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The glucose transporter-2 (GLUT2) is a low affinity dehydroascorbic acid transporter

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ABSTRACT

We studied the acquisition of dehydroascorbic acid by rat hepatocytes, H4IIE rat hepatoma cells and *Xenopus laevis* oocytes. Transport kinetics and competition and inhibition studies revealed that rat hepatocytes transport oxidized dehydroascorbic acid through a single functional component possessing the functional and kinetic properties expected for the glucose transporter GLUT2. On the other hand, rat hepatoma cells showed expression of at least two dehydroascorbic acid transporters with the expected functional and kinetic properties expected for GLUT1 and GLUT2. Expression studies of GLUT2 in *X. laevis* oocytes followed by transport kinetics and competition and inhibition studies revealed that GLUT2 is a low affinity dehydroascorbic transporter whose kinetic and functional properties match those observed for the endogenous GLUT2 transporter in rat hepatocytes and rat hepatoma cells. Therefore, GLUT2, a transporter known as a low affinity transporter of glucose and fructose and a high affinity transporter of glucosamine is also a low affinity dehydroascorbic acid transporter.

1. Introduction

Vitamin C is an essential micronutrient required for the maintenance of the normal human physiology [1,2]. Vitamin C is required for the synthesis of collagen, carnitine, as a co-factor in enzymes to maintain metal ions in their reduced form, and to protect tissues from oxidative damage by scavenging free radicals [3–5]. Also, reduced vitamin C (ascorbic acid) can recycle glutathione and vitamin E, two other important biological antioxidants [6].

Vitamin C exists in two chemically distinct forms, the reduced ionizable form (ascorbic acid, AA), and the oxidized non-ionic form (dehydroascorbic acid, DHA). Human cells acquire vitamin C by transporting both chemical forms across cell membranes with the participation of two different transporter systems that show absolute specificity for each form of the vitamin [7]. One transporter system is a low affinity, high capacity sodium independent system which includes several members of the facilitative glucose transporter family (GLUTs) [8]. These transporters show absolute specificity for oxidized vitamin C and are bidirectional transporters that transport dehydroascorbic acid down its concentration gradient. Fourteen glucose transporter isoforms (GLUT1–GLUT14) have been molecularly characterized, and there is evidence that the isoforms GLUT1, GLUT3 and GLUT4 are efficient dehydroascorbic acid transporters [9–11]. A second transport system for vitamin C is a high affinity, low capacity sodium-dependent system (SVCTs) composed of two members, the sodium–ascorbic acid co-transporters SVCT1 and SVCT2 [12–14]. These transporters show absolute specificity for reduced vitamin C and transport ascorbic acid down the electrochemical sodium gradient [15].

Humans are not capable of synthesizing vitamin C and therefore must obtain it from the diet [1,2], and supplementation studies combined with *in vitro* analysis of cellular models of the intestinal barrier have established the concept that the limiting step for vitamin C bioavailability in humans is the transcellular transport across the intestinal barrier [16–18]. On the other hand, species such as the rat obtain their supplement of vitamin C from two complementary sources; *de novo* synthesis of vitamin C and dietary sources.

We used fresh rat hepatocytes as an *in vitro* model to study how these cells obtain vitamin C from extracellular sources. These cells have been established that express the low affinity glucose transporter GLUT2 [19–23]. Regarding the capacity of the hepatocytes to obtain vitamin C, the available evidence is incomplete and controversial. In the initial study demonstrating that the glucose transporters are efficient transporters of oxidized vitamin C by direct expression in *Xenopus laevis* oocytes, GLUT1, GLUT2 and GLUT4 transported dehydroascorbic acid [9]. In contrast, studies from other laboratory first indicated that only GLUT1 and GLUT3 were dehydroascorbic acid transporter and that GLUT2 and GLUT4

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lacked this activity [11]. Although in further analyses it was demonstrated that GLUT4 transports dehydroascorbic acid, no evidence was presented supporting the concept that GLUT2 functions as a dehydroascorbic acid transporter [10]. Studies using rat hepatocytes and hepatoma cells have provided evidence indicating that these cells take up dehydroascorbic acid, but failed to address the question of the identity of the transporters involved. Given that hepatocytes and hepatoma cells show abundant expression of GLUT2, a better understanding of the physiology of vitamin C in these cells requires an answer to the question of whether GLUT2 is a *bonafide* dehydroascorbic acid transporter.

