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Site-specific antibodies as probes of the topology and function of the human erythrocyte glucose transporter

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Antibodies were raised against synthetic peptides corresponding to most of the regions of the human erythrocyte glucose transporter predicted to be extramembranous in the model of Mueckler, Caruso, Baldwin, Panico, Blench, Morris, Lienhard, Allard & Lodish [(1985) Science **229**, 941–945]. Most of the antibodies (17 out of a total of 19) recognized the intact denatured protein on Western blots. However, only seven of the antibodies recognized the native membrane-bound protein, even after its deglycosylation. These antibodies, against peptides encompassing residues 217–272 and 450–492 in the hydrophilic central and *C*-terminal regions of the transporter, bound to the cytoplasmic surface of the erythrocyte membrane. This finding is in agreement with the prediction of the model that these regions of the sequence are cytoplasmic. Antibodies against peptides from the central cytoplasmic loop of the transporter were found to inhibit the binding of cytochalasin B to the membrane-bound protein, whereas antibodies against the *C*-terminal region had no effect. The anti-peptide antibodies were then used to map the sequence locations of fragments of the transporter arising from tryptic digestion of the membrane-bound protein. This in turn enabled the epitopes for a number of anti-transporter monoclonal antibodies to be located within either the central cytoplasmic loop or the *C*-terminal region of the protein. Of those monoclonal antibodies which inhibited cytochalasin B binding to the protein, all but one were found to have epitopes within the central region of the sequence. In conjunction with the results of the anti-peptide antibody studies, these findings indicate the importance of this part of the protein for transporter function.

INTRODUCTION

Most mammalian cells take up glucose by the passive process of facilitated diffusion [1]. The transport proteins responsible for this process in different tissues differ in their kinetic and regulatory properties [2,3]. However, the recent cloning of the passive transporters from several mammalian tissues has revealed that, despite their different properties, they are members of a family of related proteins, which also includes transporters from yeasts and bacteria [4–11].

A complete understanding of the mechanism of transport will require elucidation of the three-dimensional structure of at least one of these proteins at atomic resolution. However, the only member of the family that has so far been purified to near homogeneity and upon which direct structural studies are possible is the human erythrocyte glucose transporter [12,13]. Sequence studies have shown that this protein is probably identical to a transporter encoded by a cDNA clone from the human hepatocellular carcinoma cell line HepG2, and also very closely resembles a transporter from rat brain [4,5]. The predicted M_r of the protein from its sequence is 54116, and the purified protein migrates as a broad band of average apparent M_r 55000 on SDS/polyacrylamide-gel electrophoresis as a result of heterogeneous glycosylation [4,12–14].

Hydropathic analysis of the sequence, together with the knowledge from spectroscopic studies that the protein is predominantly α-helical [15–17], enabled us to propose a model for the arrangement of the polypeptide in the membrane [4]. In this model, the protein spans the bilayer 12 times in the form of hydrophobic or amphipathic α-helices. The *N*- and *C*-terminal regions of the protein (residues 1–12 and 451–492), together with a large hydrophilic region (residues 207–271) linking proposed transmembrane helices 6 and 7, are predicted to lie at the cytoplasmic face of the membrane. We have obtained some direct evidence for this arrangement by vectorial proteolytic digestion studies [4,17]. Similarly, we and others have confirmed the cytoplasmic location of the *C*-terminus of the protein using antibodies raised against a synthetic peptide corresponding to residues 477–492 [18,19]. In the present paper we extend this approach by using anti-peptide antibodies to other regions of the sequence as structural probes.

Abbreviations used: KLH, keyhole-limpet haemocyanin; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine.

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Tryptic digestion of the transporter has also provided information about the function of different regions of the protein. One or both of the central hydrophilic and *C*-terminal regions of the sequence are important for transporter function, because transport activity is abolished if they are removed from the membrane by tryptic digestion [20]. Similarly, the affinity of the protein for the inhibitor of transport, cytochalasin B, is greatly reduced by tryptic digestion [21]. However, low-affinity D-glucose-sensitive cytochalasin B binding to the protein is still seen after extensive digestion, indicating that the two large membrane-bound fragments which result from such digestion of the transporter retain the substrate-binding site, at least in part [17,21,22]. One of these fragments is glycosylated and migrates as a broad band of apparent M_r 23000–42000 on SDS/polyacrylamide gels [21,23]. We have shown that it is derived from the *N*-terminal half of the protein, and probably comprises residues 1–212 [17,18]. The *C*-terminal half of the protein yields a fragment that migrates as a sharp band of apparent M_r 18000 [21,23]. This fragment bears the label if the transporter is photoaffinity labelled either with [^{3}H]cytochalasin B or with bis-mannose derivatives before digestion [21–24]. These reagents are thought to bind to the substrate binding site(s) at the cytoplasmic and extracellular surfaces respectively of the membrane. It is therefore probable that the substrate binding site(s) of the transporter is located at least in part within this region of the sequence.

Another way of probing the functional importance of different regions of a protein is to examine the effect of site-specific antibodies on its activity. To this end, we have previously raised two panels of monoclonal antibodies against the transporter, some of which affected its function [25,26]. Unfortunately, the antibodies did not bind to the fragments yielded by prolonged tryptic digestion of the transporter, and so it was not possible to locate their epitopes. In the present study we have been able to examine the effects of anti-peptide antibodies in order to probe the function of specific regions of the transporter. In addition, these antibodies have enabled us to map the fragments produced by short periods of digestion of the transporter with trypsin. Because some of these fragments are also recognized by the monoclonal antibodies, it has been possible to locate the relevant epitopes and correlate these with the effects of the monoclonal antibodies on transporter function.

This work was presented, in part, at the 629th Meeting of the Biochemical Society held in December 1988 at the Royal Free Hospital School of Medicine, London.

