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The Uptake of 2-deoxy-D-glucose (2dGlc) by the Endogenous Sugar Transporter(s) of *Spodoptera frugiperda* Clone 21-AE Cells and the Inhibition of 2dGlc Transport in the Insect Cells by Fructose and Cytoc halasin B

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The baculovirus/*Spodoptera frugiperda* (Sf) cell system has become popular for the production of large amounts of the human erythrocyte glucose transporter, GLUT1, heterologously. However, it was not possible to show that the expressed transporter in insect cells could actually transport glucose. The possible reason for this was that the activity of the endogenous insect glucose transporter was extremely high and so rendered transport activity resulting from the expression of exogenous transporter very difficult to detect. Sf21-AE cells are commonly employed as the host permissive cell line to support the baculovirus AcNPV replication and protein synthesis. The cells grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source, strongly suggesting the presence of endogenous glucose transporters. However, unlike the human glucose transporter, very little is known about properties of the endogenous sugar transporter(s) in insect cells. Thus, the uptake of 2-deoxy-D-glucose (2dGlc) by Sf21-AE cells and the inhibition of 2dGlc transport in the insect cells by fructose and cytochalasin B were investigated in the present work. The binding assay of cytochalasin B was also performed, which could be used as a functional assay for the endogenous glucose transporter(s) in the insect cells. Sf21-AE cells were infected with the recombinant virus AcNPV-GT or no virus, at a multiplicity of infection (MOI) of 5. Infected cells were resuspended in PBS plus and minus 300 mM fructose, and plus and minus 20 μM cytochalasin B for use in transport assays. Uptake was measured at 28℃ for 1 min, with final concentration of 1 mM deoxy-D-glucose, 2-[1,2-^{3}H]- or glucose, L-[1,^{3}H]-, used at a specific radioactivity of 4 Ci/mol. The results obtained demonstrated that the sugar uptake in uninfected cells was stereospecific, and was strongly inhibited by fructose but only poorly inhibitable by cytochalasin B. It is therefore suggested that the Sf21-AE glucose transporter has very low affinity for cytochalasin B, a potent inhibitor of human erythrocyte glucose transporter.

Key Words: Glucose transporter, Insect cell sugar transporter, Sf21 cell, GLUT1, Cytochalasin B

INTRODUCTION

Sugar transport proteins are physiologically and clinically important in humans[3,4,17] but their low abundance in cell membranes renders their isolation and study difficult. Thus, their production in large amounts by genetic means has been an important goal of many research groups throughout the world. In order to establish a system useful for the large scale production of normal and mutant mammalian passive glucose transporters for structural and functional studies, the baculovirus/*Spodoptera frugiperda* (Sf) cell expression system[15] has been exploited for the production of large amounts of the human erythrocyte glucose transporter[19], GLUT1, which has been extensively characterized[8,13]. 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 that the expressed transporter could actually transport glucose[19]. The possible reason for this was that the activity of the endogenous insect glucose transporter was extremely high and so rendered

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transport activity resulting from the expression of exogenous transporter very difficult to detect. Unlike the human glucose transporter, very little is known about the nature of the endogenous sugar transporter(s) in insect cells.

Spodoptera frugiperda (Sf) cells are commonly employed as the host permissive cell line to support the baculovirus AcNPV replication and protein synthesis[15]. Two common cell lines used for the AcNPV-based vectors are Sf9 and Sf21-AE. They were originally isolated from the pupal ovarian tissue of Sf cells (the alfalfa looper) at the USDA insect pathology laboratory (IPLB) at Beltsville, Maryland, USA[18]. Sf21-AE cells grow well on TC-100 medium that contains 0.1% D-glucose as the major carbon source. This evidence strongly suggests the presence of endogenous glucose transporters. Another evidence is rendered by the recent hexose transport study of Sf9 cells[10]. The study suggested that insect cells contain an endogenous glucose transport activity that in several aspects resembles the human erythrocyte glucose transporter. Also, the occurrence of sugar transporters homologous to GLUT1 in a wide range of organisms[1] 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 and Sf21-AE cells. Thus, activities of 2dGlc transport by Sf21-AE cells and the inhibition of 2dGlc transport in the insect cells by fructose and cytochalasin B were investigated in the present work. The binding assay of cytochalasin B[5] was also performed, which could be used as a functional assay for the endogenous glucose transporter(s) in the insect cells.

