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Localization of the forskolin photolabelling site within the monosaccharide transporter of human erythrocytes

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Chemical and proteolytic digestion of intact erythrocyte glucose transporter as well as purified transporter protein has been used to localize the derivatization site for the photoaffinity agent 3-[^{125}I]iodo-4-azido-phenethylamino-7-*O*-succinyldeacetylforskolin ([^{125}I]IAPS-forskolin). Comparison of the partial amino acid sequence of the labelled 18 kDa tryptic fragment with the known amino acid sequence for the HepG2 glucose transporter confirmed that the binding site for IAPS-forskolin is between the amino acid residues Glu^{254} and Tyr^{456}. Digestion of intact glucose transporter with Pronase suggests that this site is within the membrane bilayer. Digestion of labelled transporter with CNBr generated a major radiolabelled fragment of $M_r \approx 5800$ putatively identified as residues 365–420. Isoelectric focusing of *Staphylococcus aureus* V8 proteinase-treated purified labelled tryptic fragment identified two peptides which likely correspond to amino acid residues 360–380 and 381–393. The common region for these radiolabelled peptides is the tenth putative transmembrane helix of the erythrocyte glucose transporter, comprising amino acid residues 369–389. Additional support for this conclusion comes from studies in which [^{125}I]IAPS-forskolin was photoincorporated into the L-arabinose/H$^+$-transport protein of *Escherichia coli*. Labelling of this transport protein was protected by both cytochalasin B and D-glucose. The region of the erythrocyte glucose transporter thought to be derivatized with IAPS-forskolin contains a tryptophan residue (Trp^{388}) that is conserved in the sequence of the *E. coli* arabinose-transport protein.

INTRODUCTION

Most mammalian cells transport glucose across their plasma membrane by a process of facilitated diffusion. Understanding the molecular mechanism of glucose transport has been the focus of extensive investigation. The most characterized mammalian glucose-transport system is that of the human erythrocyte plasma membrane, which has been identified by reconstitution methods and various affinity-labelling techniques [1]. Antiserum against the purified erythrocyte glucose transporter has been used to clone the HepG2 human hepatoma [2] and adult-rat brain glucose-transport proteins [3]. The corresponding amino acid sequences have been deduced. Structural analysis of the purified erythrocyte transporter by fast-atom bombardment and gas-phase Edman degradation has demonstrated that the HepG2 and erythrocyte transporters are similar and may be identical [2]. The determination of the sequences for the HepG2 and rat brain glucose transporters has been an important advance that holds promise for providing new insight into the molecular mechanism of sugar transport.

Despite recent advances in this area, questions remain regarding the native structure and the dynamics of the glucose transporter. The definitive location of membrane-spanning domains and the identification of binding sites for glucose and glucose-transport inhibitors await further investigation. An important strategy for obtaining such information has been the application of affinity- and photoaffinity-labelling techniques in conjunction with chemical and enzymic digestion of both membrane-labelled and purified glucose transporters [4–10,10a]. The use of selective inhibitors of glucose transport has provided valuable information concerning the topography of the transport protein. Several compounds have been used as photoaffinity probes for the transporter. These include cytochalasin B [4–7,9, 10,15–17], iodoazido-derivatives of glucose [18,19], and several derivatives of mannose [8,10a,20–22]. We have reported the synthesis of a carrier-free radioiodinated phenylazide derivative of forskolin, 3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyl-deacetylforskolin ([^{125}I]IAPS-forskolin), which has been shown to have high selectivity as a photoaffinity probe for the erythrocyte glucose transporter [23] as well as glucose-transport proteins in membranes derived from several mammalian tissues and cultured cells [24]. [^{125}I]IAPS-forskolin is particularly useful because of its high affinity for the glucose transporter and its high specific radioactivity.

In the present paper we report information obtained from chemical and proteolytic digestion of both labelled intact erythrocyte glucose transporters and the labelled purified protein, which has been used to localize the site of derivatization for [^{125}I]IAPS-forskolin. Additional support for the conclusions which are drawn concerning the region covalently labelled with [^{125}I]IAPS-forskolin has come from studies examining the photoincorporation of label into the L-arabinose-transport protein of *Escherichia coli* [25–29].

EXPERIMENTAL

Materials

Cytochalasin B and CNBr were obtained from Aldrich. High-molecular-mass markers were purchased from Sigma and included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (92.4 kDa), BSA (66 kDa), ovalbumin (45 kDa)

Abbreviations used: [^{125}I]IAPS-forskolin, 3-[^{125}I]iodo-4-azidophenethylamido-7-*O*-succinyldeacetylforskolin; 5P8 buffer, 5 mM-sodium phosphate buffer, pH 8; [^{125}I]IAPA, 2-(3-[^{125}I]iodo-4-azidophenyl)ethylamine; 1-NHS-S-forskolin, 1-(*N*-hydroxysuccinimidyl)succinylforskolin; S.A.V8, *Staphylococcus aureus* V8 proteinase; TEMED, *NNN'N'*-tetramethylethylenediamine.

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and carbonic anhydrase (29 kDa). The low-molecular-mass markers were purchased from Bethesda Research Laboratories and included α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa) and the A- and B-chains of insulin (approx. 3 kDa). Isoelectric-focusing markers were purchased from Sigma, and included amyloglucosidase from *Aspergillus niger* (pI 3.5), soybean trypsin inhibitor (pI 4.6), β-lactoglobulin A from bovine milk (pI 5.2), carbonic anhydrase B from bovine erythrocytes (pI 5.9), human carbonic anhydrase (pI 6.6), horse myoglobin (pI 7.4) and lentil lectin (pI 8.2 and 8.7). Trypsin [tosylphenylalanylchloromethane ('TPCK')-treated] and soybean trypsin inhibitor were obtained from Sigma. Pronase, a proteinase from *Streptomyces griseus*, and Endoproteinase Glu-C (*Staphylococcus aureus* V8 proteinase, S.A.V8) were purchased from Calbiochem Corp. Acrylamide, bisacrylamide SDS, urea, *NNN'N'*-tetramethylethylenediamine (TEMED), Tris, glycine and ammonium persulphate were purchased from either Bio Rad (Richmond, CA, U.S.A.) or BLR (Gaithersburg, MD, U.S.A.). Spectrophotometric-grade glycerol was from Aldrich (Milwaukee, WI, U.S.A.). Carrier ampholytes and Immobilines were purchased from LKB (Bromma, Sweden). *E. coli* NM 522 was a gift from Dr. Woo-Hyeon Byeon (Department of Pharmacology, University of Wisconsin, Madison, WI, U.S.A.).

