

Photolabelling of the liver-type glucose-transporter isoform GLUT2 with an azitrifluoroethylbenzoyl-substituted bis-D-mannose

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The bis-D-mannose photolabel ATB-BMPA {2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine} has been used to radiolabel the glucose transporter present in liver plasma membranes. The labelling was inhibited by 4,6-*O*-ethylidene-D-glucose. Approx. 7% of the liver plasma-membrane protein that was photolabelled in a 4,6-*O*-ethylidene-D-glucose-inhibitable manner was specifically immunoprecipitated by either an anti-(GLUT2 C-terminal peptide) antibody or by an anti-(GLUT2 exofacial-loop peptide) antibody. After correction for non-specific labelling and precipitation, the ratio of immunoprecipitable GLUT2 to GLUT1 was $\approx 5:1$, suggesting that GLUT1 was not a major component of liver plasma membranes. The low levels of immunoprecipitation of the photolabelled transporter may be due to low antibody affinity for GLUT2 or may indicate that the photolabelling reagent has labelled another glucose-transporter-like protein. The hexose-transport inhibitors phloretin, cytochalasin B and 4,6-*O*-ethylidene-D-glucose all inhibited the photolabelling by ATB-BMPA of immunoprecipitable GLUT2. D-Glucose inhibited approx. 57% of the ATB-BMPA labelling of GLUT2. D-Fructose also inhibited the GLUT2 labelling confirming that it is a substrate for GLUT2 [Gould, Thomas, Jess & Bell (1991) *Biochemistry* 30, 5139–5145]. From photolabel displacement by a range of concentrations of non-labelled ATB-BMPA, the affinity constant (K_d) of ATB-BMPA was found to be $250 \pm 78 \mu\text{M}$, whereas the B_{max} (total number of binding sites) value was 2.1 ± 0.29 pmol of GLUT2/mg of membrane protein. Since GLUT1, GLUT4 and GLUT2 have approximately equal affinities for the external ligand ATB-BMPA, but have widely varying affinities for equilibrated and transported substrates, it is suggested that the isoforms may differ in their ability to bind hexoses at the internal site.

INTRODUCTION

Facilitative glucose transport in mammalian cells is mediated by a family of structurally similar membrane proteins (GLUT1–GLUT5). These have a tissue-specific pattern of distribution which reflects the various glucose requirements of different tissues (Gould & Bell, 1990; Mueckler, 1990).

Hepatocytes, islet β -cells, cells of the intestinal epithelium and proximal-tubule cells in the kidney all express the GLUT2 transporter isoform. The human and rat cDNA coding for this transporter was cloned and sequenced by Fukumoto *et al.* (1988) and by Thorens *et al.* (1988) respectively. The apparent molecular mass of rat GLUT2 varies from 55–61 kDa depending on the tissue in which it is expressed. In liver it has an apparent molecular mass of 53 kDa. In islet β -cells its weight is 55 kDa, in the kidney 57 kDa, and in the intestine 51 kDa (Thorens *et al.*, 1988). These differences in size may be due to glycosylation or other post-translational modifications of the protein (Thorens *et al.*, 1988).

In isolated rat hepatocytes the K_m for 3-*O*-methyl-D-glucose is ≈ 20 mM (Craik & Elliott, 1979; Okuno & Gliemann, 1986). Gould *et al.* (1991) have shown that GLUT2 expressed in oocytes has a K_m for 3-*O*-methyl-D-glucose exchange transport of 42 mM. The K_m for D-glucose transport in isolated hepatocytes is very high, about 60 mM (Elliott & Craik, 1982; Granner & Pilkis, 1990). This value is in excess of normal physiological blood glucose concentrations. This high K_m is an important feature of the specialized role played by GLUT2-containing tissues. The high K_m of liver GLUT2 ensures that the uptake of

glucose is proportional to the highest physiological extracellular glucose concentrations (Unger, 1991) and that during gluconeogenesis the intracellular glucose concentration does not become rate-limiting for glucose efflux (Granner & Pilkis, 1990). In the intestine and kidney, GLUT2 is important in the trans-epithelial transport of glucose.

In the kidney, GLUT2 is located basolaterally in the gluconeogenic cells of the proximal convoluted tubule (Thorens *et al.*, 1990b); in the small intestine it is found at the serosal surface of the enterocytes. In both these tissues GLUT2 is responsible for high-efficiency low-affinity absorption of glucose over a wide range of glucose concentrations (Thorens *et al.*, 1990a; Gould & Bell, 1990; Thorens *et al.*, 1990b).

The presence of GLUT2 in the β -cells of the pancreatic islets of Langerhans may enable the β -cell to function as a glucose sensor (Thorens *et al.*, 1988). The high capacity of GLUT2 ensures that the intracellular glucose level rapidly reflects the circulating glucose concentration, which in turn triggers the β -cell secretory mechanism to produce an appropriate insulin response. It follows that any defect in the high- K_m glucose-transport function would impair the β -cell response to hyperglycaemia. The destruction or alteration of β -cell GLUT2 has been implicated in the pathogenesis of both insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (Unger, 1991). The common symptom of both types of diabetes is that glucose fails to induce normal insulin secretion. In IDDM and NIDDM the failure of glucose-induced insulin release precedes the loss of response to alternative secretagogues (Tominaga *et al.*, 1986; Johnson *et al.*, 1990b; Orci *et al.*, 1990).

Abbreviations used: GLUT n , glucose-transporter isoform n ; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine; K_d , affinity constant; B_{max} , total number of binding sites; IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; PBS, phosphate-buffered saline (see the text for composition and pH); KLH, keyhole-limpet haemocyanin; C₁₂E₉, nona(ethylene glycol) dodecyl ether; TBS, Tris/saline buffer (for composition and pH, see the text); ECL, enhanced chemiluminescence.

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Studies on animal models have shown that in both diabetic states there is a decrease in the rate of glucose transport (Tominaga *et al.*, 1986; Johnson *et al.*, 1990b) and a decrease in the immunodetectable GLUT2 in islet β -cells (Johnson *et al.*, 1990b; Orci *et al.*, 1990).

