Functional expression and characterization of the *Trypanosoma brucei* procyclic glucose transporter, THT2

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The gene encoding THT2, one of two hexose-transporter isoforms present in *Trypanosoma brucei*, has been expressed in both *Xenopus laevis* oocytes and a stably transfected line of Chinese hamster ovary (CHO) cells. The heterologously expressed gene encodes a protein with pharmacological and kinetic parameters similar to those of the hexose transporter measured in procyclic-culture-form trypanosomes. The substrate recognition of the THT2 transporter differed from that of the THT1 isoform, which is expressed only in bloodstream forms, in that: (i) it has a relatively high affinity for substrate with a K_m of 59 μ M for 2-deoxy-D-glucose (2-DOG) and a similar high affinity for D-glucose (compared with K_m of 0.5 mM for 2-DOG in bloodstream forms); (ii) the affinity for 6-deoxy-D-glucose (6-DOG) is two orders of magnitude lower than that for D-glucose, whereas the bloodstream-form transporter recognizes D-glucose and its 6-DOG analogue with similar affinity; (iii) the

bloodstream-form transporter, but not THT2, recognizes 3-fluoro-3-deoxy-D-glucose. D-Fructose-transport capacity and insensitivity to D-galactose was also found in THT2-expressing CHO cells and procyclic trypanosomes. We conclude from these cumulative results that the THT2 gene encodes the transporter responsible for hexose transport in procyclic trypanosomes. The transport of 2-DOG in procyclic organisms was inhibited by both the protonophore, carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP), and KCN, suggesting a requirement for a protonmotive force. However, sensitivity to these reagents depended on the external substrate concentration, with uptake being unaffected at substrate concentrations higher than 2 mM. THT2 expressed in CHO cells behaved as a facilitated transporter, and was unaffected by FCCP or KCN over the whole substrate concentration range tested.

INTRODUCTION

African trypanosomes of the *Trypanosoma brucei* subgroup cause the disease sleeping sickness, plus a number of veterinary diseases in sub-Saharan Africa [1]. Forms infectious to mammals are entirely dependent on the metabolism of glucose for the provision of energy. Glycolysis is unusual in trypanosomes and related organisms of the genus *Kinetoplastida* in that the first seven enzymes are localized to unique organelles termed glycosomes [2].

Genes encoding hexose transporters have been cloned from several kinetoplastids [3–6]. *T. brucei* has two distinct families of genes, linked in the genome, called THT1 and THT2 [7], which encode putative hexose transporters. Messenger RNA from THT1 can be detected only in bloodstream-form organisms, whereas THT2 is the only form detectable in procyclic culture forms [7]. Kinetic parameters have been determined for the transport of glucose in both of these life-cycle stages [8–12], and marked differences in affinity for substrate are apparent, adding credence to the notion that the parasite expresses separate isoforms at different stages in the life cycle. The THT1 isoform has been expressed in *Xenopus laevis* oocytes, where it indeed encodes a hexose transporter with pharmacological and substrate specificities similar to those measured in bloodstream-form parasites [7]. The situation regarding the second isoform, THT2, is complicated by the fact that THT2 mRNA can be detected in both bloodstream-form and procyclic organisms [7]. Separate pieces of work measuring transport in procyclic organisms have yielded conflicting reports, suggesting either a proton-dependent

secondary active transporter [11] or a facilitative diffusion carrier [13]. Different experimental approaches were used in these different studies. The proton symport system was identified in zero-trans rapid-uptake experiments with 60 μ M 2-deoxy-D-glucose as substrate. The facilitative diffusion system, by contrast, was identified by growing trypanosomes in a chemostat, then assaying glucose uptake over 15 min rather than transport *per se*.

