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Cross-reactivity of Human Polyclonal Anti-GLUT1 Antisera with the Endogenous Insect Cell Glucose Transporters and the Baculovirus-expressed GLUT1

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Most mammalian cells take up glucose by passive transport proteins in the plasma membranes. The best known of these proteins is the human erythrocyte glucose transporter, GLUT1. High levels of heterologous expression for the transporter are necessary for the investigation of its three-dimensional structure by crystallization. To achieve this, the baculovirus expression system has become popular choice. However, *Spodoptera frugiperda* Clone 9 (Sf9) cells, which are commonly employed as the host permissive cell line to support baculovirus replication and protein synthesis, grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source, suggesting the presence of endogenous glucose transporters. Furthermore, very little is known of the endogenous transporters properties of Sf9 cells. Therefore, human GLUT1 antibodies would play an important role for characterization of the GLUT1 expressed in insect cell. However, the successful use of such antibodies for characterization of GLUT1 expression in insect cells relies upon their specificity for the human protein and lack of cross-reaction with endogenous transporters. It is therefore important to determine the potential cross-reactivity of the antibodies with the endogenous insect cell glucose transporters. In the present study, the potential cross-reactivity of the human GLUT1 antibodies with the endogenous insect cell glucose transporters was examined by Western blotting. Neither the antibodies against intact GLUT1 nor those against the *C*-terminus labelled any band migrating in the region expected for a protein of M_r comparable to GLUT1, whereas these antibodies specifically recognized the human GLUT1. Specificity of the human GLUT1 antibodies tested was also shown by cross-reaction with the GLUT1 expressed in insect cells. In addition, the insect cell glucose transporter was found to have very low affinity for cytochalasin B, a potent inhibitor of human erythrocyte glucose transporter.

Key Words: Glucose transporter, Insect cell sugar transporter, Sf9 cell, GLUT1, Cytochalasin B

INTRODUCTION

In mammals, the uptake of glucose is mediated by passive transport proteins in the plasma membranes[3,4,9,14,17]. The best known of these proteins is the human erythrocyte glucose transporter[1,10,11], GLUT1, which has been extensively characterized[2,6~8,13,15]. Recently, for detailed studies of structure-function relationships in this protein, and in particular for the investigation of its structure by crystallization, high levels of heterologous expression for the transporter has been reported by employing the baculovirus/*Spodoptera frugiperda* Clone 9 (Sf9) cell system[18]. Characterization of the expressed protein was expected to include assay of its function, including its ability to transport sugars and to bind inhibitory ligands such as cytochalasin B. 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.

Sf9 cells, which are commonly employed as the host permissive cell line to support baculovirus replication and protein synthesis[16], grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source[16,18]. This evidence strongly suggests the presence of endogenous glucose transporters. Another evidence is recently

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shown by the hexose transport study of Sf9 cells[12]. The hexose transport activity of insect cells described suggested that endogenous transporters were abundant, and the occurrence of sugar transporters homologous to GLUT1 in a wide range of organisms[1,3,4] indicated a likelihood that the insect cell transporter(s) might exhibit sequence similarity to the human protein. However, very little is known of the endogenous transporters properties of Sf9 cells. It is therefore expected that characterization and exploitation of the heterologous GLUT1 expression in the baculovirus system for various studies are heavily dependent on the usage of GLUT1 antibodies.

A number of antibodies against the human erythrocyte glucose transporter[5] are available that could potentially be used for various purpose. However, the successful use of such antibodies for characterization of GLUT1 expression in insect cells relies upon their specificity for the human protein and lack of cross-reaction with endogenous transporters. It is therefore important to determine the potential cross-reactivity of the antibodies with the endogenous insect cell glucose transporters. In the present study, cross-reactivity of a range of polyclonal, rabbit anti-human GLUT1 antisera with the endogenous insect cell glucose transporters and the baculovirus expressed GLUT1 was examined. The binding assay of cytochalasin B was also performed, which can be used as a functional assay for the endogenous glucose transporter(s) in insect cells.

