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Baculovirus-mediated expression of the Na^+/glucose cotransporter in Sf9 cells

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We have used baculovirus (AcNPV) to express the Na^+/glucose cotransporter protein in cultured Sf9 cells. We constructed a baculovirus transfer vector containing the cDNA for the rabbit intestinal Na^+/glucose cotransporter (SGLT1) under the control of the polyhedrin gene promoter. Recombinant baculovirus was obtained by cotransfection of SF9 cells with wild-type AcNPV DNA and the transfer vector. Recombinant virus was identified by Southern blotting and then purified. Recombinant infected Sf9 cells expressed a protein which was recognized by anti-peptide antibodies raised to sequences of the cloned Na^+/glucose cotransporter. This protein migrated with a molecular mass of 55 kD by SDS-PAGE, similar to the *in vitro* translation product of SGLT1. An identical protein was metabolically labeled with [^{35}S]methionine. Cells which synthesized the transport protein showed Na^+-dependent αMeGlc transport. Micromolar phlorizin inhibited transport. Uninfected and wild-type virus infected Sf9 cells did not have Na^+-dependent glucose transport. All transport protein migrated at 45% sucrose (w/w) by density gradient sedimentation, suggesting that the expressed transporter is membrane associated. We conclude that we have functionally expressed the rabbit intestinal Na^+/glucose cotransporter in Sf9 cells. The transporter is not heavily glycosylated, and this is consistent with previous work showing that glycosylation is not necessary for function. We are poised to purify and characterize this protein from a structure-function perspective.

Introduction

The Na^+/glucose cotransporter is an integral membrane protein from the brush border of intestinal and kidney epithelia [1]. This protein mediates trans-epithelial transport by coupling concentrative uptake of glucose across the apical membrane with the energetically favorable movement of sodium ions down their concentration gradient. Sugar is then transported from the epithelial cell into the blood through the basolateral facilitated glucose transporter [2,3].

The sodium-coupled transporter is thought to be less than 0.1% of the protein present in the intestinal brush border, an abundance so low as to impede biochemical studies on the cotransport protein. However, Hediger and his colleagues [4] have isolated a cDNA clone which encodes this transport protein, and this advance, together with the development of a new expression system, shows promise for overexpression of integral membrane proteins. This new system is the insect cell line *Spodoptera frugiperda*, Sf9, derived from the pupal ovary cells of the Fall armyworm [5]. These cells are avid hosts to insect baculoviruses, which have been used to introduce genes for eucaryotic proteins into Sf9 cells under the control of a highly active viral polyhedrin gene promoter [6]. Several integral membrane proteins have been expressed in Sf9 cells, such as the *Drosophila* potassium channel Shaker [7], the multidrug resistance transporter [8] and several plasma membrane localized receptors [9–11]. However, no transport protein has been expressed functionally. In this paper we show that Sf9 cells infected with recombinant baculovirus containing the cDNA for the rabbit intestinal Na^+/glucose cotransporter (SGLT1) express this protein at high levels. We show that expressed protein transports glucose in a Na^+-dependent fashion, that transport is phlorizin sensitive and that all of the expressed protein is localized to a membrane associated protein fraction.

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Methods

Preparation of a transfer vector for in vivo homologous recombination

All molecular biology was carried out by standard techniques [12,13]. Transfer vector pVLSRC, obtained from T. Roberts at Harvard University, was digested with *Bam*HI, and the 5′ overhang converted to a blunt end with the Klenow fragment of DNA polymerase I. The linearized, blunt-ended vector was digested with *Nco*I. The product was electrophoresed on a 0.6% SeaChem (FMC, Rockland, ME) low-melt agarose minigel in TBE in the presence of ethidium bromide. The major band of 10 kb was visualized by UV illumination, excised and stored at −20°C.

The rabbit Na^+/glucose cotransporter cDNA, SGLT1, was excised from pBluescript [4]. The plasmid was linearized with *Xba*I, and the 5′ overhang filled in with the Klenow fragment of DNA polymerase I. The linearized, blunt ended plasmid was digested with *Nco*I under conditions favoring partial digestion, as SGLT1 also contains an internal *Nco*I site. Aliquots were removed and the reaction quenched at 10-min intervals. Products were electrophoresed on a 0.6% SeaChem low-melt agarose minigel containing ethidium bromide. The 2.2 kb band which corresponded to the *Nco*I-*Xba*I fragment of SGLT1 from the 5′ initiation codon to the end of the 3′ untranslated region was excised from the gel.

The linearized transfer vector (pVLSRC) and the SGLT1 cDNA were ligated in low melt agarose at a 1:1 ratio with T4 DNA ligase overnight at room temperature. The resultant transfer plasmid was called pVL17H, and was used to transform DH5α competent cells (Invitrogen, San Diego, CA), which were plated on LB containing ampicillin (50 μg/ml) plates and incubated overnight at 37°C. Colonies were isolated and grown in LB + ampicillin (50 μg/ml) liquid cultures. Plasmids were isolated by the boiling mini-prep method, and analyzed by restriction analysis and Southern blotting for the presence of the SGLT1 cDNA in the proper orientation in the transfer vector (see Fig. 1 for schematic presentation). Plasmid pVL17H was identified as containing the full-length cDNA of interest in the proper orientation relative to the polyhedrin promoter.

