J. Biomed. Lab. Sci. 8 (2002) 211–215

Photoaffinity Labelling of the Human Erythrocyte Glucose Transporters Expressed in *Spodoptera frugiperda* Clone 9 (Sf9) Cells

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The baculovirus/Sf9 cell expression can be employed as a powerful system for producing large amounts of the human erythrocyte glucose transporter, GLUT1, heterologously. In order to exploit the system further, it is necessary to develop a convenient method for demonstrating that the transporter expressed in insect cells is biologically active. To achieve this, we have expressed the human GLUT1 in insect cells and photolabelled the expressed protein with [^{3}H] cytochalasin B, a potent inhibitor of the human erythrocyte glucose transporter. Subsequently, the labelled proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes labelled with [^{3}H] cytochalasin B in the presence of L-Glucose yielded a single sharp peak of labelling of apparent M_r 45,000 on SDS/polyacrylamide gels. The mobility of this peak corresponded exactly to that of the band detected by anti-glucose transporter antibodies on Western blots of membranes prepared from insect cells infected with recombinant virus. In addition, the sharpness of the radioactive peak provides further evidence for the conclusion that the expressed protein is much less heavily and heterogeneously glycosylated than its erythrocyte counterpart. No peak of labelling was seen with the membranes prepared from non-infected Sf9 cells. Furthermore, the incorporation of label into this peak was completely inhibited by the presence of 500 mM-D-Glucose during the photolabelling procedure, showing the stereoselectivity of the labelling. These evidences clearly show that human glucose transporter expressed in insect cells exhibits native-like biological activity, and that photolabelling with [^{3}H] cytochalasin B can be a convenient means for analysing the biological activity of the transport protein expressed in insect cells.

Key Words: Photolabelling, GLUT1, Baculovirus, Sf9 cell, [^{3}H] cytochalasin B

INTRODUCTION

Most mammalian cells take up glucose by the passive process of facilitated diffusion[1,7,11]. Glucose transporters are the proteins responsible for this process. The best known of these proteins is the human erythrocyte glucose transporter, GLUT1, which has been extensively characterized[2,6,8,9,15,16]. However, a complete understanding of the mechanism of transport will require elucidation of the three-dimensional structure of at least one of these proteins at atomic resolution. Thus, this type of studies would be greatly facilitated by development of heterologous expression systems capable of producing large amounts of recombinant sugar transport proteins.

As demonstrated in our previous work[12], the baculovirus/*Spodoptera frugiperda* Clone 9 (Sf9) cell expression is a powerful system for producing large amounts of the recombinant GLUT1 proteins. Characterization of the expressed protein was expected to include assay of its function, including its ability to transport sugars directly. However, it was not possible to show the transport activity of the expressed protein in the insect cells, because of the presence of endogenous transport systems[12,13]. Sf9 cells, which are commonly employed as the host permissive cell line to support baculovirus replication and protein synthesis[17], grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source[13,17], suggesting the presence of endogenous glucose transporters.

Cytochalasin B is a potent inhibitor of the human ery-

*Received: October 8, 2002
Accepted after revision: November 15, 2002

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throcyte glucose transporter, to which it binds with a K_d of approximately 0.12 μM[6]. The hexose and ligand binding experiments described previously[12,13] had revealed that the mammalian and insect transporters do differ in some of their kinetic properties, namely their affinities for fructose and for cytochalasin B. Therefore, binding of cytochalasin B could be used as a measure of the biological activity of the expressed protein, since the insect cell transporter was found to bind this transport inhibitor only very poorly. From the ligand binding study[13], it was also evident that there were D-glucose-sensitive cytochalasin B binding sites in cells infected with recombinant baculovirus containing the human GLUT1 gene. However, we did not examine that whether these binding sites actually corresponded to the protein immunologically identified on Western blots[12] as the expressed GLUT1 transporter. In order to prove it and to develope a convenient method for functional characterization of transport proteins expressed in insect cells, photoaffinity labelling experiments[10] of the expressed transporter with [^{3}H] cytochalasin B were performed in the present study.

MATERIALS AND METHODS

1. Recombinant baculovirus and culture and viral infection of insect cells

The recombinant baculovirus that is constructed to express human GLUT1 (AcNPV-GT) was produced by co-transfection of Sf9 cells with the baculovirus transfer vector containing the GLUT1 cDNA, pAcYM1-GT and wild-type AcNPV viral DNA according to the method described previously[12,17].

