the enzyme-linked immunosorbent assay (ELISA) (see Materials and methods) to be of the subclass IgG_1. The purified glucose transport protein proved to be an effective inhibitor of the binding of each of the mabs to the immobilised protein in competitive ELISA (Figure 1a), indicating that each of the antibodies could bind to the transporter in its native conformation. The ability of the glucose trans-

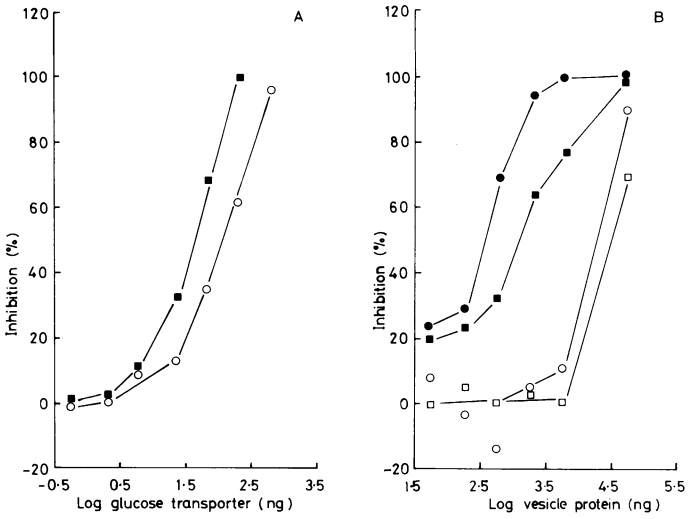

Fig. 1. Competitive ELISA. **(a)** The ability of purified glucose transporter to compete for limiting amounts of mab with 339 ng transporter bound to the surface of microtiter wells. ■, GTPR-1 (1:5000 dilution); ○, GTPR-2 (1:30 000). The amounts of transporter producing 50% inhibition of binding are 35 and 115 ng for GTPR-1 and -2, respectively. **(b)** The ability of right-side-out (○□ and inside-out (● ■) vesicles to compete with solid phase bound glucose transport protein for limiting amounts of GTPR-1 (1:5000 dilution) denoted by circles and GTPR-2 (1:30 000) denoted by squares.

port protein in erythrocyte membranes to compete for the mabs with solid phase bound protein was also investigated by incubating diluted ascitic fluid with various concentrations of right-side-out or inside-out erythrocyte membrane vesicles (Figure 1b). Enzymic assays showed that the 'inside-out' vesicle preparation comprised 65% sealed, inside-out vesicles contaminated with 35% sealed, right-side-out vesicles. The preparations of 'right-side-out' vesicles used was far less contaminated, comprising >99% sealed, right-side-out vesicles. Both preparations contained equal amounts of glucose transporter per mg of membrane protein, as measured by D-glucose-sensitive cytochalasin B binding activity. The inside-out vesicle preparation proved to be ~100-fold more effective in binding either antibody than the right-side-out preparation. This indicates that the mabs bind to epitopes exposed on the cytoplasmic surface of the erythrocyte membrane. The binding observed when very high concentrations of the right-side-out preparation was used presumably resulted from the small (<1%) contamination of this preparation with inside-out vesicles.

Western blotting

The preparation of transporter used to raised antibodies in this study contained small amounts of contaminating proteins (see Introduction). It was possible, therefore, that some of the mabs produced might have been directed against one or other of these contaminants. Confirmation that the mabs bound specifically to the glucose transporter was sought using the technique of Western blotting (Towbin *et al.*, 1979). GTPR-1 labelled a broad zone between bands 3 and 5 (nomenclature according to Steck, 1974b) on blots of human erythrocyte membrane proteins, corresponding to the mol. wt. range 43 000 – 80 000 (Figure 2a). Within this band the region occupied by bands 4.1 and 4.2 was poorly labelled. No other erythrocyte membrane proteins were labelled. In contrast to the results with human erythrocytes, labelling by GTPR-1 of a blot of pig erythrocyte membrane proteins run on the same gel was barely detectable (Figure 2a). GTPR-2 yielded labelling of blots of human erythrocyte membrane proteins identical to that of GTPR-1 (data not shown). However, when a control IgG_1 mab, anti-O^6-methyl deoxyguanosine (Wild *et al.*, 1983), was substituted for the anti-transporter mabs, no labelling was observed. Both mabs also labelled blots of the purified transporter, yielding a pattern identical to that seen by staining for protein (Figure 2b). Similarly each antibody strongly labelled the 46-kd band produced by treatment of the purified transporter with endoglycosidase F (Figure 2b).