Homologous recombination

Techniques for generating recombinant virus and handling Sf- cells were derived from Summers and Luckow [14] and Piwnica-Worms [13]. Sf9 cells were maintained in an insect culture (TNM-FH) medium composed of Grace's insect medium containing, per liter, 3.3 gm Yeastolate, 3.3 gm lactalbumin, 10% fetal calf serum (FBS), 12500 units of penicillin, 12500 units streptomycin, and 5 ml Fungizone, all purchased from Gibco (Grand Island, NY). The transfer vector, pVL17H, was purified using CsCl density gradient centrifugation. The purified plasmid and purified viral genomic DNA (AcNPV) (Invitrogen, San Diego, CA) were combined in the presence of 1 mM calcium phosphate and added to a monolayer of $3 \cdot 10^6$ Sf9 cells (Gift of Dr. Christopher Miller, Brandeis University). Cells were incubated for 1 h, the virus was removed and TNM-FH added. Cells were incubated at 27°C for 96 h and resuspended. The cell suspension was centrifuged for 10 min. at 1000 × g and the viral supernatant stored at 4°C.

Purification of recombinant baculovirus

The virus supernatant was used to generate virus plaques in an SF9 cell monolayer. Monolayers of $3 \cdot 10^6$ cells were plated in NUNC 60 mm^2 Contur plates (Cole Scientific, Calabasas, CA) in 4 ml TNM-FH medium prepared as described in Summers and Luckow [14]. Cells were attached to plates at room temperature for 1 h, and washed once with TNM-FH without fetal calf serum (SMM). Cells were infected with virus by adding 1 ml viral dilutions from 10^{-3} to 10^{-5} in SMM. After 1 h of infection at room temperature, virus was removed and the cells were covered with a layer of 1.5% SeaPlaque low-melt agarose (FMC, Rockland, ME) in TNM-FH medium. Plates were left to solidify, then incubated at 27°C for 6–7 days. After day seven, plaques were scored visually under a light microscope for recombinant plaque morphology [13,14].

Recombinant plaques were isolated and added to individual wells of a 96-well microtiter plate containing $2 \cdot 10^4$ cells in 100 μl of TNM-FH medium. The viral isolates were incubated for 72 h, after which time the viral supernatants were transferred to duplicate microtiter plates and stored at 4°C. The infected cells were lysed in 200 μl of 0.5 M NaOH, triturated to solubilize, and the pH adjusted with 20 μl of 10 M ammonium acetate. 150 μl of cell lysate was transferred to a BA85 nitrocellulose filter using a Schleicher and Schuell (Keene, NH) vacuum blotter, and the wells were rinsed with 1 M ammonium acetate, 0.02 M NaOH. The nitrocellulose sheet was washed once in 4X SSC and allowed to air dry to dampness. DNA was fixed to the nitrocellulose by UV-crosslinking (Stratagene Stratalinker, La Jolla, CA.).

Identification of recombinant baculovirus

Nitrocellulose blotted with cell lysates from putative recombinant infected cells was probed with SGLT1 gel-purified cDNA labelled with ^{32}P-dCTP using an oligolabelling kit (Pharmacia). The probe was added and the filters were hybridized at 65°C for 3–4 h in 6 × SSC, 5 × Denhardts solution, 0.1% SDS, and 0.25 mg/ml salmon sperm DNA. Washes were as follows: three washes of 10 min each in 2 × SSC, 0.1% SDS at

room temperature, and three washes of 20 min each in 0.2× SSC, 0.1% SDS at 65°C. Filters were dried and exposed to Kodak X-O-Mat film overnight at −80°C for autoradiography.

Purification of recombinant baculovirus

Positive recombinants were identified from autoradiography and appropriate viral supernatants from duplicate microtiter plates were used for further rounds of plaque purification. 100 μl samples of viral supernatant were added to 900 μl of SSM for a 1× virus stock. Serial dilutions were carried out in SMM, and used to generate viral plaques as described. Plaque isolation and Southern blot analysis with SGLT1 were repeated until all plaques were of recombinant morphology and virus infected cell lysates showed a positive Southern blot signal when probed with SGLT1 cDNA. After purification, virus was titered by 10^8-fold serial dilution in SMM and plaqued. Titer was counted as the number of plaques/ml of infecting virus multiplied by the dilution factor.

Generation of SGLT1 protein product

$3 \cdot 10^6$ cells were plated in monolayer and infected with recombinant virus at a multiplicity of infection (MOI) of 2. Infected cells were removed from the dish, centrifuged for 10 min at 1000×*g* and the supernatant removed. Cell pellets were lysed with 0.5 ml RIPA (1% NP-40, 1% deoxycholate, 0.1% SDS, 10 mM NaP_i (pH 7.2), and 150 mM NaCl) or Laemmli sample buffer (15) and stored at −20°C.