We present data indicating that rat hepatocytes and rat hepatoma cells transport oxidized vitamin C. Transport analysis, competition and inhibition studies, complemented with exogenous expression of GLUT2 in *X. laevis* oocytes revealed that DHA is transported by the glucose transporter GLUT2 which functions as a low affinity dehydroascorbic acid transporter.

2. Materials and methods

Rat hepatocytes were prepared from healthy rats by perfusion with collagenase [24,25] and used immediately. Rat hepatoma cells (H4IIE) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% L-glutamine, penicillin–streptomycin and fungizone. Cell viability was always greater than 95% as assessed by trypan blue exclusion.

For uptake assays, the cells were deprived of fetal bovine serum, and other media supplements by replacing the culture media with incubation buffer (15 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 0.8 mM $MgCl_2$) before performing the assays [26,27]. Dehydroascorbic acid assays were performed in incubation buffer containing a mix of 0.05 μCi L-[^{14}C]-ascorbic acid (specific activity 8.2 mCi/mmol, NEN-DuPont), and a final concentrations of 0.5–16 mM ascorbic acid, and incubated for 5 min at 37 °C with 1–10 U of ascorbic acid oxidase (50 U/mg protein) before adding it to the cells. Oxidation of ascorbic acid was assayed by monitoring the disappearance of the ascorbic acid peak at 265 nm. Uptake was finished by adding cold stopping solution (NaCl 160 mM, KCl 5 mM, $MgSO_4$ 0.8 mM, $CaCl_2$ 1.8 mM, $HgCl_2$ 0.2 mM). The cells were washed twice with cold phosphate saline buffer (pH 7.4), lysed in 300 μl of 10 mM Tris–HCl, pH 8.0, containing 0.2% sodium dodecyl sulfate, and the incorporated radioactivity was determined by liquid scintillation counting. Transport in the absence of sodium ions was accomplished by replacing the NaCl in the incubation media with 135 mM choline chloride. Competitors (fructose and sucrose) were added to the transport assays simultaneously with the transported substrate, or the cells were preincubated in the presence of inhibitors (cytochalasin B and cytochalasin E) prior to the uptake assay. Hexose uptake assays were similarly performed using 1 μCi of 2-[1,2-^{3}H]deoxy-D-glucose (specific activity 26.2 Ci/mmol, NEN-DuPont) and 0.1–50 mM 2-deoxy-D-glucose [26,27].

For expression in *X. laevis* oocytes we used a cDNA containing the full-length coding region of GLUT2 cloned in the plasmid pBluescript [28]. RNA was synthesized *in vitro* using the mMessage mMachine kit (Ambion) and 20 ng were injected in oocytes stage V prepared from defolliculated and collagenase treated oocytes. Three days after injection, oocytes were submitted to transport assays to determine their capacity to transport radiolabeled dehydroascorbic acid and 2-deoxyglucose.

Data are presented as the average ± the standard deviation and correspond to a minimum of three assays performed independently in triplicate. Kinetic parameters were determined directly from the Michaelis–Menten equation with the program Igor and by using the linear transformation of Eadie–Hofstee.

