Identification of the glucose transporter in mammalian cell membranes with a ^{125}I -forskolin photoaffinity label

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The glucose transporter has been identified in a variety of mammalian cell membranes using a photoactivatable carrier-free radioiodinated derivative of forskolin, 3- ^{125}I iodo-4-azidophenethylamido-7-*O*-succinyldeacetylforskolin (^{125}I IAPS-forskolin) at 1–3 nM. The membranes that were photolabelled with ^{125}I IAPS-forskolin were human placental membranes, rat cortical and cerebellar synaptic membranes, rat cardiac sarcolemmal membranes, rat adipocyte plasma membranes, smooth-muscle membranes, and S49 wild-type (WT) lymphoma-cell membranes. The glucose transporter in plasma membranes prepared from the insulin-responsive rat cardiac sarcolemmal cells, rat adipocytes and smooth-muscle cells was determined to be approx. 45 kDa by SDS/polyacrylamide-gel electrophoresis (PAGE). Photolysis of human placental membranes, rat cortical and cerebellar synaptic membranes, and WT lymphoma membranes with ^{125}I IAPS-forskolin, followed by SDS/PAGE, indicated specific derivatization of a broad band (43–55 kDa) in placental membranes and a narrower band (approx. 45 kDa) in synaptic membranes and WT lymphoma membranes. Digestion of the ^{125}I IAPS-forskolin-labelled placental and WT lymphoma membranes with endo- β -galactosidase showed a reduction in the apparent molecular mass of the radiolabelled band to approx. 40 kDa. The membranes that were photolabelled with ^{125}I IAPS-forskolin and trypsin-treated produced a radiolabelled proteolytic fragment with an apparent molecular mass of 18 kDa. ^{125}I IAPS-forskolin is a highly effective probe for identifying low levels of glucose transporters in mammalian tissues.

INTRODUCTION

Most mammalian cells transport glucose stereospecifically by facilitated diffusion mediated by a plasma-membrane protein. Recent work has focused on the identification, regulation, and structural characteristics of the glucose transporter in mammalian tissues (see [1–3] for reviews on these topics). The most extensively studied glucose-transport system is that of the human red cell, which has been identified by affinity-labelling techniques to be a heterogeneously glycosylated protein with an apparent molecular mass ranging from 40 to 70 kDa [1,4]. Determination of the sequences for the glucose-transport proteins from HepG2 human hepatoma cells [5] and the adult rat brain [6] has been an important advance in understanding the mechanism of sugar transport; however, many questions remain unanswered regarding the identification of glucose-transport proteins in other systems, as well as the determination of the native structure and the orientation of this protein within the membrane.

The glucose transporter has been identified in several mammalian tissues [7–21] by using cross-reactivity with antibodies against the purified human erythrocyte transporter, reconstitution methods and photoaffinity-labelling techniques (see [1]).

A number of compounds have been used as affinity

probes for the transporter. Cytochalasin B, a potent inhibitor of glucose transport, has proved to be a particularly useful natural photoaffinity labelling agent for studying the topology of the transport protein [22–30]. Additional photoaffinity labelling agents, which are substrate derivatives, have been reported [31–34]. Most of the currently available photoaffinity-labelling agents for the glucose transporter are tritiated compounds which are not easily detected by fluorography after SDS/polyacrylamide-gel electrophoresis (PAGE).

We have reported the synthesis of a carrier-free iodinated phenylazide derivative of forskolin, 3-iodo-4-azidophenethylamido-7-*O*-succinyldeacetylforskolin (IAPS-forskolin), which in the radioiodinated form has been shown to be a highly selective photoaffinity probe for the human erythrocyte glucose transporter [35]. The photoincorporation of ^{125}I IAPS-forskolin into the red-cell transporter is blocked by D- (but not L-) glucose, as well as cytochalasin B and forskolin. ^{125}I IAPS-forskolin is particularly useful because of its high affinity for the glucose transporter and high specific radioactivity.