Preparation of plasma membranes

Washed human erythrocytes and membrane ghosts were prepared by the method of Steck & Kant [30]. Ghost membranes in 5 mM-sodium phosphate buffer, pH 8 (5P8 buffer) were isolated by centrifugation at 17400 *g* for 15 min in a Sorvall SS-34 rotor and washed three or four times with 5P8 buffer. Membrane ghosts were frozen at −70 °C until use. All procedures were carried out at 4 °C unless otherwise noted.

Preparation of purified and reconstituted glucose transporter

The glucose transporter was purified and reconstituted by the method of Cairns *et al.* [4]. This transporter preparation can be readily centrifuged to a pellet at 30000 *g* for 60 min.

Growth conditions for *E. coli* and photoaffinity labelling

E. coli NM 522 was cultured in M9 CAA buffer [31] or in M9 CA buffer containing 10 mM-L-arabinose overnight in a shaker bather at 37 °C. The cells were collected by centrifugation for 5 min at 10000 rev./min (9148 $g_{av.}$) (Sorvall centrifuge; SS 34 rotor). The pellets were washed twice with 5 ml of 50 mM-Tris (pH 7.4)/1 mM-EDTA/130 mM-NaCl (TEN buffer) and resuspended in 1 ml of TEN buffer. *E. coli* cells (25 μl) were first preincubated in 15 ml thick-walled Pyrex tubes in the presence of either TEN buffer, or TEN buffer containing 100 μM-cytochalasin B, 500 mM-D-arabinose, 500 mM-L-arabinose or 500 mM-D-glucose for 30 min on ice. After this initial preincubation, [^{125}I]IAPS-forskolin in ethanol (1 μl, final concn. 4 nM) was added and the cells incubated for an additional 30 min on ice in the dark. The cells were then diluted with 1 ml of buffer, or buffer containing 100 μM-cytochalasin B, 500 mM-D-arabinose, 500 mM-L-arabinose or 500 mM-D-glucose. The diluted solution was immediately photolysed for 7 s as described below. After photolysis, β-mercaptoethanol (1 % final concn.) was added to the photolysed cells and the solutions centrifuged in a Microfuge (Beckman) for 8 min. The pellets were solubilized in gel-electrophoresis sample buffer and incubated at 37 °C for 30 min. The samples were then centrifuged (10 min, in an Eppendorf Microfuge at maximum setting) and the supernatants layered on to the gels for electrophoresis.

Synthesis of 2-(3-[^{125}I]iodo-4-azidophenyl)ethylamine

The synthesis, purification and characterization of this photoactive phenylamine ([^{125}I]IAPA) have been described elsewhere [32].

Synthesis of 1-(*N*-hydroxysuccinimidyl)succinylforskolin (1-NHS-S-forskolin)

Forskolin (100 mg) was dissolved in 5 ml of dry pyridine containing 300 mg of succinic anhydride and 8 mg of dimethylaminopyridine. The reaction was allowed to proceed at 45 °C overnight. The solvent was removed under vacuum and the residue was redissolved in 10 ml of methylene chloride. The organic extract was washed three times with 5 % HCl and twice with 4 M-NaCl. The organic layer was completely dried over anhydrous Na_2SO_4. The residue was dissolved in 2 ml of methylene chloride and purified by flash chromatography on a silica column with methylene chloride/ethyl acetate (4:1, v/v) as solvent. The fractions containing 1-hemisuccinylforskolin were pooled and dried.

1-Hemisuccinylforskolin (100 mg) was dissolved in 5 ml of dry methylene chloride containing 60 mg of dicyclohexylcarbodiimide and 33 mg of *N*-hydroxysuccinimide. The reaction was allowed to proceed for 4 h at room temperature. The insoluble material was removed by filtration with a sintered-glass funnel and the 1-NHS-S-forskolin was purified by flash chromatography on silica using methylene chloride/ethyl acetate (9:1, v/v) as solvent. The fractions were pooled and dried under vacuum and the 1-NHS-S-forskolin was recrystallized from light petroleum (b.p. 35–60 °C) producing white crystals that were stable in storage for up to 1 year at room temperature when kept desiccated.

^{1}H n.m.r. data at 300 MHz ([^{2}H]iodoform/tetramethylsilane) for *1-NHS-S-forskolin* were as follows: δ (p.p.m.) 5.8–5.95(dd, 1 H, H-14), 5.59(t, 1 H, H-1), 5.43(d, 1 H, H-7), 5.28(d, 1 H, H-15), 4.95(d, 1 H, H-15), 4.45(t, 1 H, H-6), 3.23(d, 1 H, H-12), 2.88–2.95(m, 2 H, -OCOCH$_2$*CH$_2$*CON-), 2.8(s, 4, N[-COCH$_2$CH$_2$CO-]), 2.6–2.75(m, 2 H -OCO*CH$_2$*CH$_2$CON-), 2.42(d, 1 H, H-12), 2.22(d, 1 H, H-5), 2.18(s, 3 H, -OCOCH$_3$), 1.78(s, 3 H, CH$_3$), 1.58(s, 3 H, CH$_3$, 1.35(s, 3 H, CH$_3$), 1.28(s, 3 H, CH$_3$), 1.02(s, 3 H, CH$_3$). The identification of the H-6, H-7, and H-1 protons was confirmed by n.m.r. decoupling experiments. The downfield shift of H-1 is consistent with succinylation at this position.

