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FUNCTIONAL AND MOLECULAR CHARACTERISTICS OF A PRIMITIVE VERTEBRATE GLUCOSE TRANSPORTER: STUDIES OF GLUCOSE TRANSPORT BY ERYTHROCYTES FROM THE PACIFIC HAGFISH (*EPTATRETUS STOUTI*)

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Summary

The characteristics of glucose transport were investigated in erythrocytes of a primitive vertebrate, the Pacific hagfish (*Eptatretus stouti*) Lockington. Transport of glucose by intact hagfish erythrocytes and by phospholipid vesicles reconstituted with *n*-octylglucoside extract of hagfish erythrocyte membranes was rapid and mediated by a saturable stereospecific mechanism sensitive to inhibition by cytochalasin B. Covalent photoaffinity labelling experiments with [^{3}H]cytochalasin B identified the hagfish glucose transporter on SDS/polyacrylamide gels as a protein with an apparent average M_r of 55 000. Amino acid sequence homology between the hagfish and human erythrocyte glucose transporters (GLUT 1) was investigated in immunoblotting experiments using a panel of 12 different antipeptide antisera and affinity-purified antibodies raised against cytoplasmic extramembranous regions of the human transporter, and with an antibody to the intact purified human protein. The latter antibody labelled a component in the membrane with the same apparent M_r as cytochalasin B. Two affinity-purified antipeptide antibodies, corresponding to residues 240–255 and 450–467 of the human erythrocyte transporter, also labelled a component in the membrane with this relative molecular mass, demonstrating localised sequence similarity between the polypeptides of the two species within the central cytoplasmic loop and within the cytoplasmic C-

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terminal region. Glucose transport by hagfish erythrocytes was not coupled to the movement of protons.

Introduction

Human erythrocytes and foetal erythrocytes of other mammalian species are capable of rapid stereoselective transport of glucose (Widdas, 1980; Kim, 1983). Pig erythrocytes, however, are glucose-transport-deficient (Kim and McManus, 1971; Young *et al.* 1985) and scavenge plasma inosine to meet their physiological energy requirements (Young *et al.* 1985, 1986). Mature adult-type erythrocytes from other mammalian species exhibit intermediate rates of stereospecific glucose transport (Mooney and Young, 1978; Kim, 1983; Wagner *et al.* 1984). In most cases, but not in rabbit erythrocytes, glucose transport is inhibited by micromolar concentrations of cytochalasin B (Albert, 1984). A stereoselective cytochalasin-B-sensitive glucose transporter has also been characterised in avian erythrocytes (Kregenow, 1975). Transport of glucose in these cells, in contrast to that in mammalian erythrocytes, is stimulated by anoxia and by catecholamines (Bihler *et al.* 1982; Simons, 1983*a*,*b*). At the molecular level, the human erythrocyte glucose transporter has been purified and its amino acid sequence determined (Mueckler *et al.* 1985). The transporter migrates on SDS/polyacrylamide gels as a broad band with an apparent average M_r of 55 000, has 492 amino acid residues with 12 transmembrane domains and a single site of glycosylation. The same glucose transporter isoform (GLUT 1) is present in mammalian placenta, brain, kidney and colon and in human HepG2 cells (Carruthers, 1990).

Much less is known about glucose transport in lower vertebrates. In all teleost species so far investigated, carrier-mediated glucose transport by erythrocytes is either slow or undetectable (Bolis *et al.* 1971; Kim and Isaaks, 1978; Ingermann *et al.* 1985; Tse and Young, 1990; Tiihonen and Nikinmaa, 1991*a*). In one species, *Anguilla japonica*, we have found what appears to be an inherited glucose transport polymorphism: erythrocytes from individual eels exhibited low but widely varying rates of cytochalasin-B-sensitive glucose transport and cells from one individual were glucose-transport-deficient (Tse and Young, 1990). Unlike the situation in mammals, fish erythrocytes and those from other non-mammalian species retain their nuclei and mitochondria and are capable of aerobic metabolism *via* the tricarboxylic acid cycle. Potential energy substrates for teleost erythrocytes in addition to glucose (and nucleosides) include amino acids and monocarboxylates (lactate, pyruvate) (Tse and Young, 1990; Tiihonen and Nikinmaa, 1991*b*).

At the far end of the vertebrate evolutionary spectrum, agnathans (jawless fish) represent the most primitive group of living vertebrates. The classification of Nelson (1984) recognises Agnatha as a superclass, comprising the class Cephalaspidomphi (lampreys) and the class Myxini (hagfishes). Of the two, Myxini are considered to be the more primitive. In marked contrast to the situation in teleosts, Ingermann *et al.* (1984) found that erythrocytes from the Pacific hagfish (*Eptatretus stouti*) have a high level of cytochalasin-B-sensitive glucose transport activity. River lamprey (*Lampetra fluviatilis*) erythrocytes, in contrast, exhibit only moderate rates of carrier-mediated glucose

transport (Tiihonen and Nikinmaa, 1991*a*). In the present study, we have taken advantage of the unexpectedly high levels of glucose transport activity in hagfish erythrocytes to undertake, for the first time, kinetic and molecular studies of a primitive vertebrate glucose transporter. We find close similarities between the hagfish transporter and that present in human erythrocytes. Our results demonstrate that there has been substantial conservation of glucose transporter structure and function during the course of vertebrate evolution.

Materials and methods

Fish

Hagfish (*Eptatretus stouti*) were trapped at 165–201 m (90–110 fathoms) in Trevor Channel, Barkley Sound, Bamfield, British Columbia, and maintained in running sea water until bled from the subcutaneous sinus into heparinised tubes.

Erythrocytes

Cells were prepared for transport experiments by washing three times with 20 vols of an incubation medium containing 500 mmol l^{-1} NaCl and 15 mmol l^{-1} Mops (titrated to pH 7.5 with KOH). The buffy coat was discarded and the washed erythrocytes were resuspended to a haematocrit of 20 % in incubation medium. The water content of hagfish erythrocytes, determined using ^3H_2O with [^{14}C]sucrose as an extracellular space marker, is 68.6 % (v/v) (Fincham *et al.* 1990).

Membrane preparation

Nucleus-free plasma membranes were prepared from washed hagfish erythrocytes by hypotonic lysis in ice-cold 3 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Mops (pH 7.0) (Ellory *et al.* 1987). The absence of nuclei (removed in a low-speed spin) was confirmed by phase-contrast light microscopy. Membranes were stored at −70 °C until required.