In the present paper we report the photoaffinity labelling of the glucose transporter with ^{125}I IAPS-forskolin in membranes derived from several mammalian tissues and cultured cells, including insulin-responsive tissues, where the glucose-transport protein is present at a low concentration.

Abbreviations used: ^{125}I IAPS-forskolin, 3- ^{125}I iodo-4-azidophenethylamido-7-*O*-succinyldeacetylforskolin; PAGE, polyacrylamide-gel electrophoresis; WT, wild-type.

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EXPERIMENTAL

Materials

Cytochalasin B was obtained from Aldrich, and forskolin was from Behring Diagnostics. High-molecular-mass markers were purchased from Sigma and included myosin (205 kDa), β -galactosidase (116 kDa), phosphorylase (92.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). The low-molecular-mass markers were purchased from Bethesda Research Laboratories and included β -lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), and bovine trypsin inhibitor (6.2 kDa). Trypsin [tosylphenylalanylchloromethane ('TPCK') treated] and soybean trypsin inhibitor were obtained from Sigma and endo- β -galactosidase from Miles Laboratories. All other reagents were obtained from Sigma.

Preparation of plasma membranes

Basal-cell membranes of human placental trophoblasts were a gift from Dr. Craig Malbon (SUNY, Stony Brook, NY, U.S.A.). These membranes were prepared using the procedure described by Kelley *et al.* [36]. The isolation and purification of cortical synaptic membranes and cerebellar synaptic membranes were performed in our laboratory by Dr. Laura Lautens [37], who used the procedure described by Gurd *et al.* [38]. Rat cardiac sarcolemmal membranes were also prepared by Dr. Laura Lautens [37], a modified version of the method of Jones *et al.* [39] being used. Rat adipocyte plasma membranes prepared from basal cells were a gift from Dr. Ian Simpson (NIH, Bethesda, MD, U.S.A.). These membranes were prepared by the procedure of Simpson *et al.* [40]. Preparation of plasma membranes from the DTT₁MF-2 smooth-muscle cell line was performed as described by Clark *et al.* [41]. S49 wild-type (WT) lymphoma-cell membranes were prepared as previously described [42] by using a modified version of the procedure of Ross *et al.* [43]. All membranes were frozen at -70°C until the day of use.

Synthesis of [^{125}I]IAPS-forskolin

The synthesis, purification, and characterization of [^{125}I]IAPS-forskolin is described elsewhere [35].

Photoaffinity labelling

[^{125}I]IAPS-forskolin photoincorporation into membranes was performed essentially as described previously [35]. The buffer used for all photolabelling experiments and enzymic digestions consisted of 50 mM-Tris and 4 mM-MgCl₂, pH 7.5. Membranes (120–300 μg , 0.8–2.0 mg/ml in buffer) were first preincubated in 15 ml thick-walled Pyrex tubes in the presence of either buffer, or buffer containing D-glucose, L-glucose, cytochalasin B or forskolin (concentrations indicated in the Figure legends) for 30 min on ice. After this initial preincubation, [^{125}I]IAPS-forskolin in ethanol (1–2 μl ; final concn. 1.0–3.0 nM) was added and the membranes incubated for an additional 30 min on ice in the dark. The concentrations of [^{125}I]IAPS-forskolin in the incubations were determined by sampling 2 μl portions before photolysis and measuring the radioactivity with a γ -radiation counter. After this time period, 1–5 ml of buffer, or buffer containing 600 mM-D-glucose, 600 mM-