Synthesis of [^{125}I]IAPS-forskolin and the derivative 1-[^{125}I]IAPS-forskolin

The synthesis, purification and characterization of [^{125}I]IAPS-forskolin is described elsewhere [23]. The synthesis of 1-[^{125}I]IAPS-forskolin was performed as follows. Approx. 700 μCi of [^{125}I]IAPA was dried under N_2, and 25 μl of 1-NHS-S-forskolin was added. The reaction was allowed to proceed at room temperature for 18 h. The radioactive product ($R_F \sim 0.3$) was then purified by t.l.c. on silica-gel plates developed with toluene/ethyl acetate (3:2, v/v). After autoradiography, the silica-containing product was extracted three times with 1 ml of ethanol and concentrated to a final concentration of approx. 0.5 μCi/μl. The product was obtained carrier-free at a theoretical specific radioactivity of 2200 Ci/mmol; the yield was 65 %.

Photoaffinity labelling

Conditions for photoaffinity labelling of membranes with [^{125}I]IAPS-forskolin were essentially the same as those previously

described [23,24]. Membranes (150 μg; 1 mg/ml in 5P8 buffer) were first preincubated in 15 ml thick-walled (2 mm) Pyrex tubes in the presence of either 5P8 buffer or 10 μM-cytochalasin B for 30 min on ice. After this initial preincubation, [^{125}I]IAPS-forskolin in ethanol (1 μl; final concns. 1.0–5.0 nM) was added and the membranes incubated for an additional 30 min on ice in the dark. After this time period, 5–10 ml of ice-cold 5P8 buffer, or 5P8 buffer containing 10 μM-cytochalasin B, were added to the respective tubes. The membranes were centrifuged at 30000 *g* for 30 min at 4 °C and the pellets were resuspended in 125 μl of the same solution used for the wash. Immediately before photolysis, 1–5 ml of the corresponding 'wash' solution was added, and the diluted membranes were photolysed in the Pyrex tubes for 7 s in ice/water at 10 cm from a 1 kW high-pressure mercury-vapour lamp (AH-6 lamp; Advanced Radiation Corp., Santa Clara, CA, U.S.A.). After photolysis, β-mercaptoethanol (final concn. 1%) was added to each tube as a scavenger for any long-lived species. The erythrocyte membranes were centrifuged again at 30000 *g* for 30 min.

For photolabelling of purified glucose transporter the same procedure as that used for membranes was employed except that the pre-photolysis 'wash' was omitted. After incubation with [^{125}I]IAPS-forskolin (2–4 nM), the purified transporter preparation (80 μg; 0.5 mg/ml) was diluted with 5 ml of ice-cold 5P8 buffer or with buffer containing 10 μM-cytochalasin B. The diluted solution was photolysed and centrifuged at 30000 *g* for 60 min. The final pellets were either subjected to enzymic digestion (see below) or solubilized in gel-electrophoresis sample buffer, and the samples were layered on to gels for electrophoresis.

Enzymic and chemical digestions

Digestions were performed with [^{125}I]IAPS-forskolin-photolabelled membranes and [^{125}I]IAPS-forskolin-photolabelled purified transporter. The approximate membrane protein concentrations were 1 mg/ml, and the purified transporter protein concentrations were approx. 0.2 mg/ml. Trypsin digestion was performed by adding trypsin to a final concentration of 0.2 mg/ml for membranes and 0.15 mg/ml for purified transporter preparations and incubating for 1 h at 37 °C. Trypsin digestions were terminated by the addition of a 5-fold excess of soybean trypsin inhibitor, followed by dilution with buffer (5 ml) and centrifugation at 30000 *g* for 30 min. Pronase digestion of [^{125}I]IAPS-forskolin-photolabelled erythrocyte membranes (approx. 100 μg) was performed by adding Pronase (1 mg/ml final concn.) in 25 mM-imidazole buffer, pH 7.2, and incubating for 1 h at 37 °C. The Pronase-treated sample was diluted with 5 ml of buffer and centrifuged at 30000 *g* for 30 min. The final pellets obtained after digestion with trypsin or Pronase were solubilized in gel-electrophoresis sample buffer.

S.A.V8 digestion of photolabelled membranes was performed by the addition of SDS (0.25% final concn.) and S.A.V8 (0.25 mg/ml final concn. in 10 mM-sodium phosphate buffer, pH 8). These samples were incubated for 20 h at room temperature, followed by solubilization in gel-electrophoresis sample buffer. CNBr cleavage of photolabelled membranes and photolabelled purified glucose transporter preparations was performed as follows. Photolabelled erythrocyte membranes (100 μg) or purified glucose transporter (40 μg) was solubilized in SDS (0.2% final concn.), followed by the addition of an equal volume of CNBr solution (30 mg/ml in 0.2 M-HCl). These samples were incubated for 20 h at room temperature and then neutralized with an equivalent amount of 0.2 M-NaOH. The samples were then freeze-dried and solubilized in gel-electrophoresis sample buffer. Under these conditions no hydrolysis of the photomoiety was observed.

Electrophoresis

SDS/PAGE of labelled membranes was performed on 1.5 mm-thick 12% (w/v)-polyacrylamide resolving/4% stacking gels using the Laemmli buffer system of Giulian *et al.* [33]. For electrophoresis of purified glucose-transporter preparations used for sequence analysis, the resolving gel was left at room temperature for 1–2 days and the stacking gel was left at room temperature for at least 3 h. Sample buffer consisted of 3% (w/v) SDS, 1 mM-EDTA, 10% (v/v) glycerol, 0.005% Bromophenol Blue, 2.5% (v/v) β-mercaptoethanol and 62.5 mM-Tris/HCl, pH 6.8 (all final concns.). The sample buffer for solubilization of samples (approx. 10 μg) used for sequence analysis consisted of ultra-pure reagents and recrystallized SDS [34]. 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, gels were stained, destained, dried on a slab dryer, and then exposed to Kodak X-OMat film with a Quanta III intensifier screen (du Pont) at −70 °C for the indicated time.