Glucose transport

Intact cells

Inward fluxes of D- and L-glucose were measured by conventional tracer techniques. Uptake was initiated by mixing cell suspension at 1 or 10 °C with an equal volume of incubation medium at the same temperature containing the appropriate concentration of hexose and was traced with D-[U-^{14}C]glucose or L-[1-^{14}C]glucose (typically 37 kBq ml^{-1}) (Amersham International, Amersham, Bucks UK). Incubations were stopped at pre-determined time intervals (2.5–60 s) by transferring 0.2 ml of the cell suspension (10 % haematocrit) to a microfuge tube (volume 1.5 ml) with 0.8 ml of ice-cold incubation medium containing 20 μmol l^{-1} cytochalasin B layered on top of 0.5 ml of ice-cold *n*-dibutylphthalate (an ‘oil tube’). The oil tube, which was positioned in the rotor of an Eppendorf 5414 microfuge, was immediately centrifuged at 15 000 ***g*** for 10 s. The aqueous medium and *n*-dibutylphthalate layers were removed by suction, leaving

the cell pellet at the bottom of the tube. After carefully wiping the inside of the centrifuge tube with absorbent dental roll, the cell pellet was lysed with 0.5 ml of 0.5 % (v/v) Triton X-100 in water and 0.5 ml of 5 % (v/v) trichloroacetic acid was added. The precipitate was removed by centrifugation (15 000 ***g*** for 2 min) and 0.9 ml of the protein-free supernatant was counted for radioactivity by liquid scintillation spectroscopy with appropriate quench correction. Correction for radioactivity trapped in the extracellular space was made by processing cell samples which had been mixed at 1 °C with [U-^{14}C]sucrose (37 kBq ml^{-1}) (Amersham International) and immediately centrifuged. Uptake values were calculated after subtraction of these 'blank' estimates. For incubations in the presence of cytochalasin B, cells were equilibrated with inhibitor for 30 min at 10 °C before addition of medium containing [^{14}C]glucose. Kinetic constants (± S.E.) for glucose uptake by intact erythrocytes and reconstituted proteoliposomes (please see next section) were determined by nonlinear regression analysis (Enzfitter, Elsevier-Biosoft).

Reconstitution studies

Hagfish erythrocyte membranes (2 mg protein ml^{-1}) were solubilised at 4 °C by suspension and stirring for 30 min in 46 mmol l^{-1} *n*-octylglucoside, 50 mmol l^{-1} Tris–HCl, 2 mmol l^{-1} dithiothreitol (pH 7.4), after which the preparation was centrifuged at 130 000 ***g*** for 1 h. The membrane-free supernatant was collected and detergent was removed by dialysis against four changes of 10 mmol l^{-1} Tris–HCl, 0.2 mmol l^{-1} dithiothreitol (pH 7.4 at 4 °C) over a period of 48 h. This solubilised crude membrane extract was reconstituted into soybean phospholipid vesicles by freeze–thaw–sonication as described previously (Tse *et al.* 1985). Radiolabelled D- and L-glucose uptake by the reconstituted proteoliposomes was measured at 15 °C by a centrifugal gel filtration method (Tse *et al.* 1985). Briefly, columns of Sephadex G-50 (fine), which had been pre-equilibrated with 10 mmol l^{-1} Tris–HCl containing 20 μmol l^{-1} cytochalasin B, were poured to the 1 ml mark in disposable 1 ml (tuberculin) syringes. The columns were centrifuged at 200 ***g*** for 2 min in the swinging bucket rotor of a bench centrifuge shortly before use. The prepared columns were kept on ice. All subsequent procedures were carried out in a cold room at 4 °C. Incubations were initiated by adding 45 μl of reconstituted vesicles (with or without 20 μmol l^{-1} cytochalasin B) to 45 μl of ^{14}C-labelled glucose (0.4–8.0 mmol l^{-1}, 74 kBq ml^{-1}) in the same 10 mmol l^{-1} Tris–HCl buffer. Uptake was terminated by the rapid addition of 20 μl of an ice-cold stopping solution containing 20 μmol l^{-1} cytochalasin B in Tris–HCl buffer, and a 75 μl sample of the reaction mixture was immediately applied to a centrifuged column. After the sample had entered the gel, 20 μl of ice-cold stopping solution was added to the syringe column, which was then recentrifuged as described above, and the eluate was collected directly into a scintillation minivial in the centrifuge bucket. Blank values for uptake assays were determined by centrifugal gel column processing of samples taken immediately after mixing ice-cold cytochalasin-B-treated vesicles and ice-cold ^{14}C-labelled glucose. These blanks were subtracted from measurements of glucose uptake by reconstituted vesicles. Protein was determined according to Lowry *et al.* (1951) and phospholipid by phosphate analysis (Kagawa and Racker, 1971).

Photoaffinity labelling of the hagfish erythrocyte glucose transporter

Cytochalasin B binding to equilibrative glucose transporters is normally reversible. However, covalent radiolabelling of transport protein occurs when site-bound [^{3}H]cytochalasin B is exposed to high-intensity ultraviolet light (Carter-Su *et al.* 1982). Hagfish erythrocyte membranes in 50 mmol l^{-1} Tris–HCl buffer, pH 7.5 at 1 °C containing either 1 mol l^{-1} sorbitol or D-glucose (volume 0.4 ml, final protein concentration 2.5 mg ml^{-1}) were equilibrated on ice for 30 min with 0.1 μmol l^{-1} [^{3}H]cytochalasin B (574 GBq $mmol^{-1}$) (Amersham International) and then flushed with N_2. Photolysis was carried out under N_2 in 0.4 ml mini-spectrometer quartz cuvettes (1 mm light path) on ice using a 450 W mercury arc lamp (Conrad-Hanovia, Newark, NJ, USA). Ultraviolet exposure was for 45 s at a distance of 6.5 cm from the lamp's silica cooling sleeve. Samples were then diluted 20-fold with 50 mmol l^{-1} Tris–HCl containing 2 μmol l^{-1} nonradioactive cytochalasin B, before recovery of radiolabelled membranes by ultracentrifugation. The membrane pellets were washed a second time in 50 mmol l^{-1} Tris–HCl, 2 μmol l^{-1} cytochalasin B to remove residual noncovalently bound radioactivity, after which they were dissolved in SDS/polyacrylamide gel sample buffer (Thompson and Maddy, 1982).

