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Identification of a novel sodium-dependent fructose transport activity in the hepatopancreas of the Atlantic lobster *Homarus americanus*

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SUMMARY

[^{3}H]Fructose and [^{3}H]glucose transport were determined in brush-border membrane vesicles (BBMV), basolateral membrane vesicles (BLMV) and isolated cells (E, R, F, B) of *H. americanus* (Atlantic lobster) hepatopancreas. Glucose transport in BBMV was equilibrative in the absence of sodium and concentrative in the presence of sodium. Sodium-dependent glucose transport by BBMV was not inhibited by a tenfold molar excess of fructose. Glucose transport by BLMV was equilibrative and sodium independent. Fructose uptake by BBMV and BLMV was equilibrative in the absence of sodium and concentrative in the presence of sodium. This enhancement was not affected by a tenfold molar excess of glucose in the presence of sodium. E-, F- and B-cells showed sodium-dependent uptake of fructose, while R-cells did not. Sodium-dependent fructose uptake by E-cells was not inhibited by a tenfold molar excess of glucose or mannose. Western blot analysis of BBMV, BLMV and E-, R-, F- and B-cells using rabbit polyclonal antibodies directed against epitopes of mammalian GLUT2, GLUT5, SGLT1 and SGLT4 indicated the presence of cross-reacting lobster proteins. Sequence alignment of the mammalian proteins with translated, lobster expressed sequence tags also indicated significant identity between species. Comparison of fructose and glucose uptake in the absence and presence of sodium by BBMV, BLMV and isolated cells indicated the presence of a distinct sodium-dependent transport activity for each sugar in the Atlantic lobster.

Key words: crustacean, sugar transport, glycosylation, symporter, sodium-dependent co-transport, GLUT, SGLT.

INTRODUCTION

Little is known about sugar absorption in arthropods. Early studies with insect gut suggested that sugars were absorbed totally by diffusion without involving carrier proteins in the process (Treherne, 1957; Treherne, 1958). More recently, several studies involving both physiology and molecular biology have described a number of sugar transporters of the GLUT family in insects from several different orders (Caccia et al., 2005; Chen et al., 2006; Escher and Rasmuson-Lestander, 1999; Pascual et al., 2006; Wang and Wang, 1993). The first report describing a detailed model for carrier-mediated sugar movement across an absorbing insect epithelium was published in 2007 (Caccia et al., 2007). These authors used a combination of radiolabelled D-glucose and D-fructose flux measurements, brush-border and basolateral epithelial membranes and immunocytochemical localization, and immunoblotting techniques to describe the sugar carrier suite present in larval parasitoid wasp (*Aphidius ervi*, Hymenoptera) midgut epithelial cells. SGLT1-like and GLUT5-like transporters were localized to the intestinal brush-border membrane, while GLUT2-like proteins were present in both basolateral and brush-border membranes, strikingly similar to the arrangement of these sugar carrier proteins in mammalian intestine (Wright and Turk, 2004; Uldry and Thorens, 2004).

The crustacean hepatopancreas is the major site of nutrient absorption from digestive products generated within the stomach and hepatopancreas. Nutrients are transferred across the epithelial lining from the hepatopancreas to the haemolymph for tissue distribution (Wright and Ahearn, 1997). The histology, ultrastructure and histochemistry of the crustacean hepatopancreas indicate that there are at least four distinct epithelial cell types classified as E-cells (embryonic), F-cells (fibrillar), R-cells (resorptive) and B-cells (blister) (Verri et al., 2001; Jacobs, 1928). The four cell types of the epithelium of the crustacean hepatopancreas act as a physiological suite, providing the functions of the vertebrate stomach, intestine, liver and pancreas (Van Weel, 1974; Paquet et al., 1993; Wright and Ahearn, 1997).

Over the past two decades, nutrient transport processes across the tubules of the hepatopancreas have been characterized for sugars (Ahearn et al., 1985; Verri et al., 2001), amino acids (Ahearn, 1992) and peptides (Thamotharan and Ahearn, 1996). Two *in vitro* methods have been applied to the study of sugar transport by crustacean hepatopancreatic epithelium: purified brush-border membrane vesicles (BBMV) and purified single cell type suspensions of hepatopancreatic epithelial cells. The use of purified BBMV of crustacean hepatopancreatic epithelial cells to investigate D-glucose transport physiology has been reported in two species, a shrimp (*Penaeus japonicus*) (Blaya et al., 1998; Verri et al., 2001) and a lobster (*Homarus americanus*) (Ahearn et al., 1985; Verri et al., 2001). Glucose transport in both species was carrier mediated, sodium dependent, and stimulated by an inside-negative membrane potential. D-Glucose influx in lobster vesicles was stimulated by an increase in proton concentration (decreased pH) as would occur *in vivo*, and a Hill coefficient of approximately 2.0 was disclosed for its dependence on external sodium concentration, suggesting a sodium:sugar transport stoichiometry of 2:1 (Ahearn et al., 1985; Verri et al., 2001). Sodium-dependent D-glucose uptake by shrimp vesicles was significantly inhibited by high concentrations (5 mmol l^{-1}) of different sugar derivatives and showed the following extent of

inhibition: phloridzin>β-methyl-D-glucopyranoside>α-methyl-D-glucopyranoside=D-glucose>6-deoxy-D-galactose=3-*O*-methyl-D-glucose=2-deoxy-D-glucose>D-galactose>glucosamine (Verri et al., 2001). All these data closely resemble those reported for sugar transport by SGLT1 in mammalian small intestine (Ikeda et al., 1989). However, as reported here, there are interesting differences with respect to mammalian hexose-transporting mechanisms. For example, lobster hepatopancreas E-cells have a unique sodium-dependent fructose co-transport activity (compared with glucose and mannose) and only an equilibrative (possibly facilitative) transport mechanism for glucose, while F-cells appear to have sodium-dependent co-transport activities for glucose (Verri et al., 2001) and fructose. We have also identified expressed lobster proteins with significant identity to mammalian sugar transporters.

MATERIALS AND METHODS

Animals

Live lobsters (*Homarus americanus* Milne-Edwards, 0.5 kg each) were purchased from a commercial dealer (Fisherman Wharf, Jacksonville, FL USA) and maintained in filtered seawater holding tanks at 15°C until needed. Lobsters were fed frozen mussel meat several times a week.

Isolation of E-, R-, F- and B-cells from the lobster hepatopancreas

Hepatopancreas was minced in 100 ml of extraction buffer (27 mmol l^{-1} sodium citrate, 396 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 5 mmol l^{-1} NaH_2PO_4, 5.6 mmol l^{-1} Na_2SO_4, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} PMSF, pH 7.1, osmolarity 900 mosmol l^{-1}) (Fiandra et al., 2006) on ice and then shaken at 110 r.p.m. on ice for 20 min. The cell suspension was filtered through two layers of cheese cloth. The filtrate was centrifuged at 100 ***g*** for 10 min. The pellet was resuspended in 2–4 ml of separation buffer (399 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 1 mmol l^{-1} NaH_2PO_4, 4 mmol l^{-1} $NaHCO_3$, 8.4 mmol l^{-1} Na_2SO_4, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, pH 7.1, osmolarity 900 mosmol l^{-1}) (Fiandra et al., 2006).