Autoantibodies that react with the surface of the β -cell and inhibit glucose-induced insulin secretion from islets have been found to be present in the serum of patients with IDDM (Kanatsuna *et al.*, 1983; Kitagawa *et al.*, 1990). Johnson *et al.* (1990a) have obtained evidence that some of the antibodies in serum from diabetics may be interacting with β -cell GLUT2 or a functionally related protein, but their role in disease pathogenesis has not yet been elucidated.

Ciaraldi *et al.* (1986) have previously described the labelling of liver glucose transporters with [3 H]cytochalasin B by using a photochemical cross-linking agent (*N*-hydroxysuccinimidyl 4-azidobenzoate). Here we report on the use of an alternative photolabelling reagent, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(*D*-mannos-4-yloxy)propyl-2-amine (ATB-BMPA) to label liver GLUT2. This compound has previously been successfully used to label GLUT1 (Clark & Holman, 1990) and GLUT4 (Holman *et al.*, 1990; Clark *et al.*, 1991).

Axelrod & Pilch (1983) have shown that, in liver membranes, now known to contain GLUT2, the affinity of cytochalasin B for glucose-sensitive binding sites is one order of magnitude lower than in erythrocyte and adipocyte membranes, now known to contain the transporter isoforms GLUT1 and GLUT4 respectively. By using oocytes expressing the different transporter isoforms separately it has been shown that GLUT2 is capable of transporting *D*-fructose, whereas GLUT1 and GLUT4 are not (Gould *et al.*, 1991). Since there are important differences between the substrate binding and kinetic properties of GLUT2 and the other transporters, we have investigated the ability of substrates and inhibitors to displace the photolabel from immunoprecipitable GLUT2. We show that ATB-BMPA has a binding constant for GLUT2 comparable with that previously calculated for interaction with the outer binding site of other transporter isoforms and suggest that the differences between isoforms in the recognition of other substrates and inhibitors reflects differences in the isoforms at the internal binding site.

MATERIALS AND METHODS

Preparation of ATB-[2- 3 H]BMPA and ATB-BMPA

The [2- 3 H]- and non-radioactive ATB-BMPA were prepared as previously described (Clark & Holman, 1990). The radio-labelled probe (sp. radioactivity 10 Ci/mmol) was stored at -20°C in phosphate-buffered saline (PBS; 140 mM-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-Na₂HPO₄, pH 7.3) at a concentration of 0.5 mM (5 mCi/ml).

Preparation of liver plasma membranes

Liver plasma membrane was isolated on a Percoll (Pharmacia) gradient essentially as described by Prpic *et al.* (1984). The liver was removed from one male 200 g Wistar rat and placed in 150 ml of ice-cold isolation medium (250 mM-sucrose/5 mM-Hepes/1 mM-EGTA, pH 7.4) containing the proteinase inhibitors aprotinin, antipain, leupeptin and pepstatin A (Sigma), each at a concentration of 3 $\mu\text{g}/\text{ml}$. The liver was minced, then homogenized with ten strokes of a loose-fitting Potter-Elvehjem homogenizer, followed by four strokes of a tight-fitting homogenizer. The homogenate was filtered through two layers of nylon mesh and centrifuged at 1464 g_{av} for 10 min at 4°C . The

resulting pellet was resuspended in isolation medium and homogenized with four strokes of the tight-fitting homogenizer. The volume was adjusted to 124.5 ml with isolation buffer and the homogenate was mixed thoroughly with 16.8 ml of Percoll and centrifuged at 34540 g_{av} for 35 min at 4°C . The plasma membrane was identified as a fluffy white layer close to the top of the gradient, lying just beneath a broader band containing nuclei, which was discarded. The plasma membrane was aspirated and washed by centrifugation at 100000 g_{av} for 30 min at 4°C in 5 mM-sodium phosphate buffer, pH 7.4, which prevented the formation of membrane vesicles. The protein content was measured using the Bio-Rad protein assay, with BSA as a standard.

Photolabelling of liver plasma membranes

Suspensions containing 100 μg of liver plasma-membrane protein were mixed with 100 μCi of ATB-[2- 3 H]BMPA and competing substrates and inhibitors as required. The final sample volume was made up to 100 μl with 5 mM-sodium phosphate buffer, pH 7.4. Samples were irradiated for 60 s in polystyrene dishes using a Rayonet RPR-100 photoreactor with RPR-3000 lamps. Labelled membranes were washed three times by centrifugation (20 min, 20000 g_{av} , 4°C) in 5 mM-phosphate buffer. Washed membranes were either analysed directly by SDS/PAGE or were used for immunoprecipitations.

Immunoprecipitations

Rabbit antibodies used were raised against synthetic peptides coupled to the carrier protein keyhole-limpet haemocyanin (KLH) by the method of Davies *et al.* (1987).

Anti-GLUT2 antibody was raised against the rat liver GLUT2 C-terminal-peptide sequence CRKATVQMEFLGSSSETV (Thorens *et al.*, 1988). A second anti-(rat GLUT2) antibody was raised against the exofacial loop region (CGVPLDDRRATINYD) between membrane-spanning helices H1 and H2 (Thorens *et al.*, 1988). Anti-GLUT2 serum raised against the C-terminal peptide of the human GLUT2 protein CHRPKAAVEMKFLGATETV (Fukumoto *et al.*, 1988), was kindly supplied by Dr. S. W. Cushman (National Institutes of Health, Bethesda, MD, U.S.A.). The anti-GLUT1 serum used was raised against the C-terminus of the human HepG2 sequence CEELFHPLGADSQV (Mueckler *et al.*, 1985). Preimmune rabbit serum was also used in control experiments. Unless otherwise indicated, the GLUT2 antiserum used routinely for immunoprecipitations was anti-(rat C-terminal peptide) antiserum.

