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Regulation of Carbohydrate Metabolism During *Giardia* Encystment[1]

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ABSTRACT. *Giardia intestinalis* trophozoites encyst when they are exposed to bile. During encystment, events related to the inducible synthesis of a novel N-acetyl-D-galactosamine (GalNAc) homopolymer, occur. Within the first 6 h of encystment, mRNA for glucosamine 6-P isomerase (GPI), the first inducible enzyme unique to this pathway appears, oxygen uptake rates double from non-encysting levels, and metronidazole (MTZ) inhibits oxygen uptake. Within 12 h, GPI and its activity are detectable and OU decreases 50% from non-encysting levels; glucose's stimulation and MTZ's inhibition of oxygen uptake cease. In contrast, aspartate uptake remained constant throughout the 40 h monitored. Two genes, *gpi* 1 and 2 encode for GPI, but only *gpi*1 is expressed during encystment. Glucosamine 6-P (GlcN6P), the synthetic product of GPI, activates UDP-N-acetylglucosamine (UDP-GlcNAc) pyrophosphorylase, a downstream enzyme, 3 to 5-fold in the direction of UDP-GlcNAc synthesis. UDP-GlcNAc is epimerized to UDP-GalNAc and UDP-GalNAc is polymerized by "cyst wall synthase" (β 1 → 3 GalNAc transferase) into a highly insoluble β 1,3-linked homopolymer. This GalNAc polysaccharide, the major component of cyst wall filaments, forms, in conjunction with polypeptides, the outer cyst wall of *Giardia*.

Key Words. Carbohydrates, encystment, *Giardia*, protozoan, regulation of metabolism.

GIARDIA intestinalis (synonym for *G. duodenalis* or *G. lamblia*) exhibits a life cycle that includes a trophozoite and a cyst (Kulda and Nohynkova 1995). Transmission is accomplished when cysts are ingested in food, water or on fomites; upon passage of cysts through the host's stomach, excystation occurs in the upper small intestine. A tetranucleate organism emerges that promptly undergoes cytokinesis to yield two binucleate trophozoites. The trophozoites colonize the host's small intestine, attaching to the epithelial brush border by an adhesive disk and multiplying by binary fission. In the presence of oxygen trophozoites respire by a flavin-iron sulfur protein-mediated electron transport system. The Embden-Meyerhof-Parnas and hexose monophosphate pathways catabolize glucose (Glc), and energy is produced, at least in part, by substrate-level phosphorylation. Substrates are incompletely oxidized to CO_2, acetate, ethanol, and alanine; the acetate formed may not come from Glc. *Giardia* trophozoites rely on preformed lipids, purines, and pyrimidines to meet these metabolic requirements (Jarroll and Lindmark 1990; Kulda and Nohynkova 1995).

Physiologic changes accompanying encystment. When *Giardia* trophozoites are exposed to bile they encyst (Gillin et al. 1987; Schupp et al. 1988). Luján et al. (1996) showed that cholesterol deprivation brought on by bile is the likely inducer of encystment rather than some component of bile itself. During encystment, trophozoites appear to develop a Golgi-like complex (Reiner, Douglas, and Gillin 1989), express cyst antigens (Gillin et al. 1987), and produce encystment specific vesicles (ESVs) (Faubert, Reiner, and Gillin 1991) which contain material apparently destined to become cyst wall filaments. Interestingly, metronidazole (MTZ), the drug most commonly used to treat giardiasis, completely inhibits oxygen uptake and motility in the trophozoites, but treatment of cysts with metronidazole had no effect on their oxygen uptake or their ability to excyst (Paget, Manning, and Jarroll 1993). After 10 h in encystment medium, trophozoites lose their ability to use exogenous Glc, which may reflect changes in metabolic activity, a change in membrane transport activity or both. Notably, during the first 10 h, trophozoites become resistant to MTZ (Paget, Macechko, and Jarroll 1998). During this same time period, ESVs, which contain cyst wall material, begin to appear in the encysting trophozoites. The number of trophozoites that exhibit ESVs and the number of ESVs per trophozoite increased with increasing time in the encystment medium (Faubert, Reiner, and Gillin 1991). In our experiments, ca. 50–70% of the encysting trophozoites exhibit ESVs during the first 24–48 h after induction although not all of these complete encystment.