The resulting mixed cell suspension was applied to a sucrose step gradient consisting of equal volumes of 30%, 40%, 50% and 80% sucrose in separation buffer and centrifuged at 50,000 ***g*** for 20 min in an SW 41 swinging bucket rotor (Beckman Coulter, Fullerton, CA, USA).

E-cells migrated to the top of the 30% sucrose step, R-cells migrated to the interface of the 30% and 40% steps, F-cells migrated to the interface of the 40% and 50% steps and B-cells migrated to the interface of the 50% and 80% steps. The identity of the separated cells at each sucrose step was determined by morphological examination on the basis of the distinct cytological features (Loizzi, 1971) of each cell type. The purity of each cell type was greater than 90%. Viability of each cell type was greater than 90% as determined by Trypan blue exclusion.

Fructose and glucose transport by lobster hepatopancreas cells

Each cell type was resuspended and washed 5 times with sodium-minus outside buffer: (410 mmol l^{-1} choline chloride, 15 mmol l^{-1} KCl, 5 mmol l^{-1} $CaSO_4$, 10 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $KHCO_3$, 5 mmol l^{-1} Hepes-KOH at pH 7.1) (Fiandra et al., 2006). Equal amounts of each cell type at 1.0 mg protein ml^{-1} (0.02 ml) were assayed for D-[^{3}H]fructose (1.0 mmol l^{-1}) or D-[^{3}H]glucose (1.0 mmol l^{-1}; E-cells only) uptake in either sodium-minus outside buffer or sodium-plus outside buffer (0.18 ml) from 0 to 10 min. Uptake was halted by the addition of 2.0 ml ice-cold stop buffer (410 mmol l^{-1} choline chloride, 15 mmol l^{-1} KCl, 5 mmol l^{-1} $CaSO_4$, 10 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $KHCO_3$, 5 mmol l^{-1} Hepes-KOH at pH 7.1, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA). Replicates (five) for each time point were filtered under vacuum through pre-wetted cellulose ester filters (0.65 μm pore size, GE, Schenectady, NY, USA). Radioactivity retained by the filters after washing was measured (c.p.m.) in a scintillation counter (LS 6500 Multi-purpose Scintillation Counter, Beckman Coulter). All transport values were corrected for a 'blank' obtained by adding the incubation mixture directly to the stop solution before filtration.

Lobster hepatopancreas BBMV preparation

BBMV were prepared as described previously (Ahearn et al., 1985), based on the Mg^{2+} precipitation technique developed by Kessler and colleagues (Kessler et al., 1978) and Biber and colleagues (Biber et al., 1981) for mammalian epithelia. Lobster hepatopancreas was homogenized for 3 min at high speed in a Waring blender in ice-cold homogenizing buffer 1 (60 mmol l^{-1} mannitol, 12 mmol l^{-1} Tris-HCl, pH 7.4, 1.64 mmol l^{-1} EGTA, 1 mmol l^{-1} PMSF). The homogenate was centrifuged at 27,000 ***g*** for 30 min at 4°C. The pellet was resuspended in ice-cold homogenizing buffer 1 and homogenized with five up and down strokes of a Potter-Elvehjem tissue grinder at high speed. The homogenate was centrifuged at 27,000 ***g*** for 30 min at 4°C. The pellet was resuspended in ice-cold homogenizing buffer 1 and homogenized with 15 up and down strokes of a Potter-Elvehjem tissue grinder at high speed. The supernatant was then centrifuged at 27,000 ***g*** for 30 min. The pellet was resuspended in homogenizing buffer 2 (60 mmol l^{-1} mannitol, 12 mmol l^{-1} Tris-HCl, pH 7.4, 6 mmol l^{-1} EGTA), homogenized with 10 up and down strokes of a Potter-Elvehjem tissue grinder at high speed; $MgCl_2$ was added to the homogenate to a final concentration of 15 mmol l^{-1}, incubated on ice for 15 min and then centrifuged at 3000 ***g*** for 15 min. The supernatant was then centrifuged at 27,000 ***g*** for 30 min. The pellet was resuspended in and washed 3 times with glucose/fructose transport inside buffer (60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 100 mmol l^{-1} KCl, 150 mmol l^{-1} choline chloride) (Verri et al., 2001). The final pellet (BBMV) was resuspended in the same buffer and then passed through a 22 gauge needle 15 times to break up vesicle clumps and then incubated on ice for 30 min.

Lobster hepatopancreas BLMV preparation

Basolateral membranes were prepared based on a previous method (Ahearn et al., 1987). Hepatopancreas was homogenized 3 min in ice-cold 25 mmol l^{-1} NaCl, 10 mmol l^{-1} Hepes-Tris pH 8.0, 2 mmol l^{-1} PMSF, using a Polytron Tissue Homogenizer (Kinematica, Bohemia, NY, USA). The homogenate was centrifuged at 2500 ***g*** for 15 min. The resulting supernatant was centrifuged at 20,400 ***g***. The pellet was resuspended in 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes-Tris pH 7.4 and 1 mmol l^{-1} magnesium-gluconate. The resuspended pellet was adjusted to 38% sorbital by the addition of 65% sorbital in 12.5 mmol l^{-1} NaCl, 0.5 mmol l^{-1} EDTA, 10 mmol l^{-1} Hepes-Tris. A sorbital (25% sorbital, 12.5 mmol l^{-1} NaCl, 0.5 mmol l^{-1} EDTA, 10 mmol l^{-1} Hepes-Tris) solution was layered on top of the 38% sorbitol-pellet solution and the tubes centrifuged at 47,750 ***g*** for 45 min. The fluffy white band at the interface of the step gradients was removed, resuspended in 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes-Tris pH 7.4 and 1 mmol l^{-1} magnesium-gluconate and centrifuged at 47,750 ***g*** for 25 min. The pellet was resuspended in and washed 3 times with glucose/fructose transport inside buffer (Verri et al., 2001). The final pellet (BLMV) was resuspended in

the same buffer and then passed through a 22 gauge needle 15 times to break up vesicle clumps and then incubated on ice for 30 min.