EXPERIMENTAL

Materials

[4-^{3}H]Cytochalasin B (18.5 Ci/mmol) and [*benzyl*-^{3}H]nitrobenzylthioinosine (NBMPR; 26 Ci/mmol) were purchased from New England Nuclear (Stevenage, Herts., U.K.). Alkaline-phosphatase-linked goat anti-rabbit IgG for e.l.i.s.a. was obtained from Miles Laboratories (Slough, Berks., U.K.). Protein A–Sepharose CL-4B, octyl glucoside and nitrobenzylthioguanosine (NBTGR) were from Sigma (Poole, Dorset, U.K.). Nitrocellulose, M_r standards for SDS/polyacrylamide-gel electrophoresis and Immuno-Blot assay kits (GAM-AP and GAR-AP) were obtained from Bio-Rad Laboratories (Watford, Herts., U.K.). Amino acid derivatives and resin for peptide synthesis by the N^{α}-fluorenylmethoxycarbonyl-polyamide solid-phase method were obtained from Milligen (Watford, Herts., U.K.). Endoglycosidase F from *Flavobacterium meningosepticum* was obtained from Boehringer (Lewes, Sussex, U.K.) and endo-β-galactosidase from *Bacteroides fragilis* was kindly donated by Dr. P. Scudder (Clinical Research Centre, Harrow, Middx., U.K.). Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) and sulpho-MBS were purchased from Pierce (Chester, U.K.). All other chemicals were from Sigma or BDH (Poole, Dorset, U.K.) and were the highest grade available. Out-dated human blood was provided by the blood bank of the Royal Free Hospital.

Peptide synthesis

All peptides corresponding to regions of the glucose transporter predicted to be exposed on the cytoplasmic face of the membrane [4] with the exception of peptide 217–232 were synthesized using the N^{α}-fluorenylmethoxycarbonyl-polyamide solid-phase method [27] and characterized exactly as described previously [18]. A cysteine residue was added to the *C*-terminus of each peptide to facilitate coupling to a carrier protein. Characterization showed that the peptides were at least 80% pure, and so they were used without further purification. The other peptides were synthesized by the solid-phase method of Barany & Merrifield [28] using t-butyloxycarbonyl amino acids in a Biosearch Model 9500 peptide synthesizer, and were purified by h.p.l.c. before use. A cysteine–glycine sequence was attached to the *N*-terminus of the peptides to facilitate coupling to the carrier protein.

Production of and characterization of antibodies

Peptides 84–98, 326–340 and 389–403, which contain no lysine residues, were coupled to bovine thyroglobulin using specially purified glutaraldehyde (grade 1, Sigma), essentially as described by Carrasco *et al.* [29]. The other peptides were coupled to keyhole-limpet haemocyanin (KLH) with MBS as described previously [18], or with sulpho-MBS, a water-soluble analogue of MBS. The compositions of the KLH– and thyroglobulin–peptide conjugates were determined by amino acid analysis. Typically, between 10 and 25 mol of peptide was bound per 100000 g of carrier protein. Antisera were then raised against the peptide conjugates exactly as described previously [18], except that in some cases the conjugates were administered to the rabbits by intramuscular injection.

Antisera were processed and screened for their anti-peptide and anti-(glucose transporter) activity by e.l.i.s.a. and by competitive e.l.i.s.a. as described previously [18]. Western blotting was also performed as described previously [18,26], except that the buffer used to block protein-binding sites on the nitrocellulose contained 5% (w/v) low-fat dried milk powder rather than gelatin. Antibodies which bound to the native glucose transporter were affinity-purified by adsorption on to protein-depleted erythrocyte membranes [18]. Monoclonal antibodies GTPR3 and GTPR4 were raised against the intact glucose transporter exactly as described previously for antibodies GTPR1 and GTPR2 [26]. Production of the other monoclonal antibodies used has already been described elsewhere [25,26].

Deglycosylation of the transporter

Deglycosylation of the transporter in the absence of detergents was achieved using a mixture of glycosidases [16]. Purified glucose transporter at a protein concentration of 2 mg/ml in 100 mM-sodium phosphate/50 mM-EDTA/75 mM-2-mercaptoethanol, pH 6.1, was incubated with 0.3 units of endo-β-galactosidase/ml and 4 units of endoglycosidase F/ml at 25 °C for 24 h. Control samples were incubated in the absence of enzymes. Samples of the digests were dialysed into 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4, before assay of cytochalasin B-binding activity and e.l.i.s.a.

Other procedures

The preparation of protein-depleted erythrocyte membranes and of the purified glucose transporter [21], immunoadsorption experiments [18,26], ligand-binding measurements [26] and other assays were all as described previously except where otherwise indicated in the text or Figure legends. Rat brain membranes were prepared by the method of Wang [30].

RESULTS

Recognition of the native and denatured transporter by anti-peptide antibodies

Antibodies were raised in rabbits against peptides corresponding to most of those regions of the transporter predicted to be extramembranous [4], as illustrated in Fig. 1. The peptides comprised residues 35–46, 46–56, 34–60, 84–98, 112–127, 144–158, 175–189, 217–232, 231–246, 240–255, 256–272, 293–306, 326–340, 389–403, 420–432, 450–467 and 460–477. (For the sake of completeness, Fig. 1 also illustrates the sequence location of peptides comprising residues 1–15 and 477–492 of the transporter. We reported the properties of antibodies raised against these regions of the sequence in a previous publication [18].) All of the antisera showed a high titre of anti-peptide antibodies when assayed by e.l.i.s.a. using the respective synthetic peptides as microtitre plate-bound antigens (results not shown). However, only the antisera raised against peptides 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 strongly recognized plate-bound purified glucose transporter in e.l.i.s.a. assays. The other antisera appeared to recognize the intact transporter weakly or not at all.

