RESEARCH COMMUNICATION

Domain assembly of the GLUT1 glucose transporter

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A full-length construct of the glucose transporter isoform GLUT1 has been expressed in Sf9 (*Spodoptera frugiperida* Clone 9) insect cells, and a photolabelling approach has been used to show that the expressed protein binds the bismannose compound 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA) and cytochalasin B at its exofacial and endofacial binding sites respectively. Constructs of GLUT1 which produce either the N-terminal (amino acids 1–272) or C-terminal (amino acids 254–492) halves are expressed at levels in the plasma membrane which are similar to that of the full-length GLUT1 (≈ 200 pmol/mg of membrane protein), but do not

bind either ATB-BMPA or cytochalasin B. When Sf9 cells are doubly infected with virus constructs producing both the C- and N-terminal halves of GLUT1, then the ligand labelling is restored. Only the C-terminal half is labelled, and, therefore, the labelling of this domain is dependent on the presence of the N-terminal half of the protein. These results suggest that the two halves of GLUT1 can assemble to form a stable complex and support the concept of a bilobular structure for the intact glucose transporters in which separate C- and N-domain halves pack together to produce a ligand-binding conformation.

INTRODUCTION

The facilitative uptake of glucose in mammalian cells is mediated by a family of at least six glucose-transporter isoforms [1,2]. The glucose transporters are highly conserved proteins, and the mammalian transporters possess high levels of sequence identity with transporters found in all organisms from *Escherichia coli* to plants and animals [3]. As well as a likely common mechanism of transport catalysis [4–6], all transporters in this family are predicted to possess the same transmembrane topology with a duplicated domain structure composed of cytoplasmic N- and C-termini and a centrally located cytoplasmic loop which divides the protein into two halves with six helical or transmembrane segments (TMs) in each half [1,2]. Studies in which GLUT1 has been photolabelled by bismannose derivatives [7,8], by cytochalasin B [7,9] and by forskolin derivatives [10,11] all suggest that the six TMs of the C-terminal domain contain both the outside- and inside-directed hexose-binding sites. Using the baculovirus/*Spodoptera frugiperida* Clone 9 (Sf9) system, we show here that the C-terminal half expressed alone is insufficient to bind photolabels. We have also examined whether the C- and N-terminal domains, when expressed from separate virus constructs infecting the same Sf9 cells, can assemble to reform a transporter than can bind ligands.

EXPERIMENTAL

Recombinant viruses

Bases 123–2487 of the rabbit GLUT1 cDNA clone [12] were subcloned into a baculovirus transfer vector pVL1392 at *Eco*R1 sites. This fragment includes 123 non-coding bases at the 5' end, the entire coding sequence of the rabbit GLUT1 and 888 non-coding bases at the 3' end. This plasmid was transfected into Sf9

cells along with linearized AcMNPV-lacZ DNA [13]. Recombinant viruses were identified by plaque assay in the presence of 5-bromo-4-chloroindol-3-yl β -*D*-galactoside (X-gal) and purified to homogeneity by three rounds of plaque purification. High-titre stocks were made of each recombinant virus, and these were used to infect Sf9 cells which were then tested for the production of GLUT1 after 48 h by Western-blot analysis. The recombinant virus producing the highest level of GLUT1 was amplified and designated 'E4.2'. The truncated GLUT1 genes were constructed by amplifying the N-terminal half (nucleotides 98–940) and the C-terminal half (nucleotides 883–1602) using oligonucleotide primers which incorporated *Eco*RI and *Bgl*II (N-terminal half), and *Bam*HI and *Xba*I (C-terminal half) restriction sites for subcloning into pVL1393 (N-terminal half) or pVL1392 (C-terminal half), plus an additional stop codon at amino acid residue 272 for the N-terminal construct and a methionine codon at amino acid residue 254 for the C-terminal construct. The amplified DNA fragments were subcloned into the transfer vector. Recombinant viruses were produced and amplified as described for the full-length GLUT1.