Glucose and fructose uptake by BBMV and BLMV

Glucose uptake was determined from 0 to 10 min for BBMV and BLMV (0.02 ml at 1 mg protein ml^{-1}) containing inside buffer (minus sodium) by incubation in outside buffer minus sodium (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 250 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]glucose, 0.1 mmol l^{-1} D-glucose or outside buffer plus sodium (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 100 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]glucose, 0.1 mmol l^{-1} D-glucose, 150 mmol l^{-1} NaCl, or outside buffer 3 (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 100 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]glucose, 0.1 mmol l^{-1} D-glucose, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} D-fructose. Uptake was halted by the addition of 2 ml of ice-cold inside buffer plus 1 mmol l^{-1} EDTA and 1 mmol l^{-1} EGTA. Replicates (five) for each time point were filtered under vacuum through pre-wetted cellulose ester filters (0.65 μm pore size). Radioactivity retained by the filters after washing was measured (c.p.m.) in a scintillation counter (LS 6500 Multi-purpose Scintillation Counter). All transport values were corrected for a 'blank' obtained by adding the incubation mixture directly to the stop solution before filtration. Fructose uptake was determined from 0 to 10 min for BBMV and BLMV (0.02 ml at 1 mg protein ml^{-1}) containing inside buffer by incubation in minus sodium outside buffer 4 (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 250 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]fructose, 0.1 mmol l^{-1} D-fructose; outside buffer 5 (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 100 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]fructose, 0.1 mmol l^{-1} D-fructose, 150 mmol l^{-1} NaCl; or outside buffer 6 (0.18 ml): 60 mmol l^{-1} mannitol, 20 mmol l^{-1} Hepes/Tris pH 7.4, 100 mmol l^{-1} choline chloride, 0.1 μCi D-[^{3}H]fructose, 0.1 mmol l^{-1} D-fructose, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} D-glucose. Uptake was halted by the addition of 2 ml of ice-cold inside buffer. Replicates (five) for each time point were filtered under vacuum through pre-wetted cellulose ester filters (0.65 μm pore size). Radioactivity retained by the filters after washing was measured (c.p.m.) in a scintillation counter (LS 6500 Multi-purpose Scintillation Counter). All transport values were corrected for a 'blank' obtained by adding the incubation mixture directly to the stop solution before filtration.

Antibodies

Rabbit anti-SGLT1 synthetic peptide corresponding to amino acids 402–420 of the putative extracellular loop of SGLT1 of the mouse/rabbit small intestine [Chemicon (Millipore), Billerica, MA, USA] was used at a 1:1000 dilution in SuperBlock T20 (TBS; Pierce, Rockford, IL, USA) or 5% non-fat dry milk, 0.05% Tween-20 to determine the presence of a cross-reacting lobster protein in hepatopancreas cells or vesicles. Rabbit anti-human SGLT-4, C-terminal synthetic peptide (Lifespan Biosciences, Seattle, WA, USA) was used at a 1:500 dilution. Rabbit anti-rat GLUT2, 16 amino acid peptide corresponding to the exoplasmic loop between helices 1 and 2 of rat liver glucose transporter (Chemicon) was used at a 1:1000 dilution. Rabbit anti-rat GLUT5, C-terminus synthetic

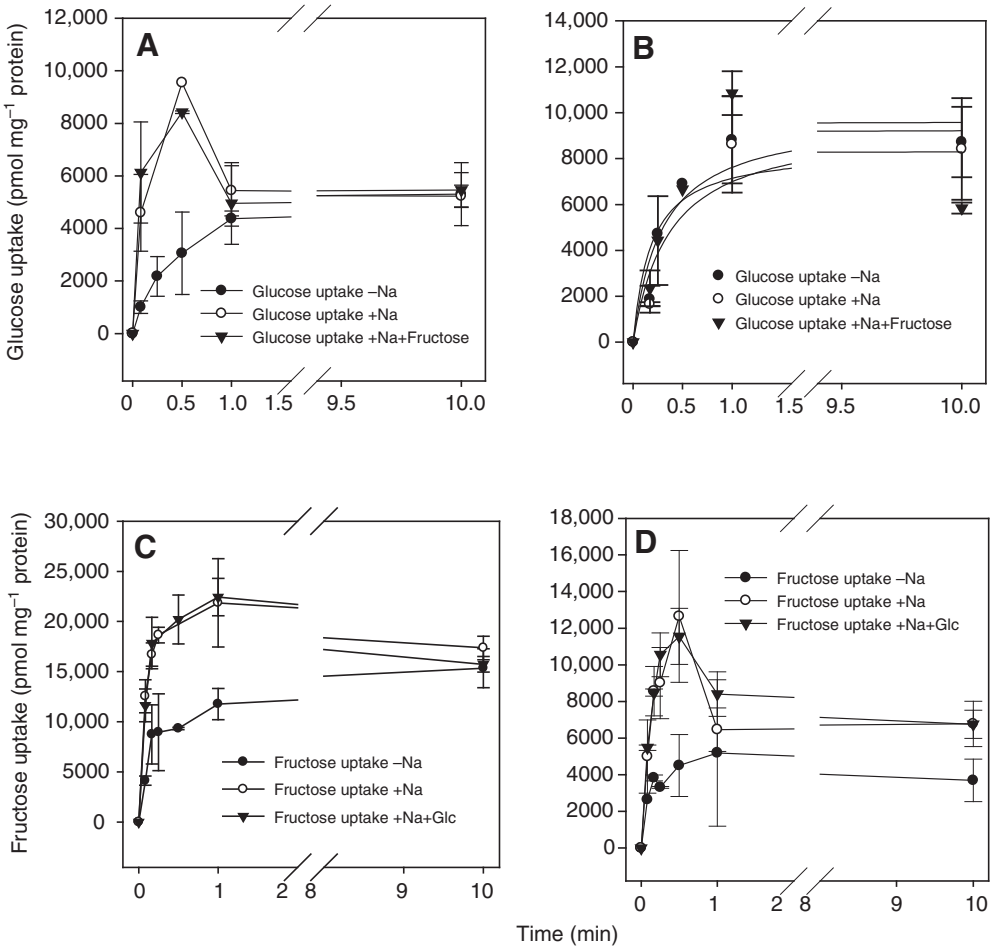

Fig. 1. (A) Glucose uptake by brush border membrane vesicles (BBMV) in the absence or presence of sodium and in the presence of sodium with the addition of a tenfold molar excess of fructose. (B) Glucose uptake by basolateral membrane vesicles (BLMV) in the absence or presence of sodium and in the presence of sodium with the addition of a tenfold molar excess of fructose. (C) Fructose uptake by BBMV in the absence or presence of sodium and in the presence of sodium with the addition of a tenfold molar excess of glucose. (D) Fructose uptake by BLMV in the absence or presence of sodium and in the presence of sodium with the addition of a tenfold molar excess of glucose.

peptide (KELNDLPPATREQ; Chemicon) was used at a 1:1000 dilution.

Immuno-blotting (western blot) procedure

Twenty or 50 µg of E-, R-, F- and B-cells, BBMV, BLMV and rat small intestine proteins were separated by SDS-PAGE. The proteins were transferred to PVDF membranes (BioRad, Hercules, CA, USA) in 10 mmol l^{-1} CAPS buffer, pH 11, 10% methanol at 75 V for 25 min. The membranes were blocked for a minimum of 1 h with 5% non-fat dry milk in PBS, 0.05% Tween-20. The blocked PVDF membranes were probed for at least 1 h with a specific antibody.