Electrophoresis of low-molecular-mass peptides

Low-molecular-mass peptides were resolved by using a modified procedure described by Fling & Gregerson [35]. Linear-gradient resolving gels (8–22% acrylamide) containing a high-molarity Tris buffer system were prepared as follows. The acrylamide solutions consisted of 0.75 M-Tris (pH 8.85)/0.1% SDS and the appropriate acrylamide/bisacrylamide [75:2, (w/v) stock solutions] concentrations (i.e. 22.0/0.8% and 8.0/0.2%). Gel solutions were degassed before polymerization with 0.04% TEMED and 0.02% ammonium persulphate. The gradient was cast and overlaid with water-saturated n-butanol. After polymerization (30–60 min), the n-butanol was removed and the top of the resolving gel rinsed with twice-distilled water. The stacking gel consisted of 0.125 M-Tris (pH 6.8), 0.1% SDS, 3%/0.08% acrylamide/bisacrylamide, 0.08% TEMED and 0.04% ammonium persulphate. The running buffer consisted of 50 mM-Tris (pH 8.5)/190 mM-glycine, and 0.1% SDS. Electrophoresis was performed at 30 mA constant current through the stacking gel (approx. 1 h) and 40 mA constant current through the resolving gel (approx. 5 h). After electrophoresis, the gels were fixed for 1 h with 10% (v/v) acetic acid/25% (v/v) propa-2-nol, stained for 3 h with 10% (v/v) acetic acid/25% (v/v) isopropanol/0.1% (w/v) Coomassie Brilliant Blue R/0.1% (w/v) cupric acetate, destained overnight with 5% (v/v) propan-2-ol/5% (v/v) glycerol/10% (v/v) acetic acid, dried on a slab-gel dryer for 2 h at 80 °C and then exposed to Kodak X-Omat film with Quanta III intensifier screen (du Pont) at −80 °C.

Purification of the radiolabelled tryptic fragment

The purified glucose-transporter preparation was photolabelled with [^{125}I]IAPS-forskolin, treated with trypsin and subjected to SDS/PAGE and autoradiography as described above. The portion of the gel which contained the radiolabelled tryptic fragment was excised and electroeluted in 50 mM-NH_4HCO_3/0.1% SDS for 5 h at 50 V, followed by electrodialysis in 50 mM-NH_4HCO_3/0.01% SDS for 18 h at 80 V [34]. The sample was then freeze-dried in a Speed-Vac apparatus and the residue resuspended in 100 μl of water (twice-distilled). In order to extract the SDS, 100% ethanol (900 μl) was added and the mixture left at −20 °C for 3 days. The sample was then subjected to centrifugation (10 min) in a Beckman Microfuge. The supernatant was removed and the pelleted residue was subjected to *N*-terminal sequence analysis.

S.A.V8 digestion of labelled tryptic fragment

The radiolabelled tryptic fragment excised from the SDS/polyacrylamide gel was electroeluted in 50 mM-NH_4HCO_3/0.01 % SDS for 5 h at 50 V. The eluted tryptic fragment was then treated with S.A.V8 (0.1–0.2 mg/ml final concn.) for 18 h at room temperature. The sample was freeze-dried, then solubilized in isoelectric-focusing sample buffer or SDS/PAGE sample buffer.

Isoelectric focusing

Analytical isoelectric focusing using a high-voltage vertical-slab polyacrylamide-gel system was performed using a modified procedure described by Giulian *et al.* [36]. Gels were cast and run vertically in a Hoefer SE-600 vertical slab cell (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). The glass plates (18.0 cm × 8.3 cm) were separated with 0.75 mm spacers. Gel solutions consisted of 5.5 %/0.15 % acrylamide/bisacrylamide 3.1 % (w/v) ampholytes pH range 3.5–10, 1.3 % (w/v) ampholytes pH range 4–6, 0.1 % (v/v) glycerol, 8 M-urea and 0.02 % Triton X-100. Sample buffer was prepared freshly and contained 6.7 M-urea, 2 % (v/v) β-mercaptoethanol, 2 % (v/v) Triton X-100 and 10 % (v/v) glycerol. Samples (20 μl) and standards (20 μl) were solubilized in 40 μl of sample buffer and loaded in the appropriate well. The upper chamber of the electrophoresis apparatus was filled with 0.02 M-NaOH and the lower chamber was filled with 0.02 M-acetic acid. The temperature of the lower chamber was maintained at 8 °C by means of a refrigerated water-bath circulator. Isoelectric focusing was performed at the following power-supply settings: 3000 V, 30 mA and 30 W. Focusing time was approx. 2 h and required 3510 V·h. The gel was fixed for 30 min [20 % (w/v) trichloroacetic acid], and then the ampholytes were removed in a solution containing ethanol/acetic acid/water (33:10:57, by vol.) with 0.25 % (w/v) SDS. The gel was rinsed four times in ethanol/acetic acid/water (33:10:57) to remove the SDS. The gel was then stained for 2 h [ethanol/acetic acid/water (33:10:57) with 0.5 % Coomassie Brilliant Blue R], destained with three changes of solution [ethanol/acetic acid/water (33:10:57)] and preserved in destain solution containing 10 % glycerol for 2 h.

***N*-Terminal sequence analysis**

The amino-acid-sequence analysis was performed on an Applied Biosystems model 477A pulsed-liquid-phase protein/peptide sequencer at the Macromolecular Structure Facility of Michigan State University.

RESULTS

Photoincorporation of forskolin photolabels into the human erythrocyte glucose transporter, followed by trypsin treatment

For trypsin studies, two forskolin photoaffinity labels were used, a carrier-free radioiodinated phenylazide derivative of forskolin, namely [^{125}I]IAPS-forskolin, which has been shown to be a highly selective photoaffinity probe for the glucose-transport proteins found in several tissues, and another forskolin derivative containing the photomoiety at the C-1 hydroxy group of forskolin, namely 1-[^{125}I]IAPS-forskolin. Fig. 1 shows an autoradiogram of the electrophoretic profile after photoincorporation of radiolabel into erythrocyte membranes. When [^{125}I]IAPS-forskolin was used, one major band exhibited significant incorporation of label into a broad region of the gel between 40 and 70 kDa (Fig. 1*a*). Specificity was determined by protection of photolabelling in the presence of 10 μM-cytochalasin B (lanes b and d). Photolabelled membranes were also subjected to trypsin treatment, followed by SDS/PAGE and autoradiography. This