L-glucose, 10 μM -cytochalasin B or 30 μM -forskolin was added to the respective tubes. The diluted membranes were immediately photolysed in the Pyrex tubes for 5 s in ice/water at 10 cm from a 1 kW high-pressure mercury lamp. After photolysis, β -mercaptoethanol (1% final concn.) was added to the photolysed membranes. For photolabelling of rat heart membranes, rat adipocyte plasma membranes, as well as for cortical and cerebellar synaptic membranes used for trypsin experiments, the same procedure was used except that a pre-photolysis 'wash' was performed. After incubation with [^{125}I]IAPS-forskolin, the membranes were diluted with 5 ml of buffer or buffer containing 600 mM-D-glucose, 600 mM-L-glucose, 10 μM -cytochalasin B or 30 μM forskolin. The diluted membranes were centrifuged at 140 000 g for 30 min at 4°C . The pelleted membranes were resuspended in 125 μl , followed by dilution with 1 ml of the same solution used for the 'wash'. The diluted membranes were then photolysed in Pyrex tubes as described above. This pre-photolysis 'wash' was performed in order to decrease the non-specific labelling [35]. All photolysed membranes were centrifuged at 140 000 g for 30 min at 4°C . The final pellets were then solubilized in gel-electrophoresis sample buffer [30], and the samples (50–250 μg of protein) were layered on to gels for electrophoresis.

Enzymic digestions

Photolabelled membranes (110–300 μg) were resuspended in buffer at a concentration of 0.75–1.5 mg/ml. Trypsin digestion was performed by adding trypsin to a final concentration of 47 $\mu\text{g}/\text{ml}$ and incubating for 60 min at 37°C . The trypsin treatment was terminated with 5 vol. of soybean trypsin inhibitor (0.188 mg/ml) in buffer, followed by the addition of 4 ml of buffer.

Endo- β -galactosidase digestions were performed at a final enzyme concentration of 27 munits/ml for 60 min at 37°C . After this time, the endo- β -galactosidase-treated membranes were diluted with 5 ml of buffer. All membranes were centrifuged at 140 000 g for 30 min at 4°C , and the resulting pellets were solubilized in sample buffer and subjected to SDS/PAGE as described below.

Electrophoresis

Electrophoresis was performed on 1.5 mm-thick 12% resolving gels and 4% stacking gels, using the modified Laemmli buffer system of Giulian *et al.* [44]. Sample buffer consisted of 3% (w/v) SDS, 1 mM-EDTA, 10% (v/v) glycerol, 0.005% Bromophenol Blue, 2.5% β -mercaptoethanol, and 62.5 mM-Tris, pH 6.8 (all final concns.). The solubilized membrane protein (100–300 μg) was loaded in the appropriate lane. Electrophoresis was performed at 30 mA constant current through the stacking gel (approx. 1 h) and 35 mA constant current through the resolving gel (approx. 4 h). After electrophoresis the gels were stained for 2 h [33% (v/v) methanol/10% (v/v) acetic acid/0.1% Coomassie Brilliant Blue R], destained overnight [33% (v/v) methanol/10% (v/v) acetic acid/5% (v/v) glycerol], dried on a slab-gel dryer for 2 h at 80°C , and then exposed to Kodak X-Omat film with a Quanta III intensifier screen (du Pont) at -70°C for the time indicated in the Figure legends.

RESULTS

Photoincorporation of [125 I]IAPS-forskolin into human placental membranes

Human placental membranes were photolysed and subjected to SDS/PAGE and autoradiography as described above. The resulting Coomassie Blue-staining protein pattern (Fig. 1a) and corresponding autoradiogram (Fig. 1b) are shown. A fairly broad specifically labelled band is seen between 43 and 55 kDa. The radiolabelled polypeptide band does not appear to be as broad as that produced by the human erythrocyte glucose transporter, which migrates with an apparent molecular mass of 40–70 kDa on SDS/PAGE (see [1,4,35]). This band does, however, exhibit properties of the transporter; that is, protection by D-glucose, cytochalasin B, and forskolin, but not by L-glucose. These results are somewhat similar to those in two previous reports of [3 H]cytochalasin B photolabelling of placental membranes, which showed D-glucose-displaceable [3 H]-cytochalasin B-labelled bands that migrated as narrow bands at approx. 52 kDa [7] and 60 kDa [8] after SDS/PAGE. Our results indicate that the [125 I]IAPS-forskolin-labelled placenta transporter is a broad band on SDS/PAGE. We have examined the effect of endo- β -galactosidase on [125 I]IAPS-forskolin-labelled placental membranes.