The blots were washed (4×15 min) in PBS, 0.05% Tween-20. The washed blots from the primary antibody reaction were then incubated for 1 h with a 1:100,000 dilution of goat anti-rabbit IgG (H+L), peroxidase-conjugated secondary antibody (Pierce) in 5% non-fat dry milk, PBS, 0.05% Tween-20. The blots were washed (6×15 min) with PBS, 0.05% Tween-20. The washed blots from the secondary antibody reaction were incubated in Supersignal West Pico Chemiluminescent substrate (Pierce) and then exposed to X-ray film (Kodak, Rochester, NY, USA) for varying times, e.g. 30 s to overnight.

Protein determination

BBMV, BLMV and cell total protein was determined by the method of Bradford (Bradford, 1976) using reagents supplied by BioRad.

Mammalian and lobster SGLT and GLUT protein sequence alignments

A translated, lobster expressed sequence tag (EST) library (Towle and Smith, 2006) (Accession no. EX471402) was submitted to BLAST (Altschul et al., 1997) analysis using human SGLT4 (Tazawa et al., 2005), rabbit SGLT1 (Hediger et al., 1987), mouse (Accession no. AAF17249) SGLT1, Pacific oyster SGLT (Huvet et al., 2004), rat GLUT2 (Fisher et al., 2008) and rat GLUT5 (Rand et al., 1993) protein sequences for identification of expressed lobster sugar transport orthologues. Lobster EST clones with significant identity to mammalian and other sugar transport proteins were aligned with mammalian SGLTs and GLUTs to indicate the degree of identity between the lobster and mammalian proteins (Tatusova and Madden, 1999; Larkin et al., 2007).

RESULTS

Hexose transport

Glucose uptake by BBMV in the absence of sodium was equilibrative, while glucose uptake in the presence of sodium was indicative of a SGLT-like, sodium-dependent uptake (Fig. 1A). The sodium-dependent uptake of glucose was not inhibited by a tenfold molar excess of fructose (Fig. 1A). Glucose uptake by BLMV was equilibrative in the presence and absence of sodium (GLUT-specific inhibitors such as cytochalasin B or phloretin were not included; Fig. 1B). Equilibrative glucose transport by BLMV in the presence of sodium was not inhibited by a tenfold molar excess of fructose (Fig. 1B). Fructose uptake by BBMV was sodium dependent and not inhibited by a tenfold molar excess of glucose in the presence of sodium (Fig. 1C). Fructose transport in the absence of sodium and GLUT inhibitors by BBMV was equilibrative (Fig. 1C). Fructose uptake by BLMV was sodium dependent and not inhibited by a tenfold molar excess of glucose (Fig. 1D).

Sugar transport analysis of the four cell types also indicated unique mechanisms of glucose and fructose transport. E-cells demonstrated both non-sodium-dependent fructose uptake and sodium-dependent uptake of fructose (Fig. 2A). A tenfold molar excess of glucose or mannose did not affect sodium-dependent fructose uptake by E-cells (Fig. 2A). Glucose uptake by E-cells was equilibrative and non-sodium dependent (Fig. 2B). The kinetics of fructose influx *vs* increasing fructose concentration were significantly enhanced in the presence of sodium, i.e. in the absence of extracellular sodium, fructose influx had a non-significant K_m of

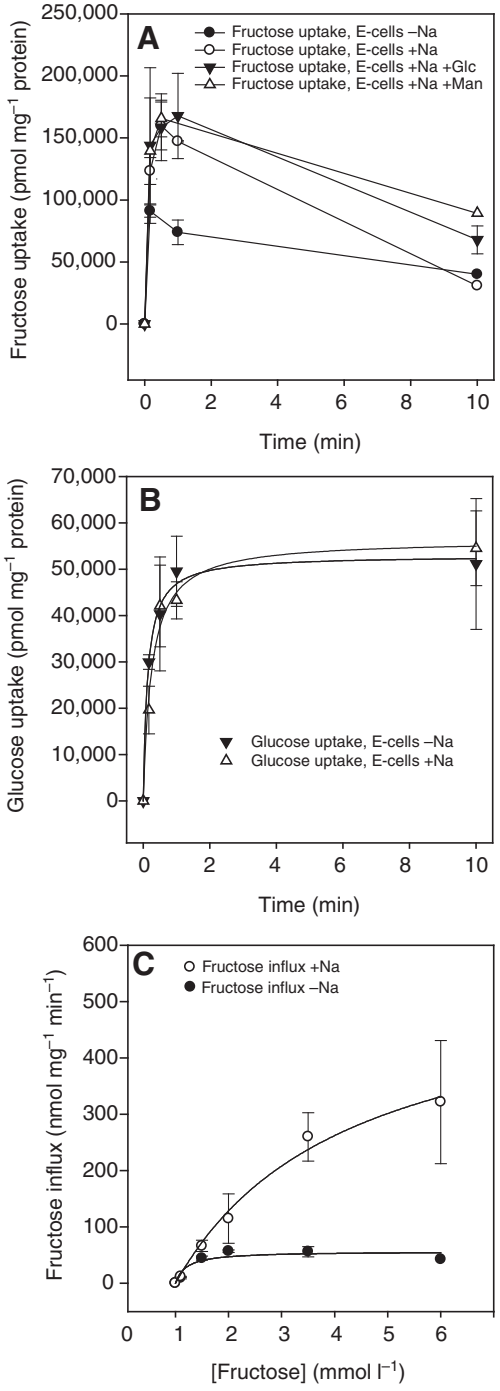

Fig. 2. (A) Fructose uptake by E-cells in the absence or presence of sodium and in the presence of sodium with a tenfold molar excess of either glucose or mannose. (B) Glucose uptake by E-cells in the absence or presence of sodium. (C) Fructose flux (per minute) by E-cells in the absence or presence of sodium as a function of increasing fructose concentration. Absence of sodium: K_m=0.20±0.15 mmol l^{-1}, J_{max}=56.60±7.92 nmol mg^{-1} protein min^{-1}. Presence of sodium: K_m=3.25±0.81 mmol l^{-1}, J_{max}=546.53±69.46 nmol mg^{-1} protein min^{-1}.

