RESEARCH COMMUNICATION

Characterization of functional human erythrocyte-type glucose transporter (GLUT1) expressed in insect cells using a recombinant baculovirus

Chong-Kee YI,*† Bambos M. CHARALAMBOUS,† Vincent C. EMERY‡ and Stephen A. BALDWIN*†§

*Department of Biochemistry, †Department of Protein and Molecular Biology and ‡Division of Communicable Diseases, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF, U.K.

The human erythrocyte-type glucose transporter (GLUT1) has been abundantly expressed in insect cells by using a recombinant baculovirus. At 4 days after infection with the virus, the insect cell-surface and intracellular membranes were found to contain > 200 pmol of D-glucose-sensitive binding sites for the transport inhibitor cytochalasin B per mg of protein. The characteristics of binding were identical with those of the erythrocyte transporter, although the two proteins differed substantially in apparent M_r , probably as a result of glycosylation differences.

INTRODUCTION

The uptake of glucose into most mammalian cells is mediated by passive transport proteins in the plasma membrane. Recent cDNA cloning studies have revealed that these proteins are members of a large family of homologous sugar transporters present in both prokaryotes and eukaryotes [1]. The best known of these proteins is the human erythrocyte glucose transporter (GLUT1), which has been purified to near homogeneity and extensively characterized [2–4]. Functional expression of small amounts of the transporter has been achieved in *Xenopus* oocytes [5] and in mammalian cells [6]. However, for detailed studies of structure–function relationships in this protein, and in particular for the investigation of its structure by crystallization, higher levels of expression would be desirable. Recently, the baculovirus expression system has been utilized to produce biologically active recombinant proteins from a variety of eukaryotic genes, including several that cannot be efficiently expressed in lower eukaryotic or prokaryotic systems [7]. In the present study we describe the successful use of this system to produce substantial amounts of functionally active erythrocyte glucose transporter suitable for structural and functional studies.

EXPERIMENTAL

Materials

Wild-type *Autographa californica* (alfalfa looper) nuclear polyhedrosis virus (AcNPV), the transfer vector pAcYM1 [8] and *Spodoptera frugiperda* Clone 9 (Sf9) cells were kindly given by Professor D. H. L. Bishop (NERC Institute of Virology and Environmental Microbiology, Oxford, U.K.). The plasmid pSGT, which contains the entire coding region of the human GLUT1 transporter [9], was gratefully received from Dr. M. Mueckler (Washington University, St. Louis, MO, U.S.A.). New England Nuclear (U.K.) supplied [4-³H]cytochalasin B, and endoglycosidase F was from Boehringer.

Construction of recombinant baculovirus

Standard recombinant DNA techniques were used throughout. The plasmid pSGT was restriction-digested with *Bam*H1 and *Hind*III, and a 1922 bp fragment containing the entire coding region of the GLUT1 cDNA was isolated. Protruding ends were filled in and then the fragment was ligated into the blunt-ended unique *Bam*H1 site of pAcYM1 to produce a recombinant (pAcYM1-GT). Recombinant baculoviruses carrying the GLUT1 cDNA were then produced by co-transfection of Sf9 cells with pAcYM1-GT and wild-type AcNPV viral DNA using the procedure of Summers & Smith [10]. Recombinants were identified and partially purified as described in [11]. Selected recombinants were then plaque-purified to homogeneity [10]. These procedures yielded three clones of recombinant virus, one of which, AcNPV-GT, was used for all the experiments described.

Culture and infection of insect cells, and preparation of membrane fractions

Sf9 cells were cultured and infected as described in [10]. For production of membranes, cells were harvested 4–5 days after infection [multiplicity of infection (MOI) 5–10] and washed three times at 20 °C with 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2. They were then resuspended in 10 mM-Tris/5 mM-MgCl₂, pH 7.4, containing proteinase inhibitors [2 mM-iodoacetamide, 0.2 mM-phenylmethanesulphonyl fluoride and pepstatin A (10 µg/ml)] and sonicated on ice for 1 min. Membranes were separated from soluble components by centrifugation for 1 h at 117000 g_{av} . For examination of the time course of GLUT1 expression, washed cells were not separated into membrane and soluble fractions before analysis by Western blotting. Alkali-stripped human erythrocyte membranes were prepared by the method of Gorga & Lienhard [12].