Cyst wall filaments. Originally, the insolubility (Manning, Erlandsen, and Jarroll 1992) of the cyst wall filaments (Fig. 1) made direct analysis impossible; however, now soluble material has been obtained by partial hydrolysis in trifluoracetic acid (Gerwig et al. 1998). Soluble material was separated by gel filtration chromatography, and one and two-dimensional ^{1}H-NMR and methylation analyses have shown that the cyst wall filaments (presumed product of CWS) of the outer cyst wall of *Giardia* is a [D-GalNAc (β1 → 3)-D-GalNAc]$_n$ homopolymer where *n* equals at least 23. The D-GalNAc accounts for 63% by weight of the filaments and fatty acids were not detected. The remaining 37% contain protein as demonstrated by amino acid analysis (Gerwig et al. 1998). In view of the relatively large amount of leucine in the amino acids associated with the polysaccharide, these could be the leucine-rich cyst wall proteins (CWPs) described by Luján et al. (1995). It is uncertain if GalNAc occurs as a polysaccharide alone or is linked to protein/peptide, but studies are underway to determine if the polysaccharide and the proteins are linked.

GalNAc synthesis. Non-encysting trophozoites lack detectable levels of GalN, but the cyst wall has a relatively large amount of this sugar (Gerwig et al. 1998; Jarroll et al. 1989; Manning, Erlandsen, and Jarroll 1992). Because *Giardia* trophozoites are well known for salvaging, rather than synthesizing essential nutrients from the growth medium (Jarroll and Lindmark 1990; Jarroll and Paget 1995; Kulda and Nohynkova 1995), the question arose as to whether the GalN is salvaged or synthesized during encystment. This question was answered by Macechko et al. (1992). Radioisotope incorporation studies using ^{14}C-labeled GalNAc precursors revealed that mainly Glc (from endogenous reserves) was incorporated into the GalN formed during encystment; labeled GalN was not incorporated in detectable quantities. The radioisotope labeling data presented, coupled with the enzyme assays performed, show that *Giar-*

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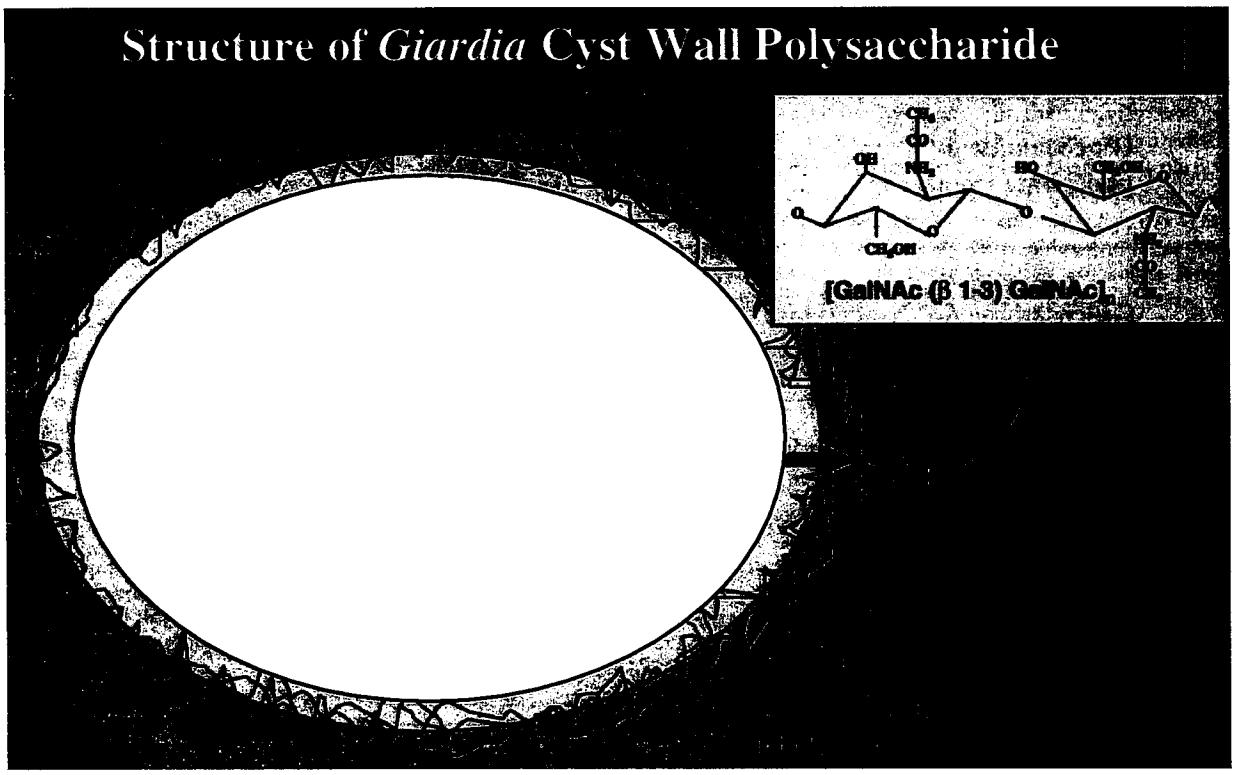