SDS/polyacryalmide gel electrophoresis

SDS/polyacrylamide gel electrophoresis was carried out in 2 mm thick 10 % (w/v) polyacrylamide slab gels by the method of Thompson and Maddy (1982). The Laemmli buffer system (Laemmli, 1970) was used. Radioactivity in the various regions of the gel was determined by slicing the gel lanes into 2 mm fractions. The ^{3}H content of these slices was measured by liquid scintillation counting (Wu *et al.* 1983).

Western blots

Antibodies against the purified human erythrocyte glucose transporter and against synthetic peptides corresponding to residues 1–15 (A), 84–98 (B), 144–158 (C), 217–232 (D), 231–246 (E), 240–255 (F), 256–272 (G), 326–340 (H), 389–403 (I), 450–467 (J), 460–477 (K) and 477–492 (L) of the transporter sequence were raised as described previously (Davies *et al.* 1987, 1990). Antibodies against peptides D, E, F, G, J, K and L, and against the intact transporter, were affinity-purified by absorption onto protein-depleted human erythrocyte membranes (Davies *et al.* 1987). Western blotting was performed using the procedures detailed in Madon *et al.* (1990) and [^{125}I]F(ab′)$_2$ donkey anti-rabbit IgG (Amersham International, Amersham, Bucks, UK) to detect bound rabbit antibodies.

Results and discussion

Glucose transport by intact hagfish erythrocytes and reconsituted proteoliposomes

Ingermann *et al.* (1984) reported that erythrocytes from the Pacific hagfish exhibit a high permeability to 3-*O*-methyl-D-glucose. In an experiment to confirm and extend this observation, we measured time courses (2.5–60 s) of D- and L-[^{14}C]glucose uptake by hagfish erythrocytes at an initial extracellular concentration of 0.2 mmol l^{-1} and a

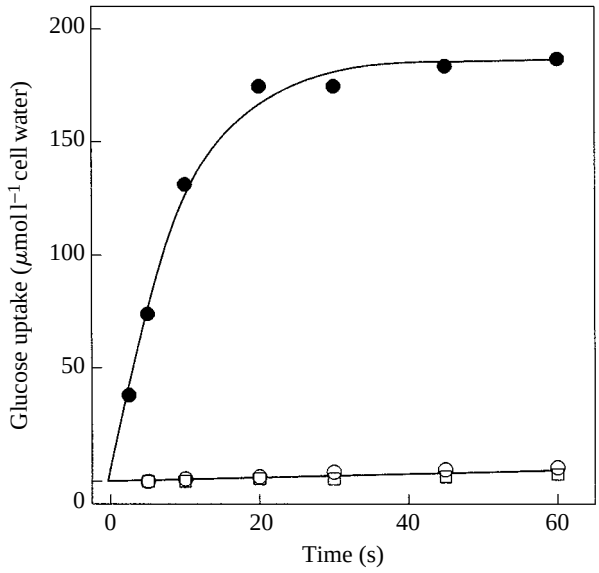

Fig. 1. Time courses of D- and L-glucose uptake by hagfish erythrocytes. Uptake of D-glucose (0.2 mmol l^{-1} extracellular concentration, 10 °C) was measured in the absence (●) and in the presence (○) of 10 μmol l^{-1} cytochalasin B as described in Materials and methods. Uptake of L-glucose at the same extracellular concentration and temperature was measured in the absence of cytochalasin B (□). Values are means of duplicate determinations.

temperature of 10 °C. As shown in the representative experiment presented in Fig. 1, uptake of D-glucose was extremely rapid, the hexose equilibrating with intracellular water within 30 s (half-time for equilibration approximately 10 s). In contrast, uptake of L-glucose occurred slowly and was linear with time (2.9 μmol l^{-1} cell water 60 s^{-1}). D-Glucose uptake in the presence of 10 μmol l^{-1} cytochalasin B was only slightly faster (5.5 μmol l^{-1} cell water 60 s^{-1}). Transport of glucose by hagfish erythrocytes is therefore both rapid and stereospecific and occurs by a mechanism sensitive to inhibition by cytochalasin B. It has previously been shown (Tse and Young, 1990) that there is an extreme variability in the D-glucose permeability of eel erythrocytes. There was no such large individual variability in the glucose permeability of hagfish erythrocytes: influxes of D-glucose (0.2 mmol l^{-1}, 10 °C) into cells from five different hagfish were 66±11 μmol l^{-1} cell water 5 s^{-1} (mean ± S.E.M., range 35–104 μmol l^{-1} cell water 5 s^{-1}).

Additional functional and molecular studies of glucose transport in this primitive vertebrate species were performed using plasma membrane preparations isolated from erythrocytes by hypotonic lysis (Ellory *et al.* 1987). For transport experiments, membranes were extracted with the detergent *n*-octylglucoside. Solubilised integral membrane proteins, including glucose transporter, were reconstituted into soybean phospholipid vesicles by freeze–thaw sonication. Fig. 2 shows representative time courses for D- and L-glucose uptake (0.2 mmol l^{-1} extravesicular concentration) by reconstituted proteoliposomes. In this and subsequent vesicle studies, we used an

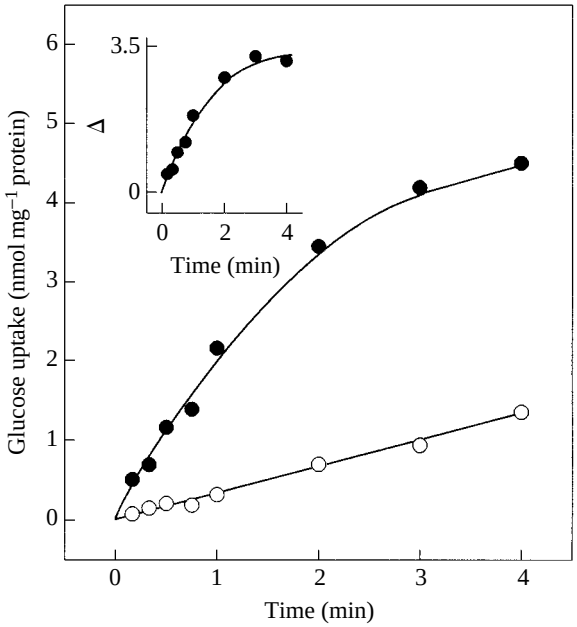

Fig. 2. Time course of D- and L-glucose uptake into vesicles reconstituted with *n*-octylglucoside membrane extract of hagfish erythrocytes. Uptake of D-glucose (●) and L-glucose (○) was measured at 15 °C and with a permeant concentration of 0.2 mmol l^{-1} as described in Materials and methods. The reconstituted preparation contained a protein:lipid ratio of 1:44 (14.7 and 650 μg per assay, respectively). The inset shows the time course of the stereoselective component of glucose uptake (Δ is the difference between D- and L-glucose uptake). Values are means of duplicate determinations.

incubation temperature of 15 °C (compared with 10 or 1 °C for intact cells) to maximise the contribution of carrier-mediated uptake of permeant *versus* diffusion across the vesicle lipid bilayer. Two routes of D-glucose uptake are apparent in the reconstituted vesicles: (a) a rapidly equilibrating component with a half-time for equilibration of approximately 50 s; and (b) a slower component that was linear with respect to time. Only the latter component of uptake was observed with L-glucose. The stereospecific entry of glucose into the vesicles is attributed to transporter-mediated influx, whereas the nonstereospecific component of uptake probably represents simple diffusion of the isotopic permeant across the lipid bilayer.