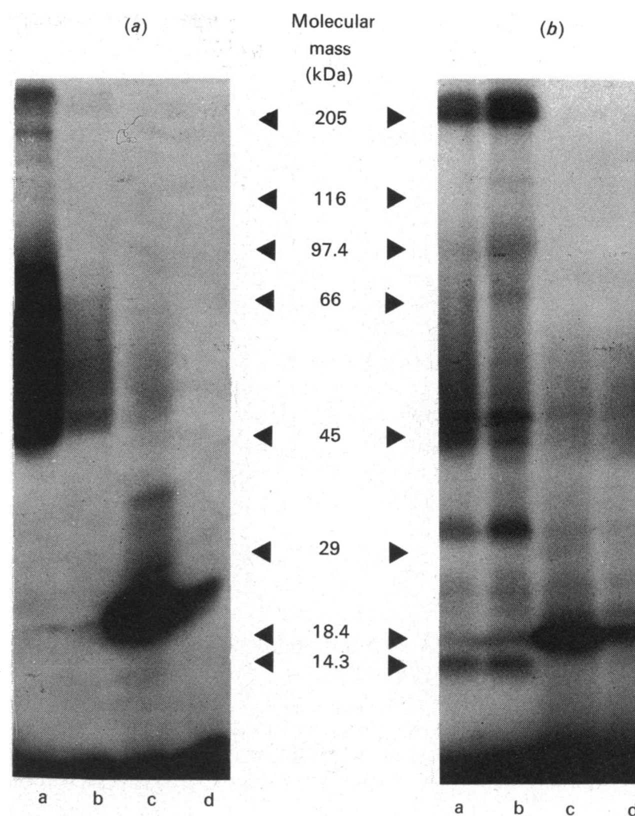

Fig. 1. Photoincorporation of [^{125}I]IAPS-forskolin and 1-[^{125}I]IAPS-forskolin into erythrocyte membranes and trypsin treatment of labelled membranes

An autoradiogram (18 h) of a polyacrylamide gel of erythrocyte membranes photolabelled with [^{125}I]IAPS-forskolin (*a*) and 1-[^{125}I]IAPS-forskolin (*b*) in the presence of buffer (lanes a and c), and in the presence of 10 μM-cytochalasin B (lanes b and d) is shown. Lanes c and d represent labelled membranes incubated in the presence of 0.2 mg of trypsin/ml for 1 h at 37 °C.

treatment shifted the radiolabelled protein from the broad region in the corresponding control to a sharp band at 18 kDa (Fig. 1*a*, lane c). Photoincorporation of 1-[^{125}I]IAPS-forskolin into erythrocyte membranes, followed by SDS/PAGE and autoradiography, resulted in several labelled proteins (Fig. 1*b*). However, when cytochalasin B (10 μM) was included before photolysis, labelling of the broad region between 40 and 70 kDa was decreased, with little change in the labelling of other proteins. Treatment of 1-[^{125}I]IAPS-forskolin-labelled erythrocyte membranes with trypsin, followed by SDS/PAGE and autoradiography, resulted in a sharp radiolabelled band at 18 kDa which exhibited protection with 10 μM-cytochalasin B (Fig. 1*b*, lane c). These results indicate that both forskolin photolabels, with the photomoiety at the opposite ends of the molecule, can be used to derivatize the erythrocyte glucose transporter and that the sites of insertion are within the 18 kDa tryptic fragment.

Peptide mapping of [^{125}I]IAPS-forskolin-labelled erythrocyte membranes

In order to localize further the derivatized forskolin-binding site, erythrocyte membranes photolabelled with the more efficacious forskolin photolabel [^{125}I]IAPS-forskolin were treated with CNBr, Pronase and S.A.V8. The treated membranes were then subjected to SDS/PAGE and autoradiography. Specificity of photolabelling in these experiments was demonstrated by the protection of covalent labelling in the presence of cytochalasin B.

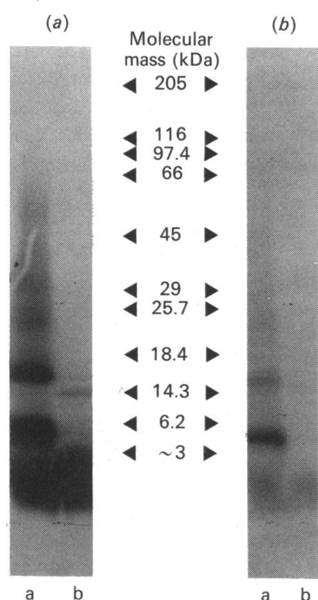

Fig. 2. CNBr treatment of labelled erythrocyte membranes and labelled purified glucose transporter

Autoradiograms (2 h) of polyacrylamide gels of CNBr-treated purified glucose transporter (*a*) and CNBr-treated erythrocyte membranes (*b*) photolabelled with [^{125}I]IAPS-forskolin (2 nM) in the presence of buffer (lanes a) and 100 μM-cytochalasin B (lanes b).

Chemical digestion of labelled membranes (Fig. 2*a*) and labelled purified glucose transporter (Fig. 2*b*) with CNBr resulted in a shift from the broad region of the holo-transporter (40–70 kDa) to a predominant radiolabelled fragment at approx. 5.8 kDa and a minor fragment at approx 16 kDa (Fig. 2).

S.A.V8 treatment of labelled membranes resulted in a radiolabelled fragment migrating at the dye front (results not shown). When the S.A.V8-treated labelled membranes were electrophoresed using a gel system for resolution of lower-molecular-mass peptides [35], the label was still observed at the dye front. This fragment was readily eluted from the gel after staining and destaining. These observations suggest that the S.A.V8 fragment is a low-molecular-mass peptide of less than 2 kDa.

The labelled membranes were also treated with Pronase, a non-specific proteinase that hydrolyses accessible amide bonds. The results (not shown) using this enzyme resembled those obtained using S.A.V8, suggesting that the labelled Pronase fragment is also a low-molecular-mass peptide of less than 2 kDa.