Fig. 1. Schematic showing the proposed carbohydrate composition of *Giardia intestinalis* cyst wall filaments.

dia synthesize rather than salvage GalNAc (Macechko et al. 1992). More interesting is the fact that all of these GalNAc synthesizing enzymes' activities increase significantly during the encystment process. This was the first report of these enzymes and of an inducible carbohydrate-synthesizing pathway in any protozoan.

Giardia trophozoites synthesize GalN primarily from endogenous Glc rather than salvage it from the environment (Macechko et al. 1992). This is supported by the demonstration of the activity of glucosamine 6-phosphate isomerase, GPI (aminase [GPIa] and deaminase [GPId], EC 5.3.1.10), GlcN 6-N-acetylase (GlcNPA, EC 2.3.1.4), phosphoacetylglucosamine mutase (PGlcNAcM, EC 2.7.5.2), uridine diphosphoacetylglucosamine pyrophosphorylase (UDP-GlcNAcPP, EC 2.7.7.23), and uridine diphosphate N-acetylglucosamine 4′-epimerase (UDP-GlcNAcE, EC 5.1.3.7) (Fig. 2). These five enzymes have been localized to the non-sedimentable (cytosolic) fraction of in vitro encysting *Giardia* (Macechko et al. 1992).

In other microbial systems where chitin is synthesized, glucosamine 6-phosphate is synthesized frequently by the activity of l-glutamine D-fructose 6-P amidotransferase (EC 2.6.1.16, GF 6-PAT). This activity was below the limits of detection in encysting or non-encysting *Giardia.* Instead, *Giardia* exhibits a reversible glucosamine-6-phosphate isomerase, GPI, a cytosolic enzyme activity with deaminase and aminase activities (Fig. 2) (Macechko et al: 1992; Steimle, Lindmark, and Jarroll 1997).

We have shown (Steimle, Lindmark, and Jarroll 1997) that the 29 kDa GPI's aminating activity exhibited a V_{max} of 86.3 ± 3.2 mmoles GlcN6P produced min^{-1} mg $protein^{-1}$ with an apparent K_m of 2.5 ± 0.24 mM for F6P and an apparent K_m of 19 ± 1.9 mM for NH_4Cl. The deaminase exhibited a V_{max} of 32.8 ± 5.3 mmoles GlcN6P consumed min^{-1} mg $protein^{-1}$ with an apparent K_m of 0.38 ± 0.16 mM GlcN6P. *Giardia*'s GlcN6PI exhibited a higher rate of anabolic activity with a V_{max} for the aminase reaction that is 2.3-fold higher than for the deaminase. D-glucose 6-P, D-mannose 6-P, and D-galactose 6-P do not substitute for D-fructose 6-P; l-glutamine and l-asparagine do not substitute for NH_4Cl. Furthermore, GlcNAc 6-P, GlcNAc, GlcN, and GalN do not serve as substrates for the deaminase. GlcNAc 6-P allosterically activates the *E. coli* and dog kidney isomerases (multimeric), but does not activate the purified *Giardia* or yeast isomerases (monomeric). Likewise, the *Giardia* isomerase was not activated or inhibited by GlcNAc 1-P, UDP-GlcNAc, or UDP-GalNAc. *Giardia*'s aminase activity was inhibited 68% by iodoacetamide (20 mM) and 61% by N-ethylmaleimide (20 mM); these compounds inhibited deaminase activity by 95%. Aminase ($K_i = 2 \times 10^{-8}$ M) and deaminase ($K_i = 2.8 \times 10^{-7}$ M) activities were inhibited by 2-amino-2-deoxyglucitol-6-phosphate, which is an analogue of GlcN6P (Comb and Roseman 1958).