These conclusions are supported by the experiment presented in Table 1, which compares the rates of D- and L-glucose uptake (30 s flux) in reconstituted and protein-free vesicles, measured both in the absence and in the presence of 10 μmol l^{-1} cytochalasin B. Uptake of glucose by protein-free liposomes was not inhibited by cytochalasin B and was not stereoselective. In these vesicles, D- and L-glucose uptake rates were similar in magnitude to the rates of L-glucose (± cytochalasin B) and cytochalasin-B-insensitive D-glucose uptake by reconstituted vesicles. For reconstituted vesicles, stereospecific glucose uptake (i.e. D-glucose − L-glucose) (0.75±0.06 nmol mg^{-1} protein 30 s^{-1}) was not significantly different in magnitude from the cytochalasin-B-sensitive component of

Table 1. *Reconstitution of glucose transport activity with* n*-octylglucoside membrane extract from hagfish erythrocytes*

	Uptake (nmol mg^{-1} protein $30\,s^{-1}$)			
	D-Glucose		L-Glucose	
	– Cytochalasin B	+ Cytochalasin B	– Cytochalasin B	+ Cytochalasin B
Reconstituted vesicles	1.05±0.05	0.40±0.03	0.30±0.04	0.37±0.07
Protein only	0	0	0	0
Lipid only	0.28±0.04	0.29±0.02	0.25±0.01	0.20±0.01

Uptake of D- and L-glucose (0.2 mmol l^{-1}) was measured at 15 °C during 30 s intervals both in the absence and in the presence of 10 μmol l^{-1} cytochalasin B, as described in the text.

The reconstituted preparation contained a protein:lipid ratio of 1:44 (14.7 and 650 μg per assay, respectively). For the 'protein only' and 'lipid only' controls, reconstitution was performed in the absence of added lipid and protein, respectively.

Values are means ± S.E.M. of triplicate determinations.

Table 2. *Effects of hexoses on* D*-glucose transport into vesicles reconstituted with* n*-octylglucoside membrane extract of hagfish erythrocytes*

Hexose	Hexose concentration (mmol l^{-1})	D-Glucose transport (% inhibition)
D-Glucose	1	65
	2	78
2-Deoxy-D-glucose	1	57
	2	84
3-*O*-methyl-D-glucose	1	39
	2	70
L-Glucose	2	0

Cytochalasin-B-sensitive uptake of 0.2 mmol l^{-1} D-[U-^{14}C]glucose (15 °C, 30 s) was measured as described in the legend to Table 1. Competing nonradioactive hexoses were added to reconstituted vesicles at the same time as permeant.

Values are means of duplicate determinations.

D-glucose uptake (0.65±0.06 nmol mg^{-1} protein $30\,s^{-1}$). The results presented in Table 1 further demonstrate that exogenous lipid was essential for the reconstitution of glucose transport activity. Table 2 shows that the cytochalasin-B-sensitive transport of D-glucose catalysed by reconstituted proteoliposomes was inhibited by 2-deoxy-D-glucose and by 3-*O*-methyl-D-glucose, but not by L-glucose.

Fig. 3 shows the concentration dependence of D- and L-glucose uptake by reconstituted proteoliposomes, measured over the concentration range 0.2–4.0 mmol l^{-1}. As predicted by the preceding experiments, L-glucose uptake was proportional to glucose concentration, while D-glucose exhibited both saturable and nonsaturable components of

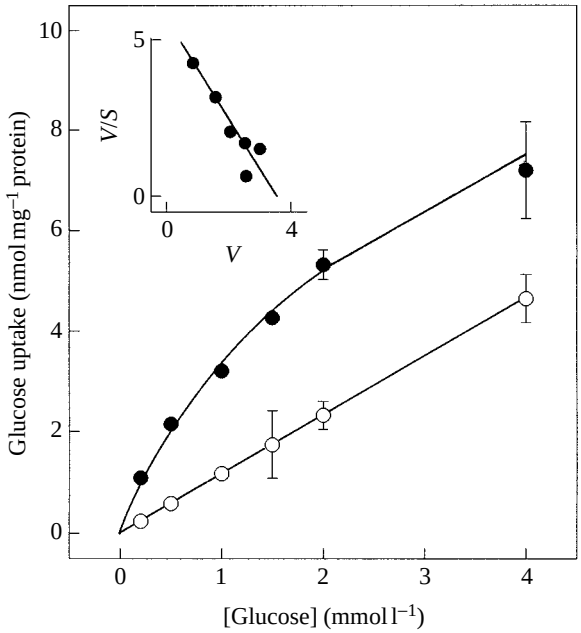

Fig. 3. Concentration dependence of D- and L-glucose uptake into vesicles reconstituted with *n*-octylglucoside membrane extract of hagfish erythrocytes. D-glucose (●) and L-glucose (○) uptake from graded concentrations of glucose during 30 s intervals was measured in vesicles prepared as described in the text and the legend to Fig. 2 (protein:lipid ratio 1:44). Error bars are S.E.M. of triplicate determinations. An Eadie–Hofstee plot of the stereospecific component of glucose uptake (*V*/*S versus V*) is shown in the inset. Kinetic constants for this component of glucose uptake, determined by nonlinear regression analysis, are given in the text.