Immunoadsorption assays

Since both the glucose and nucleoside transport proteins of human erythrocyte membranes run as broad bands of apparent mol. wt. 55 000 on SDS-polyacrylamide gel electrophoresis (Lienhard *et al.*, 1983; Wu *et al.*, 1983) Western blotting cannot establish unambiguously to which species the antibodies bind. However, the two proteins are distinguishable in that the glucose transporter specifically binds cytochalasin B, whereas the nucleoside transporter specifically binds 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR) (Lin and Spudich, 1974; Jarvis and Young, 1981). Assay of the binding of these two ligands was used to investigate the removal from solution of detergent-solubilised transporters, following incubation with antibody immobilised on protein A-Sepharose CL-4B. Two experiments were carried out: in the first, solubilised transporter was incubated either with Sepharose CL-4B as a control, or with immobilised antibody. The immobilised mabs removed ~75% of the cytochalasin B binding activity from the solution, relative to the control, but only 5 – 16% of the NBMPR binding activity (Table I). In the second experiment, non-immune mouse IgG was employed as a control. Here, the mabs GTPR-1 and 2 removed 98 – 100% of the cytochalasin B binding activity from solution (Table I). Slightly greater amounts of NBMPR binding activity were lost than in experiment 1 (10 – 18%), but substantial activity remained.

Effect of mabs on cytochalasin B binding activity

The effect of the mabs on the cytochalasin B binding activity of the glucose transport protein was investigated using protein-depleted human erythrocyte membranes. These membranes have been shown to be almost completely unsealed (Gorga and Lienhard, 1981) and so both faces of the membrane should be completely accessible to membrane-impermeant antibody molecules. In addition, the only high affinity cytochalasin B binding sites in these membranes are those associated with the glucose transporter (Baldwin *et al.*, 1979).

Non-immune IgG had no effect on the binding of cytochalasin B to the membranes (Figure 3a, b). However, GTPR-1 and 2 inhibited the binding (Figure 3a, b). Neither non-immune mouse IgG nor the mabs themselves bound cytochalasin B. A maximal inhibition of ~74% of the binding activity was obtained at concentrations of GTPR-1 of 200 μg/ml (Figure 3a), where the concentration of total membrane protein was 400 μg/ml. A somewhat higher concentration of GTPR-2 was necessary for maximal inhibition: in the experiment shown in Figure 3b, a maximal inhibition of cytochalasin B binding activity of 85% was obtained at an IgG concentration of 600 μg/ml where the concentration of membrane protein was only 200 μg/ml.

Results similar to those described above were also obtained using a purified transporter preparation (data not shown). The physical state of the membranes in such preparations has not been as well characterised as for protein-depleted erythrocyte membranes, but it is likely that they are largely unsealed (Gorga and Lienhard, 1982; Cairns *et al.*, 1984). Scatchard analysis of cytochalasin B binding to the purified transporter in the presence of a control mab (anti-O^6-methyl deoxyguanosine) and in the