Metabolic labeling studies

Cells were plated at a density of $2 \cdot 10^6$/plate in 6-well Costar (Cambridge, MA) multiwell polystyrene culture dishes and allowed to attach for 1 h at room temperature. Culture medium was removed and cells infected with 1 ml of virus at an MOI of 2 in SMM. Cells were incubated for 1 h at room temperature and the virus removed. 3 ml of TNM-FH were added per well and the plates returned to 27°C for 48 h. At 48 h post-infection the cells were washed once with methionine-free medium, and resuspended in 1 ml of methionine-free medium containing 0.25 mCi/ml [^{35}S]-methionine (Trans-Label, Amersham, Arlington Heights, IL). Plates were returned to 27°C for 3 h. Cells were then removed from the dish and centrifuged for 2 min at 1000×*g*. Cells were resuspended and washed in (in mM): 150 NaCl, 2.5 KCl, 10 Na^+/K^+ P_i (pH 7.4) (PBS). The cell pellets were lysed with 0.2 ml RIPA or 0.5 ml Laemmli sample buffer [15] and frozen at −20°C. Samples lysed in Laemmli sample buffer were sonicated for 10–30 s in a bath sonicator to reduce viscosity.

Gel electrophoresis and Western blot analysis of recombinant proteins

Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE [15]. Samples were boiled for 3' immediately before loading. Gels to be analyzed by autoradiography were fixed in 1% glycerol, 10% methanol, 1% acetic acid and dried under vacuum. Gels were exposed to Kodak X-O-Mat film overnight at −80°C without enhancement. Duplicate gels were stained with Coomassie brilliant blue.

Gels to be used for Western blot analysis were transferred to Schleicher and Schuell nitrocellulose transfer membrane BA85 [16]. Western blotting was carried out with an anti-peptide antibody raised to residues 602 to 613 in the C-terminal portion of the protein (Ab-C) as described by Hirayama et al. [17]. Blocking solution was 0.5% Carnation instant milk, 0.05% Tween 20 in PBS, heated to 65°C, cooled and filtered through Whatman No. 1 filter paper. Primary antibody dilutions were 1:200 or 1:500 from serum and secondary antibodies were Calbiochem (La Jolla, CA) anti-IgC linked to horseradish peroxidase, used at 1:200 or 1:500 dilution, with hydrogen peroxide and diaminobenzidine as substrates.

Analysis of glucose transport

Cells were infected at a MOI of 2 for 48 h. Cells were resuspended from monolayers and aliquoted to 10^6 cells/tube in 1.5 ml Eppendorf centrifuge tubes, centrifuged for 2 min at 1000×*g*. Cells were washed twice with 1 ml of glucose-free Grace's insect medium (Special order, Gibco, Grand Island, NY), unsupplemented with yeastolate, lactalbumin or FBS. The cells were then resuspended in 0.25 ml of uptake solution (see legend, Fig. 5 for detailed composition) containing 50 μM α-methyl-D-glucose (αMDG), with 2.5 μCi/ml ^{14}C-αMDG. Cells were incubated in this medium for 15 min, 1 ml of ice-cold stop solution added and the sample immediately centrifuged for 1 min at 1500×*g*. The cells were washed twice in 1 ml of ice-cold stop solution. The resulting cell pellet was lysed in 200 μl of 10% SDS and 150 μl were assayed by scintillation counting. The other 50 μl were frozen immediately at −20°C and used to measure cellular protein concentration using a modified Lowry assay [18].

Fractionation of Sf9 cells expressing recombinant SGLT1 gene product

10^7 confluent cells at 48 h post-infection were centrifuged at 1000×*g* for 10 min, and resuspended in 4 ml ice-cold phosphate-buffered saline (PBS). The suspension was homogenized on ice 2× 10 s on setting 9 of a Polytron homogenizer, with a 10 s cooling period between homogenizations. The homogenate was loaded onto two continuous sucrose density gradients from 10% to 65% sucrose (w/w). The sample was cen-

trifuged in an SW28 rotor overnight at 100000 × *g*. Fractions were collected by an autosampler and frozen immediately. Protein concentrations were determined by a modified Bradford assay (Bio-Rad, Richmond, CA) and the presence of the SGLT1 protein product was assayed by Western blot analysis as described above.