In order to verify that the THT2 gene encodes the hexose transporter responsible for uptake in procyclic-form trypanosomes and to gain more insight into its function, we report here the expression of this gene in both *Xenopus laevis* oocytes and mammalian [Chinese hamster ovary (CHO)] cells to study its pharmacological and kinetic properties, and to compare these with the properties of the hexose transporter in procyclic organisms.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals were of the highest quality available from Sigma, unless specified in the text. 2-Deoxy-D-[1-³H]Glucose, D-[U-¹⁴C]glucose and D-[U-¹⁴C]fructose were from Amersham.

Trypanosomes

Procyclic trypanosomes (strain 427) [14] were grown in SDM-79 medium, including 1 g/l D-glucose, according to standard procedures [15].

Abbreviations used: FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; 2-DOG, 2-deoxy-D-glucose; 6-DOG, 6-deoxy-D-glucose; CHO cells, Chinese hamster ovary cells.

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In vitro Transcription and oocyte microinjection

THT2 cRNA was transcribed *in vitro* from plasmid pX2a, which was prepared by the insertion of the *T. brucei* THT2 gene into plasmid vector BSDP1400H-A65 (kindly provided by H. B. Osborne, CNRS URA256, Rennes, France) as previously described [7]. *In vitro* transcription was performed using the Ambion mMessage mMachine kit. Oocytes were prepared from *Xenopus laevis* ovaries, and microinjected as described [7].

2-Deoxy-D-glucose (2-DOG) uptake in oocytes

2-DOG uptake into oocytes was carried out exactly as described for THT1 [7].

Kinetics of transport of glucose, fructose and 2-DOG in trypanosomes

Trypanosomes were harvested at mid-exponential phase and stored in transport buffer (TB) (25 mM Hepes, 120 mM NaCl, 5.4 mM KCl, 0.55 mM CaCl₂, 0.4 mM MgSO₄, 5.6 mM Na₂HPO₄, 11.1 mM glucose, pH 7.4). Before use they were washed three times then resuspended in TB without glucose at a concentration of 10⁸/ml, and warmed to 25 °C. To initiate uptake, 0.1 ml of the trypanosome suspension was added to 0.1 ml of prewarmed (25 °C) TB containing radiolabelled substrate, placed over a 0.1 ml cushion of oil [1-bromododecane, 98 % (Aldrich); final density, 1.038 g/ml] in a 0.5 ml Eppendorf tube. Uptake was terminated after 2 s, and incorporated radioactivity was counted as described [6].

K_i values were determined by measuring the uptake of 10 μ M 2-DOG in the presence of various concentrations of inhibitor. The relationship $v_0/v = 1 + [I]/K_i$ (where v_0 and v are the uninhibited and inhibited rates respectively and $[I]$ is the concentration of inhibitor) was used to calculate K_i as described [8]. When K_i values for competitive substrates (hexoses) were determined, no preincubation step was included. However, in experiments involving cytochalasin B, phloretin, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP) and KCN a 5 min preincubation before initiation of uptake was included. Uptake was allowed to proceed for 20 or 30 s.

Construction of an expression vector and expression of THT2 in CHO cells

The THT2 gene including the Kozak eukaryotic ribosome-binding site, engineered upstream of the gene, and a poly(A) addition site plus poly(A) tail, downstream of the gene, was cloned directionally into the expression vector pCDNA3 (from Invitrogen) to yield plasmid pCHOTHT2 for transfection into CHO cells.

CHO-K1 cells were maintained in Iscove's medium containing 10 % foetal calf serum. Cells were transfected with pCHOTHT2 by the calcium phosphate method [16] and selected for resistance to the neomycin derivative G418 (Gibco) at 700 μ g/ml. Cells expressing THT2 were identified by assaying transfected lines for their ability to transport 2-DOG and D-fructose in the presence or absence of 10 μ M cytochalasin B.