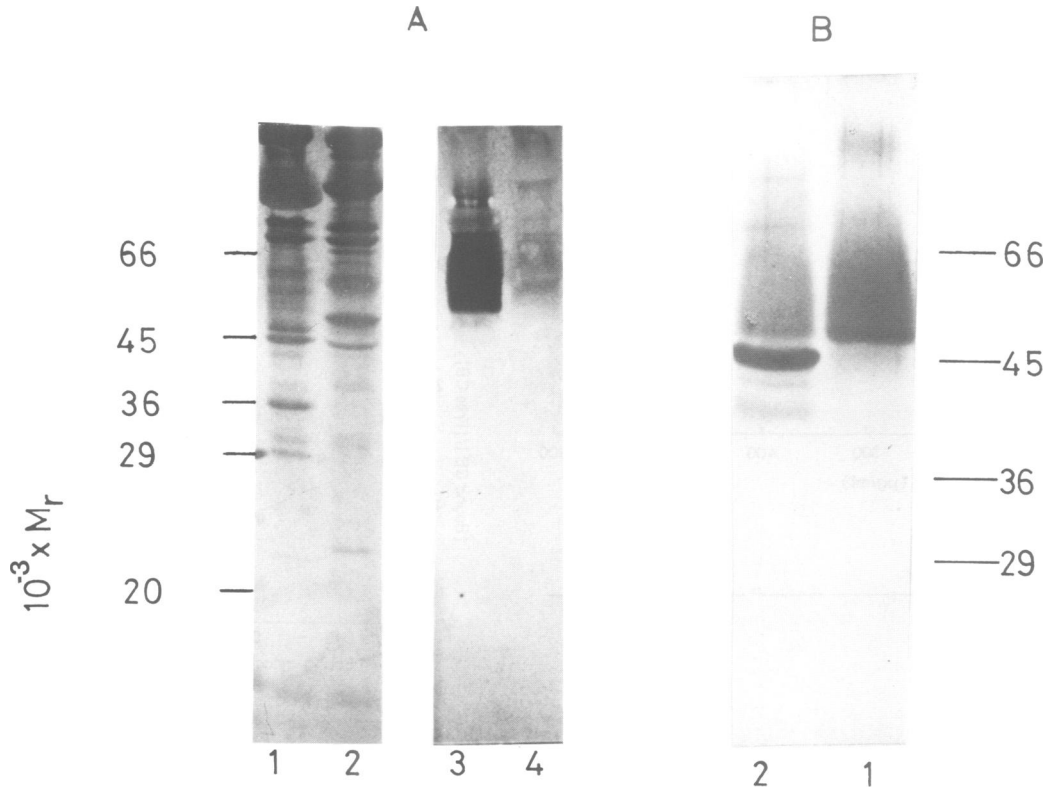

Fig. 2. Binding of mabs to the glucose transport protein on Western blots. **(a)** Binding of GTPR-1 to human **(lanes 1** and **3)** and pig **(lanes 2** and **4)** erythrocyte membrane proteins. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose as described in Materials and methods. **Lanes 1** and **2** were stained for protein with amido black; **lanes 3** and **4** were stained with GTPR-1 as described in Materials and methods. **(b)** Binding of GTPR-2 to the purified transporter before **(lane 1)** and after **(lane 2)** treatment with endoglycosidase F as described in Materials and methods. Samples were electrophoresed on a 10% SDS-polyacrylamide gel, electrophoretically transferred to nitrocellulose and then stained with GTPR-2 as decribed in Materials and methods. The following marker proteins of known mol. wt. were used: bovine serum albumin (66 000); ovalbumin (45 000); glyceraldehyde 3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); and soya bean trypsin inhibitor (20 100).

Table I. Immunoadsorption of solubilised glucose and nucleoside transporters

	Immunoadsorbent	% Ligand binding activity remaining in solution	
		Cytochalasin B	NBMPR
Experiment 1	Sepharose CL-4B[a]	100	100
	GTPR-1 (0.7 mg)[b]	24	84
	GTPR-2 (1.0 mg)[b]	25	95
Experiment 2	Non-immune mouse IgG (1.2 mg)	100	100
	GTPR-1 (1.2 mg)	2	82
	GTPR-2 (1.2 mg)	0	90

Removal of cytochalasin B and NBMPR binding activities from a solution of purified transporter solubilised in octyl glucoside. In experiment 1 the solubilised transporter was incubated either with 200 μl Sepharose CL-4B alone as a control (a), or with mab bound to 200 μl protein A-Sepharose CL-4B (b). In experiment 2, non-immune mouse IgG was used as a control, and both it and the two mabs were bound to 300 μl portions of protein A-Sepharose CL-4B. The ligand binding activities of the supernatants were measured, after removal of detergent, as described in Materials and methods.

presence of a concentration of GTPR-1 that gave maximal inhibition revealed that the antibody reduced the affinity of the transporter protein for cytochalasin B (Figure 4): the dissociation constant for binding was increased from 0.17 μM to 0.45 μM. There was no significant effect on the concentration of sites. It is likely that GTPR-2 exerts a similar effect on the affinity for cytochalasin B, but the high ratio of IgG to transporter concentration required for a maximal effect precluded Scatchard analysis of the binding in the presence of this antibody.