MATERIALS AND METHODS

1. Insect cell culture and viral infection of insect cells

Sf21-AE cells were cultured in complete TC-100 medium [TC-100 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 3.0) to 1 ml of cells and examining under a microscope. The Sf 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 the recombinant baculovirus, AcNPV-GT that is constructed to express human GLUT1, 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 Sf21-AE cells

Sf21-AE cells were cultured as described above. For the production of membranes, cells were harvested 4 days after 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 hour at 117,000 g.

3. Hexose transport assay

Tritiated sugars used were 2-(1,2-^{3}H)-deoxy-D-glucose (30.2 Ci/mmol) and L-[1-^{3}H(N)]-glucose (10.7 Ci/mmol) and were supplied by NEN. Prior to 2-deoxy-D-glucose (2dGlc) uptake measurements, Sf21-AE cells were washed twice with phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) to remove glucose. The cells were resuspended to a density of 1×10^6 cells per ml in PBS and then stored on ice until required for the experiment (up to 30 min). Assays were performed in triplicate, with 1×10^6 cells per assay. The hexose transport assay was performed as follows: 1 ml of the cell suspension was centrifuged at 6,000 × g for 15 sec in a microfuge and resuspended to a volume of 150 μl in PBS. The cells were then incubated at 28℃ for 2 min. The transport was initiated by the addition of 100 μl of 2.5 mM 2dGlc or L-glucose containing 1 μCi tritiated sugar to give a final concentration of 1 mM sugar. Following the incubation for 1 min at 28℃, the assay was terminated by adding 1 ml of ice-cold PBS containing 10 μM cytochalasin B and 0.1 mM phloretin, the potent glucose transport inhibitors, and then by centri-

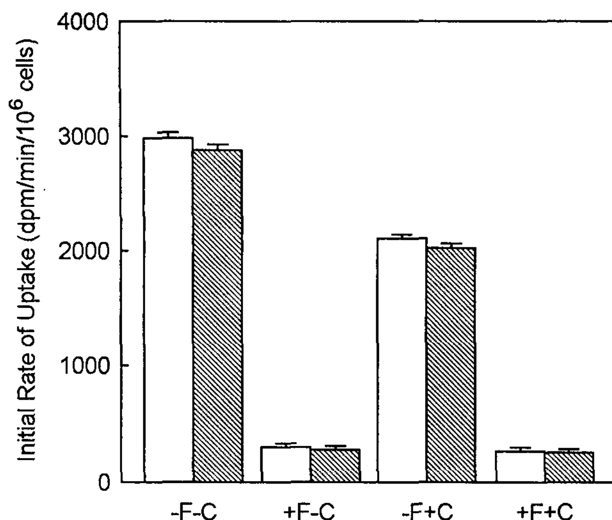

Fig. 1. Uptake of 1 mM 2-deoxy-D-glucose by noninfected and recombinant virus infected Sf21-AE cells. Cells were infected with recombinant AcNPV-GT or no virus (MOI=5) as described in Materials and Methods. After 2 days infection cells were collected and resuspended in PBS plus (+) and (−) 300 mM fructose, and plus (+) and (−) 20 μM cytochalasin B. Transport was carried out as described in Materials and Methods. Bars represent mean ± SEM for three measurements assayed in triplicate. Open bar: noninfected cells. Speckled bar: recombinant AcNPV-GT-infected cells. F: fructose. C: cytochalasin B.