For each immunoprecipitation, 5 mg of Protein A-Sepharose (Sigma) was conjugated to 50 μl of serum (unless indicated otherwise in Figure legends). The Protein A-Sepharose was swollen for 30 min in PBS, then washed by low-speed centrifugation. The antiserum was diluted in 500 μl of PBS and mixed with the beads by rotation for 3–16 h at 4°C . The antibody-Protein A-Sepharose conjugate was then washed four times in PBS.

The pellets of washed photolabelled membranes were solubilized for 1 h on ice in 500–1000 μl of 5 mM-sodium phosphate buffer, containing 2% nona(ethylene glycol) dodecyl ether (C₁₂E₉) (Boehringer Mannheim) and the proteinase inhibitors pepstatin A, leupeptin, antipain and aprotinin, each at 5 $\mu\text{g}/\text{ml}$. Any insoluble material was removed by centrifugation for 20 min at 20000 g_{av} at 4°C . In some experiments the C₁₂E₉ detergent was replaced by 1% *n*-octyl β -*D*-glucopyranoside (Sigma) or 4% Triton X-100 and 0.5% SDS. The solubilized material was incubated with the Protein A-antibody complex for 2½–3 h by a rotation at 4°C . The Protein A-Sepharose immunoprecipitates were pelleted by low-speed centrifugation. The pellets were washed five to seven times with 1 ml of ice-cold 5 mM-phosphate

buffer containing 0.2% C₁₂E₉. Labelled proteins were released from the conjugate by incubation for 30 min at room temperature in electrophoresis sample buffer containing 10% SDS, 6 M-urea, 1% Bromophenol Blue and 10% (v/v) mercaptoethanol. After a low-speed centrifugation to remove the Sepharose beads, radiolabelled proteins were analysed by electrophoresis.

In some experiments the supernatant was also saved for analysis by electrophoresis. Proteins were precipitated from the C₁₂E₉ detergent buffer using a method modified from that described by Wessel & Flügge (1984). A 1 ml sample of detergent-solubilized membranes was mixed with 3.6 ml of methanol, and 900 μ l of chloroform was added. After mixing the components, 2.7 ml of water was added and the sample was vortex-mixed. After centrifugation at 9000 g for 1 min the upper phase was removed and discarded. In order to precipitate the protein from the lower chloroform phase and interphase, 2.7 ml of methanol was added and mixed. Protein was pelleted by centrifugation for 2 min at 9000 g, and the supernatant was removed by aspiration. The precipitated proteins were solubilized in electrophoresis sample buffer containing 10% SDS, 6 M-urea, 1% Bromophenol Blue and 10% (v/v) mercaptoethanol and analysed by electrophoresis.

Electrophoresis

Solubilized membranes and immunoprecipitates were analysed on 10%-(w/v)-polyacrylamide gels by electrophoresis overnight in a Laemmli (1970) discontinuous buffer system.

To analyse photolabelled proteins, the gels were stained briefly with Coomassie Blue, then destained and cut into slices of 6.6 mm width. The slices were placed in scintillation vials and dried at 80 °C for 2 h, then solubilized for a further 1–2 h at 80 °C in 0.5 ml of H₂O₂ containing aq. 2% (v/v) NH₃ solution; 8 ml of scintillation fluid was added and the radioactivity was counted.

The position of the radiolabelled peak was compared with the molecular-mass marker proteins (myosin, 205 kDa; β -galactosidase, 116 kDa; phosphorylase, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa; Sigma). The size of the peak was quantified by summing the radioactivity measured for each slice under the peak and subtracting a background value based on the average amount of radioactivity in the slices on either side of the peak (Calderhead & Lienhard, 1988).

Western blotting

Non-radiolabelled liver plasma membranes and protein precipitated from detergent-solubilized membranes using chloroform and methanol were solubilized in electrophoresis sample buffer and run on 1.5 mm-thick 10% polyacrylamide gels. Proteins were transferred electrophoretically on to nitrocellulose paper using a semi-dry blotting apparatus (Novablot; Pharmacia LKB). The nitrocellulose was blocked for 1 h at room temperature in a Tris/saline buffer (TBS; 155 mM-NaCl/10 mM-Tris, pH 7.4) containing 5% (w/v) non-fat dried milk and 0.2% (v/v) Tween 20. Antisera diluted 1:8000 in the blocking buffer were incubated with the nitrocellulose for a further 1 h at room temperature. The nitrocellulose was washed with TBS+0.2% Tween (2 \times 10 min), TBS+0.2 M-NaCl (5 min) and finally in TBS+0.2% Tween (20 min). Bound antibodies were detected by incubation for 1 h at room temperature with alkaline-phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1:4000 in blocking buffer. After washing, the blots were developed using enhanced chemiluminescence (ECL). The protocol followed was as recommended by the manufacturers (Amersham International).

Estimation of the binding constant of ATB-BMPA for liver GLUT2

The binding constant of ATB-BMPA was determined by the displacement of radiolabelled ATB-[2-³H]BMPA by non-labelled ATB-BMPA. Photolabelling of 100 μ g samples of liver plasma-membrane protein using 100 μ Ci of ATB-[2-³H]BMPA was performed in the presence of a range of concentrations of unlabelled ATB-BMPA. The final ATB-BMPA concentrations used were 100 μ M (no added unlabelled ATB-BMPA), 300 μ M, 500 μ M, 700 μ M and 900 μ M. The radioactive and unlabelled ATB-BMPA compounds were mixed together in a final volume of 100 μ l of 5 mM-phosphate buffer before the addition of membranes. Samples were then irradiated, immunoprecipitated and subjected to electrophoresis as described above. The size of the GLUT2 peak was calculated for each concentration of ATB-BMPA. A background value was then subtracted to take account of the binding and immunoprecipitation, which was non-specific. The background value used was calculated from the average amount of radioactive protein precipitated by preimmune rabbit serum. Values for the binding constant (K_d) and the total number of binding sites (B_{max}) were obtained from non-linear regression (weighted for relative error) of the equation:

$$B = (B_{max}/K_d) \cdot F/(1 + F/K_d)$$

where B and F are the bound and free ATB-BMPA. The data were graphically presented as F/B -against- F plots. The number of pmol of ATB-BMPA bound per 100 μ g of liver plasma-membrane protein were calculated from the peak areas (in d.p.m.) which, based on the known sp. radioactivity of ATB-BMPA, were converted into mol of ATB-BMPA bound.