Despite the failure of several of the anti-peptide antibodies to recognize the plate-bound transporter, all except those against peptides 112–127 and 420–432 labelled the denatured transporter on Western blots. One possible explanation for the lack of recognition of the native protein in e.l.i.s.a. assays might be steric hindrance from the oligosaccharide chain attached at Asn-45, particularly for those antibodies directed against the region of carbohydrate attachment. In order to test this hypothesis, deglycosylated transporter was prepared as described in the Experimental section. Following treatment with glycosidases, the protein retained > 95% of its original cytochalasin B binding activity, indicating that it remained in its native conformation. The extent of deglycosylation was estimated to be > 80% by spectrophotometric scanning of Coomassie Blue-stained gels of the deglycosylated transporter. These showed primarily a sharp band of apparent M_r 46000, cor-

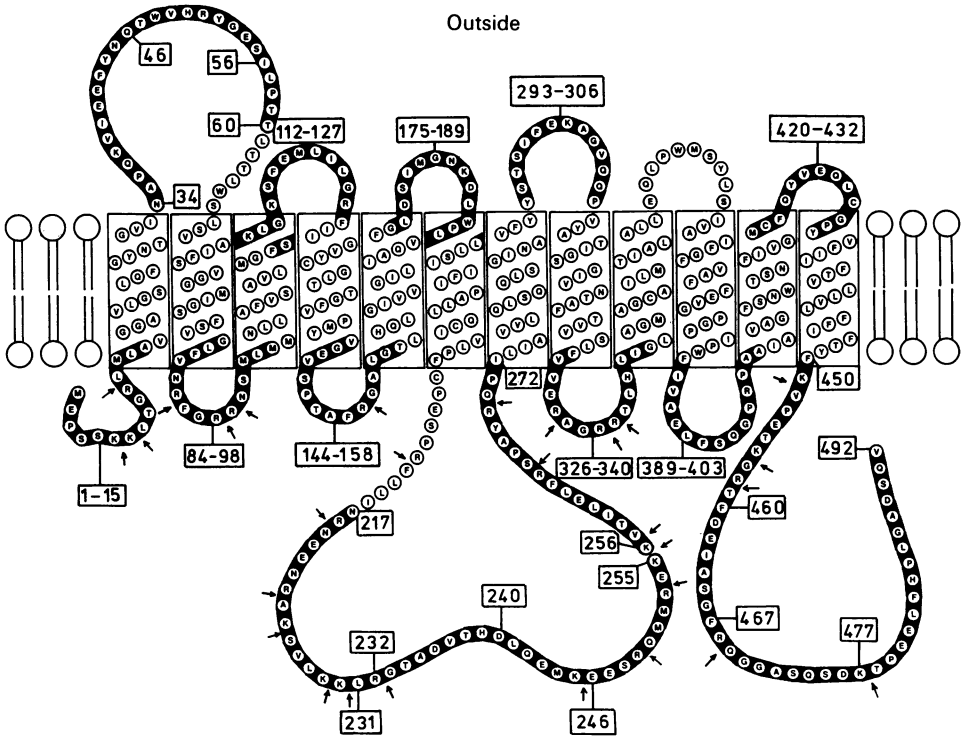

Fig. 1. Location of chemically synthesized peptides within the glucose transporter sequence

Regions of the sequence corresponding to synthetic peptides are outlined in black. Numbers refer to the sequence location of the *N*- and *C*-terminal residues of each peptide. Arrows indicate the location of potential tryptic cleavage sites. The proposed model for the arrangement of the transporter in the membrane is taken from [4].

responding to the band previously identified as the fully deglycosylated transporter [31]. A minor band of approximate M_r 48000 probably corresponded to a partially deglycosylated species. Both bands were labelled on Western blots by all of the anti-peptide antibodies except those against peptides 112–127 and 420–432. However, none of the anti-peptide antibodies reacted any more strongly with the plate-bound deglycosylated transporter than with the fully glycosylated transporter in e.l.i.s.a. (results not shown).

Antisera raised against peptides 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 strongly recognized plate-bound purified glucose transporter in e.l.i.s.a. assays. This finding suggested that they could bind to the native protein, although it is possible that the transporter is at least partially denatured upon adsorption to plastic in an e.l.i.s.a. assay. Additional evidence that (a fraction of) the antibodies in each serum recognized the native transporter was provided by the fact that antibodies could be affinity-purified from the sera by adsorption on to erythrocyte membranes, as described in the Experimental section. Yields of IgG ranged from 0.25 mg/ml of serum to 0.8 mg/ml of serum.

The affinity-purified antibodies were used in further experiments to demonstrate not only that they recognized the native glucose transporter, but also that they were specific for the latter. Such experiments were necessary because both the glucose and nucleoside transport proteins of human erythrocyte membranes migrate as broad bands of identical M_r on SDS/polyacrylamide gels [32]. Because of their similarities in size, kinetics and function, it has been suggested that the two proteins may in fact be related [33]. Furthermore, both are present not only in protein-depleted erythrocyte membranes but also in the 'purified' glucose transporter preparation [13,34]. Western blotting and e.l.i.s.a. assays cannot therefore unambiguously establish to which species the antibodies bind. However, the two transporters do differ in that the glucose transporter specifically binds the inhibitor cytochalasin B, whereas the nucleoside transporter specifically binds the inhibitor NBMPR [13,34]. Assay of the binding of these two ligands was therefore used to quantify the ability of immobilized antibodies to remove each transporter from a detergent-solubilized mixture of the two proteins, as described in the Experimental section. As shown in Table 1, when an 8-fold molar excess of antibody over glucose transporter was used, antibodies raised against peptides 231–246, 240–255, 256–272, 450–467 and 460–477 removed $\geqslant 95\%$ of the cytochalasin B binding sites from solution, as compared with $\leqslant 17\%$ of the NBMPR binding sites. (Insufficient antibody against peptide 217–232 was available for this experiment.) Not only did these results demonstrate the specificity of the antibodies, but they

Table 1. Immunoadsorption of solubilized glucose and nucleoside transporters

Samples (52 μg) of purified glucose transporter in 1 ml of 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4, were solubilized by addition of 1% *n*-octyl-β-D-glucoside, then incubated for 1 h at 4 °C with 200 μl of Protein A–Sepharose CL-4B to which 1.2 mg of the relevant non-immune or affinity-purified IgG was bound. The ligand-binding activities of the supernatants were then measured, after removal of detergent, as described previously [26]. Binding measurements were performed in triplicate and differed from the mean by < 10%.

