Facilitated Hexose Diffusion in Kinetoplastida

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Summary. The kinetoplastid glucose transporters belong to the glucose transporter superfamily, exemplified by the mammalian transporters, especially GLUT1. Some species, which undergo a life cycle in which parasitic stages are exposed to different glucose concentrations in several hosts, have evolved two different transporters to deal with this difference. While all of the trypanosome transporters also carry D-fructose, GLUTs (excluding GLUT2 and GLUT5) do not. Mammalian glucose transporters are very much more susceptible to cytochalasin B and phloretin, inhibitors of GLUTs, than are the trypanosome transporters. These properties suggest that the glucose transporter may be a good target for anti-trypanosomal drugs. The trypanosome hexose transporter might also be a vaccine candidate if it is accessible to antibodies. Genes encoding proteins involved in glucose transport have been cloned from several kinetoplastid species. Typically, the expression of hexose transporter genes is stage-regulated. The putative hexose transporter genes are highly conserved among Kinetoplastidae.

Key words: facilitated diffusion, gene expression, gene organization, hexose transporters, Kinetoplastida.

INTRODUCTION

Carbohydrates, (glucose in particular), are an important source of energy for most living organisms. However, as the lipid bilayers that make up cell membranes are impermeable to carbohydrates, carbohydrate-transport systems are required. In recent years, it has proved possible to clone two distinct molecular families of cellular transporters of glucose and other hexoses.

Cotransporters are a major class of membrane proteins that are formed by members of several gene families. They share the common property of being able to couple the electrochemical potential gradient of a cation to transport organic solutes, ions, and water "uphill" into cells. This type of transport system has been described in many organisms and includes, for example, Na⁺/coupled glucose transporters (SGLTs) in mammals (Loo et al. 1998, Rhoads et al. 1998, O’Connor and Diamond 1999). Over 35 members of the SGLT1 family have been identified from animal cells, yeast and bacteria (Turk and Wright 1997, Wright et al. 1998).

The other group of transporters conveys glucose by facilitated diffusion down glucose-concentration gradients. This group consists of seven homologous trans-
membrane proteins, GLUTs1-5, GLUT7 and GLUTX1 that are encoded by distinct genes. These proteins are also widely distributed in organisms (Pessin and Bell 1992, Gould and Holman 1993, Mueckler 1994, Sá-Noguiera and Ramos 1997, Szablewski 1998, Szablewski et al. 1999, Iberson et al. 2000). Glucose transporter genes have been cloned from several parasitic protozoa of the order Kinetoplastida (Snapp and Landfear 1997, Tetaud et al. 1997), and have been expressed functionally in Xenopus oocytes and/or Chinese hamster ovary (CHO) cells (Barrett et al. 1998).

The order Kinetoplastida comprises unicellular flagellates. Several species are important parasites, for example causing sleeping sickness in humans and a number of veterinary diseases, as in the case of African trypanosomes. Leishmania species causes a wide spectrum of disease world-wide, while Crithidia fasciulata is known as a parasite of insects.

Kinetoplastida are typically parasites with a digenetic life cycle; however a few, for example Trypanosoma equiperdum, live in one host only. Amastigotes of Leishmania are forms of the parasite that live within the macrophages of the mammalian host. The other life cycle form, the procystate, is specialized for the colonization of the insect alimentary tract. The same is true of African trypanosomes, which differentiate into several adaptive forms, the most prominent of which are the bloodstream form in the mammalian host and the procyclic form in the midgut of the tsetse fly vector. The great difference between these hosts has required the forms to adapt to differing environmental conditions. For example, they differ in their metabolism (Fairlamb and Opperdoes 1986). The availability of free glucose in serum differs greatly from that in a mammalian cells’ cytoplasm or within the midgut of insect vectors. All kinetoplastid species have specific plasma membrane transporters to facilitate the uptake of hexose. On the other hand infective bloodstream forms use glucose only as a carbon source, while the procyclic stage from the insect midgut can thrive in the absence of glucose, preferring amino acids (mainly proline) as an energy source (Parsons and Nielsen 1990, Ter Kuile and Opperdoes 1991). These characteristics suggest that glucose transport might be stage-regulated.