uptake, the latter being equivalent to that observed for L-glucose. The inset in Fig. 3 is a plot of *V*/*S* against *V*, where *V* is the rate of glucose uptake and *S* is glucose concentration, for the carrier-mediated stereospecific component of glucose uptake. The data conform to simple Michaelis–Menten kinetics, giving estimates of apparent K_m and V_{max} of 0.50 ± 0.18 mmol l^{-1} and 3.23 ± 0.34 nmol mg^{-1} protein 30 s^{-1}, respectively. In a corresponding experiment with intact hagfish erythrocytes at 10 °C, we obtained kinetic constants of 0.88 ± 0.06 mmol l^{-1} (K_m) and 0.575 ± 0.015 mmol l^{-1} cell water 5 s^{-1} (414 ± 11 mmol l^{-1} cell water h^{-1}) (V_{max}) for carrier-mediated D-glucose uptake, calculated either as the stereospecific component of glucose uptake or as the component of D-glucose uptake inhibited by 10 μmol l^{-1} cytochalasin B (Fig. 4). For comparison, estimated kinetic constants for D-glucose zero-*trans* influx by intact human erythrocytes at this temperature (10 °C) are 0.47 mmol l^{-1} (K_m) and 270 mmol l^{-1} cell water h^{-1} (V_{max}) (Lowe and Walmsley, 1986). At the higher temperature of 20 °C, river lamprey (*Lampetra fluviatilis*) erythrocytes transport glucose with an apparent K_m value of 1.6 mmol l^{-1} but with a 20-fold lower V_{max} than hagfish erythrocytes (Tiihonen and Nikinmaa, 1991*a*).

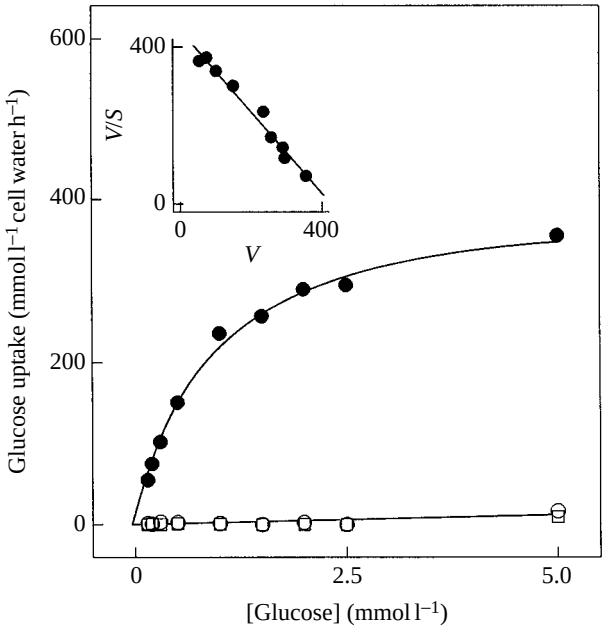

Fig. 4. Concentration dependence of D- and L-glucose uptake by hagfish erythrocytes. Initial rates of D-glucose uptake (5 s flux, 10 °C) were measured in the absence (●) and in the presence (○) of 10 μmol l^{-1} cytochalasin B as described in Materials and methods. Uptake of L-glucose under the same conditions was measured in the absence of cytochalasin B (□). Values are means of duplicate determinations. An Eadie–Hofstee plot of the stereospecific component of glucose uptake (*V*/*S versus V*) is shown in the inset. Kinetic constants for the carrier-mediated component of glucose uptake, calculated either as the difference between D- and L-glucose uptake or as the cytochalasin-B-sensitive component of the D-glucose uptake and determined by nonlinear regression analysis, are given in the text.

Photoaffinity labelling of the hagfish erythrocyte glucose transporter

The functional characteristics of glucose transport by hagfish erythrocytes, as defined by us here in studies of D- and L-glucose uptake by intact cells and reconstituted proteoliposomes and by Ingermann *et al.* (1984) in studies of 3-*O*-methyl-D-glucose uptake by intact cells, correspond closely with the properties of the GLUT 1 hexose transporter expressed in human erythrocytes (Widdas, 1980; Carruthers, 1990). The human erythrocyte GLUT 1 glucose transporter can be photoaffinity labelled with [^{3}H]cytochalasin B and migrates on SDS/polyacrylamide gels as a band 4.5 polypeptide (nomenclature of Steck, 1974) with an apparent M_r of 55 000. Photoaffinity labelling experiments with radiolabelled cytochalasin B using two separate batches of isolated hagfish erythrocyte membranes are shown in Fig. 5. In each case, a major peak of radiolabelling was observed in the band 4.5 region of the gel (between standards of relative molecular mass 66 000 and 45 000; average M_r 55 000), this labelling being almost completely abolished in the presence of D-glucose.

The extent of covalent radiolabelling of hagfish band 4.5 polypeptides by cytochalasin