Sf9 cells were cultured according to the method described by Summers and Smith[17], with some modification. Viral infection of insect cells was carried out as described[12]. Briefly, the Sf9 cells were counted and seeded into flasks or dishes at the appropriate density. The cells were then allowed to attach by leaving the dishes for 1 hour in a laminar flow cabinet. Following attachment, the medium was removed and the appropriate amount of wild type AcNPV or recombinant baculovirus AcNPV-GT[10] was added to the cells. After incubating for 1 hour at 28℃ or room temperature, the inoculum was removed. Fresh complete medium was then added to the cells, followed by incubation at 28℃ for 2 to 4 days. The infected cells were visually examined daily for cytopathic effects under a microscope. Following incubation, the culture medium was collected and centrifuged to remove residual cells at 1,000 ×g for 10 min. The extracellular virus was then harvested and stored at 4℃.

2. Preparation of plasma membranes from Sf9 cells

For production of membranes, cells were harvested 4 days after infection [mutiplicity of infection (MOI) 5 and washed three times at 20℃ with 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2. They were then resuspended in 10 mM-Tris/5 mM-$MgCl_2$ pH 7.4, containing proteinase inhibitors [2 mM-iodoacetamide, 0.2 mM-phenylmethanesulphonyl fluoride and pepstatin A (10 g/ml)] and sonicated on ice for 1 min. Membranes were separated from soluble components by centrifugation for 1 h at 117,000 g_{av}.

3. Gel electrophoresis and Western blotting

SDS-PAGE (polyacrylamide gel electrophoresis) was carried out using a discontinuous buffer system essentially as described previously[4,12]. Protein samples were routinely run on 10 or 12% polyacrylamide slab gels. Gels were run until the pyronin Y marker had migrated about 9 cm from the top of the separating gel. The gel running buffer used was 25 mM Tris, 190 mM glycine and 0.1% SDS, pH 8.3. Following electrophoresis the gels were either stained with coomassie blue or subjected to electrotransfer for Western blotting.

Western blotting was performed as previously described[12], by using antibodies against the C-terminus (residues 477-492) of GLUT1[4] and either an alkaline phosphatase conjugate of goat anti-rabbit IgG or ^{125}I-F(ab')$_2$ donkey anti-rabbits IgG as the second antibody.

4. Photoaffinity labelling

The photoaffinity labelling of GLUTI expressed in insect cells was performed using tritiated cytochalasin B, essentially as described previously[3,10]. Insect membrane samples at 1 mg protein/ml in 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA and 500 mM D-or L-Glucose, were incubated with 0.51 μM [^{3}H]-cytochalasin B on ice for 30 minutes to allow the attainment of binding equilibrium. Cytochalasin E (10 μM) was also included to inhibit cytochalasin B binding to cytoskeletal elements not

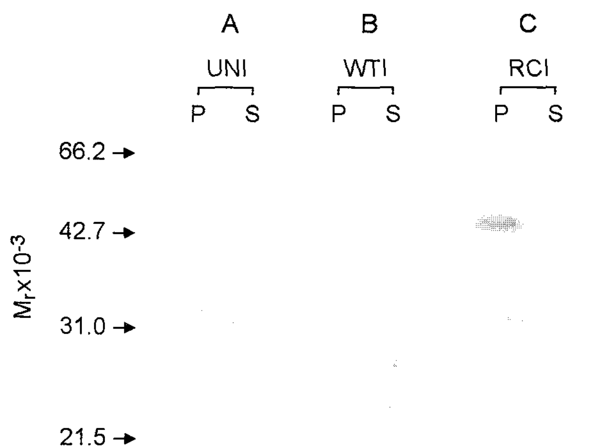

Fig. 1. Western-blot analysis of GLUT1 expresssion in insect cells. Sf9 cells were grown in the absence of virus (**A**, UNI), infected with wild-type virus (**B**, WTI) or with the recombinant virus, AcNPV-GT (**C**, RCI). After 3 days they were harvested, sonicated, and then separated into membranous (P) and soluble (S) fractions. Samples derived from equal numbers of cells (2× 10^6 cells) were then electrophoresed on an SDS/10%-polyacrylamide gel, transferred to nitrocellulose, and stained with antibodies against the C-terminus of GLUT1 and alkaline phosphatase-linked second antibody as described in Methods. The positions of proteins used as M_r markers are indicated.