3. Results and discussion

3.1. GLUT2 mediates the transport of dehydroascorbic acid in rat hepatocytes

Rat and human hepatocytes express elevated levels of the low affinity hexose transporter GLUT2, and previous evidence indicated that rat hepatocytes have the capacity of transporting dehydroascorbic acid. However, no detailed characterization of the transport process has been undertaken and the identity of the transporters involved remains controversial. Our initial studies using 2-deoxyglucose and short uptake assays revealed that uptake was highly dependent on the incubation temperature, with the transport rate increasing from 6 to 27 nmol/min × 10^6 cells (4.5-fold increase) when the temperature was increased from 4 to 22 °C, with an additional 2-fold increase (to 48 nmol/min × 10^6 cells) when the temperature was increased to 37 °C (Fig. 1A). These short uptake assays defined a temporal window of 8 s for the transport assays at which the uptake rate was constant, with no further increase in cell-associate radioactivity at extended times (Fig. 1B). We therefore used 6 s uptake assays and 22 °C to study the kinetic constants associated to transport. Control experiments revealed that the transport rate was not affected by the absence of sodium ions in the incubation medium (replaced with choline$^+$) (Fig. 1C), which is consistent with the expected properties of a facilitative type transporter. Concentration–response analysis using increasing concentrations of substrate revealed that transport approached saturation at 60 mM of 2-deoxyglucose (Fig. 1D), and Eadie–Hofstee analysis of the transport data indicated the presence of a single functional component involved in the transport of deoxyglucose by rat hepatocytes, with apparent transport K_m and V_{max} values of 18.1 ± 0.4 mM and 451 ± 4 nmol/min × 10^6 cells, respectively (Fig. 1E). Competition experiments revealed that cytochalasin B inhibited the transport of 2-deoxyglucose in rat hepatocytes in a dose-dependent manner, with no inhibition at 0.1 μM inhibitor, the concentration at which GLUT1 activity is inhibited by 50%, and instead 50% inhibition was observed at 3 μM cytochalasin B and more than 80% inhibition at 50 μM cytochalasin B (Fig. 1F). Immunolocalization experiments in liver *in situ* and in isolated hepatocytes, and immunoblotting experiments using plasma membranes purified from isolated hepatocytes confirmed the abundant expression of GLUT2 in rat hepatocytes (data not shown). Overall, the results of the transport and inhibition experiments are fully compatible with the expected functional properties of the low affinity glucose transporter GLUT2 expressed in rat hepatocytes.

We next analyzed the capacity of rat hepatocytes to transport dehydroascorbic acid. Similarly to the observations with 2-deoxyglucose, the uptake phase was composed of two time-dependent components. Short uptake assays revealed that dehydroascorbic acid uptake was highly dependent on the incubation temperature, with the transport rate increasing from 3 to 12 nmol/min × 10^6 cells (4-fold increase) when the temperature was increased from 4 to 22 °C, with an additional 2- to 3-fold increase (to 27 nmol/min × 10^6 cells) when the temperature was increased to 37 °C (Fig. 2A). These short uptake assays defined a temporal window of 20 s for the transport assays at which the uptake rate was constant. Longer uptake assays showed a secondary, slower uptake phase characterized by a linear increase in dehydroascorbic acid uptake at a constant uptake rate that lasted for 4 and 2 min at 18 and 37 °C, respectively (Fig. 2B). This secondary phase was also temperature dependent, with 4- and 2-fold increase in the uptake