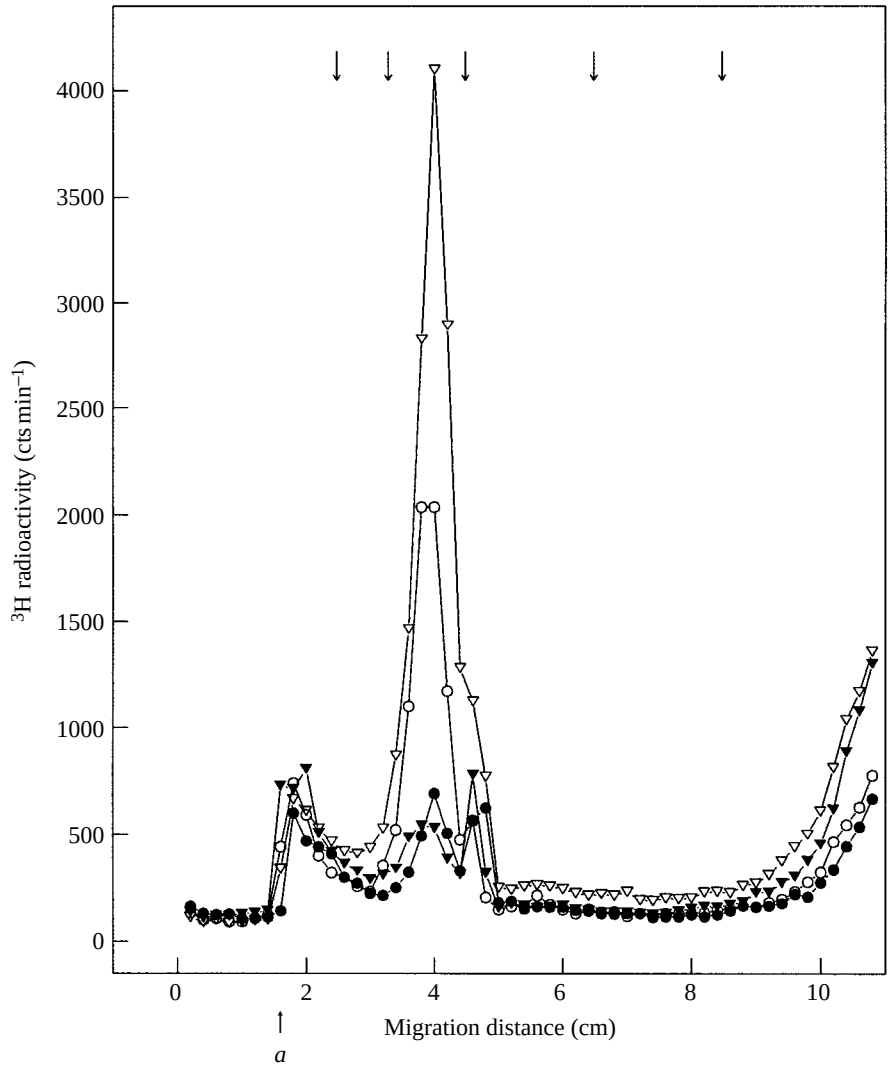

Fig. 5. Photoaffinity labelling of hagfish erythrocyte membranes with [^{3}H]cytochalasin B. Membranes were incubated at 1 °C with [^{3}H]cytochalasin B (0.1 μmol l^{-1}) in the presence of either 1 mol l^{-1} sorbitol (○, ▽) or 1 mol l^{-1} D-glucose (●, ▼) as described in Materials and methods. After equilibrium binding had been reached, the samples were irradiated under N_2, washed and subjected to SDS/polyacrylamide gel electrophoresis (12 % acrylamide). Circles and triangles refer to two different batches of hagfish erythrocyte membranes loaded onto the gel at the same protein content (0.25 mg per sample). The positions of molecular mass standards (97×10^3, 66×10^3, 45×10^3, 31×10^3 and 22×10^3 Da, left to right) are indicated by arrows and are from the same slab gel. The position of the stacking gel–running gel interface is indicated by *a*.

B in the experiment shown in Fig. 5 is similar to that reported by us previously for cytochalasin B labelling of human erythrocyte membranes under similar experimental conditions (Jarvis and Young, 1987). This observation, together with the correspondence of translocation capacities of glucose transport in human and hagfish erythrocytes at 10 °C, suggests that the two species may have similar densities of glucose transport proteins in their erythrocytes. In human erythrocytes, GLUT 1 corresponds to approximately 6 % of the integral membrane protein (approximately 5×10^5 copies per cell) (Allard and Lienhard, 1985), most of the remaining integral membrane protein being accounted for by the band 3 chloride/bicarbonate exchange transporter (approximately 10^6 copies per cell) (Wieth, 1979). Interestingly, hagfish erythrocytes are essentially deficient in chloride/bicarbonate exchange protein (Ellory *et al.* 1987). Coomassie-Blue-stained gels of hagfish erythrocyte membranes (Ellory *et al.* 1987) show no detectable band 3 protein, but do show readily visible staining in the band 4.5 region of the gel corresponding to the peak of cytochalasin B radiolabelling seen in Fig. 5. It is likely that the glucose transporter is a major component of this band.

Cross-reactivity of the hagfish erythrocyte glucose transporter with human GLUT 1 antibodies

The extent of amino acid sequence homology between the hagfish and human erythrocyte glucose transporters was investigated in experiments where hagfish erythrocyte membranes were electrophoresed on an SDS/polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then probed with a panel of 12 different antipeptide antisera and affinity-purified antibodies (A–L in Fig. 6) raised against the cytoplasmic extramembranous regions (174 out of a total of 492 amino acid

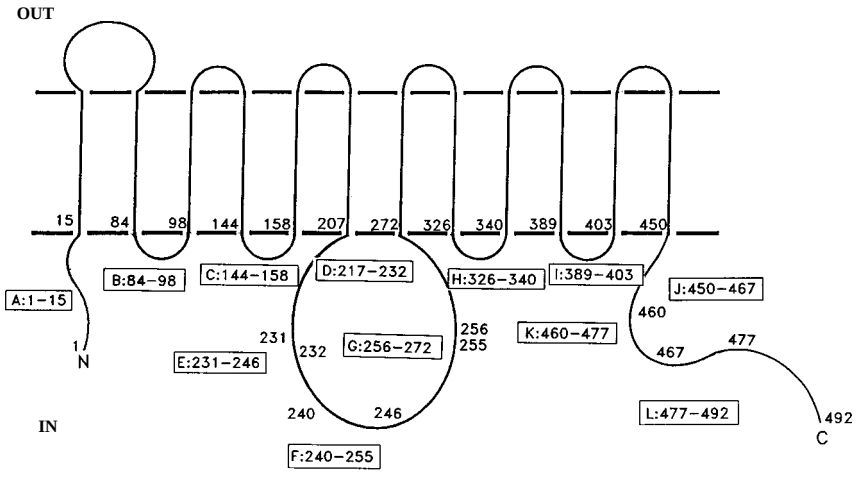

Fig. 6. Transmembrane topology of the human erythrocyte/HepG2 (GLUT 1) glucose transporter, showing locations and residue numbers of the cytoplasmic domains recognised by antipeptide antibodies (A–L). Cross-reactivity with Western blots of the hagfish erythrocyte glucose transporter was observed with antibodies F and J (please see Fig. 7).