Fig. 1(c) shows an autoradiogram of a gel after enzymic treatment of the photolabelled placental membranes and SDS/PAGE. A specifically labelled protein migrating as a broad band with an apparent molecular mass of 43–55 kDa is observed in the control (lane a). Stereospecificity was determined in all cases by the protection of photolabelling in the presence of 600 mM-D-glucose, but not in the presence of 600 mM-L-glucose. Endo- β -galactosidase caused a shift in the apparent molecular mass of the specifically labelled band from 50 kDa (peak centre) to approx. 40 kDa. Treatment with trypsin caused a shift in the apparent molecular mass of the labelled region from 50 kDa (peak centre) to a much sharper band at approx. 18 kDa. Although enzymic treatments are not complete, the results are very similar to those observed for red-cell membranes [35]. We can, therefore, conclude that this radiolabelled protein is the heterogeneously glycosylated glucose transporter in human placental membranes.

Photoincorporation of [125 I]IAPS-forskolin into rat synaptosomal membranes

We have tested the ability of [125 I]IAPS-forskolin to photolabel the glucose transporter in synaptosomal membranes from rat cortex and cerebellum. Cortical synaptic membranes and cerebellar synaptic membranes were photolysed and subjected to SDS/PAGE and autoradiography as described above. Figs. 2(a) and 2(b) (cortical synaptic membranes) and Figs. 2(d) and 2(e) (cerebellar synaptic membranes) show the Coomassie Blue-staining protein patterns (Figs. 2a and 2d) and the corresponding autoradiograms (Figs. 2b and 2e) obtained after SDS/PAGE. For both types of synaptosomal membranes a specifically labelled peptide is observed at approx. 45 kDa, which is D-glucose, cytochalasin B, and forskolin-protectable, but which is not protected by L-glucose. By comparison with the human erythrocyte glucose transporter (40–70 kDa on SDS/PAGE; see [35]) and the placental glucose transporter (43–55 kDa

Fig. 1. Photoincorporation of [125 I]IAPS-forskolin into human placental membranes and enzymic digestion

The Coomassie Blue-protein-stained electrophoretic pattern (a) and corresponding 2 h autoradiogram (b) of an SDS/PAGE gel of human placental membranes (200 μ g) photolabelled with [125 I]IAPS-forskolin (1.5 nM) in the presence of buffer (lane a), buffer and 600 mM-D-glucose (lane b), 10 μ M-cytochalasin B (lane c), 30 μ M-forskolin (lane d), and 600 mM-L-glucose (lane e) are shown. (c) A 2 h autoradiogram of a SDS/PAGE gel of placental membranes photolabelled with [125 I]IAPS-forskolin (2.8 nM) in the presence of buffer (lanes a, d and g), 600 mM-D-glucose (lanes b, e and h), and 600 mM-L-glucose (lanes c, f and i). Lanes a–c represent control membranes incubated without enzyme; lanes d–f represent membranes incubated with 27 munits of endo- β -galactosidase/ml for 30 min at 37 $^{\circ}$ C; and lanes g–i represent membranes treated with 47 μ g of trypsin/ml for 30 min at 37 $^{\circ}$ C.

Fig. 2. Photoincorporation of [125 I]IAPS-forskolin into cortical and cerebellar synaptic membranes and trypsin treatment of labelled membranes

The Coomassie Blue-protein-stained electrophoretic patterns of cortical (*a*, 300 μ g) and cerebellar (*d*, 200 μ g) synaptic membranes photolabelled with [125 I]IAPS-forskolin (3.0 and 1.5 nM respectively) in the presence of buffer (lane *a*), buffer and 600 mM-D-glucose (lane *b*), 10 μ M-cytochalasin B (lane *c*), 30 μ M forskolin (lane *d*), and 600 mM-L-glucose (lane *e*) are shown. The corresponding autoradiograms of the SDS/PAGE gel of cortex (*b*, 2 h) and cerebellum (*e*, 8 h) synaptic membranes are also shown. Also depicted is a 4-day autoradiogram of cortical (*c*) and cerebellar (*f*) synaptic membranes which were photolabelled with 1.5 nM-[125 I]IAPS-forskolin in the presence of buffer (lanes *a* and *c*) or 10 μ M-cytochalasin B (lanes *b* and *d*) and then incubated without enzyme (lanes *a* and *b*) and with 47 μ g of trypsin/ml (lanes *c* and *d*) for 60 min at 37 $^{\circ}$ C.