Expression of GLUT1 constructs in Sf9 cells

Sf9 cells were infected with E4.2, N1 and R12 viruses at 1.6 plaque-forming units/cell and left in culture at 28 °C for 48 h. Membrane samples were prepared by suspending the material from two 35 mm dishes in 1 ml of TES buffer (10 mM Tris/HCl/5 mM EDTA/250 mM sucrose, pH 7.2) and homogenizing with 20 strokes in a Teflon homogenizer. The homogenized material was transferred to 3 ml centrifuge tubes by dilution and washing with a further 2 ml of TES. The membrane material was then either sedimented at 16000 $g_{\max}$ or 100000 $g_{\max}$ to obtain plasma membrane and total membrane pellets respectively. The Sf9 cells or membrane pellets were dissolved in electrophoresis sample

Abbreviations used: GLUT1, glucose transporter isoform 1; Sf9, *Spodoptera frugiperida* Clone 9; TMs, transmembrane segments; ATB-BMPA, 2-*N*-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(*D*-mannos-4-yloxy)-2-propylamine; X-gal, 5-bromo-4-chloroindol-3-yl β -*D*-galactoside; C₁₂E₉, nonaecto(ethylene glycol) dodecyl ether.

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buffer [10 % (w/v) SDS/6 M urea/10 % (v/v) mercaptoethanol] and the proteins separated by electrophoresis on 12 %-polyacrylamide gels using a discontinuous buffer system. Proteins were transferred to nitrocellulose and immunoreactivity was determined using enhanced chemiluminescence detection. The GLUT1 constructs were detected either by using an anti-(central-loop peptide) antiserum (to residues 240–255) or an anti-(C-terminal peptide) antiserum (to residues 480–492).

Photolabelling of GLUT1 constructs

The infected Sf9 cells in 35 mm dishes were washed in PBS and incubated with 100 μ Ci (30 μ M) of 2-*N*-4-(1-azido-2,2,2-trifluoroethyl)benzoyl[2-³H]bis-(α -mannos-4-yl)-2-propylamine (ATB-[2-³H]BMPA) or 16 μ Ci (2.8 μ M) of [4-³H]cytochalasin B in the presence of 10 μ M cytochalasin E in 300 μ l of PBS at 18 °C. The cells were then irradiated for 45 s in a Rayonet photochemical reactor. Following irradiation, the cells were solubilized in 1 ml of detergent buffer containing 2 % nonaeta-(ethylene glycol) dodecyl ether (C₁₂E₉) in 5 mM sodium phosphate, pH 7.4, with the proteinase inhibitors antipain, aprotinin, pepstatin A and leupeptin, each at 1 μ g/ml. Following the removal of unsolubilized material by centrifugation at 20000 g_{max} , samples were mixed with 20 μ l of Protein A-Sepharose coupled to 100 μ l of anti-(C-terminal peptide) antisera (for E4.2 and R12) and the anti-(central-loop peptide) antiserum (for N1). The immunoprecipitates were washed six times in 0.2 % C₁₂E₉ detergent buffer and then directly solubilized in electrophoresis sample buffer containing 10 % SDS, 6 M urea and 10 % mercaptoethanol. The supernatant was subjected to electrophoresis on 10 or 12 % polyacrylamide gels. Gel slices, in scintillation vials, were dried at 80 °C for 2 h and were then dissolved in 0.5 ml of alkaline H₂O₂ [2 % (v/v) NH₄OH in 30 % H₂O₂] at 80 °C for an additional 2 h. Scintillant was added and the radioactivity counted. The positions of the photolabelled peaks were compared with the positions of molecular-mass markers in adjacent lanes. The levels of radioactivity in the gel slices were normalized to account for the different amounts of proteins recovered.