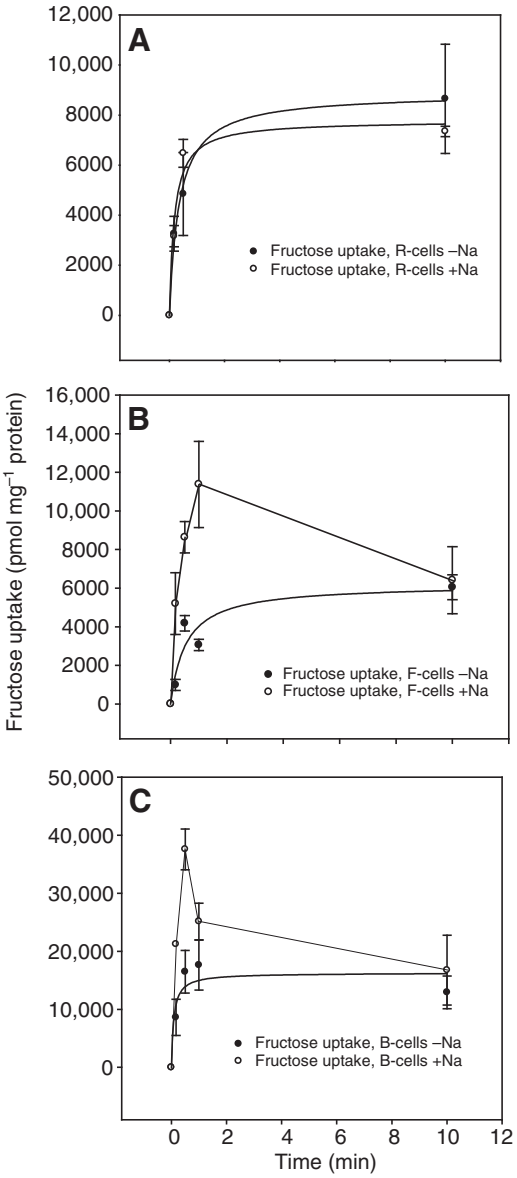

Fig. 3. (A) Fructose uptake by R-cells in the absence or presence of sodium. (B) Fructose uptake by F-cells in the absence or presence of sodium. (C) Fructose uptake by B-cells in the absence or presence of sodium.

$0.20\pm0.15\,\text{mmol}\,l^{-1}$ and a J_{max} of $56.60\pm7.92\,\text{nmol}\,mg^{-1}$ protein min^{-1}. In the presence of extracellular sodium, fructose influx had a K_m of $3.25\pm0.81\,\text{mmol}\,l^{-1}$ and a J_{max} of $548.53\pm69.48\,\text{nmol}\,mg^{-1}$ protein min^{-1} (Fig. 2C). These results indicated a novel, sodium-dependent fructose transport mechanism for E-cells.

Fructose uptake by R-cells was non-sodium dependent (Fig. 3A). F- and B-cells both demonstrated sodium-dependent fructose uptake (Fig. 3B,C).

Identification and localization of GLUT-like and SGLT-like proteins

Polyclonal antibodies to specific mammalian GLUT and SGLT family members were used for determining the presence and putative localization of orthologous lobster hepatopancreas proteins by immuno-blot (western blot) analysis. An SGLT1-like protein signal (approximately 75 kDa) was observed for R-, F- and B-cells

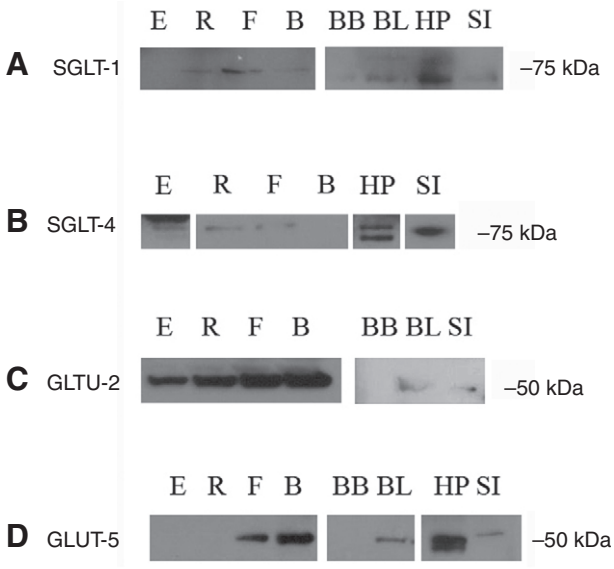

Fig. 4. (A) Western blot analysis of E-, R-, F- and B-cells and BBMV (BB), BLMV (BL), total hepatopancreas (HP) and rat small intestine (SI) using a rabbit anti-mouse/rabbit SGLT1 antibody. (B) Western blot analysis of E-, R-, F- and B-cells, total hepatopancreas and rat small intestine using a rabbit anti-human SGLT4 antibody. (C) Western blot analysis of E-, R-, F- and B-cells and BBMV, BLMV and rat small intestine using a rabbit anti-rat GLUT2 antibody. (D) Western blot analysis of E-, R-, F- and B-cells and BBMV, BLMV, total hepatopancreas and rat small intestine using a rabbit anti-rat GLUT5 antibody.

but not E-cells using a rabbit anti-mouse SGLT1 antibody (Fig. 4A). The SGLT1-like protein signal was most intense from F-cells (Fig. 4A). Purified BBMV were weakly positive for a protein of approximately 75 kDa. Purified BLMV and total hepatopancreas were also positive for an approximately 75 kDa protein as well as a slower migrating (approximately 100 kDa) protein signal (Fig. 4A). The approximately 100 kDa signal from BLMV was much weaker than that from total hepatopancreas (Fig. 4A). There was no detectable 100 kDa signal from BBMV (Fig. 4A). However, an approximately 100 kDa signal was detected in BBMV in a separate anti-SGLT-1 immunoblot (data not shown). The approximately 100 kDa signal most probably corresponds to an additionally glycosylated SGLT1-like transmembrane protein. The anti-SGLT1 immunoblot results indicate the presence of a 75 kDa, SGLT1 orthologue expressed primarily in F-cells that appears to be present in both the BBMV and BLMV.

Western blot analysis of hepatopancreas cells using a rabbit anti-human SGLT4 antibody indicated an orthologous protein predominating in E-cells that migrated with an apparent molecular mass (approximately 75 kDa) consistent with that of SGLT4 from mammals (Fig. 4B). A slower migrating cross-reacting protein of approximately 100 kDa from E-cells is believed to be an additionally glycosylated lobster-specific form of the protein (Fig. 4B). A very weak, 75 kDa, SGLT4-like protein signal from both BBMV and BLMV was also observed (data not shown). An approximately 60 kDa signal was also present in total hepatopancreas and E-cells possibly corresponding to an under-glycosylated form of the lobster SGLT4-like protein (Fig. 4B).

A GLUT2-like protein migrating at an apparent molecular mass (approximately 50 kDa) consistent with that of the mammalian protein was present in all four cell types and was localized to the BLMV using a rabbit anti-rat GLUT2 antibody (Fig. 4C).