Discussion

We have characterised two mabs raised against the human erythrocyte glucose transport protein. Their specificity for the glucose transporter was confirmed by the removal from solution of cytochalasin B binding activity seen when a detergent-solubilised transporter preparation was treated with mabs immobilised on protein A-Sepharose CL-4B. The requirement for up to a 7-fold molar excess of IgG over transporter in these experiments in order to remove $\geq 98\%$ of the activity probably reflects hindrance by the Sepharose matrix of transporter access to all the bound IgG. Even at the high concentrations of IgG used, 82% of the NBMPR binding activity (an assay for the nucleoside transport protein) remained in solution. The apparent ability of these mabs to bind a small proportion of the NBMPR binding activity may reflect a slight cross-reaction of the mabs with the nucleoside transporter. Alternatively, the small losses of activity seen may be due to some denaturation of the nucleoside transport protein during these assays.

Lack of reactivity towards the nucleoside transport protein was also evident from Western blotting experiments using pig erythrocyte membrane proteins (Figure 2a). Pig erythrocytes lack a functional glucose transporter (Kim and McManus, 1971) but possess a nucleoside transporter. This migrates on SDS-polyacrylamide gels as a broad band of apparent mol. wt. similar to that labelled

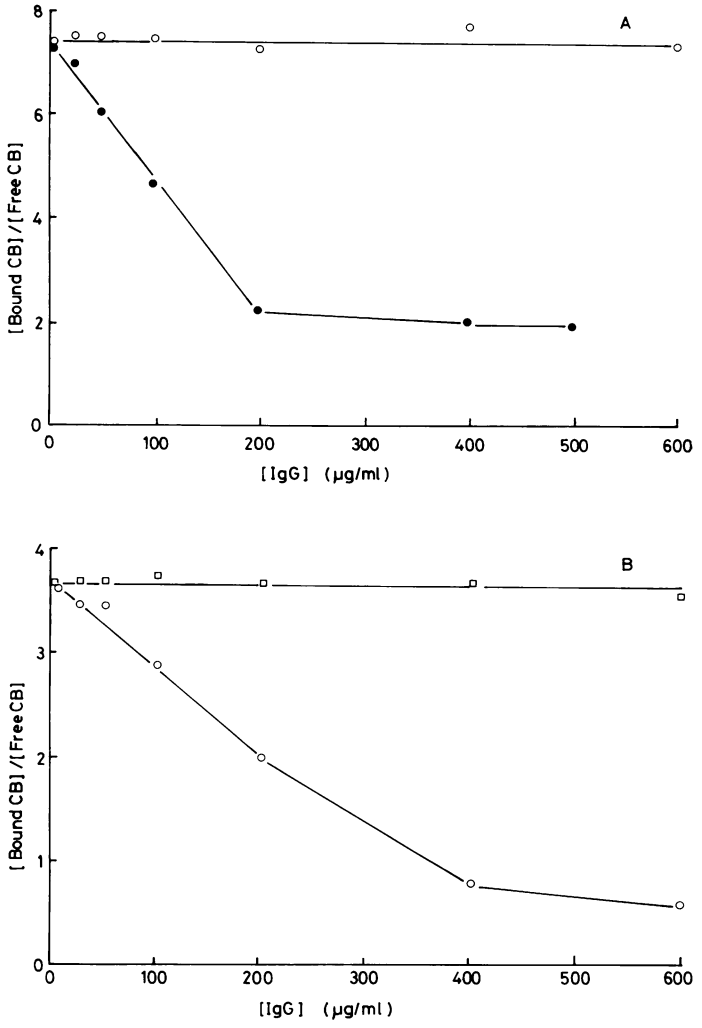

Fig. 3. Inhihition of the cytochalasin B binding activity of protein-depleted erythrocyte membranes by mabs. Protein-depleted erythrocyte membranes at a concentration of 0.4 mg/ml, (**a**) or 0.2 mg/ml (**b**) in 10 mM sodium phosphate, pH 7.2, containing 145 mM NaCl were incubated with various concentrations of the following IgGs: (**a**) non-immune mouse IgG (○), GTPR-1 (●); (**b**) non-immune mouse IgG (□), GTPR-2 (○). Cytochalasin B binding activity was measured as described in Materials and methods. The results have been corrected for non-specific binding measured after heating the membranes at 100°C for 5 min.

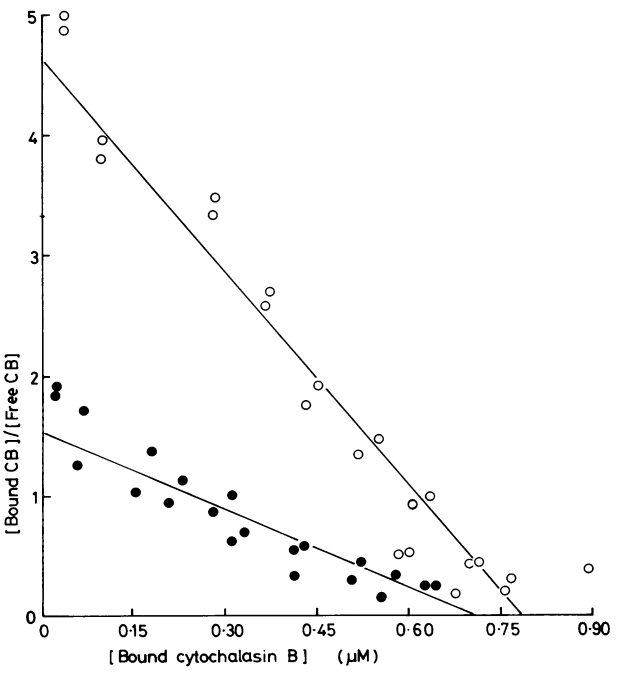