We have cloned and sequenced two genes for GPI from *Giardia* that we refer to as *gpi*1 and *gpi*2 (van Keulen et al. 1998). Evidence that only *gpi*1 represents the inducible GPI comes from: 1) There is an ORF in the *gpi*1 sequence which encodes a ca. 29 kDa polypeptide with a calculated isoelectric point comparable to that determined for the purified enzyme (see Steimle, Lindmark, and Jarroll 1997). 2) The *gpi*1 product exhibits only a 34% identity with the polypeptide subunit of the hexameric *nag*B from *E. coli* and with the single subunit *nag*1 from *Candida albicans,* but the entire active site, especially the Asp72 and His14 critical for catalytic activity of the *E. coli* isomerase (*nag*B), are conserved in both *Candida*'s *nag*1 and in *Giardia*'s *gpi*1. 3) *Giardia*'s GPI is inhibited by 2-amino-2-deoxyglucitol-6-phosphate which is a potent inhibitor of the *E. coli* isomerase. *Giardia*'s *gpi*1 conserves the same Asp72, His143, Arg172 and Lys208 that are critical for the same inhibition in *E. coli*'s isomerase by crystallography (Oliva et al. 1995). 4) The *gpi*1 ORF was cloned in frame with the

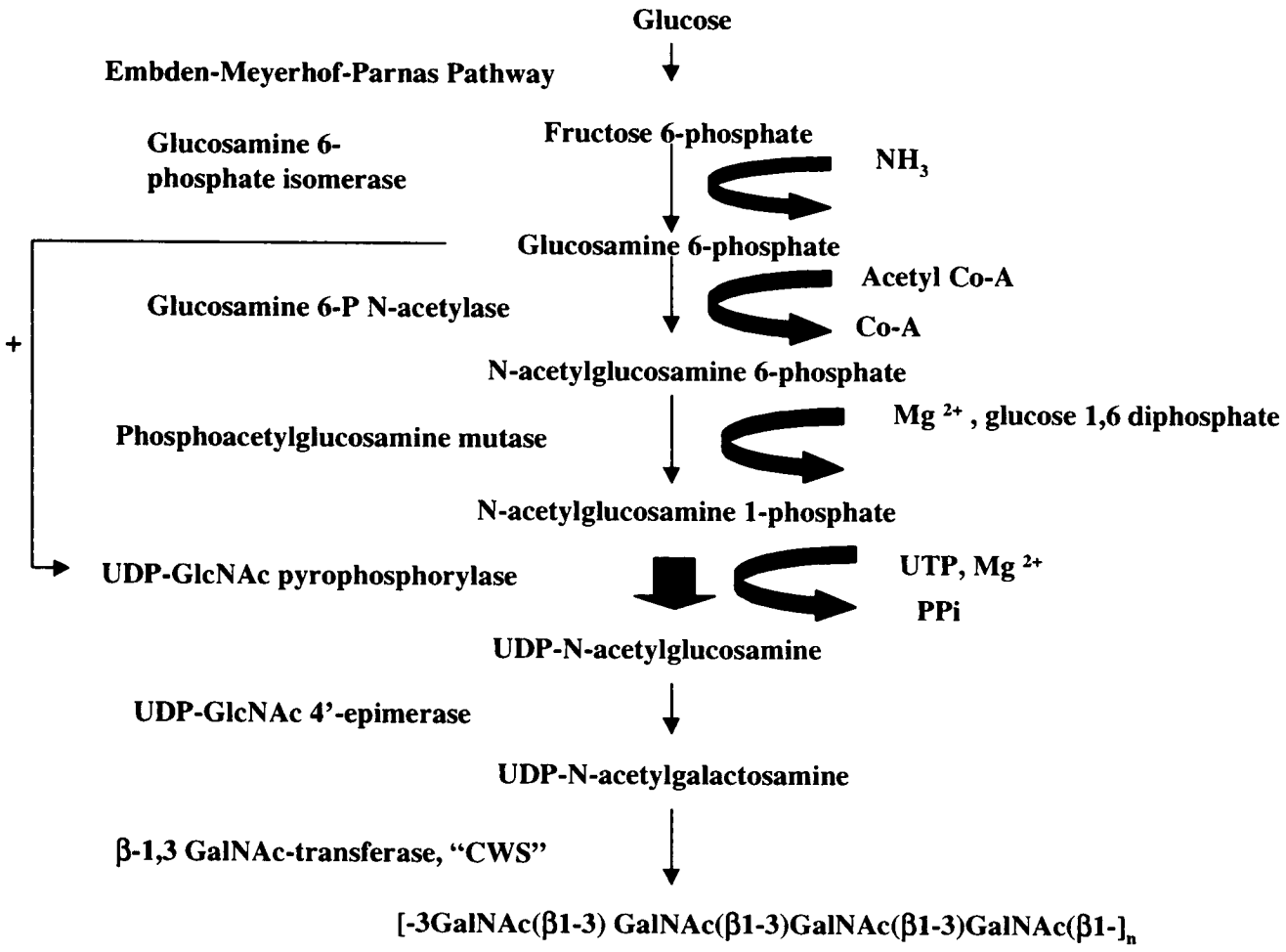

Fig. 2. The pathway of UDP-N-acetylgalactosamine synthesis that is induced when *Giardia* encyst.