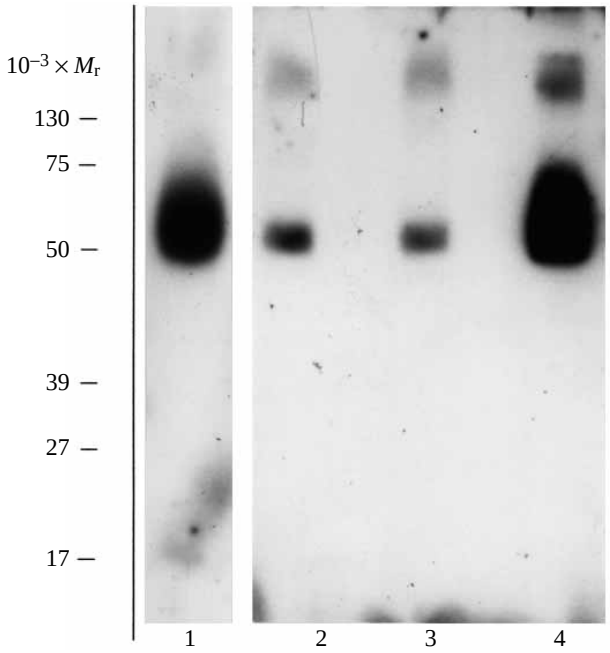

Fig. 7. Immunological detection of proteins present in hagfish erythrocyte membranes showing homology to the human erythrocyte/HepG2 glucose transporter. Samples of hagfish erythrocyte membranes in lanes 1, 2 and 3 (containing 50 μg of protein), and purified human erythrocyte glucose transporter (lane 4) (1 μg of protein), were electrophoresed on 10 % SDS polyacrylamide gels, electrophoretically transferred to nitrocellulose paper and then immunologically stained with an antibody to the intact human erythrocyte glucose transporter (lane 1), with antipeptide antibody (F) to residues 240–255 of the human erythrocyte glucose transporter (lane 2) or with an antibody (J) to residues 450–467 of the human erythrocyte glucose transporter (lanes 3, 4). The positions of relative molecular mass standards are indicated.

residues) of the human erythrocyte glucose transporter (Fig. 6), and with an antibody to the intact purified human protein.

The latter antibody was found to label a component in the membranes with an apparent average M_r of 55 000, suggesting similarity between the carbohydrate and/or polypeptide components of the human and hagfish proteins (Fig. 7, lane 1). In addition, two affinity-purified antipeptide antibodies (F and J) corresponding to residues 240–255 and 450–467 of the human erythrocyte transporter (Fig. 6), also labelled a component of similar M_r (Fig. 7, lanes 2 and 3). This finding demonstrates localised sequence similarity between the polypeptides of the two species within the central cytoplasmic loop and within the cytoplasmic C-terminal region (Fig. 6). No labelling was seen when non-immune rabbit IgG was used (not shown). The stronger labelling resulting from the use of the antibody against the intact human transporter, when compared with antipeptide antibody labelling, suggests that the former antibody may be recognising multiple epitopes, within either the oligosaccharide or polypeptide moieties of these glycoproteins, that are similar in the two

species. The relative sharpness of the hagfish transporter bands, both on photolabelling (Fig. 5) and on Western blots (Fig. 7), indicates that this protein carries less heterogeneous glycosylation than the human protein.

The two segments of the human glucose transporter sequence found to be antigenically similar to the hagfish protein are both located in functionally important regions of the protein. Residues 450–467 immediately follow predicted transmembrane helix 12 in the sequence and contain the conserved sequence motif VPETKG (residues 452–457). This motif is found not only in all members of the mammalian passive sugar transporter family (GLUT 1–5), with only very minor variations (K to R in human GLUTs 3 and 4; G to A in human GLUT 5), but also in yeast, plant and bacterial sugar transporters (Baldwin, 1992). For example, it is completely conserved in the active, H^+/xylose symporter XylE of *Escherichia coli*. Truncation of GLUT 1 within this region at residue 455 by mutagenesis completely inactivates transport, probably by locking the transporter into an inward-facing conformation (Oka *et al.* 1990). The region containing residues 240–255 is less well conserved, although glutamate residues are present at positions 243 and 247 in all known mammalian passive sugar transporters (Baldwin, 1992). However, antibodies against this region inhibit the binding of the transport inhibitor cytochalasin B to the protein, demonstrating the importance of the central cytoplasmic loop of the protein for transporter function (Davies *et al.* 1990).

Is the glucose transporter in hagfish erythrocytes proton-dependent?

It is clear from the results presented in this paper that the hagfish erythrocyte glucose transporter belongs to a gene family which, at one evolutionary extreme, includes the human erythrocyte GLUT 1 glucose transporter isoform and, at the other, encompasses yeast and bacterial H^+/sugar cotransport proteins. As far as we are aware, the possibility that the human erythrocyte glucose transporter, which, like the hagfish transport protein, functions as an equilibrative system, might exhibit vestigial proton coupling has not been rigorously addressed. In fact, such experiments would be technically difficult because the band 3 Cl^-/HCO_3^- exchange transporter in human erythrocytes would rapidly dissipate any imposed transmembrane H^+ gradient. This is not a problem with hagfish erythrocytes, which are essentially deficient in band 3 transport activity (Ellory *et al.* 1987). Previously, we have shown that experimentally induced H^+ gradients (outside alkaline) in hagfish erythrocytes persist for periods in excess of 10 min at 1 °C (Ellory *et al.* 1987).

In Fig. 8 we compare the time courses of 0.2 mmol l^{-1} D-glucose uptake by hagfish erythrocytes at 1 °C, both in normal pH 7.5 medium and in medium buffered at pH 5.5. Cells were suspended in the latter medium immediately prior to the addition of permeant. The inwardly directed H^+ gradient slightly decreased ($P<0.05$), rather than increased, the initial rate of D-glucose uptake by hagfish erythrocytes and had no effect upon the intracellular D-glucose concentration at equilibrium. At the end of the incubation period (5 min), the external pH of the acidified cell suspension was still 5.5 compared with 7.5 for the control cell suspension. A repeat experiment with erythrocytes from a second hagfish gave essentially identical results except that there was no significant difference in the initial rates of D-glucose uptake at the two pH values (data not shown).

In the converse experiment, we suspended hagfish erythrocytes at a high haematocrit of

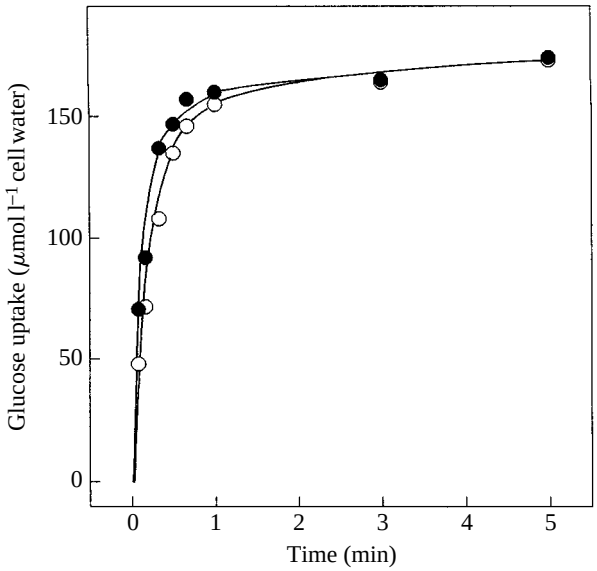

Fig. 8. Effect of external pH on D-glucose uptake by hagfish erythrocytes. Time courses of D-glucose uptake by hagfish erythrocytes (1 °C, 0.2 mmol l^{-1} extracellular concentration) were measured in normal pH 7.5 medium (●) and in medium buffered at pH 5.5 (○). Cells were suspended in the latter medium immediately prior to the addition of permeant. Values are means of triplicate determinations. Standard errors (not shown) were typically 5 % of mean values. See text for other experimental details.