Fig. 4. Scatchard plot analysis of cytochalasin B binding to the transporter in the presence of a control mab or GTPR-1. Purified glucose transporter (50 μg/ml) was incubated with anti-O^6-methyl deoxyguanosine IgG (384 μg/ml, ○), or with purified GTPR-1 (356 μg/ml, ●), in 41 mM sodium phosphate, pH 7.4, containing 110 mM NaCl and 0.8 mm EDTA. Cytochalasin B binding was measured by equilibrium dialysis over the concentration range $0.4-41 \times 10^{-7}$ M. The straight lines are computerised best fits determined by the method of Munson and Rodbard (1980).

in Figure 2a by GTPR-1 (Wu *et al.*, 1983). The slight amount of labelling seen here may represent cross-reactivity of the antibody towards the pig erythrocyte nucleoside transporter, or may have stemmed from the presence of a very small amount of glucose transport protein in the erythrocytes or other cells present in the pig erythrocyte preparation.

The pattern of labelling of blots of human erythrocyte membrane proteins found with the mabs closely resembled that previously found using polyclonal antibodies (Baldwin and Lienhard, 1980; Sogin and Hinkle, 1980), and that seen upon photoaffinity labelling of the membranes with cytochalasin B (Shanahan, 1982; Carter-Su *et al.*, 1982). The lack of labelling of the band 3 region provides further evidence against the hypothesis that the transporter is a protein of mol. wt. 100 000 (Shelton and Langdon, 1983). Western blots also indicated that the mabs bound to the protein moiety of the glycoprotein transporter, since they labelled the transporter after its digestion with endoglycosidase F. This glycosidase is known to remove the N-linked oligosaccharide chain(s) from the transporter and converts the glucose transport protein into a species that migrates with an apparent mol. wt. of 46 000 on SDS-polyacrylamide gel electrophoresis (Lienhard *et al.*, 1984). Further evidence that GTPR-1 and -2 bind to the protein moiety of the transporter was provided by competitive ELISA, where they were shown to bind to the cytoplasmic surface of the erythrocyte membrane (Figure 1b): the oligosaccharide moiety of the transporter is known to be attached to its extracellular domain (Gorga *et al.*, 1979).