Results

Production of SGLT1 recombinant baculovirus

We utilized the recombinant transfer vector pVL17H to produce a recombinant baculovirus in which the rabbit intestinal SGLT1 cDNA replaced the polyhedrin gene in the genome of a wild-type AcNPV (Fig. 1), and was thus under the control of the polyhedrin gene promoter. The 12.2 kb transfer plasmid pVL17H contained the SGLT1 cDNA including the full-length coding region from the initiation ATG, the entire 3′ untranslated region including the poly A tail and the

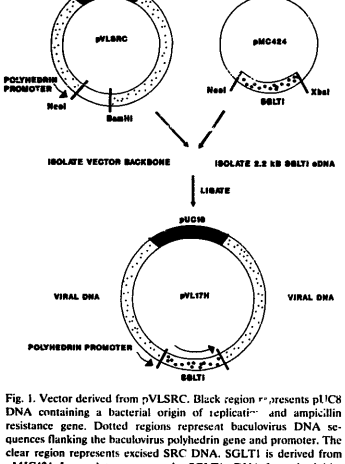

Fig. 1. Vector derived from pVLSRC. Black region represents pUC8 DNA containing a bacterial origin of replication and ampicillin resistance gene. Dotted regions represent baculovirus DNA sequences flanking the baculovirus polyhedrin gene and promoter. The clear region represents excised SRC DNA. SGLT1 is derived from pMJC424. Large dots represent the SGLT1 cDNA from the initiation 5′ ATG to the *Xba*I restriction site downstream of the SGLT1 3′ untranslated region. pVL17H is the transfer used in viral recombination, which derived from ligation of pVLSRC and SGLT1. Arrows represent direction of transcription from the polyhedrin promoter and SGLT1.

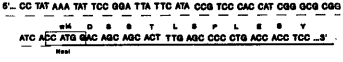

Fig. 2. Sequence of the junction between pVLSRC and SGLT1 5′ end in pVL17H. Double-stranded plasmid (pVL17H) was sequenced using a T7-sequencing kit (Pharmacia) and a SGLT1 sequence-specific primer. The dashed underlined region represents cDNA sequence derived from pVLSRC. Boxed region is the *Nco*I site joining pVLSRC with the SGLT1 5′ end. Solid underlined region is the cDNA sequence of the 5′ end of SGLT1. Asterisk denotes the SGLT1 translation initiation site. Capital letters above the cDNA sequence are single letter amino acid codes.

*Eco*RI-*Xba*I linker region from pBluescript. Fig. 2 shows the DNA sequence of pVL17H at the 5′ end of the SGLT1 insert, demonstrating that plasmid construction removes the 5′ untranslated sequence from SGLT1 while retaining the 5′ *Nco*I restriction site and the ATG initiation codon. The sequence upstream of the SGLT1 initiation codon is identical to that published for pVL941 [6], the original vector from which pVLSRC was derived [19].

pVL17H plasmid DNA and wild-type AcNPV DNA were used in a Ca^{2+}-phosphate cotransfection procedure in the Sf9 cell host [14]. The viral sequences in the transfer vector underwent homologous recombination with the AcNPV genomic DNA and gave rise to recombinants containing SGLT1. These recombinant viruses were isolated by plaque assays and confirmed by Southern blot analysis with SGLT1 as a probe. The initial screen showed 3% recombinant virus, which is consistent with predicted frequencies for cotransfection. Five rounds of recombinant screening were used to purify six individual isolates. These isolates were designated BV-SGLT1/C1 through BV-SGLT1/C6. These recombinants were also screened individually for the occlusion (-) phenotype, and by [^{35}S]methionine metabolic labeling of infection products for the absence of the wild-type polyhedrin protein. Isolates C1–C6 were selected for high purity and equivalent levels of expression. They have been used interchangeably for expression and transport studies with no discernable difference in stability or expression levels.

Expression of recombinant SGLT1 in BV-SGLT1 infected Sf9 cells

Anti-peptide antibodies raised to sequences from SGLT1 were used to monitor the appearance of SGLT1 protein expressed by recombinant infected cells. In a preliminary experiment, Sf9 cells were infected with wild-type virus or BV-SGLT1/C2 viral isolate at a MOI of 2, for 48 h. Cells were metabolically labeled with [^{35}S]methionine and solubilized. The whole cell lysates were analysed by SDS-PAGE, transferred to nitrocellulose and Western blotted with the antipeptide antibody Ab-C. The transfers were also analyzed

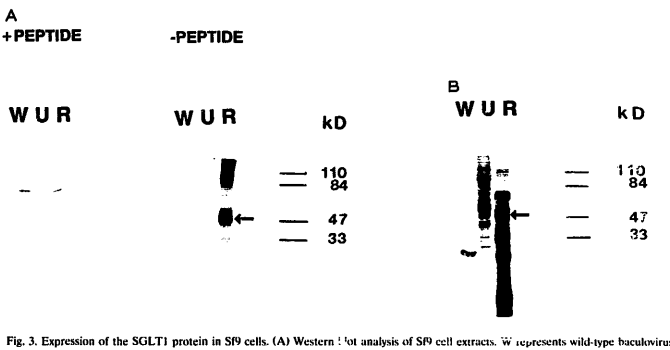

Fig. 3. Expression of the SGLT1 protein in Sf9 cells. (A) Western blot analysis of Sf9 cell extracts. W represents wild-type baculovirus infected cells, U represents uninfected cells and R is BV-SGLT1 recombinant baculovirus infected cells. Gels are 10% acrylamide, and protein samples are 1/200 of total cell extract from $3 \cdot 10^6$ cells solubilized in RIPA, mixed with equal volume of 2X Laemmli sample buffer and boiled for 3'. Western blots were probed with Ab-C at 1:200 dilution from serum, either preabsorbed with 5 μg/ml peptide (left) or unabsorbed (right). (B) Autoradiogram of nitrocellulose transfer from Fig. 3A. Cells were labeled in culture with [^{35}S]methionine for 3 h at 48 h post-infection, separated by SDS-PAGE in 10% acrylamide and detected by autoradiography as described in Methods.

