The Characterization of nucleoside transport in the intestinal protozoan parasite *Giardia intestinalis*.

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Abstract

A rapid sampling technique has been adapted and used to measure nucleoside transport in a human-derived isolate of the protozoan parasite *Giardia intestinalis* (syn. *G. lamblia*). The study was based on published findings that *Giardia* cannot produce nucleotides by *de novo* synthesis; that labelled adenine, guanine and uracil are incorporated directly into the ribonucleotide pool by the action of specific phosphoribosyltransferases; that exogenously acquired ribonucleosides are degraded rapidly within the cells by specific hydrolases to yield the respective bases; and that the parasite lacks ribonucleotide reductase and is able to produce 2'-deoxynucleotides only by direct phosphorylation of acquired 2'-deoxynucleosides. As cell membranes are effective barriers to the diffusion of nucleosides, it was considered that transporters must exist in *Giardia* to mediate their uptake.

Transport assays were conducted at 0°C to prevent loss of trophozoites by adherence, to minimize intracellular metabolism of transported nucleosides, and to slow transport for more accurate measurements. Initially, the transport of [^{3}H]thymidine was measured and this was shown to follow simple Michaelis-Menten kinetics, with an apparent K_m of 50 μM and V_{max} of 70 pmol•min^{-1}•(10^6 cells)$^{-1}$. Thymine and uracil were the most effective inhibitors of thymidine transport (K_i 's of 30 μM and 45 μM respectively), followed by thymidine, deoxyuridine and uridine (K_i 's between 64-96 μM). In contrast, cytosine, its ribonucleoside and 2'-deoxyribonucleoside derivatives, and adenosine, were not effective inhibitors, even at high concentrations. These results indicated that the transporter responsible for a major part of thymidine influx recognized the nucleoside base moiety. Recognition may require the presence of an oxygen attached to the carbon at position 4 of the pyrimidine ring, as occurs in thymine and uracil, to allow hydrogen bonding. The 5-methyl group of thymine apparently does not interfere with the recognition process. Although the furanose ring appeared not to be involved in recognition and binding to this transporter (uracil, uridine and deoxyuridine were all efficient inhibitors of thymidine transport), it may be required for the translocation step, as bases enter the cells via a separate carrier (see below).

A detailed analysis of inhibition of thymidine influx by adenosine and deoxycytidine, which exhibited a maximum (plateau) level of inhibition only 20-30% of that effected by either uracil or uridine, suggested the existence of a second nucleoside transporter with a lower affinity for thymidine. The presence of a second permease was confirmed by measuring influx of [^{3}H]deoxycytidine, [^{3}H]adenosine and [^{3}H]guanosine. The transport of each of these nucleosides was inhibited strongly by all naturally-occurring nucleosides but only poorly or not at all by nucleobases. These findings were consistent with a transporter that recognized structural features on the furanosyl moiety of ribonucleosides and 2'-deoxyribonucleosides. Both 2'- and 5'-deoxyadenosine were potent inhibitors of influx (>95% inhibition at 2 mM), 3'-deoxyadenosine was less effective (≈70% inhibition), whereas 2',3'-dideoxycytidine and cytosine arabinoside were virtually inactive (0-20% inhibition). These observations suggest that the 2' and 5' hydroxyl groups of the furanosyl ring are not necessary for recognition of nucleosides by the second transporter, whilst the 3' hydroxyl appears to be important. Michaelis-Menten constants (K_m) were calculated for the influx at 0°C of deoxycytidine (220±116 μM) and adenosine (45±24 μM), with respective V_{max} values of 13±4 and 11±2 pmol•min^{-1}•(10^6 cells)$^{-1}$. Thymidine exhibited a K_i of 205±90 μM against [^{3}H]deoxycytidine influx.

The second transporter, which has a broad specificity, would appear to be sufficient to mediate the uptake of all nucleosides and 2'-deoxynucleosides required by the parasite. The thymine/uracil-specific thymidine transporter, which has high affinity for uridine and thymidine, may be important when these compounds are scarce. No evidence could be found for the existence of any other nucleoside transporter under the assay conditions used. In work extending these studies, the presence of a distinct base transporter which has specificity for purines and pyrimidines was demonstrated.

In order to clone the genes encoding the *Giardia* nucleoside transporters, a nucleoside transport deficient strain of *E. coli* (SΦ1660) was obtained. This enabled a strategy of complementation to be employed, by selection for transformants able to utilize pyrimidine nucleosides (uridine or cytidine) as the source of carbon. Two genes, designated *bc2* and *10ca*, were cloned from a *Giardia* genomic DNA library by this technique. Sequence analysis of the cloned fragments that had been isolated by complementation revealed an open reading

frame (ORF) of 1,134 bp and 984 bp, encoding a putative 43-kDa polypeptide and a 29-kDa polypeptide for *bc2* and *10ca* respectively. Transcripts of *bc2* and *10ca* were detected as a 1.6-kb and 1.1 kb RNA species respectively on Northern blots using total RNA extracted from *Giardia* trophozoites.

The likely secondary structure of the *BC2* polypeptide determined from a consensus of the structures predicted by eight algorithms is consistent with a known class of transporter, i.e. the porins, which have been characterized from mitochondria and bacterial outer membranes. The structural model contains 12 hydrophobic or amphiphilic β-strands, each sufficiently long to span a lipid bilayer. These are connected by hydrophilic loops, which contain the 7 predicted α-helices, all comprised predominantly of hydrophilic residues. Southern hybridizations confirmed that *bc2* was of giardial and not bacterial origin, and that it was present in trophozoites as a single gene. This class of transporter may provide the structural stability required for proteins that are exposed to the lumenal environment of the gut.

In contrast, the *10CA* polypeptide shares significant sequence homology to DNA-binding proteins. This indicates that although complementation in *E. coli* strain SΦ1660 has enabled the cloning of a putative transporter gene (*bc2*), other genes may be able to complement the bacterial mutation. It will therefore be important to further analyse the genes (and their encoded polypeptide products) isolated by this technique, to confirm their role as transporters in *Giardia*.

In summary, this study has led to the discovery and kinetic characterization of two distinct nucleoside transporters in *Giardia intestinalis* and to the isolation and characterization of a gene encoding a porin-like transporter. The data provide an insight into the pathways involved in nucleoside and nucleobase transport in this organism, and of the features of the substrate molecules that are necessary for recognition in this parasite. Further study of the putative nucleoside transporter gene and its polypeptide product(s) will be needed to confirm its role as a nucleoside transporter in *Giardia* and may provide an insight into the structure and function of the transporters. The data have important implications for future design of chemotherapeutic drugs that may be used in the treatment of giardiasis.

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