It was not possible to locate the epitopes to which the mabs bound within the primary structure of the protein, because neither mab labelled the proteolytic fragments seen on Western blots after prolonged digestion of the transporter with either trypsin or chymotrypsin (data not shown) (Cairns *et al.*, 1984; Shanahan and D'Artel-Ellis, 1984; Deziel and Rothstein, 1984). It is likely that the mabs bind to epitopes that are destroyed by proteolytic digestion, or are found on peptides too small to be detected by Western blotting.

Both mabs were found to inhibit the binding of cytochalasin B to the transporter. Several lines of evidence suggest that cytochalasin B binds to a site on the transporter exposed on the cytoplasmic surface of the erythrocyte membrane (Deves and Krupka, 1978; Baldwin *et al.*, 1980). The finding that GTPR-1 and -2, which bind to the cytoplasmic domain of the transporter, also inhibit cytochalasin B binding suggests that the epitopes they recognise lie close to the cytochalasin B binding site. The mabs may thus prove useful in locating this site within the primary structure of the protein. Their inhibition of cytochalasin B binding also, of course, confirms their specificity for the glucose transporter.

In conclusion, the properties of two mabs to the human erythrocyte glucose transport protein have been investigated. They appear to be largely or wholly specific for the glucose transporter,

and so should prove invaluable in the study of transport in human and possibly in other animal cells. They should enable purification of the nucleoside transport protein, and subsequent investigation of its possible structural relationship to the glucose transporter. Studies are in progress to determine the precise location of the epitopes within the sequence of the protein and also to determine the tissue and species specificities of these and other mabs to the human erythrocyte glucose transport protein.

Materials and methods

Materials

[4-^{3}H]Cytochalasin B (10.3 Ci/mmol) was obtained from New England Nuclear (Southampton, UK). [G-^{3}H]6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine (NBMPR; 17.6 Ci/mmol) from Moravek Biochemicals, Brea, CA, was the kind gift of Dr J.D.Young. 2-Amino-6[(4-nitrobenzyl)thio]-9-D-ribofuranosylpurine (NBTGR), *p*-nitrophenyl phosphate (Sigma phosphatase substrate 104) and mouse IgG were from Sigma (Poole, Dorset, UK). Alkaline phosphatase-linked rabbit anti-mouse IgG was obtained from Miles Laboratories Ltd. (Slough, UK). Nitrocellulose membranes and Immun-Blot Assay (GAM-HRP) Kit were obtained from Bio-Rad Laboratories Ltd. (Watford, UK). All other reagents were bought from Sigma or from BDH (Poole, Dorset, UK) and were the highest grades available. Out-dated human blood was provided by the blood bank of the Royal Free Hospital.

Preparation of membranes

Membranes were prepared from human and pig erythrocytes, respectively, by the method of Steck and Kant (1974). Unsealed, protein-depleted human erythrocyte membranes were then prepared by treatment with dilute alkali as described by Gorga and Lienhard (1981). Impermeable right-side-out and inside-out erythrocyte membrane vesicles were prepared from out-dated human blood as described by Steck (1974a) and by Steck and Kant (1974). Sealed vesicles were separated from unsealed membrane fragments by centrifugation at 4°C through a barrier of 8% (w/v) dextran T70 in 0.5 mM sodium phosphate, pH 8.0, for 2.5 h at 75 000 *g* (r_{av} 8.4 cm). Membrane sidedness was then assayed by measuring the accessibility of acetylcholinesterase and of glyceraldehyde 3-phosphate dehydrogenase, as described by Steck and Kant (1974). The purified glucose transport protein was prepared from out-dated human blood by the method of Cairns *et al.* (1984).

Preparation of monoclonal antibodies

Female BALB/c mice at 6 – 8 weeks old were immunised with purified glucose transport protein emulsified with Freund's complete adjuvant. At 0, 38 and 56 days animals received 9 μg transporter i.p. followed by a similar boost without adjuvant by the tail vein 4 days prior to fusion.