30 % in lightly buffered medium (1.5 *versus* 20 mmol l^{-1} Mops, pH 7.5) at 1 °C. Addition of 4 mmol l^{-1} HCl to duplicate cell suspensions decreased the external pH to 3.6 after 10 min and to 5.1 after 30 min. As shown in Fig. 9, subsequent addition of 10 mmol l^{-1} D-[^{14}C]glucose to one of the cell suspensions after a further 10 min (external pH 5.4) had no measurable effect on medium pH over the next 20 min. During this period, uptake of D-glucose by the cells was 3.6 mmol l^{-1} cell water (Fig. 9). Addition of 10 mmol l^{-1} D-glucose to cells in pH 7.5 medium caused no measurable changes in external pH. Glucose uptake under these conditions was 3.4 mmol l^{-1} cell water in 20 min. We conclude from these experiments that glucose uptake by hagfish erythrocytes is not coupled to the movement of protons.

Conclusions

A high glucose permeability is considered to be characteristic of primate erythrocytes. Most investigations have focused on human erythrocytes, but there is evidence that monkey (*Macaccus rhesus*) erythrocytes exhibit comparable rates of glucose transfer (Andreen-Svedberg, 1933). Lemur (*Lemur fulvus*) erythrocytes also transport glucose rapidly (J. D. Young and R. C. Crompton, unpublished results). In nonprimate mammalian species, rates of carrier-mediated glucose transport are high in foetal erythrocytes, but either low (e.g. sheep, rabbit) or absent (pig) in erythrocytes from adult animals. Similarly, carrier-mediated glucose transport is either slow or undetectable in

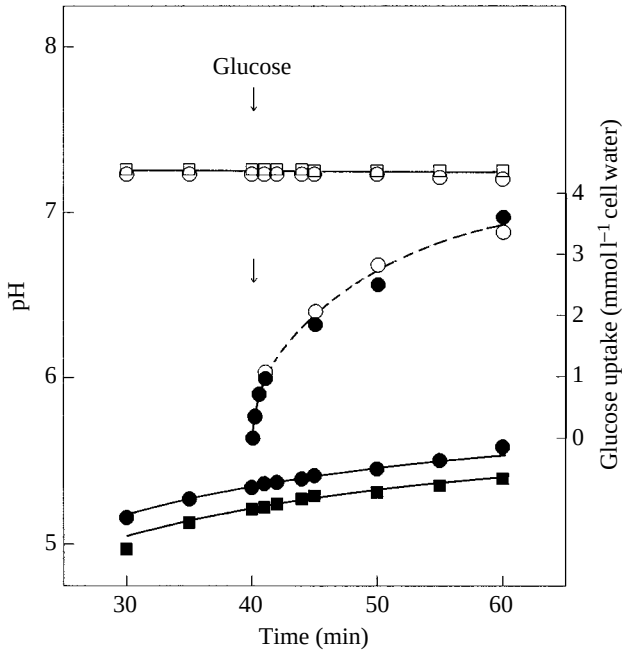

Fig. 9. Effect of D-glucose on the external pH of hagfish erythrocyte suspensions. Hagfish erythrocytes (30 % haematocrit) were suspended in 1.5 mmol l^{-1} Mops medium at 1 °C. External pH was monitored in duplicate cell suspensions for the period 30–60 min after the addition of 4 mmol l^{-1} HCl (●, ■, solid lines). One cell suspension (●) was supplemented with 10 mmol l^{-1} D-[^{14}C]glucose at the time indicated by the arrow (40 min). External pH was also monitored in duplicate cell suspensions in non-acidified medium (○, □, solid lines). Again, 10 mmol l^{-1} D-[^{14}C]glucose was added to one of the suspensions (○) after 40 min. The dashed curve, and associated symbols, shows the measured uptake of radioactive D-glucose under the two conditions (●, acidified medium; ○, non-acidified medium). See text for other experimental details.

nucleated erythrocytes from avian or teleost species. Rapid glucose transfer in foetal erythrocytes may be seen as offering a biological advantage in the carriage of glucose from the placenta to foetal tissues (Goodwin, 1954, 1956; Widdas, 1980). The retention of this property in adult erythrocytes in humans and in other primates is considered by Widdas (1980) to be an example of 'biochemical' pedomorphosis, the persistence of characteristics that were embryonic or juvenile features of ancestral types. Whether the persistence of a high glucose permeability in the erythrocytes of adult primates serves any physiological function, such as buffering of the gradient between blood and tissue or facilitation of transfer across the blood–brain barrier, requires further investigation (Jacquez, 1984).

The hagfish diverged from the main line of vertebrate evolution approximately 550 million years ago and thus occupies a key branch point on the evolutionary tree. In marked contrast to those of other nonprimate vertebrates, erythrocytes from hagfish exhibit rates of glucose transfer comparable to that of human erythrocytes. Unlike the

absence of the chloride/bicarbonate exchange transporter, band 3 (Ohnishi and Asai, 1985; Ellory *et al.* 1987), a high glucose permeability is not a general characteristic of agnathan species. Lamprey erythrocytes transport glucose much more slowly than those of hagfish; at 10 °C, the V_{max} for transporter-mediated glucose uptake by hagfish erythrocytes is still 20-fold greater than that determined for lamprey cells at the higher temperature of 20 °C. Although both hagfish and lampreys belong to the same phylogenetic superclass, hagfish exhibit more primitive features. As is the case for primates, it remains to be established whether the high glucose permeability of hagfish erythrocytes serves any physiological function.

There are very close functional and structural similarities between hagfish and human erythrocyte glucose transporters. The latter shows substantial sequence similarities to the active H^+-linked sugar transporters from a number of organisms, including plants, green algae, cyanobacteria, eubacteria and fungi (Baldwin, 1992). However, our results show no effect of an induced proton gradient on glucose transport by hagfish erythrocytes. Therefore, it appears that proton cotransport was lost before, or early in, the evolution of vertebrates.

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