RESULTS

Photolabelling and immunoprecipitation of ATB-BMPA-labelled proteins

When ATB-[2-³H]BMPA was irradiated in the presence of liver plasma membranes, analysis of the labelled proteins by electrophoresis revealed the incorporation of radiolabel into a single broad or double-peaked band migrating with a molecular mass of 45–66 kDa (Fig. 1). The reason for the labelled proteins sometimes appearing as a double peak could not be determined, but it did not occur in all experiments and within any given experiment all the samples included were found to have the same pattern of labelling, either a single or double peak. The broadness of the peak suggests reaction of ATB-BMPA with polypeptides not related to glucose transporters. However, the photolabel could be displaced from liver plasma membranes by $43 \pm 1.6\%$ (mean and S.E.M. from three independent experiments) when irradiation was carried out in the presence of 500 mM-4,6-*O*-ethylidene-D-glucose. D-Mannitol (500 mM) included as a control caused no displacement of the photolabel. In order to identify the nature of the labelled proteins in liver plasma membrane, immunoprecipitations were carried out using anti-GLUT2 antibodies. The specificity of the immunoprecipitating antisera is illustrated in Fig. 2, which shows the results obtained by Western-blotting rat liver plasma membranes. Both the anti-(rat C-terminal peptide) and anti-(rat exofacial-loop peptide) antisera strongly recognized a band with an apparent molecular mass of ≈ 57 kDa. Preabsorption of the anti-(rat GLUT2 C-terminal peptide) antiserum with the immunizing peptide removed the antibodies that bound to the ≈ 57 kDa band. The anti-(rat C-terminal peptide) antiserum was specific for GLUT2 and did not recognize any proteins when blotted against erythrocyte or adipocyte membranes (results not shown). The anti-(human GLUT2) antiserum also recognized the ≈ 57 kDa protein, albeit less strongly than the antisera raised against the rat sequence. In

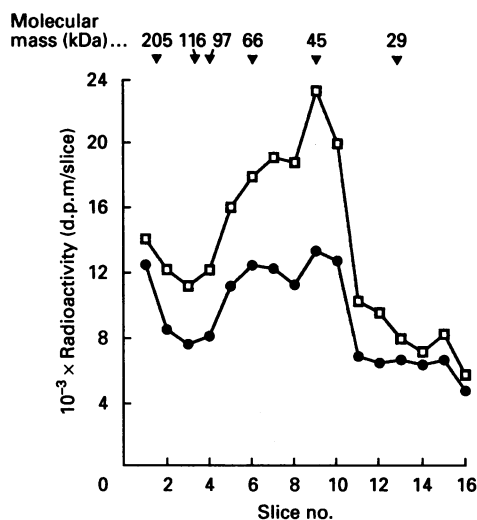


Fig. 1. Photolabelling of liver plasma membrane by ATB-BMPA

Liver plasma membranes were photolabelled by irradiation in the presence of ATB-BMPA. After washing by centrifugation, labelled proteins were analysed by SDS/PAGE, and a peak of labelled protein with an apparent molecular mass of between 45 and 66 kDa was obtained. In the illustrated example, labelled proteins appeared as a double peak; on other occasions the peak was single. Arrowheads indicate the position of molecular-mass marker proteins. Labelling was compared in the presence of 500 mM-4,6-*O*-ethylidene-D-glucose (●) and 500 mM-mannitol (□), which was included as a control. In this example 4,6-*O*-ethylidene-D-glucose caused an inhibition in ATB-BMPA binding to liver plasma membranes of 45%.

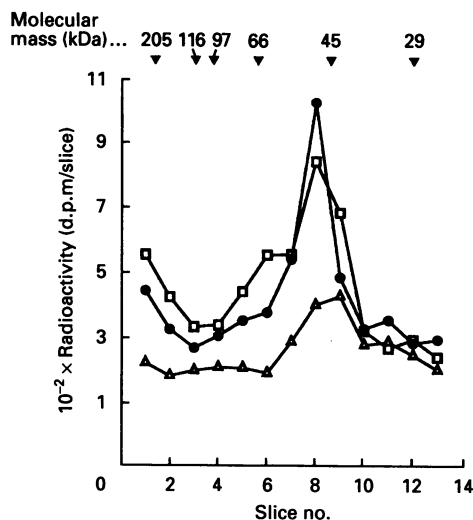


Fig. 3. Immunoprecipitation of photolabelled liver plasma membranes using different anti-GLUT2 antisera

Liver plasma membranes were photolabelled by irradiation in the presence of ATB-BMPA. The labelled membranes were washed then solubilized in buffer containing the detergent $C_{12}E_9$. Solubilized labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50 μ l of the following anti-GLUT2 antisera: anti-(rat C-terminal peptide) (●), anti-(rat exofacial-loop peptide) (□) and the anti-(human C-terminal peptide) (△). Labelled immunoprecipitates were analysed by SDS/PAGE. Both anti-(rat GLUT2) antisera precipitated identical amounts of a 45–66 kDa labelled protein. The anti-(human GLUT2) antiserum immunoprecipitated 60% of the amount of the same labelled protein precipitated by the anti-(rat GLUT2) antisera. Arrowheads indicate the positions of the molecular-mass marker proteins.