on SDS/PAGE; see Fig. 1*b*), the 45 kDa radiolabelled band found in cortical and cerebellar synaptic membranes is much narrower, which would suggest less glycosylation in this transport protein. This radiolabelled protein in both synaptosomal membranes did not appear to be sensitive to endo- β -galactosidase (results not shown). An intense non-specific band is also seen at approx. 100 kDa; the nature of this polypeptide is not known at the present time.

When the cortical and cerebellar synaptic membranes were photolabelled and trypsin-treated [cortex (Fig. 2*c*) and cerebellum (Fig. 2*f*)], it was found that the specifically labelled band with an apparent molecular mass of 45 kDa was shifted to approx. 18 kDa after trypsin treatment. Specificity is illustrated by the protection of photolabelling in the presence of 10 μ M-cytochalasin B. The generation of an 18 kDa radiolabelled fragment after trypsin treatment of these membranes is consistent

with the results obtained after trypsin treatment of photolabelled placental membranes (Fig. 1b) and photolabelled red-cell membranes [35].

Photoincorporation of [125 I]IAPS-forskolin into membranes prepared from insulin-responsive tissues

The glucose-transport proteins in heart [45], adipose tissue [2,14,15] and cultured smooth muscle [46] have been shown to be regulated by a number of factors, including insulin. We have examined the photoincorporation of [125 I]IAPS-forskolin into these membranes. Rat sarcolemmal cardiac membranes, rat adipocyte plasma membranes and DTT₁MF-2 smooth-muscle membranes were photolabelled with [125 I]IAPS-forskolin and subjected to SDS/PAGE and autoradiography. The Coomassie Blue-staining protein patterns (Figs. 3a, 3c and 3e) and corresponding autoradiograms (Figs. 3b, 3d and 3f) show the results obtained. A specifically labelled band was observed at approx. 45 kDa. Photoincorporation of [125 I]IAPS-forskolin in sarcolemmal membranes (Fig. 3b) showed protection with D- (but not L-)glucose, cytochalasin B and forskolin. Photolabelling of adipocyte plasma membranes (Fig. 3d) and smooth-muscle membranes (Fig. 3f) was protected with D-glucose and cytochalasin B. These observations provide evidence that the photolabelled 45 kDa polypeptide in these membranes is the glucose transporter.

When labelled rat cardiac sarcolemmal membranes and labelled rat adipocyte plasma membranes were subjected to trypsin treatment, an 18 kDa radiolabelled proteolytic fragment was generated (results not shown).

Photoincorporation of [125 I]IAPS-forskolin into WT lymphoma-cell membranes

The S49 lymphoma cell line (WT) and its guanine-nucleotide-binding-protein (G_s)-deficient variant (*cyc*⁻), as well as its protein kinase-deficient variant (*kin*⁻), have been used as a useful model system for the study of the

mechanism of hormonal regulation and desensitization of adenylate cyclase [41,47,48]. Forskolin has been known to activate adenylate cyclase in S49 membranes and has also been reported to bind to the glucose transporter and inhibit glucose transport in adipocytes [49,50], platelets [51], red cells [52,53], and cardiomyocytes [54]. We therefore examined the photoincorporation of [125 I]IAPS-forskolin into these membranes.