by autoradiography. Fig. 3A shows the major immunoreactive band of 50–60 kDa which was not present in uninfected or wild-type virus infected cells, or in blots probed with Ab-C preabsorbed with the peptide antigen. Two other bands of 110 kDa and 220–250 kDa were also detected. These may be aggregates of the

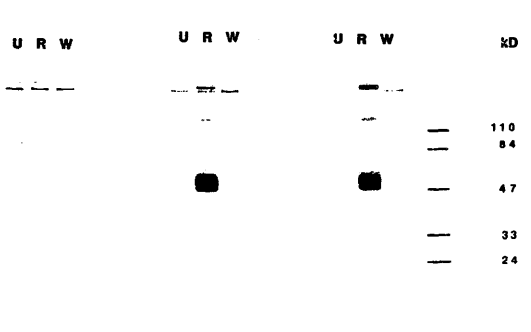

Fig. 4. Time course of SGLT1 protein expression. Cells were infected as described in Methods, and harvested at 24, 48 and 77 h post-infection. $6 \cdot 10^6$ cells were lysed with Laemmli sample buffer (0.5 ml), boiled for 3 min and 4 μl samples applied to a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose as described and probed with Ab-C at 1:500 dilution from serum. U = un infected cells. W = wild-type baculovirus infected cells and R = SGLT1 recombinant infected cells. Time is hours after viral infection. Samples were processed together from frozen lysates.

monomeric form or undisrupted multimers of native transporter, and were observed when cells were initially solubilized in non-ionic detergents. Cells solubilized by sonication in Laemmli sample buffer had vastly decreased levels of these aggregate species (see Fig. 4). The autoradiogram of this transfer in Fig. 3B shows a protein migrating at 50–60 kDa which has been labeled by [^{35}S]methionine. This band exactly corresponds to the major immunoreactive band, and was not present in wild-type or uninfected cells. The recombinant SGLT1 gene product synthesized by the Sf9 cells had a lower molecular mass than the rabbit intestinal Na^+/glucose cotransporter, but was close in size to the *in vitro* translation product of SGLT1 in the absence of microsomes, and less than the *in vitro* molecular weight in the presence of dog pancreatic microsomes [26].

Time course of SGLT1 expression in Sf9 cells

We examined the time-dependence of expression of the SGLT1 protein in Sf9 cells to determine optimal conditions for protein production. BV-SGLT1/C2 infected Sf9 cells, AcNPV-infected and uninfected control cells were harvested at 24, 48 and 77 h post-infection. Whole-cell lysates were analyzed by SDS-PAGE, transferred to nitrocellulose and probed with antibody Ab-C. Fig. 4 shows no immunoreactivity in uninfected, AcNPV infected or BV-SGLT1/C2 infected cells at 24 h post-infection. However, BV-SGLT1/C2 infected cells had high levels of immunoreactivity at 48 h post-infection. At 77 h, the immunoreactivity decreased slightly relative to the 48 h signal. No degradation products were observed on the Western blot at 77 h. The virus enters the lytic stage between 60 and 80 h post-infection, after which time over-all rate of protein synthesis is decreased, and cell lysis may cause significant loss of protein. This observed maximal protein production at day 2 post-infection is unusual for the AcNPV-mediated gene expression of foreign genes in Sf9 cells. Several groups have reported integral membrane protein accumulation up to 4 days post-infection [7,8].

Transport function of SGLT1 expressed in Sf9 cells

The SGLT1 protein synthesis has been clearly demonstrated. However, we wished to measure transport function in these cells. Sf9 cells normally take up D-glucose from the growth medium, in a saturable process with a $T_{1/2} = 15$ min, which is not dependent on external sodium, is phlorizin insensitive and inhibited by phloretin and cytochalasin B (data not shown). The equilibrium cell volume for [^{3}H]glucose uptake was $4.7 \cdot 10^{-5}$ μl/cell, which is comparable to the calculated volume of $6.5 \cdot 10^{-5}$ μl/cell for a 50 μm diameter cell. Fig. 5 shows the specific, sodium-dependent transport of ^{14}C-αMDG in uninfected, wild-type AcNPV-infected and BV-SGLT1-infected Sf9 cells.