RESULTS AND DISCUSSION

A construct was generated that placed full-length GLUT1, from bp 1 to bp 2487 and including the coding region 124–1352, under the control of the polyhedrin promoter. Baculovirus containing this construct (E4.2) was used to infect Sf9 cells, and the increase in the expression of immunodetectable GLUT1 was monitored over 7 days. Using the human erythrocyte GLUT1 as a standard to quantify the amounts of expressed protein, it was found that the level of GLUT1 produced reached a maximum of $\approx$ 150 pmol/mg of membrane protein after 48 h. All of the expressed protein could be sedimented by centrifugation at 100000 g_{max} , while $\approx$ 80 % of this protein was sedimented at 16000 g_{max} . These results suggest that a large proportion of the expressed GLUT1 protein is targeted to the plasma membrane in this system [14]. Truncated GLUT1 constructs were prepared in which the N-domain (from b 98 to b 940 and corresponding to amino acids 1–272; N1) and the C-domain (from b 883 to b 1602 and corresponding to amino acids 254–492; R12) were also expressed in the Sf9 cells and, surprisingly, were found to be targeted with very high efficiency to the plasma membrane. The C-domain and N-domain constructs were expressed in 100000 g_{max} -sedimenting membranes to a level of $\approx$ 200 pmol/mg of protein. Figure 1 shows a Western-blot analysis of these expressed constructs. The C-domain construct was detectable

with an anti-(C-terminal-peptide) antibody, but not an anti-(central-loop) antibody, while the N-terminal construct was detectable only with an anti-(central-loop) antibody and not the anti-(C-terminal peptide) antibody. Both antibodies recognized the intact GLUT1. Proteins expressed from both the N-terminal and C-terminal constructs showed dimer aggregates on gels. This was particularly evident for the C-domain construct (Figure 1).

Cultures of Sf9 cells at 48 h post infection were examined to determine whether the inner and outer ligand-binding sites of GLUT1 were present in the expressed protein. The compound ATB-BMPA, which is specific for the exofacial site of GLUT1, bound efficiently to the full-length GLUT1 protein. SDS/PAGE analysis of the labelled protein showed a peak at 50 kDa (Figure 2a). This apparent molecular mass is somewhat smaller than that of human erythrocyte GLUT1 run on the same gel, presumably reflecting the greater glycosylation of the latter protein [15]. Similarly, the Sf9-expressed full-length GLUT1 was labelled by irradiation in the presence of cytochalasin B to produce a labelled SDS/PAGE band at 50 kDa (Figure 2b). Neither the separately expressed C-terminal domain nor the N-terminal domain were able to bind these ligands, suggesting that in these protein constructs the necessary ligand-binding sites are either not present or are incorrectly assembled.

The baculovirus expression system is highly suited to studies on the assembly of domain structures of proteins, as separate domains can be produced in the same cells by simultaneously infecting with two virus constructs coding for each of the separate domains of the protein. A similar approach has been used to

Figure 1 Expression of GLUT1 recombinant protein in Sf9 cells

Sf9 cells were infected with viruses coding for GLUT1 constructs and maintained in culture for 48 h at 28 °C. Approx. 2 μ g of cell protein was then directly solubilized in electrophoresis sample buffer. The left-hand panel shows a Western blot of GLUT1 constructs as detected using an anti-(central-loop-peptide) antiserum to GLUT1 amino acids 240–255. This recognizes the protein produced from infection with the E4.2 virus (coding for full-length GLUT1), (lane 2), from the N1 virus (coding for the GLUT1 N-terminal half) (lane 3), but not from the R12 virus (coding for the C-terminal half of GLUT1) (lane 4) or from the control virus expressing only the *lacZ* gene product (lane 1) ('LZ control'). The right-hand panel shows a Western blot of GLUT1 constructs as detected using an anti-(C-terminal-peptide) antibody to GLUT1 amino acids 480–492. This recognizes the protein produced from infection with the E4.2 virus (not shown) and from the R12 virus (lane 1), but not from the N1 virus (lane 2). The blotting of GLUT1 in human erythrocyte membranes is shown (lane 3) ('RBC control'). Abbreviation: *M*, molecular mass.