	Ligand-binding activity remaining in solution (%)	
Immunoadsorbant (Ligand ···)	Cytochalasin B	NBMPR
Non-immune rabbit IgG	100	100
Anti-peptide 231–246	0	83
Anti-peptide 240–255	0	93
Anti-peptide 256–272	5	91
Anti-peptide 450–467	4	95
Anti-peptide 460–477	4	89

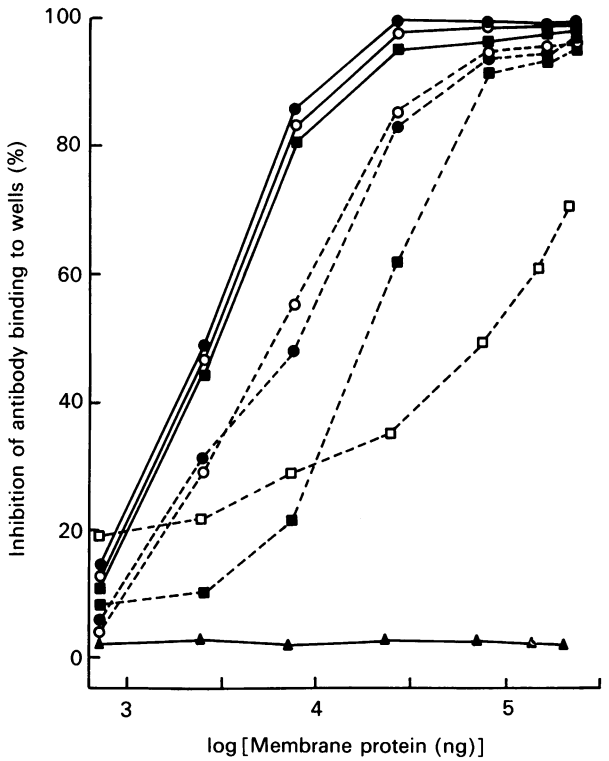

Fig. 2. Establishment of the sidedness of antibody binding by competitive e.l.i.s.a.

The ability of intact erythrocytes (▲) and unsealed erythrocyte membranes (○, ●, □, ■) to compete for a limiting amount of antibody with 600 ng of glucose transporter bound to the surface of a microtitre well was assessed. Samples (100 μl) of 1 μg/ml solutions of affinity-purified antibodies against peptides 217–232 (■– –■), 231–246 (●– –●), 240–255 (○– –○), 256–272 (□– –□), 450–467 (■—■), 460–477 (○—○) and 477–492 (●—●) were incubated with the amount of erythrocyte membrane protein indicated. The amount of free antibody remaining in the supernatant after centrifugation was then assessed by e.l.i.s.a. as described previously [26]. For incubations with unsealed erythrocyte membranes, each point is the mean of triplicate determinations. The values yielded by intact erythrocytes did not differ significantly for each antibody, and so are given as the mean for all seven antibodies.

also confirmed the ability of the antibodies to recognize the native protein, because only the latter is detectable in ligand-binding assays.

Sidedness of anti-peptide antibody binding to erythrocyte membranes

In order to determine at which surface of the erythrocyte membrane their epitopes were exposed, the affinity-purified antibodies were tested in competitive e.l.i.s.a. experiments using unsealed erythrocyte membranes and intact erythrocytes as the competing antigen, as described previously for monoclonal antibodies [26]. Incubation of each of the six antibodies with protein-depleted membranes, which are unsealed [35], inhibited the subsequent binding of antibodies to the plate-bound transporter in an e.l.i.s.a. assay (Fig. 2). Under the experimental conditions used, essentially complete inhibition of the binding of 100 ng of anti-peptide antibodies against peptides 450–467 and 460–477 was produced by incubation with 25 μg of membranes. Almost identical results obtained with antibodies against peptide 477–492, which we described previously [18], are included in Fig. 2 for comparison. Almost complete inhibition (> 90%) was also seen for antibodies against peptides 217–232, 231–246 and 240–255, although somewhat larger amounts of membranes were required to achieve this (Fig. 2). Inhibition of the binding of anti-(peptide 256–272) was substantial (up to 70%) but was not complete at the highest concentration of membranes used in the experiment.

In complete contrast with the results obtained with unsealed membranes, intact erythrocytes containing equivalent amounts of transporter had no effect on the binding of any of the antibodies to the plate-bound glucose transporter (Fig. 2). This finding indicates that the epitopes for all six antibodies are exposed at the cytoplasmic surface of the membrane. The observed recognition of the epitopes in unsealed membranes also provides additional confirmation of the ability of the antibodies to recognize the native membrane-bound conformation of the glucose transporter. The differences in the amounts of membranes required for inhibition of the different antibodies may reflect differences in antibody affinity.

Effects of antibodies on cytochalasin B binding activity

The effect of the anti-peptide antibodies on the function of the glucose transporter was examined by investigating the effect of the six affinity-purified antibodies on cytochalasin B binding to protein-depleted erythrocyte membranes. The latter were used in preference to the purified transporter because they are completely unsealed [35]. Antibodies against peptides 450–467 and 460–477 had no effect on cytochalasin B binding (Fig. 3). However, antibodies to peptides 231–246 and 240–255 did inhibit the binding as measured using a fixed low concentration of cytochalasin B (4×10^{-8} M). A maximum inhibition of about 60% was seen when membranes at a concentration of 0.25 mg of protein/ml were incubated with 0.2 mg of IgG/ml (Fig. 3). This concentration corresponds to about a 3-fold molar excess of antibody over glucose transporter. No inhibition was seen using a corresponding concentration of non-immune IgG (Fig. 3). The mechanism of the inhibition was examined further by measuring the effect of a maximally inhibitory concentration of IgG on cytochalasin B binding over a range