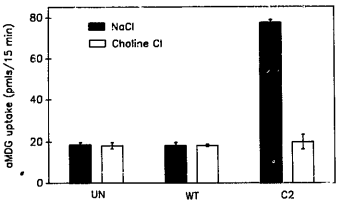

Fig. 5. Functional expression of the Na^+/glucose cotransporter in recombinant infected Sf9 cells. Na^+-dependent α-methyl-D-glucopyranoside (αMDG) uptake into Sf9 cells as described in Methods. Na^+ uptake solution is (mM) 122 NaCl, 10 Mes (pH 6.5) with Tris, 3 $CaCl_2$, 5 $MgCl_2$, 5 fructose, 50 μM αMDG, and 2.5 μCi/ml ^{14}C-αMDG. Choline uptake solution is identical, with 122 mM choline chloride substituted for NaCl. Stop solution is choline chloride uptake solution without αMDG at 4°C. Transport is reported as ^{14}C-αMDG taken up in 15 min at 22°C. Data are the mean ± S.D. of triplicate samples.

The uninfected and wild-type infected cells showed only sodium-independent transport, while the BV-SGLT1-infected cells showed dramatically elevated levels of sodium-dependent transport. After incubation for 15 min, the intracellular concentration of αMDG was calculated to be 1.22 μM, nearly 50-fold lower than the equilibrium value of 50 μM, suggesting that we were measuring initial rates of transport. Transport rates in Sf9 cells were ≈ 1300 pmol/mg per h, which are comparable to initial rates observed in *Xenopus* oocyte transport experiments [4], when normalized to a literature value of 300 μg protein/oocyte [20].

Phlorizin, a specific, high affinity inhibitor of the Na^+/glucose cotransporter in kidney and intestine [21],

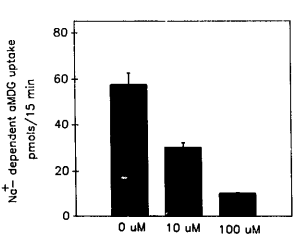

Fig. 6. Phlorizin inhibition of expressed Na^+/glucose cotransport. Phlorizin transport inhibition experiments are carried out in NaCl or choline-Cl uptake solution with the appropriate concentration of phlorizin dissolved directly into the aqueous uptake solution. Transport is described as the difference between αMDG uptake in NaCl and choline-Cl uptake solution after 15 min at 22°C. Data are the mean ± S.D. of triplicate samples. A K_i of 10 μM is estimated.

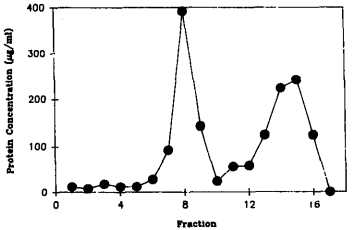

Fig. 7. Fractionation of Sf9 cells expressing SGLT1. Cells were harvested at 48 h post-infection and washed once with PBS. Cells were resuspended in 4 ml of PBS (approx. $2 \cdot 10^6$ cells/ml), homogenized by polytron on ice for two times 10 s, and fractionated by sucrose gradient density centrifugation from 15 to 65% as described. The figure shows the distribution of protein as determined from a modified Bradford protein assay of sucrose density gradient fractions. Fraction 3 corresponds to 15% and fraction 17 to 65% sucrose.

was observed to inhibit sodium-dependent αMDG transport in cells infected with BV-SGLT1/C7. Fig. 6 shows the inhibition at 10 μM phlorizin to be 50% of control values. This concentration approximates the published K_i of 8–11 μM for αMDG transport inhibition in intestinal brush border membrane vesicles [21].

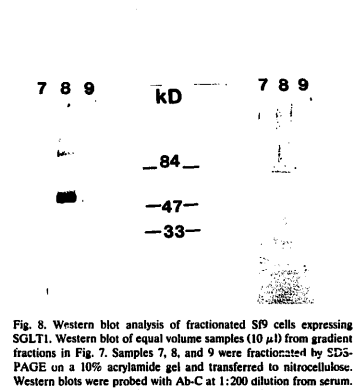

Fig. 8. Western blot analysis of fractionated Sf9 cells expressing SGLT1. Western blot of equal volume samples (10 μl) from gradient fractions in Fig. 7. Samples 7, 8, and 9 were fractionated by SDS-PAGE on a 10% acrylamide gel and transferred to nitrocellulose. Western blots were probed with Ab-C at 1:200 dilution from serum. All specific immunoreactivity was present in Fraction 8, which corresponds to a density of 45% sucrose (w/w).

Phlorizin had no effect on αMDG transport in uninfected or wild-type infected cells (data not shown).