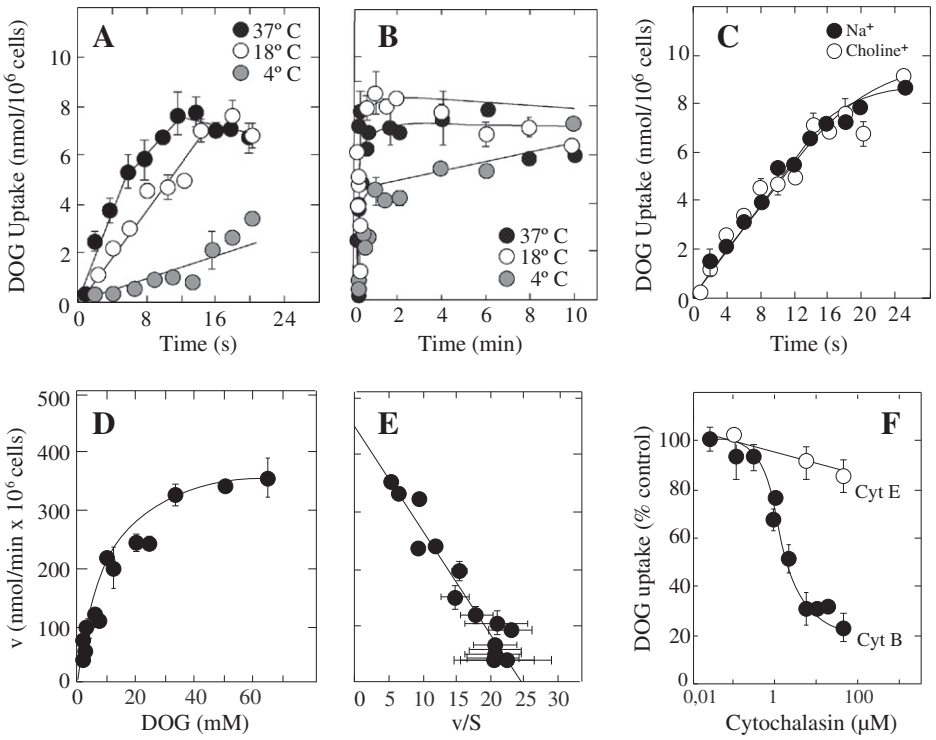

Fig. 1. 2-Deoxyglucose (DOG) transport in rat hepatocytes. (A and B) Time-course of the uptake of DOG at 37 (●), 22 (○), or 4 °C (●). (C) Time-course uptake of DOG in the presence (●) or in the absence (○) of NaCl (replaced with choline chloride). (D) Dose–response analysis of DOG transport. (E) Eadie–Hofstee plots of the substrate dependence of DOG transport. (F) Effect of cytochalasin B (●) and cytochalasin E (○) on the uptake of DOG. Uptake experiments were performed at room temperature. Data represent the mean ± SD of experiments performed in triplicate.

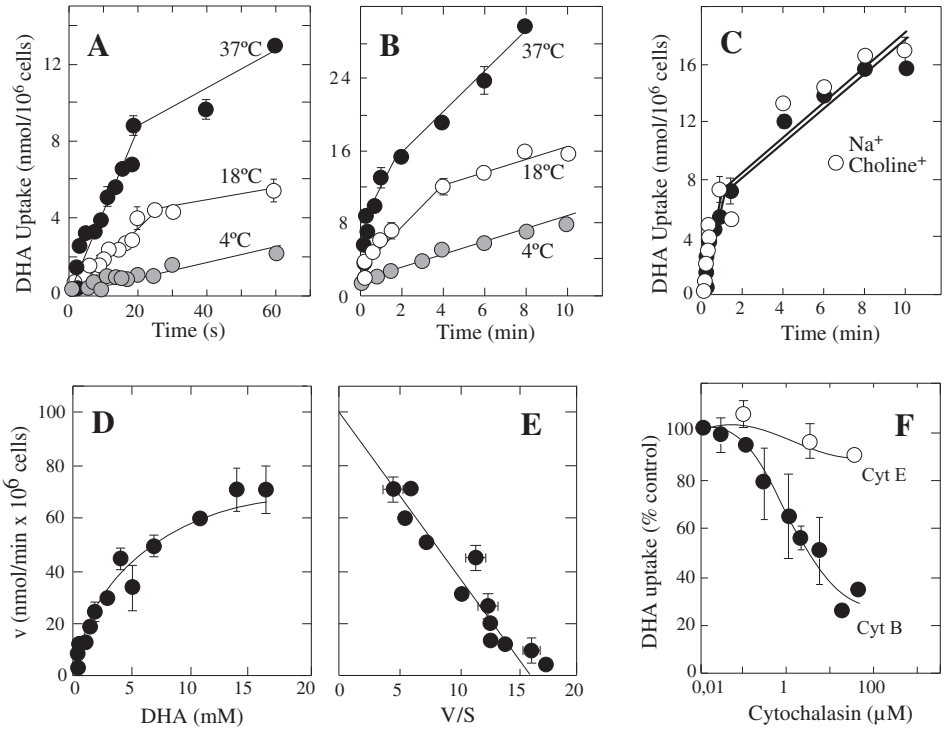

Fig. 2. Dehydroascorbic acid (DHA) transport in rat hepatocytes. (A and B) Time-course of the uptake of DHA at 37 (●), 22 (○), or 4 °C (●). (C) Time-course uptake of DHA in the presence (●) or in the absence (○) of NaCl (replaced with choline chloride). (D) Dose–response analysis of DHA transport. (E) Eadie–Hofstee plots of the substrate dependence of DHA transport. (F) Effect of cytochalasin B (●) and cytochalasin E (○) on the uptake of DHA. Uptake experiments were performed at room temperature. Data represent the mean ± SD of experiments performed in triplicate.

rate when the temperature increased from 4 to 18 °C and from 18 to 37 °C, respectively (Fig. 2B).