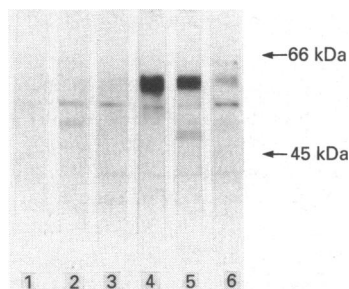


Fig. 2. Immunochemical detection of glucose transporters in liver plasma membrane

Liver plasma-membrane proteins were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with the following antisera as described in the Materials and methods section. Preimmune serum (lane 1), anti-(GLUT1 C-terminal peptide) antiserum (lane 2), anti-(rat GLUT2 C-terminal peptide) antiserum preabsorbed with the immunizing peptide (lane 3), anti-(rat GLUT2 C-terminal peptide) antiserum (lane 4), anti-(rat GLUT2 exofacial-loop peptide) antiserum (lane 5) and anti-(human GLUT2 C-terminal peptide) antiserum (lane 6). The anti-GLUT2 antisera detected a band with an apparent molecular mass of \approx 57 kDa. This was recognized more strongly by antisera raised against the rat sequence than the human sequence. A non-specific band with an apparent molecular mass of \approx 51 kDa was also detected by all sera. In addition, the anti-(GLUT1 exofacial-loop) and (GLUT2 exofacial-loop) sera detected non-specific bands of apparent molecular masses 48 and 47 kDa respectively. Arrowheads indicate the positions of the molecular-mass marker proteins.

addition to the specific GLUT2 band, all sera tested, including preimmune serum and anti-GLUT1 antiserum, also bound to a protein with an apparent molecular mass of \approx 51 kDa.

When used to immunoprecipitate photolabelled liver plasma membrane, both the anti-(C-terminal peptide) antiserum and anti-(exofacial-loop peptide) antiserum raised against the rat GLUT2 sequence immunoprecipitated the same amount of a labelled protein from liver plasma membrane. This protein appeared on polyacrylamide gels as a single- or double-peaked band between the 45–66 kDa markers (Fig. 3). Again, the reason for the appearance of the double peak in some experiments was unknown, but it occurred consistently in all samples analysed on the same occasion. Anti-human GLUT2 antiserum was found to precipitate about half the amount of the same labelled protein that was precipitated by the anti-(rat GLUT2 C-terminal peptide) antiserum.

The specificity of the precipitation was checked using pre-immune serum. Fig. 4 shows that preimmune serum precipitated a peak of similar molecular mass to that precipitated by the anti-GLUT2 antiserum. On average, non-specific immunoprecipitation, as calculated from precipitates obtained with preimmune serum, was found to account for $32 \pm 9.2\%$ (mean \pm S.E.M. from six independent experiments) of the total amount of precipitation by anti-rat GLUT2 antiserum.

Anti-GLUT1 antiserum was also found to precipitate radio-labelled protein amounting to $47 \pm 13.9\%$ (mean \pm S.E.M. from four independent experiments) of the quantity precipitated by GLUT2 antiserum. When corrected for non-specific immunoprecipitation, only 15% of the protein precipitated by anti-GLUT1 antiserum was specific, compared with 68% of the immunoprecipitated GLUT2. The ratio of immunoprecipitable GLUT2 to GLUT1 is therefore \approx 5:1. This confirms the results of the Western blotting, namely that GLUT1 is not a major component of liver plasma membrane.

The amount of immunoprecipitated GLUT2 was low; on

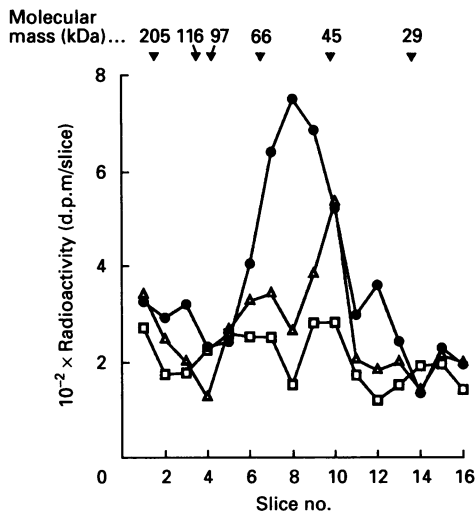


Fig. 4. Specificity of the immunoprecipitation of photolabelled proteins from liver plasma membrane

Liver plasma membranes were photolabelled with ATB-BMPA, washed, then solubilized in buffer containing the detergent $C_{12}E_9$. Solubilized labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50 μ l of anti-(rat GLUT2 C-terminal peptide) antiserum (\bullet), anti-GLUT1 antiserum (Δ) or rabbit preimmune serum (\square). Labeled proteins were analysed by PAGE. All sera precipitated some labelled protein with an apparent molecular weight of 45–66 kDa. In the illustrated example, non-specific immunoprecipitation by preimmune serum accounted for $\approx 27\%$ of the labelled protein precipitated by the anti-GLUT2 antiserum. When corrections were made for non-specific precipitation, anti-GLUT2 serum precipitated ≈ 3 -fold more labelled protein than did anti-GLUT1 serum. Arrowheads indicate the positions of the molecular-mass marker proteins.

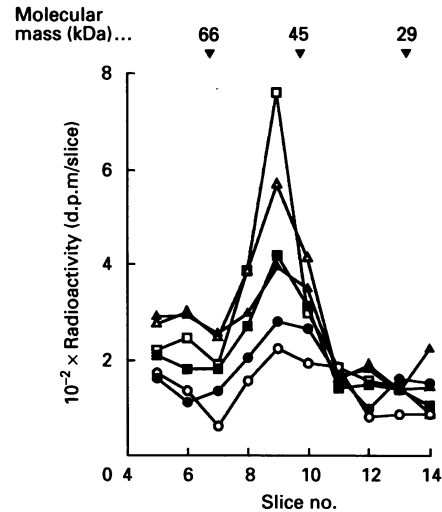


Fig. 6. Displacement of ATB-BMPA from immunoprecipitates of liver GLUT2

Liver plasma membranes were photolabelled with ATB-BMPA in the presence of the inhibitors 500 mM-D-glucose (Δ), 100 μ M-cytochalasin B (\blacktriangle), 500 mM-D-fructose (\blacksquare), 500 mM-4,6-O-ethylidene-D-glucose (\bullet) and 100 μ M-phloretin (\circ). Peak areas were compared with that obtained in a control sample in which irradiation was carried out in the presence of 500 mM-D-mannitol (\square). After washing and solubilization, the labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50 μ l of anti-(GLUT2 C-terminal peptide) antiserum. Labeled proteins were analysed by SDS/PAGE. Arrowheads indicate the positions of the molecular-mass marker proteins. For clarity, only the glucose-transporter region of the gel is shown. There are no other labelled peaks in other regions of the gel.