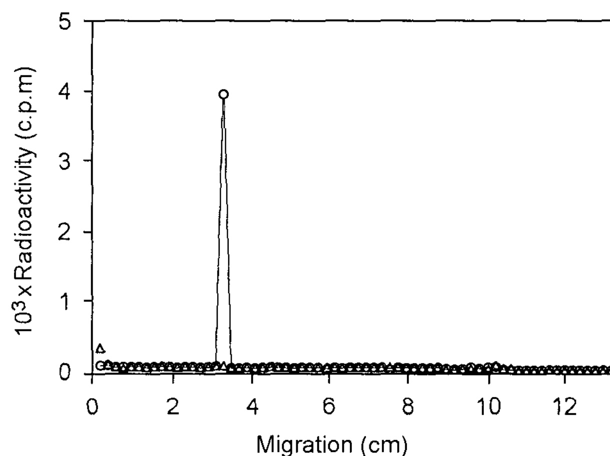

Fig. 2. Electrophoretic profile of photoaffinity-labelled GLUT1 expressed in insect cells. Membranes were labelled with [^{3}H] cytochalasin B in the presence of 500 mM-L-glucose (○) or 500 mM-D-glucose (△). Samples were electrophoresed on a SDS/12% polyacrylamide gel. The radioactivity of 2 mm gel slices were determined by liquid scintillation counting. Arrows indicates the positions of M, markers.

associated with glucose transport. The samples were transferred to 1 ml quartz cuvettes, flushed with N_2, stoppered and then irradiated on ice for 10 minutes with a 100 W UV lamp at a distance of 10 cm. The irradiated samples were then transferred to ultracentrifuge tubes (Beckman Instru-

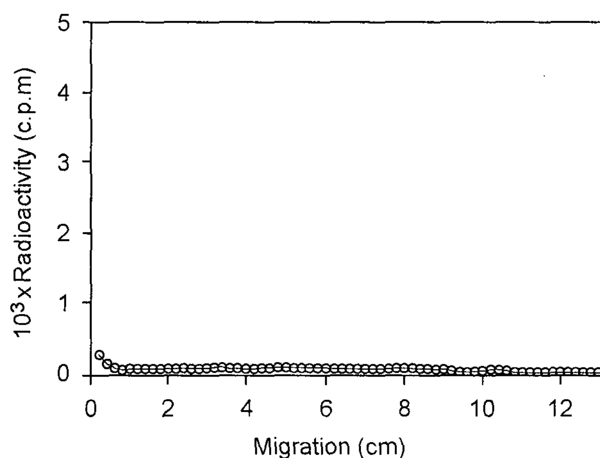

Fig. 3. Electrophoretic profile of photoaffinity labelling of uninfected insect cells. Non-infected Sf9 cell membranes were photoaffinity-labelled with [^{3}H] cytochalasin B in the presence of 500 mM-L-glucose, as described in the legend to Fig. 2.

ments, Inc., Palo Alto, CA, U.S.A) and washed twice with 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA pH 7.4, containing 20 μM unlabelled cytochalasin B by centrifugation at 126,000×g for 10 min at 4℃ to remove non-covalently-bound [^{3}H]-cytochalasin B. The supernatants were discarded each time. After washing, samples (100 μg) were electrophoresed on a 3 mm thick, SDS/12% polyacrylamide gel. The gel was then fixed, stained with Coomassie Blue and then destained. Relevant tracks were cut out to a width of 1 cm using a long blade and then cut into 2 mm slices with a gel slicer. Four blank slices were also taken from non-radioactive part of the gel to determine backgrounds. The slices were then placed in scintillation vials and solubilized by incubation with 1 ml 50% (v/v) Solvable (Du Pont GMBH, Hamburg, Germany) for 3 hours at 50℃. Finally, 4 ml of scintillation fluid was added to each vial and the radioactivity was counted by using a Beckman LS 5,000 CE scintillation counter.

RESULTS

The nature of the human glucose transporter expressed in insect cells was analysed by Western blotting using antibodies against residues 477-492 of GLUT1. The Western analysis revealed that a single immunoreactive band was present in cells infected with a recombinant baculovirus, but not in the negative controls (uninfected cells and those infected with the wild-type virus, Fig. 1). Expressed transporter was confined to the membrane fraction of the infected

cells and was absent from the soluble fraction as reported previously.