There are currently no satisfactory drugs to use against these parasites, and no vaccines exist. It is likely that the glucose transport system might provide an alternative target for chemotherapy, as might gateways that allow for the targeting of other toxic molecules of these parasites. Moreover, such transporters represent potential targets for immunotherapy.

THE STRUCTURE OF GLUCOSE TRANSPORTERS

In 1985, Mueckler et al. cloned and sequenced a gene encoding a human facilitated glucose transporter (GLUT1). Analysis of the sequence using a hydrophobicity profile program (Kyte and Doolittle 1982) led to a proposed model for the secondary structure. According to these authors, the transporter is composed of twelve putative hydrophobic transmembrane segments separated by hydrophilic loops. The N and C termini are located on the cytoplasmic side of the plasma membrane. Two large hydrophilic loops, one external between the putative transmembrane domains 1 and 2, and the other internal between transmembrane helices 6 and 7, were found. An extracellular loop of 33 amino acids (the first loop) is the location of a single asparagine (Asn) linked oligosaccharide addition.

In the case of the THT (for Trypanosoma Hexose Transporter), the hydrophathy plot of the sequence, determined by the above mentioned method, is similar to that of the human glucose transporter (Bringaud and Baltz 1992). The THT contains hydrophilic N and C termini. The largest extracellular loop in the THT resembles the LTP (for Leishmania Transporter Protein [Cairns et al. 1989, subsequently designated Pro1 (Langford et al. 1992)], in containing a large number of cysteines (6 over 50 amino acids and 8 over 83, respectively) while the loop between transmembrane domains 6 and 7 is relatively small (Tetaud et al. 1997). The cysteines are probably involved in disulfide bonds. This conformation is consistent with a stable folded structure resistant to protease digestion - a property that may be important in the biological functioning of this molecule in promastigote forms found in the midgut of insects (Bringaud and Baltz 1992). These cysteine-rich protein segments are not found in the other known sugar transporters.

Amino acid analysis of the various kinetoplastid glucose transporters has revealed significant differences in the occurrence of potential N-linked glycosylation sites (Barrett et al. 1998). Asn-69 in T. brucei THT2, Asn-81 in T. cruzi TcrHT1, and Asn-90, and Asn-91 in T. vivax TvHT1 are the only potential exofacial N-glycosylation sites in these transporters, all located within the first extracellular loop.
The *T. brucei* bloodstream form transporter, THT1, lacks potential N-linked glycosylation sites on any of the predicted extracellular loops. THT1 does contain one N-glycosylation consensus sequence (Asn-7), although this is located on the amino terminal tail and is predicted to face the cytoplasm. THT2 (Asn-7) and TvHT1 (Asn-10) also have an additional N-glycosylation consensus sequence at similar positions (Bringaud and Baltz 1992). In *Leishmania* spp., no potential N-linked glycosylation site was identified in the first extracellular loop, so characteristic for GLUTs.

The different kinetoplastid glucose transporter amino acid sequences are all highly homologous with one another. Alignments of the amino acid sequences of the different transporters from *T. brucei* (THT1 and THT2), *Leishmania* (Pro1-Iso-1, Pro1- Iso-2 and D2), *T. cruzi* (TcrHT1) and *T. vivax* (TvHT1) reveal the great similarity of all of these proteins (30-85% similarity, with most conservation in the central part and towards the C-terminus of the protein) (Tetaud et al. 1997). For example, comparison of the sequence of amino acids in the THT with the sequence of Iso-2 revealed 46.5% identity between the two proteins, and a level of 68.3% similarity when conservative amino acid substitutions were considered (Bringaud and Baltz 1992). The *L. donovani* D2 transporter is the most divergent, suggesting a distinct physiological role consistent with its extremely high $K_m$ for D-glucose (Langford et al. 1995).