glutathione transferase of the expression vector pGEX-4-T-1. The expressed protein was purified and showed glucosamine 6-P isomerase activity in both the aminating and deaminating direction with K_m and V_{max} comparable to the native enzyme. The inhibitor 2-amino-2-deoxyglucitol-6-phosphate showed competitive inhibition with the recombinant enzyme in the same manner as it does with the native enzyme, showing unequivocally that the cloned gene is that of the isomerase. Additionally, monospecific, polyclonal antibody (1:500) made to the expressed GPI recognizes the purified GPI and immunoprecipitates GPI activity. Evidence that *gpi*2 is not expressed includes 1) the *gpi*2 sequence shows one ORF but two possible start codons that are 54 bp apart. One ORF would be 263; the other 244 amino acid residues. The longer ORF is too long to account for a mRNA start site further upstream, even if the gene were expressed (van Keulen et al. 1998). In neither case is the molecular weight 29 kDa (28.7 and 26.8 kDa), nor do the calculated isoelectric points (5.8 and 5.9) approximate that of the purified enzyme (7.1 and 7.3). Additionally, probes specific for *gpi*2 do not show mRNA present in encysting or nonencysting trophozoites; whereas, *gpi*1 specific probes demonstrate, using RT-PCR, that mRNA for the isomerase is detectable within 5–6 h post-induction with bile.

Initial characterization of GlcNPA (Fig. 2) reveals that it is a cytosolic enzyme activity in *Giardia* with a pH optimum of ca. 5. In non-encysting trophozoites the enzyme activity was < 0.2 mU mg crude protein^{-1} but the activity increases at least 20-fold by 20 h into encystment. N-acetylation of glucosamine is also detected with an activity of < 0.5 mU mg crude protein^{-1} in non-encysting cells and ca. 1 mU mg crude protein^{-1} in encysting cells (Macechko et al. 1992).

Partial purification (Lindmark and Schmidt 1992) and initial characterization of PGlcNAcM (Fig. 2) (Lindmark and Schmidt 1992; Macechko et al. 1992) show that it requires Mg^{2+}, Glc 1,6-diphosphate, and diethyldithiocarbamate. The optimal pH was 8 for this non-sedimentable activity. The activity increased by 8 to 20-fold by 20 h into encystment. The mutase has been purified 24-fold by a combination of differential sedimentation, ammonium sulfate precipitation, HPLC-DEAE chromatography, and Affi-green (Cibacron Blue F3GA gel, Spectrum) chromatography. The mutase requires Mg^{2+} and Glc 1,6-bisphosphate, and it is protected by DTT during purification. SDS-PAGE of partially purified Affi-green fractions shows a unique band at 42 kDa, a molecular weight similar to that for the PGlcNAcM from pig submaxillary gland.

The non-sedimentable activity of UDP-GlcNAcPP (Fig. 2) increased by 8-fold in encysting over non-encysting cell extracts (Macechko et al. 1992). Non-specific pyrophosphorylase activity was *not* detected. UDP-GlcNAcPP has now been purified to apparent homogeneity as assessed by 1- and 2-dimensional SDS-PAGE using a combination of ammonium sulfate precipitation, ion exchange HPLC, hydroxyapatite HPLC, and