Fig. 4(a) shows the Coomassie Blue-staining protein pattern, and Fig. 4(b) shows the corresponding autoradiogram of the photoincorporation of radiolabel into the S49 WT membrane polypeptides. In the control and in the presence of 600 mM-L-glucose a specifically labelled polypeptide was observed in a narrow band at approx. 45 kDa that migrated slightly ahead of the Coomassie Blue-staining actin band. This 45 kDa band was protected by D-glucose, cytochalasin B and forskolin. Another polypeptide, at approx. 52 kDa, showed a large amount of photoincorporation of label. Labelling of the 52 kDa band was partially inhibited by 140 μ M-forskolin and completely protected by 160 μ M-cytochalasin B; however, no significant protection was observed in the presence of either 600 mM-D-glucose or 600 mM-L-glucose. The nature of this protein is not known at the present time. In the region of the gel between the 52 kDa and the 45 kDa bands a diffuse band of radioactivity was observed which was protected by D-glucose, cytochalasin B and forskolin, but not by L-glucose. The diffuse band and the 45 kDa polypeptide most likely represent the S49 WT glucose transporter. This experiment was repeated with lower concentrations of cytochalasin B (10 μ M) and forskolin (10 μ M), and a similar pattern of protection was observed in the 45 kDa region (results not shown). When *cyc*⁻ and *kin*⁻ lymphoma cell membranes were photolabelled and subjected to SDS/PAGE and autoradiography, it was found that a 45 kDa and a 52 kDa protein were specifically labelled in a manner similar to that observed in WT membranes (results not shown).

Fig. 3. Photoincorporation of [125 I]IAPS-forskolin into various insulin-responsive cells

The Coomassie Blue protein-stained electrophoretic patterns of rat cardiac sarcolemmal membranes (a, 120 μ g), rat adipocyte plasma membranes (c, 139 μ g), and smooth-muscle membranes (e, 200 μ g) photolabelled with [125 I]IAPS-forskolin (3.0, 1.5 and 0.7 nM respectively) in the presence of buffer (lane a), 600 mM-D-glucose (lane b), 10 μ M-cytochalasin B (lane c), 30 μ M-forskolin (lane d) and 600 mM-L-glucose (lane e) are shown. The corresponding autoradiogram of the SDS/PAGE gel of labelled cardiac sarcolemmal membranes (b, 8 h), adipocyte plasma membranes (d, 2 h) and smooth-muscle membranes (f, 19 h) is also depicted.

Fig. 4. Photoincorporation of $[^{125}\text{I}]\text{IAPS}$ -forskolin into WT membranes and enzymic digestion

The Coomassie Blue-protein-stained electrophoretic patterns of WT membranes (a, 200 μg) photolabelled with $[^{125}\text{I}]\text{IAPS}$ -forskolin (2.8 nM) in the presence of buffer (lane a), buffer and 600 mM-D-glucose (lane b), 160 μM -cytochalasin B (lane c), 140 μM -forskolin (lane d) and 600 mM-L-glucose (lane e) is shown. The corresponding autoradiogram (2 h) of SDS/PAGE gels of labelled WT membranes (b) is also shown. Also depicted is a 1.5-h autoradiogram (c) of an SDS/PAGE gel of WT membranes (300 μg) photolabelled with $[^{125}\text{I}]\text{IAPS}$ -forskolin (3.0 nM) in the presence of buffer (lanes a, d and g), 600 mM-D-glucose (lanes b, e and h), and 600 mM-L-glucose (lanes c, f and i) is also depicted. Lanes a-c represent control membranes incubated without enzyme, lanes d-f represent membranes incubated with 27 munits of endo- β -galactosidase/ml for 60 min at 37 °C and lanes g-i represent membranes treated with 550 munits of trypsin/ml for 60 min at 37 °C.

In order to evaluate better which one of the photolabelled polypeptides in WT membranes had characteristics similar to the glucose transporter in other cells, the photolabelled membranes were subjected to