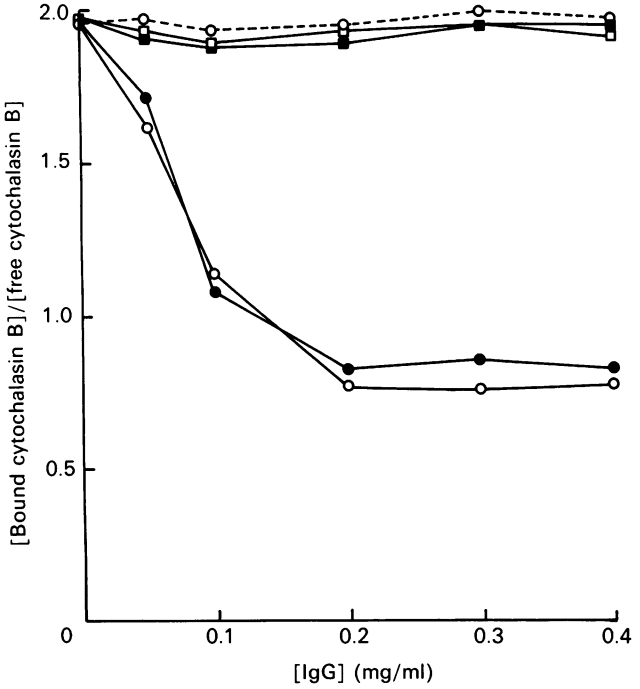

Fig. 3. Inhibition of the cytochalasin B binding activity of protein-depleted erythrocyte membranes by anti-peptide antibodies

Protein-depleted erythrocyte membranes at a concentration of 0.25 mg/ml in 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2, were incubated with various concentrations of non-immune rabbit IgG (○--○), or affinity-purified antibodies against peptides 231–246 (●), 240–255 (○—○), 450–467 (□) and 460–477 (■). Cytochalasin B binding activity was then measured as described in the Experimental section. Each point is the mean of triplicate determinations.

of cytochalasin concentrations. The results are shown in the form of Scatchard plots in Fig. 4. Analysis of the results by the LIGAND procedure [36] showed that neither antibody had a significant effect on the concentration of cytochalasin B binding sites. Instead, antibody binding decreased the K_a for cytochalasin B binding from $8.4 \pm 0.9\ \mu M^{-1}$ to $3.4 \pm 0.6\ \mu M^{-1}$ for antibody against peptide 231–246 and to $3.5 \pm 0.4\ \mu M^{-1}$ for antibody against peptide 240–255.

Identification of the membrane-bound tryptic fragments of the glucose transporter

The availability of the anti-peptide antibodies allowed us to determine the sequence locations of the membrane-bound fragments produced by tryptic digestion of the glucose transporter more precisely than was previously possible. Tryptic digestion of the transporter yielded the same pattern of fragments as reported previously (results not shown), i.e. a glycosylated fragment of average apparent M_r 35000, and a non-glycosylated fragment of apparent M_r 18000 [18,21]. The M_r-18000 fragment was preceded at early times of digestion by precursors of apparent M_r 25500, 23500 and 21000. The latter was formed in small amounts, and had not previously been noted by us [21].

In a previous study, we showed that the amino acid composition of the glycosylated fragment was consistent

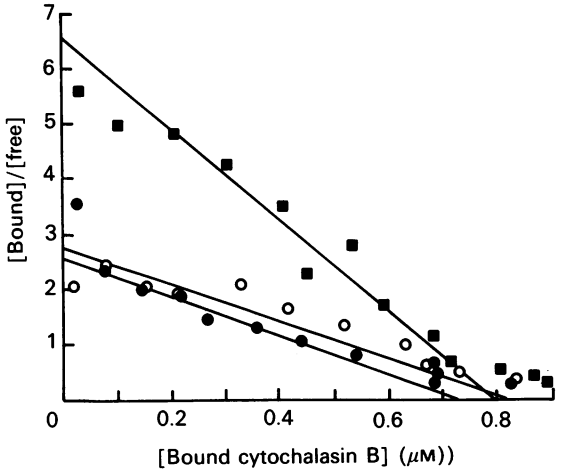

Fig. 4. Scatchard plot analysis of cytochalasin B binding to protein-depleted erythrocyte membranes in the presence of control or anti-peptide antibodies

Protein-depleted membranes (0.5 mg/ml in 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2) were incubated with non-immune rabbit IgG (■, 0.4 mg/ml) or with affinity-purified IgG (0.4 mg/ml) against peptides 231–246 (●) and 240–255 (○). Cytochalasin B binding was measured over the concentration range $(0.5–75.5) \times 10^{-7}$ M, as described previously [13]. Each point is the mean of triplicate determinations. The straight lines are computerized best fits determined by the LIGAND procedure [36].

with its comprising residues 1–212 of the glucose transporter [17]. We also showed that it was labelled on Western blots by antibody against a peptide corresponding to residues 1–15 of the transporter [18]. In the present study the same result (not shown) was obtained with antibodies against sequences 34–60, 84–98, 144–158 and 175–189, confirming that this fragment contains the first five putative membrane-spanning helices of the transporter [4,17]. Because it is produced by tryptic cleavage at the cytoplasmic surface of the membrane, it is likely also to contain the sixth membrane-spanning sequence. However, it was not labelled on Western blots by antibodies against peptide 217–232, a finding consistent with its formation by tryptic cleavage at Arg-212 (results not shown).