Localization of the labelled tryptic fragment

Purified and reconstituted glucose-transport proteins were photolysed with [^{125}I]IAPS-forskolin, treated with trypsin and subjected to SDS/PAGE and autoradiography (see the Experimental section). The radiolabelled tryptic fragment (18 kDa) was excised from the gel, electroeluted and ethanol-precipitated as described in the Experimental section. The pelleted sample

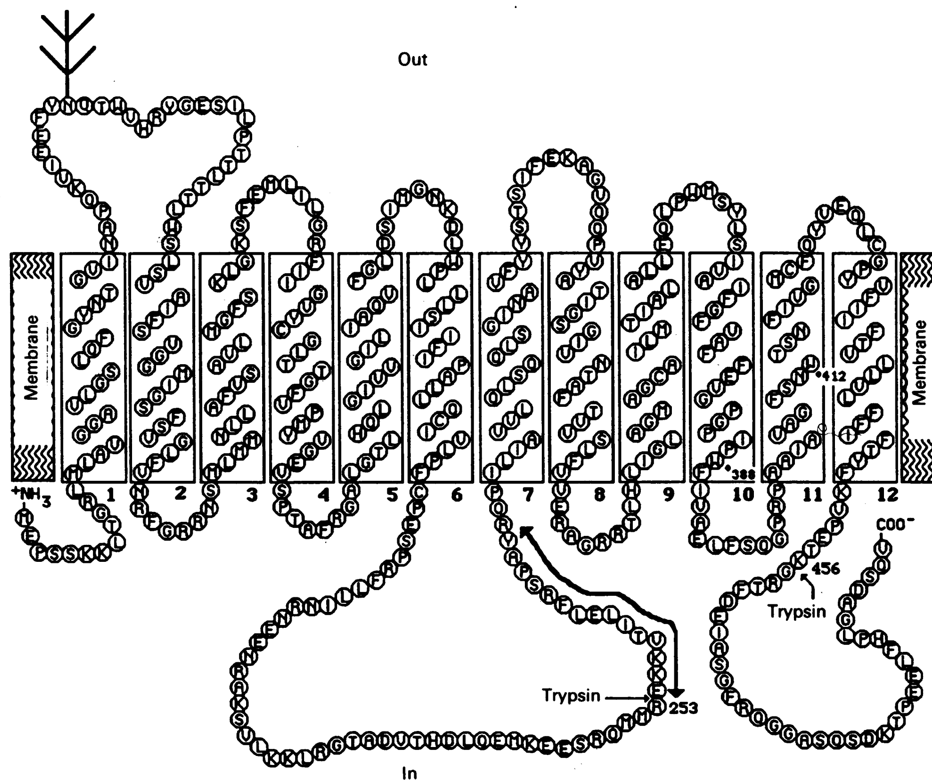

Fig. 3. Location of the [^{125}I]IAPS-forskolin-labelled-tryptic fragment within the proposed model of the glucose-transport protein

The 12 putative membrane-spanning domains are numbered and shown as rectangles. Amino acids are identified by the single-letter code. The location of the sequence *N*-terminus of the radiolabelled tryptic fragment is depicted by a double arrow line (⚹). Tryptophan residues 388 and 412 are shown with an asterisk. The Figure is modified from the model previously described [1].

was then subjected to *N*-terminal sequence analysis. The sequence for the first 15 residues was as follows:

(EKK)VTILELFRSPAY.

corresponding to residues Glu^{254}–Tyr^{268} (inclusively) of the transporter. The first three amino acid residues, Glu-Lys-Lys (EKK) could not be analysed, owing to high background and possibly a result of some minor cleavage at one of the other basic residues in this region. This sequence was localized in the large cytoplasmic loop within the known sequence for the HepG2 human hepatoma glucose transporter (Fig. 3) deduced by Mueckler and colleagues [2].

Isoelectric focusing of the labelled S.A.V8-cleaved fragment

Purified glucose-transporter preparations were photolabelled, trypsin-treated and subjected to SDS/PAGE and autoradiography. The resulting tryptic fragment was electroeluted from the gel, treated with S.A.V8 and subjected to SDS/PAGE and isoelectric focusing as described in the Experimental section. An autoradiogram of the 'wet' SDS/polyacrylamide gel shows the radiolabelled tryptic fragment migrating at 18 kDa (Fig. 4*a*, lane a). S.A.V8 treatment of the tryptic fragment produces a radiolabelled band migrating with the dye front (Fig. 4*a*, lane b). An autoradiogram of the 'wet' isoelectric-focusing gel showed a labelled S.A.V8-cleaved peptide which focused at approx. pH 4.5 (Fig. 4*b*, lane b). Under the conditions used for proteolytic digestion, S.A.V8 hydrolyses preferentially at glutamic acid residues. The known amino acid sequence for the HepG2 transporter contains eight glutamate residues within the 18 kDa tryptic fragment. Assuming complete hydrolysis of the tryptic fragment with S.A.V8, nine peptides with various molecular masses and net charges should be generated. Most of these peptides have expected pI values that are neutral or higher. Fragments 360–380 and 381–393 contain a glutamine and valine *N*-terminus respectively and both contain a glutamate residue at the *C*-terminus which would contribute to the net charge of the peptides. Fragment 394–426 contains a leucine *N*-terminus, an arginine residue and a cysteine residue within the peptide, and a glutamate residue at the *C*-terminus. These three peptides are likely candidates for the radiolabelled peptide which focused at pI 4.5, since they possess a slightly acidic net charge. It seems more likely that site of insertion of [^{125}I]IAPS-forskolin is within fragment 360–380 or fragment 381–393, not fragment 394–426. This conclusion is based on the observation that the radiolabelled peptide migrates at the dye front on SDS/PAGE. The two smaller fragments (360–380 or 381–393) would be expected to migrate with the dye front, whereas the larger fragment (394–426) should migrate above the dye front (i.e. theoretical molecular mass of approx. 3.6 kDa). Attempts to define the amino acid residue derivatized by IAPS-forskolin by sequence analysis of the S.A.V8 cleaved peptide proved unsuccessful, suggesting a blocked *N*-terminus or the presence of contaminants interfering with sequencing.