Western blotting

Antibodies against the human erythrocyte glucose transporter

Abbreviations used: GLUT1, human erythrocyte-type glucose transporter; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* Clone 9; MOI, multiplicity of infection.

§ To whom all correspondence should be addressed.

Fig. 1. Confocal microscopy of non-infected and baculovirus-infected insect cells

At 2 days after infection, cells were collected, fixed and incubated first with affinity-purified anti-(glucose transporter) antibody (50 $\mu\text{g}/\text{ml}$) and then with a fluorescein conjugate of goat anti-rabbit IgG, before examination using a confocal laser scanning microscope. The panels show immunofluorescent images of cells infected with recombinant AcNPV-GT (panel a), wild-type AcNPV (panel b), no virus (panel c) or cells infected with recombinant AcNPV-GT, but omitting the primary antibody (panel d). The corresponding phase-contrast images are shown in panels A–D. The scale bar represents 50 μm .

were raised in rabbits against a synthetic peptide corresponding to the C-terminal sequence (residues 477–492) as detailed in [13]. Western blotting was performed as previously described, by using either an alkaline phosphatase conjugate of goat anti-rabbit IgG [13] or ^{125}I -F(ab')₂ donkey anti-rabbit IgG [14] as the second antibody. The concentration of transporter within membrane samples was determined by γ -radiation counting of the ^{125}I -labelled bands and comparison with human erythrocyte membrane standards of known cytochalasin B-binding-site content run on the same gel, as previously described [14].

Other methods

Routine assays for cytochalasin B-binding activity of membrane samples were performed by equilibrium dialysis using 40 nM-[^3H]cytochalasin B [3]. Correction for non-specific binding to membrane lipids was made by performing the binding experiments in the presence or absence of 400 mM-D-glucose. More accurate estimation of the concentration and affinity of cytochalasin B-binding sites was obtained by measuring binding over a range of cytochalasin concentrations by the method previously described [3]. Glycosylation analysis was carried out as previously described [14]. The procedure used for indirect immunofluorescence staining was also as described [15], except that samples were examined using a Bio-Rad MRC600 confocal laser scanning microscope. The same anti-transporter antibodies were used as for Western blotting.

RESULTS AND DISCUSSION

The expression of human glucose transporter in insect cells

was examined by confocal immunofluorescence microscopy using anti-transporter antibodies and a fluorescein-labelled secondary antibody. Fig. 1 shows that, 2 days after infection with recombinant baculovirus, the cells exhibited bright fluorescence, indicative of an abundance of transporter. In contrast, non-infected cells and those infected with wild-type virus showed no fluorescent staining. Serial confocal 0.6 μm optical sections through the fluorescently labelled infected cells showed that the expressed transporter was present not only at the cell surface but also in membranous structures throughout the cytoplasm, except for the region occupied by the nucleus (results not shown). Previous studies have shown that heterologous expression of other membrane proteins using the baculovirus/insect-cell system induces a proliferation of cytoplasmic membranes [8].

Western-blot analysis using the same anti-transporter antibodies confirmed the presence of immunologically cross-reactive transporter in cells infected with recombinant baculovirus, but not in non-infected cells or those infected with wild-type virus (Fig. 2). Expressed transporter was confined to the membrane fraction of the infected cells and was absent from the soluble fraction (Fig. 2). The native erythrocyte protein is heterogeneously glycosylated and migrates on SDS/polyacrylamide gels as a broad band of apparent M_r 45000–65000, which is converted into a sharp band of M_r 46000 upon enzymic deglycosylation [3,16] (Fig. 3). By contrast, the transporter expressed in insect cells migrated as a single fairly sharp band of M_r 45000, almost identical with that of its deglycosylated erythrocyte counterpart. The presence of less-extensive and more homogeneous glycosylation was confirmed by the fact that treatment with endoglycosidase F only slightly increased the