enzymic digestion. The membranes were photolabelled with $[^{125}\text{I}]\text{IAPS}$ -forskolin in the presence of buffer, D-glucose, or L-glucose, as described previously. After photolysis and centrifugation the membranes were treated with endo- β -galactosidase and trypsin. Fig. 4(c) shows an autoradiogram of the results obtained after SDS/PAGE and autoradiography. Both the 45 kDa and the 52 kDa polypeptide were observed in the control and in the presence of L-glucose, with only the lower band (45 kDa) showing protection by D-glucose. Treatment of the radiolabelled membranes with glycosidase resulted in a shift of the 45 kDa polypeptide to approx. 40 kDa, which was very similar to the apparent molecular mass of the photolabelled placental transporter (Fig. 1b) and the red-cell transporter when subjected to endo- β -galactosidase [35]. When photolabelled WT membranes were trypsin-treated, the 45 kDa radiolabelled polypeptide was shifted to approx. 18 kDa. This result is consistent with those obtained when the glucose transporter was photolabelled in other membranes and trypsin-treated (Figs. 1c, 2c, 2f; [39]); therefore we conclude that the 45 kDa polypeptide is the glucose transporter in S49 lymphoma cells. The 52 kDa radiolabelled polypeptide was glycosidase- and trypsin-insensitive. Further investigation will be required to determine whether this 52 kDa polypeptide is related to the 45 kDa glucose transporter in S49 lymphoma cells.

DISCUSSION

$[^{125}\text{I}]\text{IAPS}$ -forskolin is a very selective photoaffinity label for the glucose-transport proteins in many mammalian tissues. In the present report we have identified the glucose transporter in human placental membranes, rat cortical and cerebellar synaptic membranes, rat cardiac sarcolemmal membranes, rat adipocyte plasma membranes, DTT₁MF-2 smooth-muscle membranes and S49-WT and *cyc*⁻ lymphoma-cell membranes. Table 1 summarizes the results obtained after photolysis of these membranes with $[^{125}\text{I}]\text{IAPS}$ -forskolin, SDS/PAGE and autoradiography. This photoinsertion was blocked by D- (but not L-)glucose, cytochalasin B, and forskolin. The protection of photolabelling by D-glucose, but not L-glucose, illustrates the stereospecificity exhibited by the transport protein. The inhibition of photolabelling in the presence of cytochalasin B or forskolin, two potent inhibitors of glucose transport in many cell types ([15,56] and [49–55]), is another feature characteristic of the glucose transporter.

We have previously shown that $[^{125}\text{I}]\text{IAPS}$ -forskolin is a potent photoaffinity label for the human red-cell D-glucose transporter and purified red-cell glucose-transporter preparations [35]. The red-cell transport protein migrated as a broad band (40–70 kDa) on SDS/PAGE and could be sharpened to a much narrower band around 40 kDa after endo- β -galactosidase treatment. Treatment of $[^{125}\text{I}]\text{IAPS}$ -forskolin-labelled placental (Fig. 1c) and WT lymphoma cell membranes (Fig. 4c) with endo- β -galactosidase resulted in a shift in the apparent molecular mass of the radiolabelled band to approx. 40 kDa, which is very similar to the apparent molecular mass of the partially deglycosylated red-cell glucose transporter. Endo- β -galactosidase did not appear to have any effect on the photolabelled transporter in other membranes (results not shown); however, this

Table 1. Summary of membranes photolabelled with [¹²⁵I]IAPS-forskolin

The various membranes were photolabelled with [¹²⁵I]-IAPS-forskolin and subjected to SDS/PAGE and autoradiography as described in the Experimental section. Several photolabelled membranes were treated with endo- β -galactosidase and trypsin, followed by SDS/PAGE and autoradiography. The apparent M_r values for the photolabelled glucose-transport proteins are shown. Abbreviations: NE, no effect; NT, not tested.

Membrane	$10^{-3} \times$ Apparent M_r of the transporter	$10^{-3} \times$ Apparent M_r of the transporter after treatment with:	
		Endo- β -galactosidase	Trypsin
Red cell [35]	40–70	40	18
Placenta	43–55	40	18
Cortex	45	NE	18
Cerebellum	45	NE	18
Heart	45	NE	18*
Adipocyte	45	–*†	18*
Smooth muscle	45	NT	NT
S49 WT	45	40	18
S49 <i>cyc</i> [–]	45*	NT	NT
S49 <i>kin</i> [–]	45*	NT	NT

* B. E. Wadzinski, M. F. Shanahan & A. E. Ruoho, unpublished work.