MATERIALS AND METHODS

1. Insect cell culture and viral infection of insect cells

Sf9 cells were maintained according to the method described by Summers and Smith[16], with some modification. The cells were cultured in complete TC100 medium [TC100 medium (Gibco-BRL), 10% (v/v) fetal calf serum (Flow), 1% of antibiotics (penicillin 5,000 units/ml + streptomycin 5,000 μg/ml, Gibco-BRL)] at 28℃. Cell viability was checked by adding 0.1 ml of trypan blue (0.4% stock, pH 3) to 1 ml of cells and examining under a microscope[16,18].

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[16] or recombinant baculovirus, AcNPV-GT that is constructed to express human GLUT1[18], 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 x g for 10 min. The extracellular virus was then harvested and stored at 4℃.

2. Preparation of plasma membranes from Sf9 cells

Sf9 cells were cultured as described above. Cells were harvested 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. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a discontinuous buffer system essentially as described previously[5,18]. Briefly, protein samples were routinely run on 10 or 12% polyacrylamide slab gels. The slab gel comprised a 2 cm stacking gel of high porosity and a 10 cm separating gel of low porosity. Prior to loading the proteins were solubilized in a loading buffer [40 mM Tris-HCl, pH 6.8, 0.8 mM ethylene diamine tetraacetic acid (EDTA), 0.8% SDS, 4 mM dithiothreitol, 10% (v/v) glycerol, and 0.12% (w/v) pyronin Y]. Between 5 and 50 μg of each of the solubilized samples was then loaded into each track of a 1.5 or 3 mm-thick gel. Low molecular weight range markers (M_r 14,400-97,400, Bio-Rad) were run alongside the sample tracks. Electrophoresis was carried out as described previously[5,18]. 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.

4. Western blotting

Antibodies against the human erythrocyte glucose transporter employed were originally raised in male New Zealand White rabbits against synthetic peptides, which were coupled to Keyhole limpet hemocyanin using maleimidobenzoyl-N-hydroxysuccinimide ester, corresponding to the *C*-terminal (residues 477-492), the *N*-terminal (residues 1-15) and the central cytoplasmic loop regions of GLUT1 (residues 240-255) as detailed in [18]. Western blotting was performed as previously described[1], by using either an alkaline phosphatase conjugate of goat anti-rabbit IgG or ^{125}I-F(ab')$_2$ donkey anti-rabbits IgG as the second antibody.

5. Cytochalasin B binding assay

Cytochalasin B is a potent inhibitor of the human erythrocyte glucose transporter. The binding of cytochalasin B could be used as a functional assay for the endogenous glucose transporter(s) in insect cells and was measured by equilibrium dialysis using [4-^{3}H] cytochalasin B essentially according to the methods described previously[2].

RESULTS

The potential cross-reactivity of the human GLUT1 antibodies with the endogenous insect cell glucose transporters was examined by Western blotting. Insect cell membranes were analysed by SDS-PAGE and then transferred to nitrocellulose for immunoblotting. The blots were immunostained with antisera raised against intact GLUT1 or against the *C*-terminus of GLUT1. The bound primary antibodies were then detected with secondary antibody as previously described[2,18]. As shown in Fig. 1, neither the antibodies against intact GLUT1 nor those against the *C*-terminus labelled any band migrating in the region expected for a protein of M_r comparable to GLUT1 (lanes C and E), but some cross-reactive bands with higher M_r were detected in the insect cell membranes by the antibody against the whole protein. One of these bands was also present when pre-immune serum was used to stain the blot. However, such bands were not detected when the antibodies against the *C*-terminus of GLUT1 were used.

In contrast, specificity of the human GLUT1 antibodies[5]