When compared with GLUT1, THT has almost the same percentage identity (19.2%) and similarity (42.5%) as LTP has with GLUT1 (21.7% and 44.4%, respectively) (Cairns et al. 1989). In addition, comparison of the THT sequence with other sugar transporters, i.e. arabinose/H$^+$ and xylose/H$^+$ transporters from *Escherichia coli* (Maiden et al. 1987), the hexose carrier from *Chlorella* (Sauer and Tanner 1989), the yeast SNF3 glucose transporter (Celenza et al. 1988) and the yeast GAL2 galactose transporter (Nehling et al. 1989), indicates the presence of 15 amino acids strictly conserved in their specific positions (Bringaud and Baltz 1992). These findings are consistent with the notion that these residues serve critical and related functions in all of these proteins (Cairns et al. 1989).

There are several blocks of sequences conserved between the THT and GLUT1. A first block consisting of QLTGINA V (315-322) is also present in LTP (Cairns et al. 1989) and two blocks VGSMVGS (130-136) and PMYVNE (197-202) are more specific to THT and GLUT (Mueckler et al. 1985). These three blocks of sequences are also very conserved in the five other sugar transporters mentioned above (Nehling et al. 1989, Sauer and Tanner 1989). The other sequences of the GLUT (residues 89-93 and 330-334), which are conserved in many known transporters and are only moderately conserved at the same relative position in LTP, are not present in THT. Similarly, several highly-conserved regions of many of the known sugar transporters are not conserved in the THT, most notably PESPR and PETKG (residues 208-212 and 454-458 of GLUT) (Bringaud and Baltz 1992). The three consensus ATP-binding sequences found in GLUT (Carruthers and Helgerson 1989) and partly conserved in the other mammalian glucose transporters are also not present, at least not in the same relative position, in the THT (Bringaud and Baltz 1992).

Arginine is shown to play a role in the interaction of transporters with substrate (Tetaud et al. 1996). A comparison of the different sequences of kinetoplastid hexose transporters (Pro-1, D2, THT1, THT2, TcrHT1 and TvHT1) reveals four conserved arginine residues. The residues are located in transmembrane segment 4 and between helices 5/6, 8/9 and 10/11. Three of these residues (those located in transmembrane helix 4 and between transmembrane helices 8/9 and 10/11) are highly conserved in other members of the glucose transporter superfamily (Baldwin 1993), and one or more of these may be critical in substrate binding (Tetaud et al. 1997). The arginine residue located in the loop between transmembrane domains 8/9 of the GLUT4 plays a direct role in glucose uptake (Wandel et al. 1995).

Differential subcellular localization has been noted for the *Leishmania* transporters. Pro1-Iso-1 is found principally in the flagellar membrane, while Pro1-Iso-2 occurs in the plasma membrane and flagellar pocket. The different N-terminal sequences may target the different isoforms (Piper et al. 1995). The 130 amino acid NH$_2$-terminal cytoplasmic domain of the isoform 1 glucose transporter is sufficient to target a non-flagellar integral membrane protein into the flagellar membrane (Snapp and Landfear 1999).

An essential flagellar targeting signal is located between amino acids 20 and 35 of the N-terminal sequence (Snapp and Landfear 1999). The Iso-2 associates with the microtubular cytoskeleton that underlies the cell body membrane. The second isoform (Iso-1, flagellar membrane isoform) does not associate with the cytoskeleton (Snapp and Landfear 1997). These transporters are struc-
naturally similar to other members of the glucose transporter superfamily, and can be expressed in heterologous systems.

**GENOMIC ORGANIZATION OF THE HEXOSE TRANSPORTERS**

Glucose transporter genes from several kinetoplastids have been cloned and expressed functionally in *Xenopus* oocytes and/or CHO cells (Cairns et al. 1989, Tetaud et al. 1997). Some kinetoplastids contain a multigenic family encoding two isoforms of glucose transporters (Stack et al. 1990, Barrett et al. 1998, Bringaud et al. 1998). In contrast, there are also species which express a single isoform encoded by tandemly-repeated genes (Bringaud and Baltz 1992, Bringaud and Baltz 1993, Tetaud et al. 1994, Waitumbi et al. 1996). Some of these glucose transporters are regulated developmentally. The results suggest that kinetoplastids have a high level of conservation in gene organization (Bringaud et al. 1998), indicating an important role for these proteins in the parasite life cycle.