Additional experiments revealed that the uptake rate was not affected by the absence of sodium ions in the incubation medium (replaced with choline$^+$) (Fig. 2C), which is consistent with the concept that dehydroascorbic acid is transported by a facilitative type transporter.

In the next experiments we used 6 s uptake assays at 22 °C to study the transport kinetic constants without interference from the secondary events associated to the intracellular reduction of the recently transported dehydroascorbic acid [26]. Concentration–response analysis using increasing concentrations of substrate revealed that transport failed to reach saturation at 17 mM dehydroascorbic acid (Fig. 2D), and linearization of the data indicated the presence of a single functional component involved in the transport of dehydroascorbic acid by rat hepatocytes, with apparent transport K_m and V_{max} values of 6.4 ± 0.4 mM and 100 ± 3 nmol/min × 10^6 cells, respectively (Fig. 2E). Competition experiments revealed that cytochalasin B inhibited the transport of dehydroascorbic acid in rat hepatocytes in a dose-dependent manner, with 50% and 80% inhibition observed at 3 and 50 μM cytochalasin B, respectively, while no inhibitory effect was observed at 0.1 μM cytochalasin B and elevated concentrations of cytochalasin E had only a minor effect on dehydroascorbic acid transport (Fig. 2F).

Overall, the above indicate that rat hepatocytes acquire oxidized vitamin C with the participation of a single transporter characterized by a transport K_m of 6 mM, clearly different from the respective K_m values described for GLUT1 (<1 mM), GLUT3 (<2 mM) and GLUT4 (<2 mM), but is similar to the transport K_m of the lower affinity transporter. Moreover, the hepatocyte transporter of dehydroascorbic acid demonstrated a low sensitivity to the inhibitory effect of cytochalasin (IC_{50} > 2 μM), therefore discarding the participation of GLUT1 (cytochalasin B IC_{50} < 0.2 μM).

3.2. Two transporters mediate dehydroascorbic acid transport in rat hepatoma cells

It has been described that human hepatoma cells express at least two glucose transporters, GLUT1 and GLUT2, two transporters that possess different kinetic properties. GLUT1 is considered a high affinity glucose transporter, with an apparent K_m for the transport of 2-deoxy-D-glucose in the range of 2–4 mM, while GLUT2 is a low affinity transporter with a K_m for the transport of 2-deoxy-D-glucose greater than 20 mM. Time-course experiments revealed that rat hepatoma cells transported 2-deoxy-D-glucose efficiently, with a transport rate of 40 nmol/10^6 cells × min for the first 30 s of uptake (Fig. 3A). Concentration–response experiments using increasing concentrations of 2-deoxyglucose revealed that the transport rate approached saturation at 60 mM 2-deoxy-D-glucose (Fig. 3B) and Eadie–Hofstee analysis of the transport data indicated that transport was mediated by two kinetic components of different affinity (Fig. 3C). The calculated apparent K_m and V_{max} of the lower affinity component were 26 mM and 79 nmol/min × 10^6 cells, respectively, and for the higher affinity component 3.5 mM and 35 nmol/min × 10^6 cells, respectively (Fig. 3C). Expression of GLUT1 and GLUT2 in the hepatoma cells was confirmed by immunolocalization experiments showing positive immunoreactivity with anti-GLUT1 and anti-GLUT2 antibodies, and by RT-PCR and quantitative Real Time PCR that confirmed expression at the mRNA level (data not shown).