Photoaffinity labelling of the L-arabinose transporter

The L-arabinose-inducible transport protein from *E. coli* has been cloned and the corresponding amino acid sequence determined. This protein is similar in structure (40%) to the HepG2 glucose-transport protein. Because of this similarity to the mammalian glucose transporter, it seemed reasonable to ascertain whether a forskolin-binding site existed on the *E. coli* arabinose transporter. If this was indeed the case, then one might expect to find a sequence in the arabinose transporter similar to the putative binding site within the erythrocyte glucose transporter. Intact *E. coli* cells grown in the presence (induction

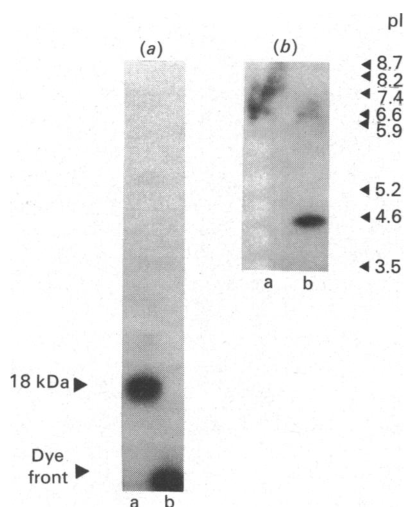

Fig. 4. SDS/PAGE and isoelectric focusing of the [^{125}I]IAPS-forskolin-labelled tryptic and S.A.V8 fragments

(*a*) Autoradiogram (18 h) of a polyacrylamide gel of purified [^{125}I]IAPS-forskolin-labelled tryptic fragment (lane a) and purified labelled tryptic fragment treated with S.A.V8 (lane b). (*b*) Autoradiogram (18 h) of an isoelectric-focusing gel of purified [^{125}I]IAPS-forskolin-labelled tryptic fragment (lane a) and purified labelled tryptic fragment treated with S.A.V8 (lane b).

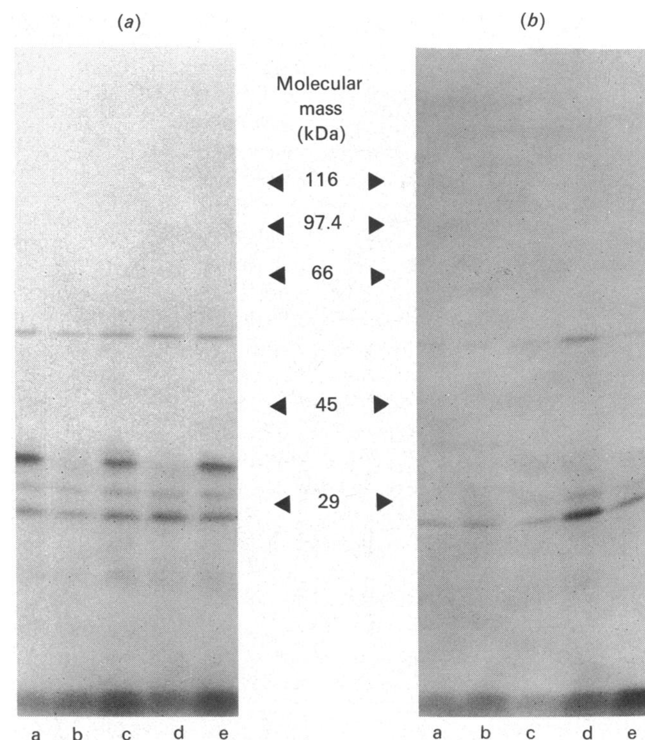

Fig. 5. Photoincorporation of [^{125}I]IAPS-forskolin into the *E. coli* L-arabinose transporter

E. coli grown in the presence (*a*) or absence (*b*) of L-arabinose were photolabelled with [^{125}I]IAPS-forskolin (3 nM) in the presence of buffer (lanes a), or buffer and 10 μM-cytochalasin B (lanes b), 500 mM-L-arabinose (lanes c), 500 mM-D-glucose (lanes d), and 500 mM-D-arabinose (lanes e). The autoradiogram (3 day) of the polyacrylamide gel is shown.

condition) or absence of L-arabinose were photolysed with [^{125}I]IAPS-forskolin and subjected to SDS/PAGE and autoradiography as described in the Experimental section. The autoradiogram (Fig. 5*a*) shows a specifically labelled protein migrating with an apparent molecular mass of approx. 36 kDa in the control (lane a), in the presence of 500 mM-L-arabinose (lane c) and in the presence of 500 mM-D-arabinose (lane e). Protection of photolabelling was seen in cells photolysed with 500 mM-D-glucose (lane d) and 10 μM-cytochalasin B (lane b). No specifically labelled proteins were observed when photolabelling was performed in *E. coli* grown in the absence of L-arabinose (Fig. 5*b*). The apparent molecular mass of the [^{125}I]IAPS-forskolin-labelled protein corresponds to the reported size of the L-arabinose-transport protein [25–29]. These observations provide evidence that the [^{125}I]IAPS-forskolin-labelled protein is the L-arabinose transporter.

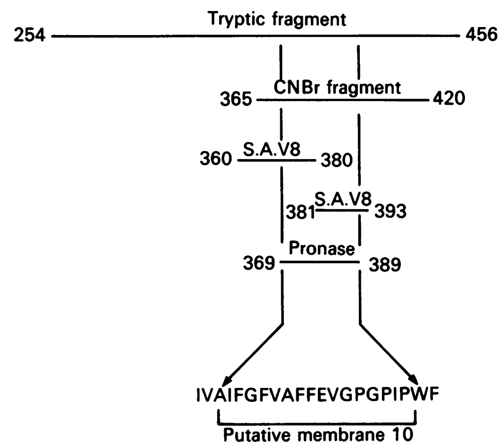

Fig. 6. Putative locations of the radiolabelled tryptic, CNBr, S.A.V8 and Pronase fragments

The proposed locations of the radiolabelled fragments within the glucose-transport protein are shown. The putative location of the binding site for IAPS-forskolin is within the tenth transmembrane domain.