*Leishmania enriettii* contains the gene Pro-1 encoded LTP (for *Leishmania* Transporter Protein) (Cairns et al. 1989). Detailed analysis of the 5’ repeat units has revealed the presence of two isoforms (Iso-1 and Iso-2), which differ only in the size, and sequence of the N-termini (Stack et al. 1990). Partial sequence analysis reveals the presence of one Iso-1 copy followed by eight Iso-2 copies (Stein et al. 1990). This gene is regulated developmentally. The mRNA from Pro-1 accumulates to a much higher level in the promastigote stage of the parasite life cycle in the gut of the insect than in the amastigote stage of the parasite that lives inside the macrophage of mammalian cells (Cairns et al. 1989).

Similar organization was found in *L. donovani*. Analysis of the obtained results revealed the presence of one Iso-1 copy followed by four Iso-2 copies (Bringaud et al. 1998). Two further genes, called D1 and D2, with identity to the glucose transporter family were also cloned from *L. donovani* (Langford et al. 1992). Both genes are present as single copies. D2 is very similar to Pro-1. In contrast, D1 is structurally quite different from either D2 or Pro-1 and is more similar in sequence to the mammalian transporter GLUT1. The functional expression of the D1 gene in *Xenopus* oocytes revealed it to encode a plasma membrane myo-inositol/H⁺ symporter rather than a hexose transporter (Drew et al. 1995). Both D2 and Pro-1 are developmentally-regulated genes, which are expressed, primarily in the insect stage of the parasite life cycle, when the concentration of sugar reaches very high levels and so a high Kᵣ would be advantageous (Langford et al. 1995). In contrast, D1 is not regulated during the parasite life cycle. All three genes are located on different chromosomes in *L. donovani* (Langford et al. 1992).

The five species of Salivarian trypanosomes (also called African trypanosomes: subgenus *Trypanozoon* or *Trypanosoma brucei* group) i.e. *Trypanosoma brucei brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. equiperdum* and *T. evansi*, contain a multigenic family encoding two isoforms of glucose transporters referred to as THT1 and THT2 (for *Trypanosoma* Hexose Transporter). These two isoforms are 89% identical (Bringaud and Baltz 1992, Bringaud and Baltz 1993). Genes are arranged in a head to tail fashion with a cluster of THT2 genes following a cluster of THT1 genes with copy number varying between strains. For example, *T. brucei* contains six copies of THT1 and five copies of THT2 (Bringaud and Baltz 1994, Barrett et al. 1996), while in *T. congolense* TcoHT1 and TcoHT2 genes alternate (Bringaud et al. 1998). Analysis of the polymorphism in gene-copy number for both isoforms in numerous strains has revealed them to be present in multiple copies in tandem arrays, with copy number varying in a strain-specific manner.

These genes are regulated developmentally. In *T. brucei* the THT1 isoform is a low-affinity transporter and is expressed in bloodstream forms (40-fold more THT1 than THT2), whereas procyclics express THT2 (high-affinity form), but no detectable (or very low levels of) THT1 mRNA, depending on the strain. The bloodstream forms contain about 40-times more stable mRNA encoding THT1 than THT2 (Bringaud and Baltz 1993). In contrast, *T. vivax* and *T. cruzi* express a single isoform (TvHT1 and TcrHT1, respectively) encoded by tandemly repeated genes (Tetaud et al. 1994, Waitumbi et al. 1996).