A

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RSGLT1         351-CERYCGTRVGCTNIAFPTLVVELMPNGLRGLMLSVMMASLMSSLTSIFNSASTLFTMDIYTK
MSGLT1         351-CQKYCGTPVGCTNIAYPTLVVELMPNGLRGLMLSVMMASLMSSLTSIFNSASTLFTMDIYTK
EX471402           ------SESGCTNVAYVELVLNLLPTGLSGLMLAVMMAALMSSLTSIFNSSSTIFSIDIWTR
                         :  ****:*:  **::*:*.** ****:****:***********:**:*::**:*:
RSGLT1             IRKKASEKELMIAGRLFMLFLIGISIAWVPIVQSAQSGQLFDYIQSITSYLGPPIAAVFLLA
MSGLT1             IRKKASEKELMIAGRLFILVLIGISIAWVPIVQSAQSGQLFDYIQSITSYLGPPIAAVFLLA
EX471402           IRKKATDVELLIVGRVFVLVMVVVSVIWIPVIQNSANSQLFVYIQSISSFLSPPICAIYLLA
                   *****:: **:*.**:*:*.:: :*: *:*::*.: ..*** *****:*:*.***.*::***
RSGLT1             IFWKRVNEPGAFWGLVLGFLIGISRMITEFAYGTGSCME------PSNCPTIICGVHYLYFA
MSGLT1             IFCKRVNEQGAFWGLILGFLIGISRMITEFAYGTGSCME------PSNCPKIICGVHYLYFA
EX471402           IFWERTTEPGAFWGLIVGLVIGMIRFIMEFAFVVPVCGSEDQDKRPEWIKIIVGNVHYLHFG
                   ** :*..* ******::*::**: *:* ***: .  * .      *.    *: .****:*.
RSGLT1             IILFVISIITVVVVSLFTKPIPDVHLYRLCWSLRNS-558
MSGLT1             IILFVISVITILIISFLTKPIPDVHLYRLCWSLRNS-558
EX471402           CILFLIVLVITIVVSYLTEPIPKKCIQRLTYWTRHS
                    ***:* ::  :::* :*:***.  : ** :  *:*
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B

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RSGLT1                   402-STLFTMDIYTKIRKKASEK-420
MSGLT1                   402-STLFTMDIYTKIRKKASEK-420
EX471402                     STIFSIDIWTRIRKKATDV
                             **:*::**:*:*****::
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C

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FD483413  4    FVLMLLGWIFVPVYMSSGIYTMPEYLRERFGGQRIRVYLSCLALILSIFTKISADLYAGA  183
               ++L+ LGW+FVPVY+++G+ TMP+YL++RFGGQRI+VY+S L+LIL IFTKIS D+++GA
HSGLT4    141  WLLLALGWVFVPVYIAAGVVTMPQYLKKRFGGQRIQVYMSVLSLILYIFTKISTDIFSGA  200
FD483413  184  LFIQQMLNKNSVEWMYLSILILLAIASIFTITGGLTAVIWTDFVQTILMVVGACILMGMS  363
               LFIQ  L  N    +YLS  ILL + +++TI GGL AVI+TD +QT++MV GA +LM +
HSGLT4    201  LFIQMALGWN----LYLSTGILLVVTAVYTIAGGLMAVIYTDALQTVIMVGGALVLMFLG  256
FD483413  364  FHAVGGYENLVQDYFYAIPNRTRYSIDNSTNCGEPPSYAMNLFRSAKPGESDLPWPGMLF  543
               F  VG Y  L Q Y  AIPN T   + N+T C  P   A ++ R   P   D+PWPG++F
HSGLT4    257  FQDVGWYPGLEQRYRQAIPNVT---VPNTT-CHLPRPDAFHILRD--PVSGDIPWPGLIF  310
FD483413  544  GVTISGIWY*CTDQVIVQRTLSSKNM  621
               G+T+   W  CTDQVIVQR+LS+K++
HSGLT4    311  GLTVLATWCWCTDQVIVQRSLSAKSL  336
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D

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OSGLT      90 FILMMLGWLFVPVYVAAGVFTMPEYLKKRFGDSRIRVYLSVLALLLYVFTKISADLFSGA  149
              F+LM+LGW+FVPVY+++G++TMPEYL++RFG  RIRVYLS LAL+L +FTKISADL++GA
FD483413    4 FVLMLLGWIFVPVYMSSGIYTMPEYLRERFGGQRIRVYLSCLALILSIFTKISADLYAGA  183
OSGLT     150 LFILRTFKGFD---LYLAIIILLIIAALFTITGGLTAVIWTDSIQVLIMVIGAFILMVMS  206
              LFI +          +YL+I+ILL IA++FTITGGLTAVIWTD +Q ++MV+GA ILM MS
FD483413  184 LFIQQMLNKNSVEWMYLSILILLAIASIFTITGGLTAVIWTDFVQTILMVVGACILMGMS  363
OSGLT     207 FVRVGGYEEVVRRFFEAYPNTTLLNYNNASYPYWKCGIPPENSMNLVRAYDDG--SLPWP  264
              F  VGGYE +V+ +F A PN T  + +N++     CG PP  +MNL R+   G   LPWP
FD483413  364 FHAVGGYENLVQDYFYAIPNRTRYSIDNST----NCGEPPSYAMNLFRSAKPGESDLPWP  531
OSGLT     265 GIFFGLTISSVWYWCSDQVIVQRALAAKNI  294
              G+ FG+TIS +WY C+DQVIVQR L++KN+
FD483413  532 GMLFGVTISGIWY*CTDQVIVQRTLSSKNM  621
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E

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OSGLT  C-term 633-VTSVNAVILMTLAVFLWGFYA-653
                  V ++NAV+L+ + +FLWG++A
HSGLT4 C-term 686-VCNINAVLLLAINIFLWGYFA-706
```

F

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OSGLT  C-term 633-VTSVNAVILMTLAVFLWGFYA-653
                  V +VN +IL+T+AVF   ++A
hSGLT1 C-term 644-VLNVNGIILVTVAVFCHAYFA-664
```

Fig. 5. (A) Clustal multiple alignment of mouse/rabbit SGLT1peptide sequences (amino acids 351–558) with translated lobster EST clone EX471402. Asterisk indicates amino acid identity. Colon indicates a positive amino acid substitution. Dot indicates a neutral amino acid substitution. There is 54% amino acid identity between EX471402 and mouse SGLT1 (mSGLT1) and 52% identity between EX471402 and rabbit SGLT1 (rSGLT1). EX471402 is 75% and 74% positive to the mouse and rabbit SGLT1, respectively. (B) Clustal alignment of mouse/rabbit SGLT1 amino acids 402–420 (epitope for rabbit anti-SGLT1) with corresponding, translated EX471402 amino acid sequence. There is 58% identity between this region of EX471402 and amino acids 402–420 of the mouse/rabbit SGLT1 sequence with a total of 95% positives. (C) BLAST sequence alignment of translated lobster EST clone FD483413 (nucleotides 4–621) with the human SGLT4 protein sequence (hSGLT4; amino acids 141–336). There is 52% amino acid identity between FD483413 and hSGLT4 with a total of 72% positive amino acids. (D) Blast alignment of translated FD483413 (nucleotides 4–621) with the Pacific oyster SGLT (oSGLT) protein sequence (amino acids 90–294). There is 58% identity between FD483413 and the oSGLT protein sequence with a total of 77% positives. (E) BLAST alignment of oSGLT C-terminus protein sequence (amino acids 633–653) with hSGLT4 C-terminus protein sequence (amino acids 686–706). There is 48% identity between the oSGLT C-terminus and the hSGLT4 C-terminus with 86% positives. (F) BLAST alignment of oSGLT C-terminus protein sequence (amino acids 633–653) with human SGLT1 C-terminus protein sequence (hSGLT1; amino acids 644–664). There is 48% identity between the oSGLT C-terminus and the hSGLT1 C-terminus with 76% positives.