DISCUSSION

In the present study we have localized the position of covalent attachment of IAPS-forskolin into the human erythrocyte glucose transporter. The region where this photoaffinity label inserts into the transporter is most probably within the tenth putative transmembrane helix. This conclusion is based upon analysis of chemical and proteolytic digestion of the labelled erythrocyte transport protein.

The *N*-terminal half of the IAPS-forskolin-labelled glucose-transporter tryptic fragment was found to be at Glu-254 on the basis of *N*-terminal-sequence analysis. The *C*-terminal end of this tryptic fragment is thought to be at Tyr-456 [12], i.e. the site of covalent attachment for IAPS-forskolin is likely between amino acid residues Glu^{254} and Tyr^{456}. In order to determine whether the insertion site is within the membrane or outside the lipid bilayer, specifically labelled membranes were treated with Pronase, a non-specific proteinase. A low-molecular-mass peptide ($\approx$ 2 kDa) remained with the membrane fraction, with no radiolabel being released into the supernatant. Thus it is likely that the site of derivatization is within the membrane bilayer (i.e. the region protected from Pronase digestion).

Chemical digestion of the IAPS-forskolin-labelled transport protein with CNBr (hydrolysis at methionine residues) resulted in a major radiolabelled fragment migrating with a molecular mass of 5.8 kDa. The most likely CNBr fragment of this size within the IAPS-forskolin-labelled tryptic fragment of the transporter comprises residues 365–420. Another possibility is a 7.1 kDa fragment (residues 421–492), although the observed migration was clearly below the 6.2 kDa standard peptide marker. In addition, isoelectric-focusing data for S.A.V8 cleavage products (see below) strongly suggest that the only S.A.V8 fragment which overlaps with the 7.1 kDa fragment (i.e. residues 394–426) should not focus in the region which was observed experimentally.

Additional analysis by isoelectric focusing of the purified tryptic fragment treated with S.A.V8 showed a radiolabelled peptide focusing with an apparent pI of 4.5. The two S.A.V8 peptides of interest which should focus in this region are amino acid residues 360–380 and 381–393.

The putative chemical and enzymic fragments containing the insertion site for IAPS-forskolin are shown in Fig. 6. The common region for these radiolabelled peptides is the transmembrane domain comprising amino acid residues 369–389 (i.e. the tenth putative transmembrane domain). The proposed location of the IAPS-forskolin insertion site within this hydrophobic domain is consistent with the observation that this forskolin derivative is very lipophilic [23].

Structural comparisons of glucose and inhibitors of glucose transport (cytochalasin B [38,39] and forskolin [40]) have led some investigators to suggest that both glucose and glucose-transport inhibitors share a common binding site within the glucose-transport protein. On the basis of these observations and the results presented here, it is proposed that the forskolin portion of IAPS-forskolin may be oriented in a binding pocket [37] in a manner which allows the photoactive side chain to come in close proximity to the tenth putative transmembrane domain.

The amino acid sequence for the arabinose/H^+ and xylose/H^+ membrane-transport proteins of *E. coli* have recently been determined [29]. These proteins are similar to each other and also to the glucose transporters of human hepatoma [2] and rat brain [3]. Comparisons of sequences and hydropathic profiles of bacterial and mammalian transport proteins have provided new information which may point to significant evolutionary relationships between functionally important residues [29].

In the present paper we have shown that the *E. coli* arabinose-inducible arabinose/H^+ transporter can be photolabelled with [^{125}I]IAPS-forskolin. The photoincorporation of [^{125}I]IAPS-forskolin into this protein was protected by cytochalasin B and D-glucose but, interestingly, not by L-arabinose. The lack of protection of photolabelling by L-arabinose may be a result of a poorly generated proton gradient which is necessary to drive transport. Alternatively, one could speculate that this protein contains a binding site for glucose which is different from the L-arabinose-binding site. Further investigation is needed to address this question.

As mentioned above, the *E. coli* L-arabinose transporter and the human HepG2 glucose transporter are similar. Both of these proteins are thought to contain a binding site for cytochalasin B. The erythrocyte glucose transporter, which is most likely identical with the HepG2 transporter, has been photolabelled with [^{3}H]cytochalasin B [4–7,9,10,15–17]. Several workers [17,41] have proposed that photolabelling proceeds via photoactivation of an aromatic amino acid residue on the transporter rather than by photoactivation of the cytochalasin B molecule itself. Furthermore, Deziel and colleagues [41] have suggested that Trp-412 photolabels cytochalasin B, since maximum labelling occurs at the absorbance maximum of tryptophan (280 nm) and not that of cytochalasin B (214 mm). Cairns *et al.* [12] and Baldwin & Henderson [41a] have stated that the *E. coli* L-arabinose transporter can also be photolabelled with [^{3}H]cytochalasin B. It has

been proposed that a likely candidate for derivatization with cytochalasin B is Trp[412] within the glucose transporter (transmembrane span 11), since this is a conserved residue within the regions containing cytochalasin B for both transport proteins. Holman & Rees [10a] have proposed that the photoincorporation of a bis(D-mannose) derivative occurs near an exofacial site on the outside of transmembrane span 9 and the cytochalasin B labelling site on the inside of the hydrophobic span 10.

The region of the erythrocyte glucose-transport protein thought to be derivatized with IAPS-forskolin contains a tryptophan residue (Trp[388]) which is conserved in the sequence of the *E. coli* arabinose-transport protein. Other similar sugar-transport proteins also contain a conserved tryptophan residue near the *C*-terminus of the protein [29,42]. Amino-acid-sequence analysis of other proteins photolabelled with iodoazidoaryl azides [43], as well as photochemical studies using these compounds [44], have shown that tryptophan residues may be preferentially derivatized. On the basis of the results presented here it is tempting to speculate that Trp[388] is the site of covalent insertion of IAPS-forskolin into the erythrocyte glucose transporter. However, definitive identification will require amino acid sequencing of the IAPS-forskolin-labelled peptide.

Note added in proof (received 10 October 1990)

It has been brought to our attention that the possibility may exist that the sugar-protectable polypeptide photolabelled by [^{125}I]IAPS in *E. coli* could possibly be the GalP sugar-transport protein. Further investigation will be necessary to resolve this issue.

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