Mouse myeloma cell lines 653 (Kearney *et al.*, 1979) and NS1 (Kohler *et al.*, 1976) were grown in Dulbecco's minimal essential medium supplemented with 20% horse serum and 2% foetal calf serum (Flow Laboratories). Conditions for hybridoma production followed the method of Galfre *et al.* (1977) as described in detail previously (Strickland *et al.*, 1980; Saffhill *et al.*, 1982). GTPR-1 was produced by fusion with myeloma cell line 653 and GTPR-2 by fusion with the NS1 cell line. Hybridomas were cloned and cultured in medium supplemented with heat-inactivated serum.

Enzyme-linked immunosorbent assays

For screening well supernatants and determining antibody titres, FB well strips (Titertek) were coated with antigen by adding 80 μl of purified glucose transport protein (13 – 18 μg protein/ml) per well in 50 mM Na carbonate buffer, pH 9.6. After incubation overnight at room temperature, wells were washed five times with PBSA (Oxoid) containing 0.05% Tween 20 (PBSA-T). Unoccupied charged sites were blocked by addition of 200 μl blocking buffer (PBSA-T + 5% foetal calf serum) with incubation for 60 min at 37°C. Wells were again washed five times with PBSA-T, after which 100 μl of neat well supernatant or antibody diluted in blocking buffer was added and incubated for 90 min at 37°C. After washing five times with PBSA-T, 100 μl per well of a working dilution in blocking buffer of rabbit anti-mouse IgG conjugated to alkaline phosphatase (Miles) was added and incubated for 90 min at 37°C. After washing five times, any residual PBSA-T was removed by banging the inverted well strip onto absorbent tissue. The substrate, *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) at 1 mg per ml in 10 mM diethanolamine buffer, pH 9.8, 1 mM $MgCl_2$, was added at 100 μl per well and incubated at 37°C until a yellow colour had formed which was measured at 405 nm using a Titertek Multiskan plate reader. All buffers contained 0.1% (w/v) thiomersol to prevent microbial spoilage.

Using ascitic fluid from the growth of hybridomas *in vivo*, competitive ELISA were developed from the above protocol by optmising reagent concentrations and incubation time. Glucose transport protein was adsorbed onto the surface of the ELISA plate by incubating 339 ng protein per well for 6 h at 37°C. After excess antigen had been washed off, a limiting dilution of mab was mixed with competitor diluted in blocking buffer at room temperature and 100 μl of the mixture was added to the ELISA plate. The optimum binding of different batches of enzyme-linked 2° antibody varied with batch and incubation was for either 90 min or overnight at 37°C. The absorbance of the reaction product was measured 18 h after addition of enzyme substrate.

In the experiments involving vesicles of known sidedness, mabs and vesicles were diluted in PBSA + 0.1% gelatin + 5% foetal calf serum. Mixtures were prepared and incubated at room temperature for 15 – 30 min before separation of bound and free antibody by centrifugation for 4 min at 10 000 *g*. Free antibody in the supernatant was applied to the ELISA plate as the 1° antibody.

For determination of immunoglobulin subclass, affinity-purified rabbit anti-mouse antisera against total IgG, IgG_1, IgG_{2_a}, IgG_{2_b}, IgG_3 and IgM (Litton Bionetics) were applied to wells following binding of specific mabs to glucose transporter protein as described above. After incubation for 60 min at 37°C and washing five times with PBSA-T to remove excess subclass antiserum, goat anti-rabbit total Ig conjugated with alkaline phosphatase (Miles) was added to the sandwich.

Purification of mabs

Mabs were purified from ascitic fluid by chromatography on a protein A-Sepharose CL-4B column (2 ml) equilibrated with 10 mM sodium phosphate, pH 7.2, containing 145 mM NaCl. Bound IgG was eluted at 4°C with 0.1 M acetic acid containing 150 mM NaCl, immediately neutralised by addition of 2 M Tris, and then dialysed extensively at 4°C against 10 mM sodium phosphate, pH 7.2, containing 145 mM NaCl.