Similar results, indicating the presence of at least two functional transporters, were obtained when analyzing the transport of dehydroascorbic acid by the hepatoma cells. Time-course experiments revealed that rat hepatoma cells transported dehydroascorbic acid efficiently, with a transport rate of 1 nmol/10^6 cells × min for the first 30 s of uptake, followed by a secondary, slower phase of uptake (Fig. 3D). Concentration–response experiments using increasing concentrations of dehydroascorbic acid revealed that

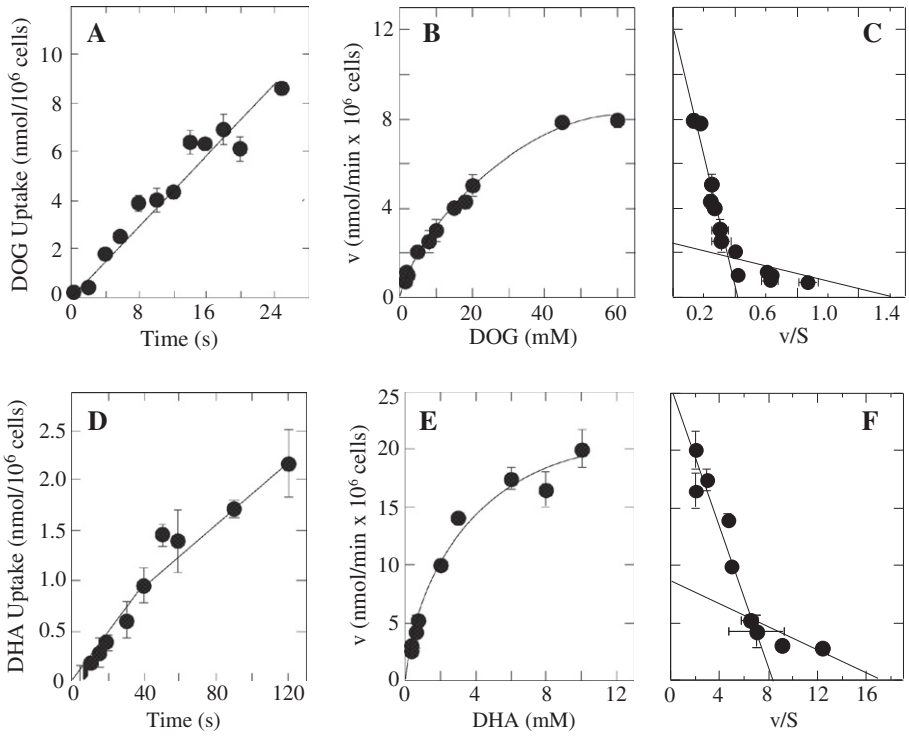

Fig. 3. Kinetic properties of 2-deoxyglucose (DOG) and dehydroascorbic acid (DHA) transport in rat hepatoma cells. (A and D) Time-course of DOG uptake (A) and DHA uptake (D). (B and E) Dose–response analysis of DOG (B) and DHA (E) transport. (C and F) Eadie–Hofstee plots of the substrate dependence of DOG (C) and DHA (F) transport. Data represent the mean ± SD of experiments performed in triplicate.