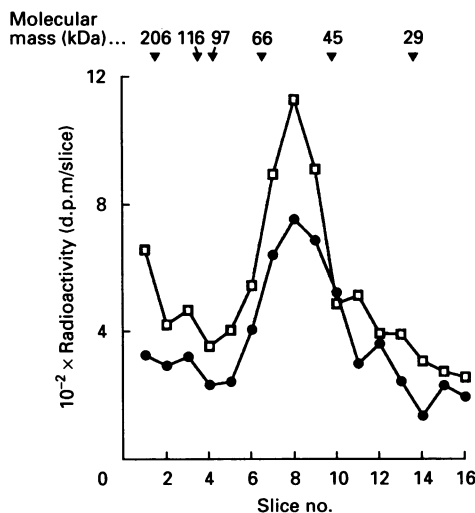


Fig. 5. Effect of increasing the amount of anti-GLUT2 antiserum used to immunoprecipitate photolabelled liver plasma membranes

Liver plasma membrane proteins were photolabelled with ATB-BMPA, washed, then solubilized in buffer containing the detergent $C_{12}E_9$. Solubilized labelled proteins were immunoprecipitated by either 5 mg of Protein A-Sepharose + 50 μ l of anti-(GLUT2 C-terminal peptide) antiserum (\bullet) or 20 mg of Protein A-Sepharose + 200 μ l of the same serum (\square). Labeled proteins were analysed by electrophoresis. Arrowheads indicate the positions of the molecular-mass marker proteins. Increasing the amount of immunoprecipitating antibody 4-fold resulted in an $\approx 10\%$ increase in the amount of immunoprecipitated GLUT2 protein.

average only $4.6 \pm 0.86\%$ (mean \pm S.E.M from five independent experiments) of the proteins radiolabelled by ATB-BMPA in total liver plasma membrane were immunoprecipitated by anti-GLUT2 antiserum. When corrections were made to account for both non-specific immunoprecipitation and non-4,6-O-ethylidene-D-glucose-inhibitable labelling by ATB-BMPA, then approx. 7% of the proteins labelled in a 4,6-O-ethylidene-D-glucose-inhibitable manner were immunoprecipitated by anti-GLUT2 antiserum. When estimated from the amount of radiolabelled protein solubilized from liver plasma membranes and precipitated by chloroform and methanol, the total amount of GLUT2 immunoprecipitated was $12 \pm 1.5\%$ (mean and S.E.M. from five independent experiments).

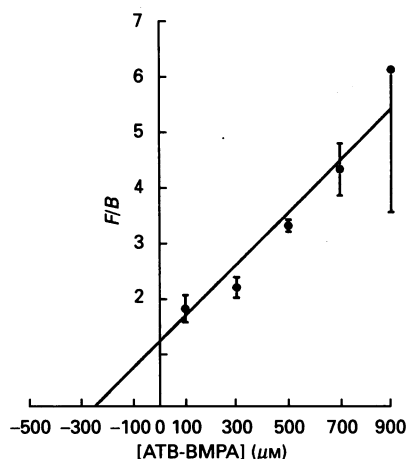
A 4-fold increase in the amount of antiserum and Protein A-Sepharose used in the immunoprecipitation resulted in only a further 1.1-fold increase in the area of the precipitated peak recovered with the anti-GLUT2 antiserum (Fig. 5).

The possibility was considered that the low level of precipitation was caused by incomplete detergent solubilization of the labelled GLUT2 in liver plasma membranes. However, when solubilized liver plasma-membrane proteins were precipitated by chloroform and methanol, subjected to SDS/PAGE and Western-blotted, they were found to contain most of the immunodetectable GLUT2. Western blotting of GLUT2 in the non-solubilized liver pellet showed only very weak bands (results not shown). The presence of high levels of rabbit IgG obscured the immunodetection of GLUT2 in either immunopellets or supernatants. Attempts at quantifying the immunoprecipitation efficiency with affinity-purified antibody were also unsuccessful. Other solubilization buffers were investigated in an attempt to increase the yield of photolabelled transporter recovered in anti-GLUT2 immunoprecipitates. These included the use of 1% n-octyl β -D-

Table 1. ATB-BMPA photolabelling of liver GLUT2 in the presence of inhibitors of glucose transport

Photolabelling of liver plasma membranes was carried out in the presence of the inhibitors as described in the Materials and methods section. Labeled membranes were solubilized in $C_{12}E_9$ detergent buffer and immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50 μ l of anti-(GLUT2 C-terminal peptide) antiserum. Precipitated proteins were analysed by SDS/PAGE, and a peak of labeled protein with an apparent molecular mass of between 45 and 66 kDa was obtained. The peak area was compared with that obtained in a control sample in which irradiation was carried out in the presence of D-mannitol. The level of non-specific immunoprecipitation was determined from the amount of photolabelled protein precipitated by preimmune serum. The peak areas obtained in the presence of inhibitors were calculated as a percentage of a control which contained D-mannitol and are means \pm S.E.M. for three to nine independent experiments, as indicated. In some experiments D-mannitol was omitted, but this did not alter the amount of ATB-BMPA binding (results not shown).