Analysis of sugar uptake in CHO cell lines transfected with THT2

Transfected CHO-K1 cells were maintained in Iscove's medium containing 10 % foetal calf serum. Cells were seeded at 10⁵ or 5 $\times$ 10⁵ cells per well in 24-well plates. For uptake assays, cells were washed three times with PBS [1.8 mM KH₂PO₄, 5 mM K₂HPO₄, 0.9 % (w/v) NaCl, pH 7.4] and incubated in 0.3 ml of PBS containing 10 μ M cytochalasin B for 5 min. 2-DOG uptake

was initiated by the addition of 100 μ l of 0.4 mM 2-DOG (1 μ Ci of 2-deoxy[1-³H]-D-glucose). Uptake at 22 °C was terminated after 5 min by aspirating uptake medium, then washing three times with ice-cold PBS containing 10 mM D-glucose. Cells were lysed with 0.2 ml of 2 % SDS, and incorporated radioactivity was assayed by scintillation counting. To test the effects of inhibitors and competitive substrates, cells were preincubated with the relevant concentration of test compound for 5 min before initiation of transport.

RESULTS

Zero-trans net uptake kinetics of D-glucose and 2-DOG and specificity of the transporter in procyclic trypanosomes

A transporter with K_m values for 2-DOG and D-glucose of 59 and 52 μ M respectively was measured in procyclic-form *T. brucei*, in good agreement with previous measurements [10,11]. V_{max} values for 2-DOG and D-glucose were 2.46 nmol/min per 10⁸ cells and 2.56 nmol/min per 10⁸ cells respectively. Since both of these molecules are substrates for phosphorylation by hexokinase, difficulties remain in distinguishing true transport from uptake (involving transport and subsequent metabolism). In bloodstream-form *T. brucei*, such a distinction was made using the glucose analogue 6-deoxy-D-glucose (6-DOG), which cannot be phosphorylated by hexokinase and is recognized by the bloodstream-form transporter with very similar affinity to D-glucose [8].

The procyclic transporter, however, recognized 6-DOG very poorly with an apparent affinity (7 mM) over two orders of magnitude lower than for 2-DOG and D-glucose. 6-DOG therefore was unsuitable as a substrate to probe transport kinetics in these cells. Experiments were therefore performed using either 2-DOG or D-glucose.

The affinity of the transporter for other glucose analogues, substituted at all carbon positions, was also tested (Table 1). A

Table 1 Inhibition constants (K_i) for D-glucose analogues

Uptake of 2-DOG was measured in procyclic forms at 25 °C for 20 s in the presence of the different competitors indicated.

Position substituted	Analogue	K_i (mM)	
		Procyclic form	Bloodstream form
C-1	1-Fluoro-1-deoxy-D-glucose	0.750	2.89†
C-2	D-Mannose	0.031*	0.67†
	2-DOG	0.068‡	0.53†
	2-Fluoro-2-deoxy-D-glucose	0.188	0.44†
	D-Glucosamine	0.433	21.34†
	N-Acetyl-D-glucosamine	0.519	11.11†
C-3	3-Deoxy-D-glucose	> 50	> 250†
	3-Fluoro-3-deoxy-D-glucose	> 50	2.31†
C-4	D-Galactose	33	> 250†
C-5	5-Thio-D-glucose	0.419	11.67†
C-6	D-Fructose	2.54	2.56†
	6-Deoxy-D-glucose	7.3	1.54†‡
	6-Chloro-6-deoxy-D-glucose	1.7	0.68†
	6-Fluoro-6-deoxy-D-glucose	0.314	—

* Value taken from ref. [11].

† Values taken from ref. [8].

‡ K_m not K_i .

Figure 1 Kinetics of initial D-fructose uptake in procyclic trypanosomes

D-Fructose transport was measured over the concentration range 0.03–10 mM at 25 °C. Assays were conducted by determining initial rate of transport (20 s time point), using centrifugation through oil. Values are expressed as means $\pm$ S.D. ($n = 3$). The inset shows a Hanes plot of the data.

similar study [11] noted that the C-4 epimer of D-glucose (D-galactose) interacted with the procyclic-form transporter, giving a K_i against 2-DOG uptake of 590 μ M. Using ultrapure D-galactose (from Sigma), however, very little effect on the transport of 2-DOG was noted in this study. A different stock of D-galactose (from Rhone-Poulenc) clearly did inhibit transport of 2-DOG. We conclude that the procyclic-form transporter, in common with the bloodstream-form transporter, does not recognize D-galactose [8], and the purity of this product is vital in obtaining clear results.