The differential expression of kinetoplastid stage-regulated genes is well documented (Borst 1986, Gibson et al. 1988, Wirtz et al. 1991), but there are few examples of genes whose expression is regulated by specific environmental agents. The parasites therefore have two transporter genes that are expressed in a fashion allowing maximal exploitation of the host’s extracellular environment. Bloodstream forms express predominantly a high-capacity, low-affinity transporter to exploit the high con-
centration of glucose in mammalian serum. Procyclic forms of *Trypanosoma* express the higher-affinity transporter in the insect midgut where glucose is relatively scarce, and amino acids become the major energy source (Barrett et al. 1998). For example, a $K_m$ of the THT1 for D-glucose of 0.053 mM was reported (Tetaud et al. 1997). Other species, for example *T. cruzi*, have a single hexose transporter gene isoform, which is expressed at similar levels in epimastigotes, which live in the insect midgut, and trypomastigotes which live transiently in the bloodstream of mammals (Barrett et al. 1998). Interestingly, *T. cruzi* expresses a high-affinity glucose transporter, consistent with the fact that at least part of its life cycle occurs in the low glucose environment of the cell interior (Barrett et al. 1998). A $K_m$ of TcrHT1 for D-glucose of 0.08-0.3 mM has been reported (Tetaud et al. 1997).

**THERAPEUTIC TARGETS**

Currently there are no satisfactory drugs for use against described parasites, and no vaccines exist. The mode of action of many reagents is unknown, and currently-used compounds were derived empirically. To increase the chances of success, the development of new drugs should be aimed at those steps in the metabolic pathway, which are either absent, or differ from analogous steps in the host. In the case of trypanosome glucose transporters significant differences can be identified in terms of both the pharmacology and substrate recognition profiles, when compared to the GLUT. All of the trypanosome glucose transporters also recognize D-fructose, which distinguishes them from the main mammalian glucose transporter, GLUT1 (Barrett et al. 1998). However, at least 6 other mammalian plasma membrane hexose transporters are expressed in different tissues, with a range of substrate specificities, including two isoforms (GLUT2 and GLUT5) (Burant et al. 1992, Colville et al. 1993) which also recognize D-fructose, high-lighting the difficulties in pinpointing unique features and compromising their utility as chemotherapeutic targets. However, differences between mammalian and kinetoplastid hexose transporters have been described. For example, GLUT1 transports D-fructose with 1000-fold less efficiency than D-glucose (Eisenthal et al. 1989) and is 93% inhibited by 5 $\mu$M cytochalasin B (Kasahara and Hinkle 1977), while the THT1 transports fructose and is only 53% inhibited by 300 $\mu$M cytochalasin B (Bringaud and Baltz 1993). All of the kinetoplastid hexose transporters are also relatively insensitive to the classical inhibitor of GLUT1 transport, phloretin (Tetaud et al. 1997).

The *T. brucei* bloodstream form transporter does not make hydrogen bonds with the hydroxyls at positions 2 and 6 of the glucose ring (Eisenthal et al. 1989). These sites are considered available for the attachment of other chemical constituents, which would not interfere with recognition by the transporter. In the case of the C-6 position, a strict limit on the size of substituent groups has been noted (Barrett et al. 1998). Relatively large replacements could be added at position C-2. At least one compound containing a substituent group at position C-2 has been developed, being toxic to bloodstream form parasites grown *in vitro* (Barrett et al. 1998). Fructose analogues have also been developed, and toxic examples are known (Page et al. 1996).

In the case of *T. cruzi*, the glucose transporter does not recognize C-3 or C-6 analogues of D-glucose. Glucose molecules substituted at C-6, might therefore be useful in the treatment of African sleeping sickness, but unfortunately not Chagas’ disease (Tetaud et al. 1996). The capacity of these parasite transporters to transport D-fructose with a high affinity compared with mammalian hexose transporters may represent a more useful means of developing toxic molecules specific for Kinetoplastida (Fry et al. 1993).

As surface molecules, the transporters may be immunogenic and exposed to the immune system. The parasite and host glucose transporters have important amino acid differences in their exofacial loops. The immunogenicity of the specific epitopes of these glucose transporters will be tested to determine potentials as eventual vaccines (Bringaud and Baltz 1993, Tetaud et al. 1997).

**REFERENCES**


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