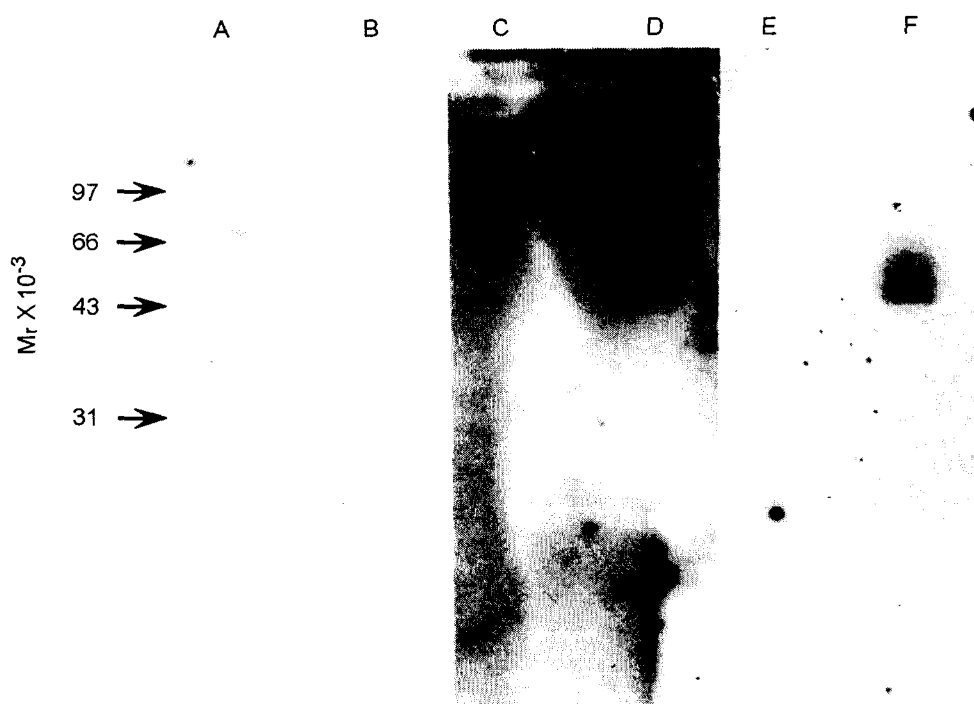

Fig. 1. Immunological examination of the endogenous glucose transporter(s) of Sf9 cells with antibodies to the human erythrocyte transporter. Samples containing 5 μg of Sf9 cell membranes (**A, C, E**) or of protein-depleted human erythrocyte membranes (**B, D, F**) were electrophoresed on an SDS/10% polyacrylamide gel, transferred to nitrocellulose and stained with either antibodies against intact GLUT1 (**C** and **D**), against the *C*-terminus (**E** and **F**) or pre-immune (**A** and **B**) as described in Methods. The positions of M_r markers are indicated.

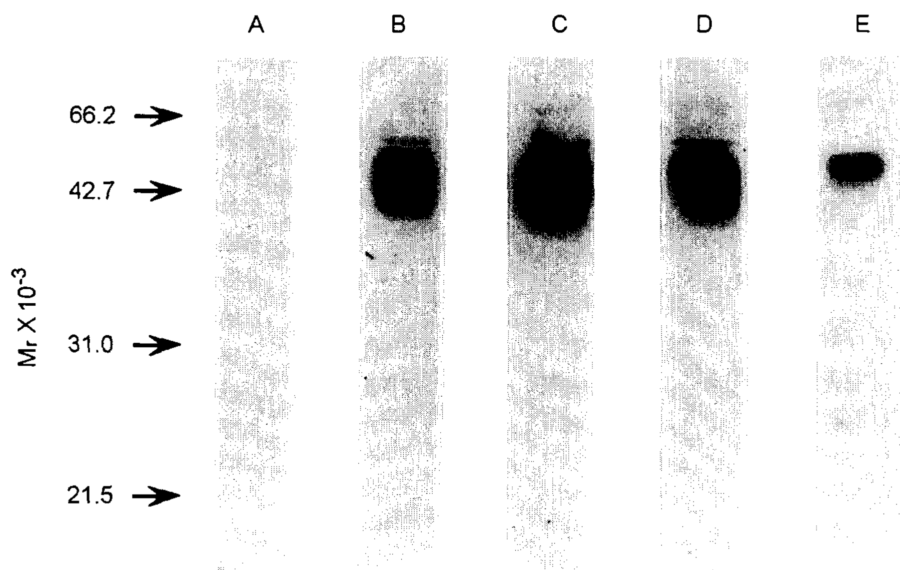

Fig. 2. Cross-reaction of the baculovirus-expressed GLUT1 with a range of polyclonal antibodies against human GLUT1. Samples (5 μg) of ACNPV-G7-infected[18] cell membranes were electrophoresed on an SDS/10%-polyacrylamide gel and subjected to Western blotting using anti-transporter antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG as described before. The primary antisera used were: Lane **A** (pre-immune serum), **B** (anti-intact GLUT1), **C** (anti-*C*-terminus of GLUT1, residues 477-492), **D** (anti-middle loop of GLUT1, residues 240-255), **E** (anti-*N*-terminus of GLUT1, residues 1-15).