Comparison of the affinity of D-glucose with other substituents in both bloodstream-form and procyclic trypanosomes showed that the largest difference is related to recognition at position C-6. Halogenated analogues at this position restored affinity in a manner dependent on the electronegativity of the substituent halogen, suggesting that a hydrogen bond is donated by the transporter to the sugar oxygen. Recognition of the D-glucose analogue lacking the C-3 oxygen is lost by both bloodstream-form and procyclic transporters. Addition of a fluorine to this position restores recognition to the bloodstream-form transporter [8], but not that present in procyclic cells.

Pharmacologically we derived similar K_i values for both phloretin (135 μ M) and cytochalasin B (264 μ M) as those previously described for bloodstream-form organisms [9].

Kinetics of D-fructose uptake in procyclic trypanosomes

D-Fructose has been shown to be transported by the same transporter as D-glucose in bloodstream-form *T. brucei* [17]. Kinetics of D-fructose transport were studied in procyclic organisms, revealing an apparent K_m of 2.54 ± 0.1 mM and a V_{max} of 2.8 nmol/min/ 10^8 cells (Figure 1). D-Glucose and 2-DOG both inhibited D-fructose transport with K_i values of 83 μ M and 64 μ M respectively, similar to their respective K_m values for the hexose transporter. Cytochalasin B and phloretin also had similar inhibition constants for D-fructose (180 μ M and 291 μ M respectively) to those for D-glucose. These results together indicate that the procyclic transporter can transport D-fructose.

Figure 2 Substrate-concentration-dependent inhibition of 2-DOG uptake by FCCP in procyclic trypanosomes

Cells were preincubated at 25 °C with or without 5 μ M FCCP for 5 min before initiation of the uptake assay by the addition of radiolabelled 2-DOG at a final concentration of between 10 μ M and 10 mM. After 30 s the uptake was terminated by spinning the cells through oil. Incorporated radioactivity was counted by liquid scintillation. Inhibition by FCCP is expressed as percentage 2-DOG uptake compared with that in control cells without inhibitor. Since FCCP was dissolved in ethanol, controls contained 0.5% (final concn.) ethanol ($n = 3$). Inset: linearization of the inhibition data between 10 μ M and 2 mM by logarithmic expression.

Substrate-concentration-dependent inhibition of transport with FCCP and KCN

The ability of FCCP (5 μ M) and KCN (2 mM) to inhibit transport of both D-glucose and 2-DOG as a function of substrate concentration was tested. FCCP significantly inhibited transport at low 2-DOG concentration, but inhibition decreased exponentially until a sugar concentration of 2 mM after which no significant inhibition was seen (Figure 2). Experiments using D-glucose led to similar observations (results not shown). Similarly, 2 mM KCN inhibited 2-DOG uptake up to 50% at the lowest substrate concentration used (50 μ M), and inhibition declined logarithmically to 25% at 1 mM. At concentrations of 2-DOG above 2 mM, no significant inhibition was detected. These results suggest that transport depends on a proton gradient at low substrate concentration, but not at higher concentrations. This process, termed 'slippage', has been described for other systems [18], and a mechanistic explanation has recently been given for uncoupling of lactose transport from a proton gradient with increasing substrate concentration in mutants of the *Escherichia coli* lactose permease [19]. This observation explains the conflict in the literature about whether transport is facilitative or active which has arisen from conclusions based on experiments using different external hexose concentrations [10–13].