Rabbit anti-rat GLUT5 detected a signal migrating at an apparent molecular mass (approximately 50 kDa) consistent with that of the mammalian protein in F- and B-cells (Fig. 4E). However, the lobster GLUT5-like protein was localized to the BLMV, which is contrary to the localization of the mammalian GLUT5 in BBMV (Fig. 4D).

Alignment of mammalian SGLT1, SGLT4, GLUT2 and GLUT5 amino acid sequences to translated lobster ESTs

BLAST analysis using full-length mammalian sugar transporter protein sequences to identify expressed lobster orthologues resulted in the identification of multiple lobster ESTs with significant identity to mammalian sodium-dependent and facilitative sugar transport family member proteins. Selected, translated lobster EST clones were aligned with mammalian sugar transport proteins to demonstrate the significant identity between lobster proteins and mammalian proteins (Figs 5–7). These results indicated that there are expressed lobster proteins that are members of both the facilitative and sodium-dependent sugar transporter families.

DISCUSSION

Glucose transport was shown to be sodium dependent and phloridzin sensitive in BBMV (Ahearn et al., 1985; Verri et al., 2001). Glucose uptake by BBMV in the absence of sodium was equilibrative (Ahearn et al., 1985). These observations and the ones reported here are consistent with an SGLT1-like glucose transporter in BBMV. However, the localization of an anti-SGLT1 cross-reacting protein to the BLMV is not consistent with the sodium-dependent transport model of glucose transport across epithelia in mammals (Suzuki et

A

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Rat GLUT2   4 DKITGTLAFTVFTAVLGSFQFGYDIGVINAPQEVIISHYRHVLGVPLDDRRATINYDING 63
              + +T  L + +F AVLG FQFGY+ GVINAPQ VI     + +G   D  +   N +I G
CN951353  254 EGLTCFLCYAIFAAVLGMFQFGYNTGVINAPQSVI----ENFIG---DCWKERFNKNIEG 412

Rat Glut2  64 TDTPLIVTPAHTTPDAWEEETEGSAHIVTMLWSLSVSSFAVGGMVASFFGGWLGDKLGRI 123
              +                              +LWS++VS FA+GGM+    GG +G+K GR
CN951353  413 SKQ-------------------------DLLWSIAVSIFAIGGMIGGICGGSVGNKFGRK 517

Rat Glut2 124 KAMLAANSLSLTGALLMGCSKFGPAHALIIAGRSVSGL  161
              K +L  + L + GA LMG S+   ++ ++I GR V G+
CN951353  518 KGLLLKHLLGVGGACLMGFSQMSYSYEMLILGRLVIGI  631
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B

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Rat GLUT2   1 MSEDKITGTLAFTVFTAVLGSFQFGYDIGVINAPQEVIISHYRHVLGVPLDDRRATINYD 60
              M++  +T  L + +F AVLG FQFGY+ GVINAPQ VI
EH116561  162 MADLGLTCFLCYAIFAAVLGMFQFGYNTGVINAPQSVI---------------------- 275

Rat GLUT2  61 INGTDTPLIVTPAHTTPDAWEE----ETEGSAHIVTMLWSLSVSSFAVGGMVASFFGGWL 116
                        +   D W+E      EGS     +LWS++VS FA+GGM+    GG +
EH116561  276 ------------ENFIGDCWKERFNKNIEGSKQ--DLLWSIAVSIFAIGGMIGGICGGSV 413

Rat GLUT2 117 GDKLGRIKAMLAANSLSLTGALLMGCSKFGPAHALIIAGRSVSGLYCGLISGLVPMYIGE 176
              G+K GR K +L  N L + GA LMG S+   ++ ++I GR V G+ CGL + LVPMYI E
EH116561  414 GNKFGRKKGLLLNNLLGVGGACLMGFSQMSYSYEMLILGRLVIGINCGLNTSLVPMYISE 593

Rat GLUT2 177 IAPTTLRGALGTLHQLALVTGILISQIAGLSFILGNQDYWHILLGLSAVPALLQCLLL  234
              IAP  LRG LGT++QLA+  G+L+SQI G+  +LG    W ILLGL+ VPA++Q +LL
EH116561  594 IAPLNLRGGLGTVNQLAVTVGLLLSQILGIEQLLGK*HAWPILLGLAIVPAIMQMVLL  767
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Fig. 6. (A) Blast sequence alignment of rat GLUT2 protein sequence (amino acids 4–161) with translated lobster EST clone CN951353 (nucleotides 254–631). There is 35% identity between rat GLUT2 and CN951353 with 51% total positives. (B) Blast sequence alignment of rat GLUT2 protein sequence (amino acids 1–234) with translated lobster EST clone EH116561 (nucleotides 162–767). There is 42% identity between the rat GLUT2 and EH116561 with 57% total positives.

al., 2006). The presence of an SGLT1-like protein in both BBMV and BLMV may be regulated by post-translational glycosylation. Glucose transport in the presence of sodium was not inhibited by a tenfold molar excess of fructose indicating that this putative SGLT1-like transporter has a marked preference for glucose over fructose. Glucose transport in lobster cells was sodium independent with the exception of F-cells in which sodium-dependent and phloridzin-sensitive transport was observed (Verri et al., 2001). In the present study F-cells expressed the highest level of an anti-SGLT1 cross-reacting protein consistent with sodium-dependent glucose uptake by these cells.

The observed sodium-dependent fructose uptake by BBMV is in contrast to the mammalian paradigm, in which fructose uptake by BBMV would be expected to involve a GLUT5-like facilitative transporter. This study has tentatively localized an anti-GLUT5 cross-reacting protein to F- and B-cells and to the BLMV. Fructose transport by BLMV was also sodium dependent. This observation is consistent with a fructose, sodium-dependent transporter in BLMV in contrast to the mammalian fructose transport paradigm that involves GLUT5 and SGLT4 present in the BBMV of mammalian intestinal and renal epithelia (Mahraoui et al., 1992; Tazawa et al., 2005). It should be noted that sodium-dependent fructose transport activity was observed in both BBMV and BLMV from the whole hepatopancreas and not the BBMV and BLMV from isolated E-, R-, F- and B-cells. Thus one cannot assign sodium-dependent fructose transport activity to both the BBMV and BLMV of any particular cell type. The tentatively

A

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Rat GLUT5 284 MAGQQLSGVNAIYYYADQIYLSAGVKSNDVQYVTAGTGAVNVFMTMVTVFVVELWGRRNL 343
              M  Q+ SG NA  +YA  I   A V  N         G + +   MV+  +++  GR  L
EH117199    3 MVFQKFSGANAFNFYAVPILTEAFVGINPYSAAVV-VGLLQILAGMVSSVLIDTVGRLPL 179