Table 1. Cytochalasin B binding to Sf9 cell membranes

Sample (1 mg/ml)	Cytochalasin B (B/F)		
	(−) D-Glucose	(+) D-Glucose	*Specific B/F
Sf9 cell membranes	0.048	0.047	0.001
Erythrocyte membranes**	8.055	0.619	7.436

The binding of cytochalasin B was measured at a single low concentration (40 nM), in the absence (−) and presence (+) of 400 mM D-glucose, as described previously[2]. Cytochalasin B binding activity (•) was calculated as described before[6]. Human erythrocyte membranes (••) were prepared as described previously[6]. B/F = [bound cythochalasin B] / [free cytochalasin B]

was shown by demonstrating the cross-reactivity of the GLUT1 expressed in insect cells towards a range of polyclonal, site-directed antibodies. As presented in Fig. 2, antisera[5] raised against intact GLUT1 (Fig. 2, lane B, residues 1-492), against the *C*-terminal peptide (lane C, residues 477-492), the *N*-terminal peptide (lane E, residues 1-15) and against the central cytoplasmic loop of GLUT1 (lane D, residues 240-255), all recognised the recombinant protein on Western blots. However, no immunostaining was seen with pre-immune serum (lane A).

Cytochalasin B binding activity was assayed by equilibrium dialysis using a single, final concentration of [4-^{3}H] cytochalasin B of 40 nM, in the absence and presence of 400 mM D-glucose[2]. The insect cell membranes used were prepared as described in Materials and Methods. Alkali-stripped human erythrocyte membranes[6] were used as a positive control. Cytochalasin binding activity was calculated by subtracting the value of the ratio of bound cytochalasin B to free cytochalasin B obtained in the presence of D-glucose from the equivalent value obtained in the absence of D-glucose[2]. The corrected bound-to-free ratio is approximately equal to the ratio of the concentration of cytochalasin B binding sites on glucose transporters to the dissociation constant for cytochalasin B. Thus, it is proportional to the concentration of binding sites[2]. The cytochalasin B binding activity for the insect cell and erythrocyte membranes at the concentration of 1 mg per ml were 0.001 and 7.436, respectively (Table 1).

DISCUSSION

Sf9 cells grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source, suggesting the presence of endogenous glucose transporters in the insect cells[12,16,18]. It is also likely that the insect cell transporter(s) might exhibit sequence similarity to the human protein, because sugar transporters homologous to GLUT1 occur in a wide range of organisms[1,3,13]. It is therefore important to establish the potential cross-reactivity of the antibodies for the human erythrocyte transporter with the endogenous insect cell glucose transporters. As shown in Fig. 1, specificity of the two antibodies used was confirmed by the fact that both, as expected, recognised a broad band of apparent M_r 45,000-65,000 on blots of protein-depleted human erythrocyte membranes. Thus, it appears that uninfected insect cell membranes do not contain transport proteins immunologically cross-reactive with antibodies directed against the *C*-terminus of the mammalian glucose transporter, although the nature of the bands of higher M_r that were recognised by antibodies against whole GLUT1, remains unclear. Lack of immunological cross-reactivity suggests that even if the endogenous insect cell glucose transporter is homologous to GLUT1, the mammalian and insect cell proteins share only very limited sequence similarity. Consequently, the presence of endogenous glucose transporter(s) should not interfere with the immunological detection of human GLUT1 expression in insect cells, at least if anti-*C*-terminal antibodies are used to probe for the expressed protein. This expectation has been proved to be true by the results presented in Fig. 2. All polyclonal antibodies tested recognised the recombinant protein on Western blots, indicating that the GLUT1 expressed in insect cells corresponded to a full-length form of GLUT1, and that no frame-shifts had occurred during construction of the recombinant virus. However, no immunostaining was seen with pre-immune serum. Therefore, a number of antibodies against the human glucose transporter could potentially be useful for various analysis, including 3-D studies of the GLUT1, in particular for the investigation of its structure by crystallization. Furthermore, binding of cytochalasin B could be used as a measure of the biological activity of the GLUT1 in insect cell, since the insect cell glucose transporter was found to have very low affinity for cytochalasin B, a potent inhibitor of human erythrocyte glucose transporter.

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