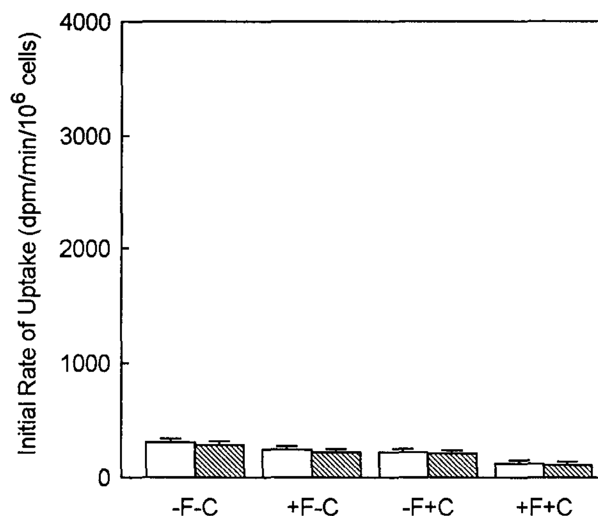

Fig. 2. Uptake of 1 mM L-glucose by noninfected and recombinant virus infected Sf21-AE cells. Cells were infected with recombinant AcNPV-GT or no virus (MOI=5) as described in Materials and Methods. After 2 days infection cells were collected and resuspended in PBS plus (+) and (−) 300 mM fructose, and plus (+) and (−) 20 μM cytochalasin B. Transport was carried out as described in Materials and Methods. Bars represent mean ± SEM for three measurements assayed in triplicate. Open bar: noninfected cells. Speckled bar: recombinant AcNPV-GT-infected cells. F: fructose. C: cytochalasin B.

fuging at 12,000 × g for 20 seconds in a microfuge. The cells were washed in this fashion for two more times and then solubilized in 200 μl of 10 % SDS by vortexing. Finally, 150 μl of the solubilized cells were dispensed to a vial containing 4 ml of scintillant using a Microman pipette and radioactivity was then determined by liquid scintillation counting (Bookman LS 5,000 CE). All results were expressed as the means of triplicate estimations, which routinely differed from the mean by less than 10%.

4. Cytochalasin B binding assay

Cytochalasin B is a potent inhibitor of the human erythrocyte glucose transporter. The binding of cytochalasin B was 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[10].

RESULTS

Sf21-AE cells were infected with the recombinant virus AcNPV-GT or no virus, at a MOI of 5[15]. They were harvested for use in transport experiments two days after infection, because infected cells become leaky at the time of maximal transport expression (4~5 days post infection; data not shown). High concentrations of unlabelled fructose were included in some 2dGlc uptake assays in order to suppress the activity of the endogenous insect cell transporters and therefore enhance detectability of GLUT1-catalysed transport in infected cells. In addition, expression of human GLUT1 should give rise to a component of the 2dGlc uptake activity of the cells that is more sensitive to inhibition by cytochalasin B, because the insect and mammalian transporters have differential susceptibility for inhibition by this ligand. The cytochalasin B also binds to the GLUT1 much more tightly than glucose[5]. Infected cells were therefore resuspended in PBS plus and minus 300 mM fructose, and plus and minus 20 μM cytochalasin B for use in transport assays. Uptake was measured at 28℃ for 1 min, with final concentration of 1 mM deoxy-D-glucose, 2-[1,2-^{3}H]- or glucose, L-[1,^{3}H]-, used at a specific radioactivity of 4 Ci/mol. The assay was terminated by the addition of the ice-cold stop solution, plus or minus or fructose as appropriate. As shown in Figs. 1 and 2, the sugar uptake in uninfected cells was stereospecific, and was strongly inhibited by fructose but only poorly inhibitable by cytochalasin B.