Functional expression of the *T. brucei* THT2 isoform in *Xenopus laevis* oocytes and CHO cells

cRNA copies of the THT2 gene were injected into *Xenopus laevis* oocytes as previously reported for the *T. brucei* THT1 gene [7], the *Trypanosoma cruzi* TcrHT gene [6] and two *Leishmania* transporter genes [20]. Subsequent transport of 2-DOG was measured up to 3 days after injection. Oocytes were assayed by incubation in 2 mM 2-DOG for a 2 h period, during which time

Figure 3 2-DOG and D-fructose uptake in control and transfected CHO cells

CHO-K1 control and THT2-transfected CHO cell lines were assayed for 2-DOG and D-fructose uptake as described in the Materials and methods section. CHO-K1, non-transfected control; CHO-Neo, control expressing the neomycin-resistance gene; THT2-7 and THT2-9, two different clones transfected with the THT2 gene. Values are expressed as means $\pm$ S.D. ($n = 3$). 1, 2-DOG uptake; 2, 2-DOG uptake in the presence of 5 μ M cytochalasin B; 3, D-fructose uptake; 4, D-fructose uptake in presence of 5 mM D-glucose.

the rate of uptake was linear (results not shown). At 3 days after injection with THT2, eggs had the capacity to transport 393 ± 38 pmol of 2-DOG/h, some 12-fold higher than transport in control oocytes injected with water. In the oocyte system, transport of 2 mM 2-DOG was measured in the presence of several sugar analogues and inhibitors of transport (each at 250 mM concentration). Percentage inhibition of 2-DOG transport was as follows: D-glucose, 92.3%; D-mannose, 99.7%; D-fructose, 81.7%. In contrast, L-glucose gave only 2.7% inhibition. It was concluded that the measured uptake was due to stereospecific uptake. Cytochalasin B (300 μ M) inhibited transport by 47%, and phloretin (300 μ M) inhibited transport by 38%. These values correlate qualitatively well with the recorded substrate recognition in procyclic cells (Table 1).

Subsequently we developed a second expression system in CHO cells to measure kinetic constants of the THT2 transporter expressed heterologously, thus circumventing difficulties frequently found in deriving kinetic parameters from the oocytes system (see for example ref. [20]).

THT-2-expressing CHO cells were tested for their ability to transport 2-DOG in the presence and absence of 10 μ M cytochalasin B (which inhibits the endogenous GLUT transporter present in CHO cells [21]), and also to transport D-fructose (which is a substrate for THT2 but not for the endogenous transporter in CHO cells). Two positive clones were identified (Figure 3). The overall transport rate in these clones was approximately five times higher than that in non-transfected cells, which is in good agreement with the expression of mammalian transporters reported for the same expression system [22].

A K_m of 151 ± 4 μ M for 2-DOG transport was measured in the presence of cytochalasin B (Figure 4). This value is similar to that observed for the glucose transporter in procyclic trypanosomes. The presence of an apparently unsaturable component in transport studies in this system can be accounted for by residual activity of the endogenous GLUT transporter. Cytochalasin B is competitive with D-glucose, hence at higher substrate concentrations, even though the THT2 transport component is saturated, the endogenous GLUT transporter contributes increasingly to overall uptake. The apparent K_m measured for THT2 is therefore probably an overestimate.

Figure 4 Kinetics of initial 2-DOG uptake in CHO cells expressing THT2

The rate of 2-DOG transport was measured over the concentration range 0.015–1 mM at 22 °C in the presence of 10 μ M cytochalasin B (THT2-9 clone). Assays of initial sugar uptake were carried out after 5 min (means $\pm$ S.D., $n = 6$), the inset shows a Hanes plot of the data.

The specificity of THT2 expressed in CHO cells was also determined by measuring the percentage inhibition of 2-DOG uptake (0.1 mM) in the presence of an excess concentration (5 mM) of competitor (Figure 5). The THT2 transporter expressed in CHO cells shared a qualitatively similar profile of substrate recognition when compared with procyclic trypanosomes including poor inhibition by 6-DOG and C-3 analogues (in contrast with bloodstream forms), as well as inhibition by cytochalasin B and phloretin (Figure 5 and Table 1).