Several distinct patterns were seen for the labelling of the non-glycosylated tryptic fragments by antibodies directed against the central and *C*-terminal hydrophilic regions of the transporter sequence. The results are summarized in Table 2. The M_r-25500 fragment, which is also labelled by antibodies against peptide 477–492 [18], was strongly labelled by antibodies against peptide 231–246, but weakly labelled, if at all, by antibodies against peptide 217–232. From the distribution of tryptic cleavage sites in the central cytoplasmic region of the transporter sequence, it is clear that this fragment must contain residues 233–492. It may contain some residues which are *N*-terminal to residue 233, but its *N*-terminus cannot precede Ala-224. Antibodies against peptide 217–232 did faintly stain an apparent precursor to the glycosylated fragment seen after short times of digestion (results not shown) and so it is likely that one or more of the cluster of five basic residues between Arg-223 and

Table 2. Patterns of reactivity of anti-peptide and monoclonal antibodies with tryptic fragments of the glucose transporter on Western blots

	Labelling of tryptic fragment			
Antibody	M_r-25500	M_r-23500	M_r-21000	M_r-18000
Anti-peptides:				
217–232	−	−	−	−
231–246	+	−	+	−
240–255	+	−	+	−
256–272	+	+	+	+
450–467	+	+	−	+
460–477	+	+	−	−
Monoclonals:				
GTPR1	+	−	+	−
GTPR2	+	−	+	−
GTPR3	+	−	+	−
GTPR4	+	+	−	−
G1	+	−	+	−
G2	+	+	−	−
G3	+	−	+	−
G4	+	+	−	−

Arg-232 represents a particularly sensitive site for tryptic cleavage of the transporter. The fragment of M_r 23500, which is also labelled by antibodies against peptide 477–492 [18], was not labelled by antibodies against peptide 240–255, although it was labelled by those against peptide 256–272. From the distribution of potential tryptic cleavage sites in the central region of the transporter sequence, it must contain residues 265–492. It may contain residues *N*-terminal to residue 265, but its *N*-terminus cannot precede Glu-246. A likely candidate for its *N*-terminal residue is Val-257 (Fig. 1). The M_r-21000 fragment was not labelled by antibodies against peptide 450–467, unlike the M_r-18000 fragment. The latter is known to terminate at either Lys-456 or Arg-458 (see below), and so the M_r-21000 fragment must terminate either at Lys-456 or, more likely, at Lys-451. The fragment resembles the M_r-25500 fragment in being strongly labelled by antibodies against peptide 231–246 but not by those against peptide 217–232. It therefore probably shares the same *N*-terminus as the latter fragment, i.e. somewhere between residues 224 and 233. The M_r-18000 fragment was labelled by antibodies against peptide 450–467, but not by those against peptide 460–477. In conjunction with our previous finding [17] that a peptide corresponding to residues 459–468 is released from the membrane upon prolonged digestion with trypsin, this observation indicates that the M_r-18000 fragment must terminate at Lys-456 or Arg-458. In addition, labelling of this fragment with antibodies against peptide 256–272 suggests that (a component of) the fragment contains residues *N*-terminal to Gln-270. The latter was suggested to be the likely *N*-terminus of the fragment from a determination of its amino acid composition by Cairns *et al.* [17]. However, fragments containing an additional five or even 13 residues at the *N*-terminus, and which might therefore be recognized by antibodies against peptide 256–272, might not be resolved from a fragment comprising residues 270–456/458 on SDS/polyacrylamide gels.

Location of epitopes for monoclonal antibodies against the glucose transporter

We have previously reported the sidedness of binding to erythrocyte membranes of a number of different monoclonal antibodies raised against the glucose transporter [25,26]. All bound to the cytoplasmic face of the erythrocyte membrane. The two additional antibodies used in the present study, GTPR3 and GTPR4, also bound to the cytoplasmic face of the membrane (J. M. Boyle & K. Meeran, unpublished work). In order to locate the epitopes for all of these antibodies, we investigated their ability to recognize tryptic fragments of the glucose transporter on Western blots. The results are summarized in Table 2. Five of the antibodies (GTPR1, GTPR2, GTPR3, G1 and G3) labelled the fragments of M_r 25500 and 21000 but not those of M_r 23500 or 18000. This pattern of labelling was identical to that found for antibodies against peptides 231–246 and 240–255 (Table 2). Failure to recognize the M_r-23500 fragment, which contains the intact *C*-terminus of the transporter, indicates that the epitopes for these antibodies cannot be within the *C*-terminal hydrophilic region of the transporter sequence. Lack of labelling of the M_r-18000 fragment, whose *N*-terminus is either Gln-270 or precedes this residue in the sequence, indicates that the epitopes must lie within the central hydrophilic region of the transporter sequence, probably between Ala-224 and Arg-269. In contrast, monoclonal antibodies GTPR4, G2 and G4 labelled only those fragments, of M_r-25500 and -23500, which contained the intact *C*-terminus of the transporter (Table 2). Lack of labelling of the M_r-21000 fragment indicates that the epitopes do not lie within the central hydrophilic region of the transporter sequence: the M_r-21000 and -25500 fragments probably share the same *N*-terminal sequence which includes much of this region (see above). In conjunction with the lack of labelling of the M_r-18000 fragment, these findings indicate that the epitopes recognized by antibodies GTPR4, G2 and G4 lie within the *C*-terminal region of the transporter sequence, between residues 459 and 492.

A more precise location for the epitopes recognized by the monoclonal antibodies was sought by investigating their ability to recognize synthetic peptides in e.l.i.s.a. assays. The peptides tested corresponded in sequence to residues 217–232, 231–246, 240–255 and 256–272 in the central hydrophilic region of the transporter and to residues 450–467, 460–477 and 477–492 in the *C*-terminal hydrophilic region of the transporter. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3 recognized only one plate-bound peptide, that corresponding to residues 231–246 of the transporter sequence (Fig. 5*a*). In addition, they did not recognize a synthetic peptide corresponding to residues 231–246 of the rat brain glucose transporter in e.l.i.s.a. (results not shown). This peptide differs in sequence from that of the human only by replacement of His-239 with an arginine, and so it is likely that this residue forms part of, or is very close to, the epitope. The human peptide, but not the rat peptide, also competed with plate-bound glucose transporter for monoclonal antibody binding in a competitive e.l.i.s.a. assay (Fig. 6*a*). However, in order to inhibit binding by 50%, a 200–300-fold molar excess of peptide over glucose transporter was required, suggesting that the antibodies have a much greater affinity for the intact transporter than for corresponding short segments of the polypeptide. This finding may reflect the different conformations of these molecules and/or the likelihood that additional residues make up the complete epitope in the folded protein.

Monoclonal antibodies G2 and G4 also recognized only one plate-bound peptide, that corresponding to residues 477–492 of the glucose transporter (Fig. 5*b*).