Table 1. Cytochalasin B binding to Sf21-AE cell membranes

Sample (1 mg/ml)	Cytochalasin B (B/F)		
	(−) D-Glucose	(+) D-Glucose	*Specific B/F
Sf21-AE cell membranes	0.043	0.042	0.001
Erythrocyte membranes**	8.027	0.596	7.431

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[10]. Cytochalasin B binding activity (*) was calculated as described before[5]. Human erythrocyte membranes (**) were prepared as described previously[5]. B/F = [bound cythochalasin B] / [free cytochalasin B]

The binding of cytochalasin B was examined as a functional assay for the glucose transporter expressed in insect cells and was measured 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[5]. The insect cell membranes of Sf21-AE cells were prepared as described in Materials and Methods. Alkali-stripped human erythrocyte membranes[2] were included 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[2,5]. 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[5]. The cytochalasin B binding activity for the insect cell and erythrocyte membranes at the concentration of 1 mg per ml were 0.001 and 7.431, respectively (Table 1).

DISCUSSION

Up till the present the bacterium *Escherichia coli* has been the most commonly used prokaryotic host system for foreign gene expression. However, use of this expression system yielded only very small amounts of functional protein of the mammalian passive glucose transporter GLUT1 isoforms[14,16]. To overcome problems that can be posed by the eukaryotic GLUT1 gene expression in a prokaryotic environment, considerable efforts have been made to express eukaryotic proteins in an eukaryotic environment. Successful expression has been reported for several mammalian glucose transporter isoforms in a variety of eukaryotic expression systems. These systems have included *Xenopus* oocytes[6,9], mammalian cells[7], and transgenic mice[12]. However, for detailed studies of structure and function relationships in these proteins, and in particular for the investigation of their structures by crystallization, higher levels of expression are prerequisite. Recently, the baculovirus/ *Spodoptera frugiperda* cell system has been exploited to produce large amounts of the GLUT1 for structural and functional studies[19]. 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 that the expressed transporter could actually transport glucose, because of the presence of endogenous transport systems.

Spodoptera frugiperda (Sf) cells are commonly employed as the host permissive cell line to support the baculovirus AcNPV replication and protein synthesis[15] The fact that Sf21-AE cells grow well on TC-100 medium strongly suggests the presence of endogenous glucose transporters in the insect cells, since the TC-100 media contains 0.1% D-glucose as the major carbon source. It is also likely that the insect cell transporter(s) might exhibit sequence similarity to the human protein, because a large family of sugar transporters homologous to GLUT1 found in wide range of organisms[3]. However, very little is known of the nature of the endogenous sugar transporter(s) in the insect cells. The human GLUT1 has a broad substrate specificity[11], involving a large number of simple sugars. The rank order of apparent affinities is as follows: 2-deoxy-D-glucose > D-glucose > D-mannose > D-galactose > D-xylose > L-arabinose > D-fucose > L-fucose > L-rhamnose >> L-glucose. As is apparent from this rank order, the GLUT1 displays a particularly strong specificity for D-stereoisomers of sugars[11]. It is therefore interesting to establish if 2-deoxy-D-glucose (2dGlc) transport could be measured in Sf21-AE cells, and if fructose and cytochalasin B could inhibit 2dGlc transport in the insect cells. The results shown in Figs 1 and

2 demonstrated that the sugar uptake in uninfected Sf21-AE cells was stereospecific, and was strongly inhibited by fructose but only poorly inhibitable by cytochalasin B, which is the most potent inhibitors of glucose transport. Surprisingly, the AcNPV-GT-infected cells showed an essentially identical pattern of transport inhibition, and the rate of 2dGlc uptake was in fact somewhat less than that seen for the uninfected cells. There was thus no evidence for any contribution of expressed GLUT1 to 2dGlc uptake by the infected cells; in particular there was no appearance of a novel cytochalasin B-sensitive but fructose-insensitive component. From the cytochalasin binding result for the Sf21-AE, it is suggested that the absence of the binding activity in the Sf21-AE cells results either from the low concentration of binding sites on the insect transporter(s), or from the low affinity for the transporter, or both. Therefore, the assay of cytochalasin B binding can be a useful measurement for functional properties of the GLUT1 expressed in insect cells because 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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