Rat GLUT5 344 LLI----------GFSTCLTACIVLTVALALQNTISWMPYVSIVCVIVYVIGHAVGPSPI 393
              L++           GF T L       V       + W+P   + C +++ +  A+G SPI
EH117199  180 LIVSNLLMSTALAGFGTFLYVTGSNGVDAGPGPGLDWIP---LTCALIFQVAFALGISPI 350

Rat GLUT5 394 PALFITEIFLQSSRPSAYMIGGSVHWLSNFIVGLIFPFIQVGLGPY-SFIIFAIICLLTT 452
                ++I E+F    R     I  SV +  +F     F   Q+ LG Y +F ++A I L+
EH117199  351 AWIYIGELFPLKHRGLG-AIANSVSYACSFASVKTFVDFQLLLGLYGAFWLYAGISLIGL 527

Rat GLUT5 453  IYIFMVVPETKGRTFVEINQIFAKKNKVSDVYPEK  487
              ++  ++VPETKGR   E+ Q +  + + + +Y E+
EH117199  528  VFTVVLVPETKGRGLQEM-QKYTPQERYTPIYNEQ  629
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B

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Rat GLUT5 12  LTLVLALATFLAAFGSSFQYGYNVAAVNSPSEFMQQFYNDTYYDRNKENIESFTLTLLWS  71
              LT  L  A F A  G  FQ+GYN   +N+P   ++ F  D + +R  +NIE     LLWS
EH116561  177 LTCFLCYAIFAAVLGM-FQFGYNTGVINAPQSVIENFIGDCWKERFNKNIEGSKQDLLWS 353

Rat GLUT5 72  LTVSMFPFGGFIGSLMVGFLVNNLGRKGALLFNNIFSILPAILMGCSKIAKSFEIIIASR 131
              + VS+F  GG IG +  G + N  GRK  LL NN+  +  A LMG S+++ S+E++I  R
EH116561  354 IAVSIFAIGGMIGGICGGSVGNKFGRKKGLLLNNLLGVGGACLMGFSQMSYSYEMLILGR 533

Rat GLUT5 132 LLVGICAGISSNVVPMYLGELAPKNLRGALGVVPQLFITVGILVAQLFGLRSVLASEEGW 191
              L++GI  G+++++VPMY+ E+AP NLRG LG V QL +TVG+L++Q+ G+  +L     W
EH116561  534 LVIGINCGLNTSLVPMYISEIAPLNLRGGLGTVNQLAVTVGLLLSQILGIEQLLGK*HAW 713

Rat GLUT5 192 PILLGLTGVPAGLQLLLL  209
              PILLGL  VPA +Q++LL
EH116561  714 PILLGLAIVPAIMQMVLL  767
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Fig. 7. (A) Blast sequence alignment of rat GLUT5 protein sequence (amino acids 284–487) with translated lobster EST clone EH117199 (nucleotides 3–629). There is 27% identity between rat GLUT5 and EH117199 with 46% total positives. (B) Blast sequence alignment of rat GLUT5 protein sequence (amino acids 12–191) with translated lobster EST clone EH116561 (nucleotides 72–713). There is 44% identity between the rat GLUT5 and EH116561 with 64% total positives.

characterized sodium-dependent fructose transport activity reported here can be compared with that of the mammalian SGLT4 in that it has been shown to transport fructose in a sodium-dependent manner (Tazawa et al., 2005).

The non-sodium-dependent uptake of fructose is interesting in that R-cells were positive for SGLT1- and SGLT4-like proteins, albeit the signals for each were not as strong as those for F-cells and E-cells, respectively. Also, a previous study did not indicate sodium-dependent uptake of glucose by R-cells (Verri et al., 2001). The lack of sodium dependency for either glucose or fructose uptake in the presence of sodium may be due to the presence of both the SGLT1-like and the lobster sodium-dependent fructose transporter proteins in an inactive form, e.g. the extent of glycosylation.

Fructose uptake experiments with B-cells indicated sodium dependency. An anti-SGLT4 cross-reacting protein was not detected in B-cells. However, an anti-SGLT1 cross-reacting protein was detected. At least two lobster ESTs (EX471402 and FD483413) have been identified with approximately 50% identity to both the mouse SGLT1 and human SGLT4. The clones may derive from the same gene or separate genes. Hence, it may be that a lobster SGLT-like protein in B-cells is responsible for the observed sodium-dependent fructose uptake. B-cells did not demonstrate sodium-dependent glucose uptake (Verri et al., 2001). F- and B-cells were the only cell types to express a GLUT5-like protein that was localized to the BLMV.

An anti-GLUT2 cross-reacting protein was present in all four cell types and localized to the BLMV, indirectly indicating that glucose uptake by BLMV in the absence of sodium may be facilitative. The present experiments did not include the GLUT2 inhibitors cytochalasin B or phloretin. Also, glucose uptake by BLMV in the absence of sodium was not determined in the presence of excess fructose. Tentative localization of the lobster GLUT2-like protein to the BLMV alone does not coincide with the mammalian system in that GLUT2 can be present in both the BBMV and BLMV and is capable of transporting both glucose and fructose (Wright and Turk, 2004; Uldry and Thorens, 2004). In this study, non-sodium-dependent glucose uptake by BBMV appeared to be equilibrative. However, we have observed that lobster intestinal, mucosal to serosal glucose transport in the absence of sodium is phloretin sensitive (data not shown). We cannot exclude the role of a GLUT5-like facilitative carrier in the BLMV of F- and B-cells as a fructose transporter given mammalian GLUT5 significantly prefers fructose as a substrate (Buchs et al., 1998). Furthermore, while antibodies to mammalian GLUT2 (first extracellular loop) and GLUT5 (C-terminus) cross-reacted with proteins from the cells with different profiles for positive signals, BLAST analysis of the translated lobster EST library with rat GLUT2 and rat GLUT5 protein sequences showed significant identity to the same lobster clone, EH116561. However, we have not determined whether these clones represent the same or separate lobster expressed genes. Thus, one must keep in mind that although comparison of sugar uptake in lobster cells is made to known mammalian sugar transporters, there are obvious differences in sugar transport physiology and function between the lobster and mammalian proteins. Further analysis aimed at verifying the cellular localization and physiology of the lobster GLUT2-like and GLUT5-like proteins is underway.

The data presented here indicate that E-cells and possibly F- and B-cells contain a sodium-dependent hexose transport activity, with a preference for fructose over glucose, that is potentially located in either the BBMV or BLMV, or possibly both. Differential expression of this sodium-dependent transporter in either the BBMV or BLMV may depend on its degree of glycosylation or other post-translational modification. The question arises as to why these cells have what appears to be a sodium-dependent fructose transporter. A tentative answer may simply be that *H. americanus* evolved with separate glucose and fructose sodium-dependent symporters, such having been dictated by the availability of specific hexoses and the energy requirements of the different hepatopancreas cells.

LIST OF ABBREVIATIONS

B	blister cells
BBMV	brush-border membrane vesicles
BLMV	basolateral membrane vesicles
E	embryonic cells
F	fibrillar cells
HP	hepatopancreas
R	resorptive cells

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