Fig. 2. Western-blot analysis of GLUT1 expression in insect cells

Sf9 cells were grown in the absence of virus (mock infection; lanes A and B), infected with wild-type virus (lanes C and D) or with AcNPV-GT (lanes E and F). After 4 days they were harvested, sonicated, and then separated into membranous (lanes A, C and E) and soluble (lanes B, D and F) fractions. Samples derived from equal numbers of cells (2×10^6 cells) were then electrophoresed on an SDS/10%-polyacrylamide gel and subjected to Western blotting using affinity-purified antibody against the C-terminus of GLUT1, and alkaline phosphatase-linked second antibody as described in [13]. G, human erythrocyte membrane control. The positions of proteins used as M_r markers are indicated.

Fig. 3. Endoglycosidase F treatment of the glucose transporter expressed in Sf9 cells

Samples ($20 \mu\text{g}$) of insect cell membranes (lanes A and B) and of alkali-stripped human erythrocyte membranes (lanes C and D) were incubated with (+) or without (-) 0.25 unit of endoglycosidase F (Endo F) for 18 h at 25°C as described in [14]. Samples ($2 \mu\text{g}$) were then electrophoresed on an SDS/12%-polyacrylamide gel and subjected to Western blotting, using anti-transporter antibodies as described in [14]. The positions of proteins used as M_r markers are indicated.

electrophoretic mobility of the recombinant protein (Fig. 3). The insect cell is known to perform *N*-linked glycosylation of expressed proteins at authentic sites. However, it lacks galactosyl- and sialyl-transferases [7] and so cannot process the *N*-linked high-mannose oligosaccharides to the complex carbohydrate chains known to occur on the erythrocyte protein [17]. It should be noted that the transporter possesses a single glycosylation site (Asn⁴⁵) [4].

Infection of insect cells with a recombinant baculovirus encoding the human erythrocyte glucose transporter led to the expression of considerable quantities of the transport protein.

Fig. 4. Time course of GLUT1 expression in insect cells

Sf9 cells were infected at a MOI of 5–10 with the recombinant virus, collected at various intervals after infection and sonicated. Samples ($10 \mu\text{g}$) of the resultant cell lysates were assayed for GLUT1 by Western blotting as described in the Experimental section. Maximal levels of GLUT1 expression typically corresponded to approx. 1.4 nmol/mg of protein.

Fig. 5. Scatchard-plot analysis of D-glucose-inhibitable cytochalasin B binding to recombinant GLUT1 expressed in Sf9 cells

Binding of cytochalasin B to infected cell membranes (3 mg/ml) was measured in the presence (○) or absence (●) of 400 mM-D-glucose as described in the Experimental section. A derived Scatchard plot (■) was generated by subtracting, along radial axes, the curve obtained in the presence of D-glucose from that obtained in its absence. Linear regression analysis was used to fit the resultant data points to the best straight line.

The transporter was stable and accumulated until the cells lysed, 4–5 days after infection (Fig. 4). Quantitative Western blotting of two separate preparations showed that, 4 days after infection, 1.41 and 1.47 nmol of transporter respectively were present per mg of membrane protein, values which represent almost 8% (w/w) of the total membrane protein. Comparable levels of expression have been reported in the baculovirus system for other membrane glycoproteins, although higher levels, equivalent to that of polyhedrin itself, i.e. 35–50%, have been reported for some non-membrane proteins [7].