In contrast with 2-DOG transport in procyclic trypanosomes, FCCP and KCN had no effect on transport of 2-DOG by THT2 expressed in CHO cells regardless of substrate concentration.

DISCUSSION

Glycolysis represents the sole energy-generating pathway in bloodstream-form African trypanosomes. The procyclic form of these parasites, however, lives in the tsetse fly midgut which is rich in the amino acid proline. Accordingly procyclic forms preferentially utilize L-proline as a carbon source [13,23], although the ability to transport D-glucose is retained [10,11,13].

Two different genes encoding proteins with great homology to the glucose transporter superfamily have been identified in *T. brucei* [7]. We show here that the THT2 gene expressed in *Xenopus* oocytes and CHO cells encodes a hexose transporter similar to that measured in procyclic trypanosomes. In bloodstream-form parasites the low abundance of THT2 mRNA (one-fortieth of that of THT1 mRNA [7]) may explain why only a single relatively low-affinity transporter corresponding to THT1 was measured in these cells [8,9,12].

We were obliged to use D-glucose and commercially available 2-DOG to study the transport kinetics of THT2, as other commonly used analogues were not suitable. Difficulties exist in distinguishing between the kinetics of the transporter itself and a combined effect of the transporter and subsequent metabolism. It has been suggested that in both bloodstream-form [12,24] and procyclic [13] parasites transport is the rate-limiting step in glucose metabolism at submillimolar concentration, suggesting that our kinetic measurements do represent transport. The

Figure 5 Specificity and inhibition of 2-DOG transport in THT2-expressing CHO cells

Uptake of 2-DOG in the THT2-expressing CHO cells (THT2-9 clone) was determined in the presence of competing sugars (50 mM) or inhibitors at the concentrations indicated. 1-F-1-DOG, 1-fluoro-1-deoxy-D-glucose; 2-F-2-DOG, 2-fluoro-2-deoxy-D-glucose; 3-DOG, 3-deoxy-D-glucose; 5-Thio, 5-thio-D-glucose; 6-C-6-DOG, 6-chloro-6-deoxy-D-glucose; 6-F-6-DOG, 6-fluoro-6-deoxy-D-glucose; Cyt. B, cytochalasin B. Velocity of 2-DOG uptake by the THT2-9 transporter was calculated and expressed as a percentage of the value for uninhibited uptake (means $\pm$ S.D., $n = 3$).

relatively low K_m measured in procyclic cells and that of THT2 expressed in CHO cells, compared with the glucose transporter of bloodstream-form parasites, strongly suggests that THT2 has a significantly higher substrate affinity than THT1.

The subject of whether transport in procyclic cells depends on a protonmotive force has been controversial. Different experimental approaches have yielded different conclusions [10,11,13]. We have shown here that inhibition of transport by both the protonophore FCCP and the respiratory inhibitor KCN is dependent on substrate concentration, decreasing as sugar concentration increases. This intrinsic 'slip' activity where the requirement for protons depends on substrate concentration resembles that reported for the lactose permease in *E. coli* [18,19].

The ability of FCCP to inhibit transport via the THT2 protein heterologously expressed in CHO cells was lost. Auxiliary factors in the parasite membrane similar to that described for the coupling of glucose transport to a protonmotive force in the yeast *Pichia ohmeri* [25] might be responsible for this observation. Changes in conformation of the transporter in the heterologous system might equally explain this discrepancy and we cannot rule out the possibility that the effects of FCCP and KCN occur at a level involving complex metabolism but not transport *per se*.

We are currently investigating the biochemical mechanism for the apparent substrate-concentration-dependent coupling of protons to glucose transport in procyclic-form trypanosomes, and extending these studies to other organisms, such as *Leishmania*, in which a similar controversy over whether transport is facilitative or proton-dependent exists [26–28].

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