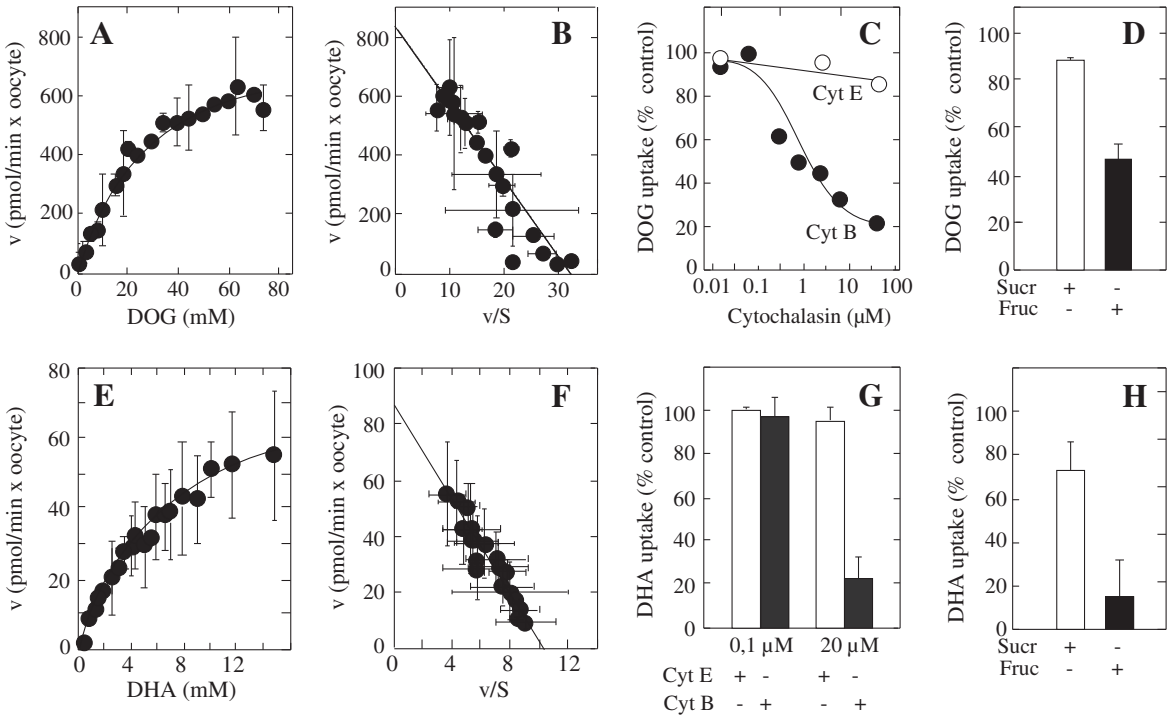

Fig. 4. 2-Deoxyglucose (DOG) and dehydroascorbic acid (DHA) transport in GLUT2-expressing *Xenopus laevis* oocytes. (A and E) Dose–response analysis of DOG (A) and DHA (E) transport. (B and F) Eadie–Hofstee plots of the substrate dependence of DOG (B) and DHA (F) transport. (C and G) Effect of cytochalasin B (●, ■) and cytochalasin E (○, □) on the uptake of DOG (C) and DHA (G). (D and H) Effect of 200 mM fructose (■) or sucrose (□) on the uptake of DOG (D) and DHA (H). Uptake experiments were performed at room temperature. Data represent the mean ± SD of experiments performed in triplicate. Sucr, sucrose; Fruc, fructose.

the transport rate failed to reach saturation at 10 mM dehydroascorbic acid (Fig. 3E), and linearization of the transport data revealed that transport was mediated by at least two kinetic components of different affinity (Fig. 3F). The calculated apparent K_m and V_{max} of the lower affinity component were 3 mM and 17 nmol/min × 10^6 cells, respectively, and for the higher affinity component were 0.6 mM and 8 nmol/min × 10^6 cells, respectively (Fig. 3F).

Combined with the data from rat hepatocytes, the immunolocalization, PCR and the kinetic properties of the two transport systems detected in the hepatoma cells can be interpreted as indicating that the low and high affinity transporters of dehydroascorbic acid expressed by the hepatoma cells are GLUT2 and GLUT1, respectively.