Figure 2 Ligand labelling of GLUT1 baculovirus constructs expressed in Sf9 cells

In (a) the Sf9 cells in 35 mm dishes were infected with E4.2 (●), N1 (○) and R12 (▲) viruses each at 1.6 plaque-forming units/cell and maintained in culture for 48 h at 28 °C. They were then labelled with (a) ATB-BMPA (100 μ Ci) or (b) cytochalasin B (16 μ Ci) in 300 μ l of PBS. The labelled material was then subjected to immunoprecipitation using either anti-(C-terminal peptide) antibody (E4.2 and R12) or anti-(central-loop peptide) antibody (N1) and resolved by electrophoresis. Experiments with the E4.2, N1 and R12 viruses were carried out separately, and the indicated positions of the molecular-mass (M) markers represent an average position from the three gels. The results shown are from single experiments representative of three similar experiments for each condition.

Figure 3 Ligand labelling of GLUT1 assembled from N- and C-terminal halves in doubly infected Sf9 cells

Sf9 cells were doubly infected with 1.6 plaque-forming units of both the N1 and R12 viruses/cell and maintained in culture for 48 h at 28 °C. Cells were then labelled with (a) ATB-BMPA (100 μ Ci) or (b) cytochalasin B (16 μ Ci) in 300 μ l of PBS either in the absence (●) or presence (▲) of 400 mM D-glucose. The labelled material was then subjected to immunoprecipitation using anti-(GLUT1-C-terminal peptide) antibody. Results are from a single experiment representative of three. No labelled peaks were obtained when immunoprecipitation was carried out using the antibody that recognizes the GLUT1 N-terminal half (results not shown). Abbreviation: M , molecular mass.

examine the domain packing in the *Escherichia coli* lac permease, but the domains were expressed from a single plasmid [16]. We have applied the double-infection approach to determine whether the non-functional C- and N-domains of GLUT1 can assemble to re-form a functional ligand-binding site. *Spodoptera* cells were co-infected with viruses N1 and R12, and Western-blotting confirmed that, in the population of cells, the two halves were produced at the same level as occurred following infection with either virus alone (≈ 200 pmol/mg of membrane protein). The cells were maintained in culture for 48 h, and then the ability of

the expressed proteins to bind ATB-BMPA (Figure 3a) and cytochalasin B (Figure 3b) was examined. The Figure shows that ATB-BMPA labelling in the doubly infected cells is clearly detectable as a 25 kDa peak. The labelling is $\approx 30\%$ of the labelling of the intact GLUT1. The position of the peak corresponds to the position of the C-terminal domain protein in Western blots and not to the position of the N-terminal domain protein. Furthermore, the 25 kDa peak was immunoprecipitated only by the anti-(C-terminal) antibody and not the anti-(central-loop) antibody (results not shown). The double-infected cells

were also efficiently labelled by cytochalasin B to a level which was $\approx 30\%$ of that of the intact GLUT1.

The relatively short linker regions of the C- and N-terminal domains of GLUT1 constrain the possible packing patterns for the TMs. We have suggested that six TM helices/segments in each half are packed so that TM 1 is next to TM 6 in the N-terminal half, while TM 7 is packed against TM 12 in the C-terminal half [17,18]. The results presented here provide support for the concept that glucose transporters are stable as bilocular structures composed of closely packing N- and C-terminal domains. Both domains are sufficiently stable to be targeted to the plasma membrane and to recognize the corresponding other protein half in the membrane. While the C-terminal half alone is insufficient to maintain ligand-binding capability, assembly with the N-terminal half facilitates this function. The ability of the N-terminal half of GLUT1 to restore the ligand labelling of the C-terminal half may be attributed to the provision of a packing surface which allows the exposure of the ligand-binding sites in the C-terminal half. Alternatively, amino acid side chains in the N-terminal half may contribute to a ligand-binding site composed of both halves. The latter possibility seems less likely, as the N-terminal half is not labelled by active-site ligands [7–11]. Further resolution of the domain packing in GLUT1 may now be possible using the double-infection approach described here.

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