Precipitating antiserum	Inhibitor	Inhibitor concentration (mM)	Peak area (% of control) [mean \pm S.E.M. (n)]	Inhibition corrected for non-specific precipitation (%)
Anti-(GLUT2 C-terminus)	D-Mannitol	500	100	0
	D-Glucose	500	75 \pm 3.8 (3)	57
	Cytochalasin B	0.1	67 \pm 10.7 (3)	65
	D-Fructose	500	55 \pm 6.2 (4)	77
	4,6-O-Ethylidene-D-glucose	500	40 \pm 2.2 (9)	92
	Phloretin	0.1	30 \pm 3.5 (3)	\approx 100
Preimmune	D-Mannitol	500	32 \pm 9.2 (6)	–

**Fig. 7. Determination of the binding constant K_d and B_{max} for ATB-BMPA and GLUT2**

The displacement of ATB-[2- 3 H]BMPA from immunoprecipitated liver GLUT2 was measured at a range of concentrations of unlabelled ATB-BMPA as described in the Materials and methods section. The size of the GLUT2 peak was calculated for each concentration of ATB-BMPA. A background value of 32% was then subtracted to take account of the binding and immunoprecipitation, which was non-specific. Values for the binding constant (K_d) and the total number of binding sites (B_{max}) were obtained from non-linear regression (weighted for relative error) of the equation given in the Materials and methods section. The data are presented as F/B -against- F plots. From this analysis B_{max} was calculated as 2.1 ± 0.29 pmol/mg of liver plasma-membrane protein and the binding constant (K_d) was calculated as 250 ± 78 μ M. The results are based on the mean F/B and F values from four separate experiments, and the error bars shown represent \pm S.E.M.

glucopyranoside and 4% Triton X-100 with 0.5% SDS. Under these conditions no increase in the amount of immunoprecipitated GLUT2 was found (results not shown).

Displacement of ATB-BMPA from immunoprecipitable GLUT2

As shown in Fig. 6 and Table 1, ATB-BMPA binding to, and labelling of, GLUT2 could be reduced by using several inhibitors of glucose transport. When corrected for non-specific immunoprecipitation, 500 mM-4,6-O-ethylidene-D-glucose caused an in-

hibition in ATB-BMPA binding to precipitable GLUT2 of 92%, whereas 500 mM-D-glucose inhibited specific ATB-BMPA labelling by only 57%. The amount of labeled protein detected when photolabelling was carried out in the presence of phloretin was not significantly different from the amount of labeled protein precipitated non-specifically by preimmune serum. Therefore 100 μ M-phloretin was able to displace all of the specific ATB-BMPA binding to liver GLUT2; 100 μ M-cytochalasin B and 500 mM-D-fructose inhibited 65 and 77% respectively of the ATB-BMPA labelling of liver GLUT2.

The displacement of radiolabelled ATB-BMPA from liver plasma membranes by non-labelled ATB-BMPA was used to calculate the binding constant of ATB-BMPA for liver GLUT2. Fig. 7 shows a plot of F/B against F . Binding parameters were derived from non-linear regression of the equation given in the Materials and methods section. The binding constant (K_d) for ATB-BMPA binding to GLUT2 was 250 ± 78 μ M, and the amount of binding sites (B_{max}) was calculated as 2.1 ± 0.29 pmol/mg of membrane protein. These values were based on mean F/B and F values from four separate experiments.

DISCUSSION

It has previously been shown that the bis-D-mannose compound ATB-BMPA can photolabel the glucose-transporter isoforms GLUT1 (Clark & Holman, 1990) and GLUT4 (Holman *et al.*, 1990). The affinity constant of this exofacial ligand is ≈ 200 μ M for both the GLUT1 and GLUT4 isoforms (Clark & Holman, 1990; Holman *et al.*, 1990; Palfreyman *et al.*, 1992). The photoactive group has also been shown to be selective enough to enable highly specific labelling of these glucose-transport proteins to be obtained (Clark & Holman, 1990).

Isolated liver plasma membranes have previously been labelled in a glucose-displaceable manner by the ligand cytochalasin B when used in conjunction with a photochemical cross-linking agent (Ciaraldi *et al.*, 1986). In that study approx. 50% of the binding was inhibited by 500 mM-D-glucose. Hellwig & Joost (1991) failed to detect any glucose-inhibitable binding of cytochalasin B to liver plasma membranes. They suggested that cytochalasin B has a much lower affinity for GLUT2 than it does for the transporter isoforms GLUT1 and GLUT4 and that this indicated important structural differences between the isoforms in the specific domains responsible for cytochalasin B binding.

Since cytochalasin B is an endofacial ligand (Basketter & Widdas, 1978; Deves & Krupka, 1978; Holman & Rees, 1987), this indicates that variations at the endofacial hexose-binding site may be responsible for functional differences between the transporter isoforms.

The present paper has described the use of ATB-BMPA as an alternative photoligand to cytochalasin B to label the glucose transporter in liver plasma membranes. An ATB-BMPA binding constant of 250 μM has been measured for GLUT2, which is similar to the value previously reported for GLUT1 and GLUT4 (Palfreyman *et al.*, 1992). This suggests that the ATB-BMPA-binding site is conserved in all transporter isoforms. This contrasts with the cytochalasin B-binding site, where differences in binding affinities between the transporter isoforms are evident. Recent studies indicate that, on irradiation, ATB-BMPA labels the exofacial region of helix 8 and that helices 7, 8 and 9 are important in exofacial ligand binding (A. F. Davies, A. Davies, R. A. J. Preston, A. E. Clark, G. D. Holman & S. A. Baldwin, unpublished work).

We have measured the number of GLUT2 ATB-BMPA-binding sites as 2.1 pmol/mg of membrane protein, which is lower than the number of cytochalasin B-binding sites measured in previous reports. The number of cytochalasin B-binding sites in liver plasma membranes has been measured as 130 pmol/mg of membrane protein and 42 pmol/mg by Axelrod & Pilch (1983) and Ciaraldi *et al.* (1986) respectively. In comparing the number of binding sites estimated either from ATB-BMPA or cytochalasin B binding, several factors need to be considered. First, some variation may be due to the different methods used to prepare liver plasma membranes in each case. Secondly, our method of ATB-BMPA labelling is followed by specific immunoprecipitation of labelled GLUT2, whereas cytochalasin B-binding-site estimates have only been measured in liver membranes and have not been shown to be specific for GLUT2. However, the value of 2.1 pmol/mg is likely to be an underestimation of the number of GLUT2-binding sites. As discussed below, it is probable that the immunoprecipitation of GLUT2 was incomplete. G. W. Gould (personal communication) has also found poor immunoprecipitation efficiencies for GLUT2. Only 7% of the 4,6-*O*-ethylidene-D-glucose-inhibitable labelling of liver membranes could be precipitated by anti-GLUT2 antiserum. If an assumption is made that the remaining 93% of the 4,6-*O*-ethylidene-D-glucose-inhibitable binding is also to the GLUT2 protein, but which the anti-GLUT2 antiserum failed to precipitate, then a value of 30 pmol/mg of membrane protein can be obtained for the number of binding sites. This is closer to the value obtained by Ciaraldi *et al.* (1986) for the number of cytochalasin B-binding sites in whole-liver plasma membranes, but is based on a correction which assumes that all the 4,6-*O*-ethylidene-D-glucose-displaceable ATB-BMPA labelling of liver plasma membrane is specific for GLUT2.