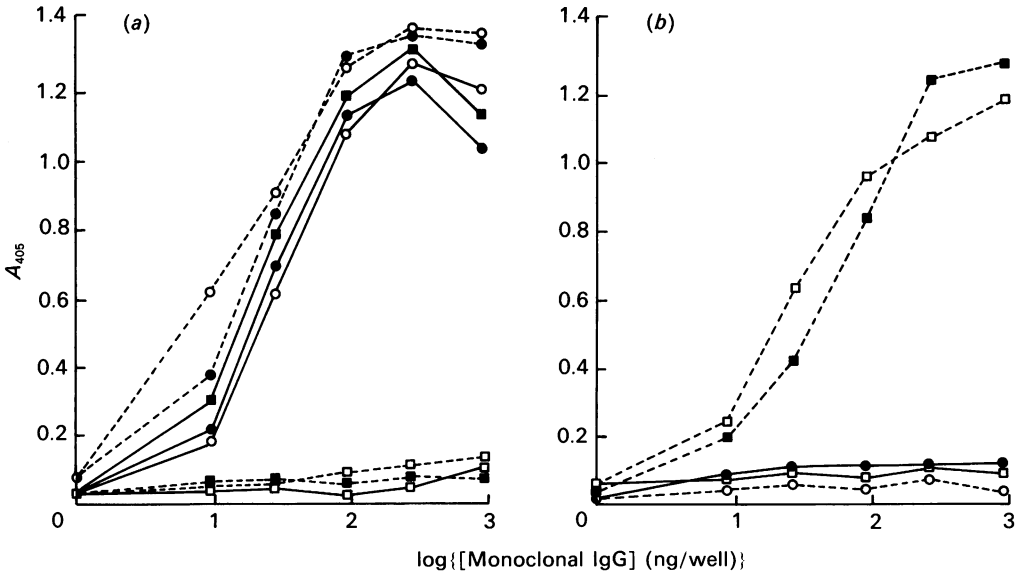

Fig. 5. Ability of anti-(glucose transporter) monoclonal antibodies to recognize plate-bound synthetic peptides in e.l.i.s.a. assays

Microtitre plates were coated with 20 ng of synthetic peptides/well, corresponding to residues 231–246 (*a*) or residues 477–492 (*b*) of the glucose transporter. Plates were then incubated with the amounts of monoclonal antibody shown, followed by alkaline-phosphatase-linked second antibody and *p*-nitrophenyl phosphate as chromogenic substrate. Each point is the mean of triplicate measurements. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○- -○), G2 (■- -■), G3 (●- -●) and G4 (□- -□).

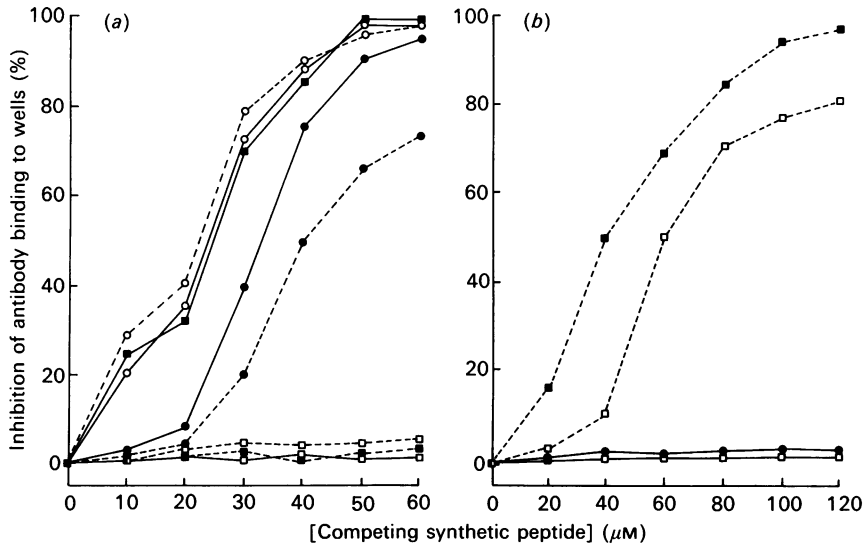

Fig. 6. Competitive e.l.i.s.a. assay of the ability of solution-phase peptides to bind monoclonal antibodies

The ability of synthetic peptides corresponding to residues 231–246 (*a*) and residues 477–492 (*b*) to compete for a limiting amount of monoclonal antibody with 600 ng of glucose transporter bound to the surface of a microtitre well was measured. Samples (100 μl) of the monoclonal antibodies (3 μg/ml) were incubated for 2 h at 25 °C with the concentrations of peptides indicated. The amount of free antibody was then measured by e.l.i.s.a., as described in the Experimental section. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○- -○), G2 (■- -■), G3 (●- -●) and G4 (□- -□).

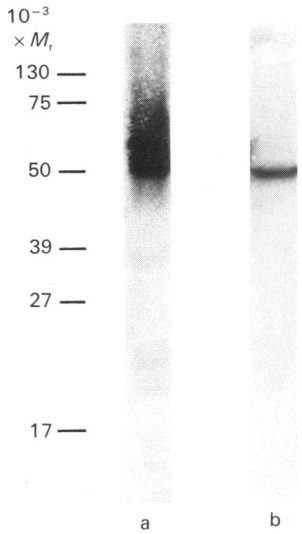

Fig. 7. Cross-reaction of anti-(human glucose transporter) monoclonal antibody with rat brain glucose transporter

Samples of purified human erythrocyte glucose transporter (*a*) and rat brain membranes (*b*) were electrophoresed on an SDS/12% polyacrylamide gel, transferred electrophoretically to nitrocellulose paper and then immunologically stained with monoclonal antibody GTPR4 as described in the Experimental section. The positions of M_r markers are indicated.