Fractionation of SGLT1 gene product expressed in Sf9 cells

Sf9 cells infected for 48 h were harvested, homogenized and fractionated by sucrose density gradient centrifugation, on a 10–65% gradient (w/w). Fig. 7 shows a bimodal protein distribution through the sucrose density gradient. A broad peak containing 53% of the total protein was present in the low density region of the gradient. This peak contains the Sf9 cell cytosolic proteins. The peak fraction, at $\approx$45% sucrose, contained 25% of the total protein. This peak, Fraction 8, contained all of the immunoreactivity in the BV-SGLT1-infected cells (Fig. 8). The fractions flanking this sharp peak contained the remaining 22% of the cellular protein and showed no immunoreactivity. The high density, immunoreactive fraction was pelletable in the ultracentrifuge at $100\,000 \times g$, and immunoreactivity pelleted with the protein. This fractionation represented a 4-fold enrichment of immunoreactive protein over whole-cell homogenate.

Discussion

We wished to establish a high level expression system for the rabbit intestinal Na^+/glucose cotransporter suitable for protein purification and transport studies. This protein has been cloned and functionally expressed in *Xenopus* oocytes and COS cells, but has not been purified to homogeneity, nor has it been expressed in a system from which it can be readily purified. This report describes the functional expression of the SGLT1 protein in Sf9 cells using the baculovirus expression system, and provides a preliminary characterization of the expressed protein. To our knowledge, this is the first report of a functioning eucaryotic ion-dependent transport protein expressed in Sf9 cells.

pVL17H represents a new baculovirus transfer vector suitable for introducing foreign genes into the AcNPV genome. It contains an *Nco*I cloning site adjacent to the polyhedrin promoter region, identical to pVLSRC. However, pVLSRC contains a limited number of cloning sites 3′ of the gene sequence. pVL17H contains a portion of the polylinker region of pBluescript (Stratagene) with restriction sites for *Pst*I, *Sma*I, *Bam*HI and *Spe*I, increasing the number of available unique cloning sites significantly.

The SGLT1 protein was expressed at maximum levels at 48 h post-infection. The protein does not continue to accumulate past 48 h, but remained at a roughly constant level through 77 h. We see no evidence of immunoreactive degradation products at later time points. This result suggests that the protein is

stable in these cells, but does not continue to accumulate. The limitation in the expression level of SGLT1 may be overcome with changes in the growth medium to eliminate substrates for the glucose transporter, which may be causing the cellular osmotic load to become unbalanced due to accumulation of sodium and glucose. In preliminary experiments using light and electron microscopy, cells infected with BV-SGLT1 appeared swollen. We estimate from intensity of Western blots compared to brush border membrane vesicles that the protein is now expressed at ≈ 0.5% of the Sf9 cell protein. This level of expression is 100-fold higher than the protein level in rabbit intestinal mucosal cells.

Preliminary experiments show that, while a monomeric form of the transporter is expressed, higher molecular forms are also observed upon Western blot analysis. Two predominant forms are seen. One band is approximately twice the molecular mass of the monomer, and the other is a higher molecular mass of ≈ 240 kDa. These polymeric forms may represent aggregation products due to the non-denaturing solubilization conditions used to lyse the cells. They may also represent stable homopolymeric forms of the transporter. Radiation inactivation studies have suggested that the functional protein is a homotetramer [22]. Danblot et al. [23] observe a similar phenomenon, where solubilization in non-ionic detergents produced monomeric, dimeric and tetrameric forms of a Na^+-glutamate cotransporter from rat brain plasma membranes. These multimers were not reduced to monomers when proteins were boiled with 2% SDS in Laemmli sample buffer. We plan to pursue crosslinking experiments on this protein to verify the existence of a homotetrameric functional unit in the plasma membrane.

The molecular mass of the transporter is ≈ 70 kDa in the brush border plasma membrane. Substrate protected FITC labeling [24] and immunoreactivity with anti-peptide antibodies raised to the amino acid sequence of the clone [17] both detected a single broad band of molecular mass 70 kDa. A functionally related protein purified from pig kidney cells also shares this molecular mass [25]. The protein expressed in Sf9 cells migrated as a broad band with an apparent molecular mass in SDS-PAGE of 55 kDa. This discrepancy in molecular mass between the intestinal protein and the SGLT1 gene product was initially surprising, considering that the primary sequence of the gene codes for a protein of 73 kDa. However, Hirayama and Wright [26] have shown that the native rabbit intestinal protein is heavily glycosylated with N-linked carbohydrates, and that these carbohydrates can be removed chemically and enzymatically. The protein detected after chemical or enzymatic deglycosylation has a molecular mass of measured for *in vitro* translation product of the SGLT1 cRNA (45–50 kDa). The anomalous enhanced migration of integral membrane proteins on SDS-PAGE compared to soluble proteins has been well documented, particularly in the eel electroplax sodium channel [27]. Therefore, we conclude that the molecular weight of the protein in Sf9 cells is consistent with the intestinal brush border transporter and the SGLT1 gene product after limited or no glycosylation.

These data suggest that the extensive glycosylation of the transporter in rabbit intestine is not critical for function, since the expressed protein is functional with minimal, if any glycosylation. Other experimental evidence corroborates this conclusion. Enzymatic deglycosylation of native brush border membrane vesicles does not inhibit transport activity [26] and altering the cDNA sequence of SGLT1 to eliminate the arginine employed in N-linked glycosylation does not eliminate the Na^+-dependent glucose transport activity [28]. This raises interesting questions about the role of such extensive glycosylation in the intestinal brush border membrane.