Endoglycosidase digestion

Purified glucose transporter was digested with endoglycosidase F from *Flavobacterium meningosepticum* essentially as described by Lienhard *et al.* (1984) except that the concentration of transporter used was 0.4 mg/ml and that of Triton X-100 1.2% (v/v).

SDS-polyacrylamide gel electrophoresis and electrophoretic blotting

SDS-polyacrylamide gel electrophoresis was carried out by the procedure of Laemmli (1970) using 10% or 12% acrylamide gels. Electrophoretic transfer to nitrocellulose paper was performed in 25 mM Tris, 192 mM glycine, pH 8.3 containing 20% (v/v) methanol, using a Bio-Rad Trans-Blot apparatus. Transfer was carried out at 4°C overnight at 30 V and then for 6 h at 70 V. Proteins were detected on nitrocellulose sheets by staining with 0.1% (w/v) amido black in 25% (v/v) propan 2-ol, 10% (v/v) acetic acid, in water.

Western blot analysis

The binding of mabs to electrophoretically transferred proteins on nitrocellulose sheets was detected using an Immun-Blot assay kit. Briefly, after transfer nitrocellulose sheets were rinsed in 20 mM Tris-HCl, pH 7.5, containing 500 mM NaCl (TBS) and then incubated for 1 h in 3% (w/v) gelatin in TBS, in order to block all remaining protein-binding sites on the membrane. They were then incubated overnight in 1% (w/v) gelatin in TBS, containing 0.3% (v/v) ascitic fluid. The sheets were next washed in TBS containing 0.05% (v/v) Tween-20 (TTBS), and then incubated for 1 h in 1% (w/v) gelatin in TBS, containing 0.033% (v/v) goat anti-mouse IgG-horseradish peroxidase conjugate. After a further two washes in TTBS the sheets were stained with HRP Color Development Solution (containing 4-chloro-1-naphthol) for ~45 min, or until strong purple bands were visible. The sheets were then washed in distilled water and air dried.

Ligand binding assays

The binding of cytochalasin B to the purified glucose transporter and to protein-depleted erythrocyte membranes was measured in triplicate by equilibrium dialysis using 4×10^{-8} M [4-^{3}H]cytochalasin B as previously described (Zoccoli *et al.*, 1978). A correction was made for the very small amount of non-specific binding to these preparations either by measuring the binding in the presence of 400 mM D-glucose or by making measurements after heating the membranes to 100°C for 5 min. The corrected ratio of bound to free cytochalasin B obtained from these assays 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). Measurements of [4-^{3}H]cytochalasin B binding to erythrocyte membranes and vesicles were made in the presence of cytochalasin E (10 μM) to minimise the binding to sites other than the glucose transport protein. 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 analysed by the LIGAND computer program of Munson and Rodbard (1980).

The nucleoside transporter present in preparations of purified glucose transporter was quantified by measuring the binding of [G-^{3}H]NBMPR, essentially as described by Jarvis and Young (1981). A saturating concentration (30 nM) of NBMPR was used, and binding measured by equilibrium dialysis in the presence and absence of NBTGR (5 μM) in order to correct for non-specific binding.

Immunoadsorption assays

For immunoadsorption experiments, the purified glucose transport protein preparation (52 μg protein/ml in 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl and 1 mM EDTA) was solubilised at 4°C by the addition of octyl glucoside to a concentration of 1% (w/v). Samples (1 ml) of the solubilised transporter were then incubated at 4°C for 1 – 2 h, with frequent swirling, with protein A-Sepharose CL-4B (0.2 – 0.3 ml) to which purified mab (0.7 – 1.2 mg) was bound. As a control, samples were incubated either with Sepharose CL-4B alone or with protein A Sepharose CL-4B to which non-immune mouse IgG was bound. After centrifugation to remove the Sepharose, dithiothreitol (100 mM) was added to the supernatant to give a final concentration of 2 mM. The resultant solution was then dialysed at 4°C against 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl and 1 mM EDTA, in order to remove the detergent. The reconstituted supernatants were then assayed for NBMPR and cytochalasin B binding activity, respectively.

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 solubilise membranous samples.

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