This peptide also competed with plate-bound transporter for antibody binding in a competitive e.l.i.s.a. assay (Fig. 6*b*), indicating that the epitope(s) for these antibodies is located within the *C*-terminal 16 residues of the protein. In contrast, monoclonal antibody GTPR4 did not recognize any plate-bound peptide. The epitope for this antibody had been predicted to be located within the *C*-terminal hydrophilic region of the transporter from the pattern of labelling of membrane-bound tryptic fragments (see above). Since it was lost upon extensive tryptic cleavage of the protein, it must either lie near a cleavage site or be located upon a small water-soluble peptide liberated from the *C*-terminal region upon digestion. Failure of the antibody to recognize synthetic peptides 450–467, 460–477 or 477–492 indicates that the epitope is not located wholly within the peptides 459–468, 469–477 or 478–492 which are liberated by tryptic digestion of the transporter [17]. Lack of recognition of synthetic peptide 460–477 would not be expected if the epitope were destroyed by cleavage at Arg-467. Similarly, lack of recognition of synthetic peptide 450–467 would not be expected if the epitope were destroyed by cleavage at Lys-456 or Arg-458. It follows that the epitope is probably located close to the only other tryptic cleavage site in the *C*-terminal region of the transporter, Lys-477, although further studies will be required to confirm this.

Additional support for the proposed locations of epitopes was provided by examining the ability of the antibodies to recognize the rat brain glucose transporter on Western blots. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which recognize the human erythrocyte glucose transporter on Western blots, did not label any protein bands on Western blots of rat brain membranes (results not shown). This species specificity is explicable if the epitope contains, or is close to, His-239 in the human sequence, because this is replaced by arginine in the rat [4,5]. In contrast, monoclonal antibodies GTPR4, G2 and G4 did label a sharp band of apparent M_r 50000 on Western blots of rat brain membranes (illustrated for GTPR4 in Fig. 7). Other workers have identified the rat brain glucose transporter as a sharp band on Western blots stained with polyclonal antibodies against the human erythrocyte

glucose transporter [30]. The sharpness of the labelled band in comparison with the diffuse nature of that seen for the human erythrocyte glucose transporter (Fig. 7) probably stems from a lesser degree of heterogeneity in glycosylation of the brain protein. The finding in the present study that monoclonal antibodies GTPR4, G2 and G4 recognized the rat brain transporter is consistent with the proposed locations of the epitopes for these antibodies within the hydrophilic *C*-terminal region of the transporter: the amino acid sequence of this region is identical in rat brain and human erythrocyte glucose transporters [4,5].

DISCUSSION

Antibodies against synthetic peptides corresponding to residues 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 were all found to recognize the native glucose transporter and to bind at the cytoplasmic face of the erythrocyte membrane. Our previous study reported identical results for an antibody against residues 477–492 [18]. These findings are consistent with the prediction of our model [4] that the central hydrophilic region of the transporter sequence, encompassing residues 207 to 271, and the *C*-terminal region of the sequence from residues 451 to 492 are located at the cytoplasmic side of the erythrocyte membrane. The cytoplasmic epitopes for a number of monoclonal antibodies, which we were able to locate in the present study, also all lay within these two regions. In a similar study by Andersson & Lundahl [37], another monoclonal antibody that bound to the *C*-terminal peptide (residues 478–492) of the transporter also bound to the cytoplasmic surface of the membrane. The lack of binding to the native protein of any of the antibodies raised against putative extracellular regions of the transporter, against the predicted short loops connecting transmembrane sequences at the cytoplasmic surface of the membrane, and against the *N*-terminal sequence [18] may have several explanations. In the case of the extracellular regions, it may stem from the fact that there are very few sequence differences between these regions in the rabbit and human glucose transporters [38]. In addition, the prediction of the model that certain regions of the sequence lie outside the membrane may be incorrect or they may be constrained in conformations too different from those available to the peptide immunogens. Studies on water-soluble proteins have found that anti-peptide antibodies against highly mobile regions tend to react strongly with the native protein, whereas anti-peptide antibodies against well-ordered regions do not [39]. Alternatively, the short loops may be too closely associated with the phospholipid head groups or with other parts of the protein to allow antibody access. Similar findings have been reported for the lactose transporter of *Escherichia coli* [29,40].

In previous studies, we have shown that removal of the central hydrophilic and *C*-terminal regions of the transporter by tryptic digestion destroys the ability of the protein to transport hexose, and lowers its affinity for cytochalasin B [20,21]. However, it was not possible to examine the roles of each of these regions of the sequence in transporter function independently. This has now become possible using anti-peptide antibodies. None of the anti-peptide antibodies which bound to the *C*-terminal region of the protein had any effect on cytochalasin B binding, suggesting that this region is not intimately involved in this function. In contrast, antibodies against peptides 231–246 and 240–255 in the central cytoplasmic region of the transporter did inhibit cytochalasin B binding. It is therefore likely that the central cytoplasmic region of the protein is important for transporter function.

The effects of the anti-peptide antibodies on transporter function were largely paralleled by the effects of monoclonal antibodies which bound to the same regions of the sequence. Thus monoclonal antibody GTPR4, which appears to bind to the *C*-terminal region of the transporter, had no effect on the cytochalasin B binding activity of the protein (A. Davies, unpublished work). Monoclonal antibody G2, which binds to the *C*-terminal peptide comprising residues 477–492, had, if anything, a slight stimulatory effect on binding [25]. However, antibody G4, which also binds to the *C*-terminus, inhibited binding [25]. Such differences between the effects of monoclonal antibodies which appear to bind to the same region of the transporter sequence may of course stem from their epitopes being partially discontinuous and so involving more than one region of the transporter sequence. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which bound to the central hydrophilic region of the transporter sequence, also inhibited cytochalasin B binding ([25,26]; for GTPR3, K. Meeran & S. A. Baldwin, unpublished work).

The site-specific polyclonal and monoclonal antibodies characterized in the present study have enabled direct evidence to be obtained for several features of the transporter structure. They have also enabled us to begin to dissect out the function of different regions of the protein. We envisage that they will be powerful tools for the future investigation of the structure, function and regulation of mammalian sugar transport proteins.

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