It was not possible to determine the transport activity of the

expressed protein in the insect cells, both because of the presence of endogenous transport systems which allow the cells to grow on glucose, and because infected cells become leaky at the time of maximal transporter expression. Fortuitously, however, the insect-cell transporter(s) was found neither to cross-react with antibodies to the mammalian protein nor to bind cytochalasin B. The binding of this inhibitor could therefore be used as a measure of the biological activity of the expressed protein. Scatchard-plot analysis, after correction for non-specific binding to membrane lipids, showed that infected cell membranes contained a single class of specific sites which bound cytochalasin with a K_d of 284 nM, almost identical with the K_d of 282 nM which we have previously reported for the native erythrocyte transporter [14]. Binding was completely inhibited by 400 mM-D-glucose (Fig. 5), but not by L-glucose (results not shown), indicating that the expressed protein bound not only the inhibitor, but also the transported substrate. Non-infected cells and cells infected with the wild-type virus exhibited no D-glucose-inhibitable binding of cytochalasin B (results not shown). Thus it is clear that human glucose transporter expressed in the baculovirus system exhibits native-like biological activity, although purification and reconstitution will be required to demonstrate that it can actually transport glucose. However, the concentration of cytochalasin B-binding sites detectable in two separate preparations of infected cell membranes (0.23 and 0.29 nmol/mg of protein respectively), was less than the concentration of immunologically cross-reactive protein (1.41 and 1.47 nmol/mg of protein respectively). This phenomenon resembles the reported differences in functional activity of baculovirus-expressed and native human multidrug transporter [18], and its origin requires investigation.

Although some non-functional protein is apparently present, expression of *biologically active* human glucose transporter in the baculovirus system is nonetheless abundant, amounting to between 1 and 2% of the total membrane protein. We therefore conclude that this system will be of great value in the study of structure-function relationships in mammalian glucose-transport proteins by site-directed mutagenesis and for the large-scale production of these proteins for biochemical and mechanistic studies.

We are grateful to the Science and Engineering Research Council, the Wellcome Trust, the Peter Samuel Fund and the Nuffield Foundation for support. C.-K.Y. is the recipient of an Overseas Research Student Award.

REFERENCES

1. Baldwin, S. A. & Henderson, P. J. F. (1989) *Annu. Rev. Physiol.* **51**, 459–471
2. Kasahara, M. & Hinkle, P. C. (1977) *J. Biol. Chem.* **252**, 7384–7390
3. Baldwin, S. A., Baldwin, J. M. & Lienhard, G. E. (1982) *Biochemistry* **21**, 3836–3842
4. Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E. & Lodish, H. F. (1985) *Science* **229**, 941–945
5. Gould, G. W. & Lienhard, G. E. (1989) *Biochemistry* **28**, 9447–9452
6. Oka, Y., Asano, T., Shibasaki, Y., Lin, J.-L., Tsukuda, K., Katagiri, H., Akanuma, Y. & Takaku, F. (1990) *Nature (London)* **345**, 550–553
7. Emery, V. C. (1991) *Rev. Med. Virol.* **1**, 11–17
8. Matsuura, Y., Possee, R. D., Overton, H. A. & Bishop, D. H. L. (1987) *J. Gen. Virol.* **68**, 1233–1250
9. Mueckler, M. & Lodish, H. F. (1986) *Cell (Cambridge, Mass.)* **44**, 629–637
10. Summers, M. D. & Smith, G. E. (1987) *Tex. Agric. Exp. Stn. Bull.* No. 1555
11. Fung, M.-C., Chiu, K. Y. M., Weber, T., Chang, T.-W. & Chang, N. T. (1988) *J. Virol. Methods* **19**, 33–42
12. Gorga, F. R. & Lienhard, G. E. (1981) *Biochemistry* **20**, 5108–5113
13. Davies, A., Meeran, K., Cairns, M. T. & Baldwin, S. A. (1987) *J. Biol. Chem.* **262**, 9347–9352
14. Madon, R. J., Martin, S., Davies, A., Fawcett, H. A., Flint, D. J. & Baldwin, S. A. (1990) *Biochem. J.* **272**, 99–105
15. Widnell, C. C., Baldwin, S. A., Davies, A., Martin, S. & Pasternak, C. A. (1990) *FASEB J.* **4**, 1634–1637
16. Lienhard, G. E., Crabb, J. H. & Ransome, K. J. (1984) *Biochim. Biophys. Acta* **769**, 404–410
17. Endo, T., Kasahara, M. & Kobata, A. (1990) *Biochemistry* **29**, 9126–9134
18. Germann, U. A., Willingham, M. C., Pastan, I. & Gottesman, M. M. (1990) *Biochemistry* **29**, 2295–2303

Received 3 February 1992/12 February 1992; accepted 26 February 1992