In erythrocytes and adipocytes ATB-BMPA binding is fully glucose-displaceable (Clark & Holman, 1990). In contrast, 500 mM-D-glucose inhibited the specific ATB-BMPA binding to GLUT2 by only 57%. Since the K_m for D-glucose transport in hepatocytes is 60 mM (Granner & Pilkis, 1990), 500 mM-D-glucose would be expected to inhibit ATB-BMPA labelling by more than 80%. Thus the K_i for D-glucose displacement of ATB-BMPA from GLUT2 may be even higher than the K_m for glucose transport by hepatocytes. The conservation of the ATB-BMPA binding in transporter isoforms which have different affinities for glucose suggests the low affinity for glucose in GLUT2 occurs because of interaction with the internal site of the transporters. The ability to bind a non-transported ligand at the outside site is kinetically a simpler reaction than that involved in binding and transporting a substrate. The apparent K_m for equilibrium sugar

binding or exchange is dependent on rate constants for the membrane-translocation step and the affinity constant at the inside site, and it is these parameters that may vary between different transporter isoforms.

The hexose 4,6-*O*-ethylidene-D-glucose, which like ATB-BMPA has bulky groups at the C-4 and C-6 positions (Holman, 1989), has a specific affinity for the outside binding site of the transporter. This hexose was able to inhibit $\approx 92\%$ of the specific ATB-BMPA binding to immunoprecipitable GLUT2. Inhibition with this sugar, which cannot be translocated, is a reflection of the affinity of binding at the exofacial hexose binding site only. Phloretin, an outside-specific ligand (Basketter & Widdas, 1978), gave an inhibition of $\approx 100\%$. D-Fructose caused a 77% inhibition in ATB-BMPA binding to GLUT2. This is in accordance with the observations made by Okuno & Gliemann (1986), who found that D-fructose is transported with low affinity by the glucose transporter in hepatocytes, and by Gould *et al.* (1991), who found that oocytes expressing GLUT2 can transport D-fructose.

The present study of liver GLUT2 has been affected by difficulties with the ATB-BMPA immunoprecipitation and photolabelling specificity, which were not a problem in previous studies of GLUT1 and GLUT4 (Clark & Holman, 1990; Holman *et al.*, 1990).

Compared with the level of labelling in liver plasma membrane, the amount precipitated by anti-GLUT2 antiserum was low ($\approx 7\%$) and could not be increased significantly by increasing the quantity of precipitating antiserum. It was considered possible that this low recovery could have been due to poor detergent solubilization of the labelled GLUT2 or a low antibody affinity for the detergent-solubilized protein. However, Western blotting showed that GLUT2 was detectable in solubilized liver plasma membranes. The use of three different detergents did not increase the percentage of immunoprecipitated proteins. Proteolytic activity, which could have cleaved the C-terminus of the transporter, resulting in a loss of immunoreactivity, was prevented by the inclusion of proteinase inhibitors. However, we consider that it is unlikely that proteolysis is responsible for the low recovery of immunoprecipitated GLUT2, since proteolysis would be unlikely to reduce the immunoprecipitation by the anti-(loop peptide) antibody. The results have shown that both antisera precipitated identical quantities of GLUT2 protein. Although the possibility of a weak antibody interaction with the detergent-solubilized antigen is a likely explanation of the poor immunoprecipitation efficiency, one would not expect this to have affected both the anti-(loop peptide) and anti-(C-terminal peptide) antibodies to the same extent.

Because GLUT2 immunoprecipitation efficiency could not be accurately quantified by Western blotting, an alternative explanation for the discrepancy between the amount of labelled protein in liver plasma membranes and the amount of precipitated GLUT2 cannot be ruled out. Thus some of the ATB-BMPA binding may have been non-specific, that is, due to labelling of a protein other than GLUT2. However, the observation that 4,6-*O*-ethylidene-D-glucose displaced 43% of the ATB-BMPA binding to whole-liver plasma membranes does suggest that at least this amount of labelling is specific to a glucose-transport protein. The possibility that the non-GLUT2-precipitable but 4,6-*O*-ethylidene-D-glucose-inhibitable binding of ATB-BMPA to liver plasma membranes is due to binding to another glucose-transporter-like protein remains to be determined.

In summary, we have shown that ATB-BMPA labels liver GLUT2 with a similar binding constant to that previously calculated for GLUT1 and GLUT4 and provides an alternative to the cytochalasin B label, which has a much lower affinity for GLUT2 compared with the other transporter isoforms. Our

results suggest that the low affinity of GLUT2 for D-glucose is a reflection of structural differences at the internal binding site of GLUT2. The use of ATB-BMPA to label GLUT2 at the surface of whole liver cells has not been investigated, but the use of the label in intact cells may be useful in investigating how levels of GLUT2 alter under different metabolic conditions. In addition, the label may be of use in studying the cell-surface availability of GLUT2 in pancreatic β -cells. However, since islets can usually only be isolated in low numbers, some resolution of the reasons for the technical difficulties that may have been responsible for the poor recovery of photolabelled GLUT2 will be needed before attempts can be made to use the label in this tissue.

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