The transporter is functionally expressed in cells infected with BV-SGLT1 by two criteria. First, the cells carry out Na^+-dependent transport of αMDG, a specific substrate, only when infected with recombinant virus. Second, the transport is inhibited by phlorizin, a specific inhibitor of the Na^+/glucose cotransporter, in the micromolar range. The Na^+-dependent transport rate was ≈ 1300 pmol/mg per h, which is comparable to expression of SGLT1 cRNA in *Xenopus* oocytes. This expression level suggests to us that we have developed an excellent system for further characterization of the functional transport protein.

To this end we have begun fractionation experiments, in which we have achieved a 4-fold purification of the transporter from whole-cell homogenates. Knops et al. [29] have reported the functional purification of β-amyloid precursor protein expressed in the Sf9 cell system using standard membrane fractionation techniques. Our goal is to purify large amounts of functional Na^+/glucose cotransporter protein to homogeneity for structural studies, and to use the purified protein to produce polyclonal antibodies for immunohistochemistry and structure-function studies on this prototypic ion-coupled solute transport protein.

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References

1 Stevens, B.R., Kaunitz, J.D. and Wright, E.M. (1984) Annu. Rev. Physiol. 46, 417–433.
2 Crane, R.K. (1962) Fed. Proc. 21, 891–895.
3 Crane, R.K. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 99–159.
4 Hediger, M.A., Coady, M.J., Ikeda, T.S. and Wright, E.M. (1987) Nature 33, 379–381.
5 Luckow, V.A. and Summers, M.D. (1988) Biotechnology 6, 47–55.
6 Luckow, V.a. and Summers, M.D. (1989) Virology 170, 31–39.
7 Klaiber, K., Williams, N., Roberts, T.M., Papazian, D.M., Jan, L.Y. and Miller, C. (1990) Neuron 5, 221–226.
8 Bermann, U.A., Willingham, M.C., Pastan, I. and Gottesman, M.M. (1990) Biochemistry 29, 2295–2303.
9 George, S.T., Arbabian, M.A., Ruoho, A.E., Kiely, J. and Malbon, C.C. (1989) Biochem. Biophys. Res. Commun. 163, 1265–1269.
10 Atkinson, A.E., Earley, F.G.P., Beadle, D.J. and King, L.A. (1990) Eur. J. Biochem. 192, 451–458.
11 Sissom, J. and Ellis, L. (1989) Biochem. J. 261, 119–126.
12 Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.
13 Piwnica-Worms, H. (1990), in Current Protocols in Molecular Biology. Supplement 10, pp. 16.8.1–16.11.7.
14 Summers, M.D. and Smith, G.E. (1987) A manual of methods for baculovirus and insect cell culture procedures. Texas Agricultural Experimental Station Bulletin No. 1555, College Station, Texas.
15 Leamnli, U.K. (1970) Nature. 227, 680–685.
16 Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci., USA 76, 4350–4354.
17 Hirayama, B.A., Wong, H.C., Smith, C.D., Hagenbuch, B.A., Hediger, M.A. and Wright, E.M. (1991) Am. J. Physiol. 261, c296–C304.
18 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
19 Piwnica-Worms, H., Williams, N.G., Cheng, S.H. and Roberts, T.M. (1990) J. Virology 64, 61–68.
20 Wall, D.A. and Patel, S. (1989) J. Memb. Biol. 107, 189–201.
21 Ikeda, T.S., Hwang, E.S., Coady, M.J., Hirayama, B.H., Hediger, M.A., and Wright, E.M. (1989) J. Membr. Biol. 110, 87–95.
22 Stevens, B.R., Fernandez, A., Hirayama, B., Wright, E.M. and Kempner, E.S. (1990) Proc. Natl. Acad. Sci. USA 87, 1456–1460.
23 Danbolt, N.C., Pines, G. and Kanner, B.I. (1988) Biochemistry 29, 6734–6740.
24 Peerce, B.P. and Wright, E.M. (1984) Proc. Natl. Acad. Sci. USA 81, 2223–2226.
25 Wu, J.S. and Lever, J.E. (1987) Biochemistry 26, 5958–5962.
26 Hirayama, B.A. and Wright, E.M. (1992) Biochim. Biophys. Acta 1103, 37–44.
27 Thornhill, W.B. and Levinson, S.R. (1987) Biochemistry 26, 4381–4388.
28 Hediger, M.A., Mendlein, J., Lee, H.-J. and Wright, E.M. (1991) Biochim. Biophys. Acta 1064, 360–364.
29 Knops, J., Johnsonwood, K., Schenk, D.B., Sinha, S., Leiberburg, I. and McConlogue L. (1991). J. Biol. Chem. 266, 7285–7290.