† Sharpened band.

enzyme is specific for galactose residues, and our observation remains to be confirmed with other endoglycosidases.

Trypsin treatment of all of the [¹²⁵I]IAPS-forskolin-labelled transporters examined in here showed an apparent 18 kDa radiolabelled proteolytic fragment. The present results agree with previous trypsin studies using [³H]cytochalasin B [22–24,26,27], [³H]forskolin [55], and [¹²⁵I]IAPS-forskolin [35] on the human red-cell glucose transporter. These observations support the view that most, if not all, mammalian systems that transport glucose by facilitated diffusion have a very similar membrane transporter.

Birnbaum *et al.* [6] have cloned and characterized a cDNA encoding the glucose transporter from the rat brain. Their work has revealed a 492-amino-acid protein with a predicted molecular mass of 56133 Da that demonstrates 97.6% identity with the human hepatoma transporter. They have shown that two transporter species are present in rat brain which, when immunoblotted with antisera against the human red-cell glucose transporter, migrated with apparent molecular mass of 55 and 46 kDa after SDS/PAGE. They postulated that the two species represent transporters with various amounts of glycosylation, since endoglycosidase F treatment produced a single polypeptide that co-migrated with the deglycosylated human red-cell transporter. A 55 kDa glucose-transport protein has also recently been observed in bovine cerebral-cortex microvessels, which was reduced in size to an apparent molecular mass of 46 kDa after treatment with endoglycosidase F. It has been suggested, by using [³H]-cytochalasin B binding and photolabelling, that two

types of rat brain glucose transporters exist: a 45 kDa protein which is present in neuronal and glial cells [12] and a 55 kDa protein localized in rat microvessels [9,10]. It is possible that the 55 kDa microvessel glucose transporter is a glycosylated form of the 45 kDa neuronal and glial transporter. These results are consistent with our observation that [¹²⁵I]IAPS-forskolin labelled a 45 kDa neuronal (or synaptosomal) glucose transporter. Depending on the purity of the synaptic membrane preparation which is used for photolabelling, we occasionally observed a faintly labelled 52 kDa polypeptide, which might represent microvessel contamination of the purified synaptosomes.

The mammalian tissues which contain hormonally sensitive glucose transporters are important systems for studying the regulation of sugar transport. Some of the hormonally sensitive transport systems include: rat and human adipose cells [2], skeletal muscle [57], cardiac myocyte [45,58] and cultured smooth-muscle cells [46]. Here we have shown specific photolabelling of the insulin-responsive rat cardiac sarcolemmal, rat adipose and DTT,MF-2 smooth-muscle glucose transporters with [¹²⁵I]IAPS-forskolin and find that they do not differ in size from the non-insulin-controlled tissues. Cells from rat cortex and cerebellum and from human placenta have not been reported to exhibit insulin-sensitive glucose transport. The regulation of sugar transport by insulin in the S49 lymphoma cell line has not been studied.

The S49 lymphoma cell line (WT), the G_s -deficient variant (*cyc*[–]) and the kinase-deficient variant (*kin*[–]) have been used as a model system for dissecting the mechanism of hormonal regulation of adenylate cyclase. Another S49 lymphoma mutant, namely 14T-g, which has lost capacity to transport thymidine, has been used for the genetic segregation of the glucose and nucleoside transporters [27]. Recent work has shown that stimulators and inhibitors of adenylate cyclase have counter-regulatory effects on insulin-stimulated glucose transport in the rat adipose cell (see [2] and [59] for reviews on these topics) and cardiac myocyte [54]. The mechanism by which these regulators of adenylate cyclase affect glucose transport appears to involve a modulation of glucose transporter intrinsic activity [60,61] and/or the insulin-signalling mechanism. The availability of the lymphoma cell lines and the highly selective photoaffinity label for the glucose transporter, [¹²⁵I]IAPS-forskolin, should make it possible to examine the relationship of the glucose transporter to the adenylate cyclase system.

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