3.3. Transport of dehydroascorbic acid in X. laevis oocytes expressing GLUT2

The capacity of GLUT1 to transport dehydroascorbic acid has been previously demonstrated under different experimental conditions. The novelty of the present contribution is that the transport and inhibition data presented in the previous sections are compatible with the concept that the dehydroascorbic acid transporter present in rat hepatocytes is GLUT2. To directly prove that GLUT2 is a dehydroascorbic acid transporter, *X. laevis* oocytes were injected with *in vitro* synthesized GLUT2 mRNA, and the injected oocytes were tested for their deoxyglucose and dehydroascorbic acid transport capacity. Time-course transport experiments revealed that injected oocytes transported 2-deoxyglucose at a rate that was about 10-fold greater than uninjected oocytes (data not shown), and concentration–response assays revealed that the transport rate showed a hyperbolic response to increasing 2-deoxy-D-glucose concentrations (Fig. 4A). Eadie–Hofstee analysis of the transport data revealed that 2-deoxyglucose transport was mediated by a single functional component with an apparent transport K_m of 26 mM and a V_{max} of 830 pmol/min × oocyte (Fig. 4B). Competition experiments revealed that cytochalasin B inhibited the transport of 2-deoxyglucose in GLUT2-expressing *X. laevis* oocytes in a dose-dependent manner (Fig. 4C). No inhibition was observed at 0.1 μM cytochalasin B, the concentration at which GLUT1 activity is inhibited by 50%, and instead 50% inhibition was observed at about 4 μM cytochalasin B, with approximately 80% inhibition at cytochalasin B concentrations greater than 50 μM (Fig. 4C). The specificity of the inhibitory effect is indicated by the finding that similar cytochalasin E concentrations failed to inhibit the transport of 2-deoxyglucose by GLUT2-expressing oocytes (Fig. 4C). To further characterize the functional activity of GLUT2 in *X. laevis* oocytes, we tested whether fructose, a known GLUT2 substrate, affected the transport of 2-deoxy-D-glucose in GLUT2-expressing oocytes. These experiments indicated that elevated concentrations of fructose efficiently competed with the transport of 2-deoxyglucose in GLUT2-expressing oocytes, with transport decreasing more than 55% at 200 mM fructose (Fig. 4D). Parallel control experiments revealed that sucrose at similar concentrations had no effect on 2-deoxyglucose transport, indicating the specificity of the fructose effect (Fig. 4D).

Similar results, a single low-affinity component inhibited by cytochalasin B and competed by fructose, were obtained when studying the transport of dehydroascorbic acid in GLUT2-expressing *X. laevis* oocytes. Injected *X. laevis* oocytes showed a 5-fold increase in dehydroascorbic uptake rate *versus* uninjected oocytes (data not shown). Concentration–response assays revealed that the transport rate showed an hyperbolic response to increasing dehydroascorbic acid concentrations (Fig. 4E), and Eadie–Hofstee analysis of the transport data revealed that dehydroascorbic acid transport was mediated by a single functional component with an apparent transport K_m of 8.4 mM and a V_{max} of 87 pmol/min × oocyte (Fig. 4F). Competition experiments indicated that

0.1 μM cytochalasin B failed to affect dehydroascorbic acid transport, but approximately 80% inhibition was observed at 20 μM cytochalasin B (Fig. 4G), and cytochalasin E failed to affect dehydroascorbic acid transport, results that are similar to the specific effect of cytochalasin B on 2-desoxy-D-glucose transport. Moreover, elevated concentrations of fructose had a marked effect on dehydroascorbic acid transport by GLUT2-expressing oocytes, with transport decreasing by more than 80% at 200 mM fructose, while similar concentrations of sucrose had no effect on dehydroascorbic acid transport (Fig. 4H).

The general conclusion from all of the above experiments is that the low affinity glucose transporter GLUT2 is also a low affinity transporter of dehydroascorbic acid. The GLUT family of facilitative glucose transporters is currently composed of 14 members classified in three classes according to sequence similarity (I, II and III). From these, members of class I (GLUT1, 2, 3 and 4) have been the most studied from a structural, functional and regulatory perspective and are considered as *bonafide* glucose transporters, although GLUT2 is also a fructose and a glucosamine transporter. Class II transporters (GLUT5, 7, 9 and 11) have been less studied but the concept is emerging that they are probably fructose transporters. Class III transporters (GLUT6, 8, 10, 12, 13 and 14) are the less known in functional terms and there is no clear definition on a shared substrate. The capacity to transport dehydroascorbic acid, a substrate clearly different from glucose from a structural perspective, score the multifunctional nature of the glucose transporters. Our demonstration that GLUT2 is a dehydroascorbic acid transporter provides evidence for an additional shared function of the class I transporters, which can be looked at as bifunctional transporters with the capacity to transport both glucose and dehydroascorbic acid. There is no evidence that the class II transporters have the capacity to transport dehydroascorbic acid, but GLUT10, a member of the class III is also a dehydroascorbic transporter. Therefore, the question of how many members of the GLUT family of facilitative glucose transporters are also dehydroascorbic acid transporters will remain us such until every single GLUT member is submitted to the test.

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