Photoaffinity labelling experiments with [^{3}H] cytochalasin B were carried out according to the methods of Kasanicki *et al.*[10]. Cytochalasin B binding to the transporter in the human erythrocyte is normally reversible. However, exposure of the protein to ultraviolet light in the presence of tritiated cytochalasin B results in covalent binding to the transporter[3]. Membranes were prepared from Sf9 cells infected with the recombinant AcNPV-GT or the wild-type AcNPV (MOI=5) 4 days post infection. The membrane samples were then photolabelled with [^{3}H] cytochalasin B in the presence of either 500 mM-D-or L-glucose, as detailed in the Method. Subsequently, the labelled proteins were analysed by SDS-PAGE. The position of the radiolabelled peak was compared with the measured migration distance of the molecular weight markers. Membranes labelled with [^{3}H] cytochalasin B in the presence of L-Glucose yielded a single sharp peak of labelling of apparent M_r 45,000 on SDS/polyacrvlamide gels (Fig. 2). The mobility of this peak corresponded exactly to that of the band detected by anti-glucose transporter antibodies on Western blots of membranes prepared from insect cells infected with recombinant virus. No peak of labelling was seen with the membranes prepared from non-infected Sf9 cells (Fig. 3). In addition, the sharpness of the radioactive peak shown in Figure 1 provides further evidence for the conclusion that the expressed protein is much less heavily and heterogeneously glycosylated than its erythrocyte counterpart[12]. In case of photolabelled human erythrocyte membranes, it is reported that the labelling occurs as a broad band of apparent M_r 45,000~70,000 on SDS/polyacrylamide gels[14]. Furthermore, the incorporation of label into this peak was completely inhibited by the presence of 500 mM-D-Glucose during the photolabelling procedure, showing the stereoselectivity of the labelling.

DISCUSSION

For detailed studies of structure-function relationships in the human erythrocyte glucose transporter, and in particular for the investigation of its structure by crystallization, it would be desirable to develop a high-level expression system. Recently, we have reported the use of the baculovirus expression system to produce substantial amounts of the human erythrocyte glucose transporter suitable for structural and functional studies[12].

Three attempts can be possibly made to demonstrate whether the transporter expressed in insect cells is biologically active. Firstly, by performing binding assays with a radiolabelled specific inhibitor of the GLUT1. Secondly, by photolabelling of the expressed protein with [^{3}H] cytochalasin B. Lastly, by measuring the glucose transport activity of the recombinant protein expressed in Sf9 cells. However, it was not possible to show the transport function of the expressed protein in the insect cells, because of the presence of highly active, endogenous transport systems which allow the cells to grow on glucose[12]. Fortuitously, however, the insect-cell transporter was found neither to cross-react with antibodies to the mammalian protein nor to bind cytochalasin B, a potent competitive reversible inhibitor of glucose transport. The binding of this inhibitor could therefore be used as a measure of the biological activity of the expressed protein in the previous work[12,13]. However, the procedure of reversible cytochalasin B binding assay is rather labor-intensive and tedious.

From the ligand binding study[13], it was clear that there were D-glucose-sensitive cytochalasin B binding sites in cells infected with recombinant baculovirus containing the human GLUT1 gene. In order to assess whether D-glucose-sensitive cytochalasin B binding sites in cells infected with the recombinant baculovirus AcNPV-GT actually corresponded to the protein immunologically identified on Western blots as the expressed GLUT1 transporter, photoaffinity labelling experiments with [^{3}H] cytochalasin B were carried out with another aim to develope a convenient method for functional characterization of the expressed protein. Cytochalasin B binding to the transporter in the human erythrocyte is normally reversible. However, exposure of the protein to ultraviolet light in the presence of tritiated cytochalasin B results in covalent binding to the transporter[3]. The mechanism of photolabelling is not yet understood, but it appears to proceed via activation of an aromatic amino acid residue on the transporter rather than by photoactivation of the ligand itself[5]. D-glucose and other transported sugars, but not L-glucose, have been reported to inhibit photolabelling of the erythrocyte protein by displacing non-covalently-bound cytochalasin prior to photolabelling[3]. A similar inhibition was seen in the present study, and confirmed the ability of the expressed protein to

bind its substrate, D-glucose. These evidences clearly show that human glucose transporter expressed in insect cells exhibits native-like biological activity, and demonstrates that the photolabelling with $[^3H]$ cytochalasin B can be an alternative, convenient means for determining functional activity of the recombinant human GLUT1 expressed in the baculovirus/Sf9 cell system.

It is therefore concluded that photolabelling with $[^3H]$ cytochalasin B, together with binding assay of a specific inhibitor of the GLUT1, could eliminate tedious purification and reconstitution procedures[2,6], which are otherwise required to show the functionality of human glucose transporters.

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