gel filtration HPLC. The enzyme has been purified 120-fold, which exhibits a molecular mass of ca. 66 kDa (native) (Bulik and Jarroll 1998). The apparent K_m (mM), V_{max} (mmoles min^{-1} mg $protein^{-1}$) (in the degradative direction of fructose 6-phosphate): UDP-GlcNAc−0.4, 4.5; PPi− 0.57, 4.9; Mg^{2+}−0.13, 3.35, respectively. UMP competitively inhibits the enzyme *only* in the degradative direction (K_i = 0.25 mM), and noncompetitively inhibits with respect to PP_i (K_i = 3.5 mM). Uridine, UDP, UTP, and AMP inhibit the enzyme activity ca. 50% at 5 mM. The enzyme exhibits no activity when other UDP-sugars are used as substrates. In the synthetic direction (towards GalNAc) the apparent K_m for UTP is 1.21 mM with a V_{max} of 1.55 mmoles min^{-1} mg $Protein^{-1}$. UDP-GlcNAcPP exhibits partial activity with GalNAc 1-P (58%) and Glc 1-P (53%). Glucosamine 6-P, but no other pathway intermediate, stimulates the activity of UDP-GlcNAcPP 3-fold only in the synthetic direction and only with UDP-GlcNAc 1-P as substrate (Bulik et al. 2000). Hill plots to assess the dynamics of this activation showed cooperativity. Such an effect has not been reported for this enzyme in other systems. Monospecific, polyclonal antibody has been made to this enzyme, and a Western blot (Bulik et al. 2000) on which it has been used suggests that UDP-GlcNAcPP is present in encysting and non-encysting trophozoites. The activity of the enzyme in non-encysting cell homogenates, which is just at or below the level of detection, can be increased up to 8-fold by the addition of as little as 3 μM GlcN6P. Amino acid analyses of the immunopurified UDP-GlcNAcPP from encysting and non-encysting cells revealed that indeed it was the same enzyme. Thus, UDP-GlcNAcPP is a potential regulatory point in this pathway.

Partial purification and initial characterization of UDPGlcNAcE (Fig. 2) show that the epimerase activity is nonsedimentable and it exhibits a pH optimum of 7 (Macechko et al. 1992). UDP-Glc and UDP-Gal were unable to substitute for UDP-GlcNAc and UDP-GalNAc in partially purified fractions. This observation is contrary to that for other epimerases reported to date. The epimerase activity has been partially purified (163-fold) from encysting cells. We have cloned a portion of *Giardia*'s epimerase, containing the N-terminal 150 amino acids, in pGEX-4-T-1, and we have purified the expressed protein for antibody production (Lopez, Jarroll, and van Keulen unpubl.).

Polysaccharide assembly. Since our report on the inducibility of the GalNAc synthetic pathway (Macechko et al. 1992), we have detected a previously undescribed β 1,3 GalNAc transferase activity which we tentatively have named "cyst wall synthetase" (CWS) (Karr, Lindmark, and Jarroll 1994) (Fig. 2). CWS activity is not detected in non-encysting trophozoites, but increases in encysting *Giardia* to detectable levels after about 8 h of encystment. Like the previously described activities, CWS is induced when encystment is induced and it reaches peak activity (ca. 1,245-fold increase) by approximately 36 h into the encystment process; unlike the other activities described, CWS is particle-associated. CWS activity (using isopycnic fractions as a source of CWS activity, see below) increases with increasing protein as well as with increasing assay time for up to 60 min. It has a temperature optimum of 30–37 °C after which the activity drops precipitously. The optimal pH is at 7.5, but CWS is active (at least 50% of maximal activity) over the pH range 5.5–9. Using a partially purified fraction, CWS activity exhibited an apparent K_m of 0.064 ± 0.015 mM UDP-GalNAc and a V_{max} of 0.70 ± 0.085 nmoles UDP-GalNAc min^{-1} mg $protein^{-1}$ incorporated into the SDS-insoluble, ethanol precipitate. CWS activity exhibits a requirement for Mg^{2+} or Ca^{2+} with the activity increasing by 11-fold with the addition of either 10 mM Ca^{2+} or Mg^{2+} over the level of activity without the exogenous metal ions. At 10 mM, Co^{2+} stimulated activity 7-fold, but Mn^{2+} and Zn^{2+} stimulated activity only about 2-fold above activity without metal ions. EDTA (1 mM) inhibited activity by 99%, but the activity was restored with the addition of excess Mg^{2+}. DTT is not required for maximal activity. CWS activity has been localized to a particle population with a density in sucrose of 1.15–1.18 (lysosome-like organelles have a peak density in sucrose of 1.18) (Lindmark 1988), after isopycnic centrifugation. Encystment in trophozoites is asynchronous and these vesicles are more variable in density than lysosomes. CWS activity increases correspondingly to the increase in the number of ESV's and cysts both of which stain with cyst wall-specific antibody (Erlandsen et al. 1990; Erlandsen et al. 1996). We have succeeded in partially purifying CWS from the isopycnic particle fraction with the highest CWS activity.

The particle-associated CWS activity from encysting cells is specific for UDP-GalNAc; there was no detectable incorporation of UDP-GlcNAc, UDP-Gal, UDP-Glc, GalN, or GlcN using isopycnic fractions as the source of CWS. When S-fractions (cytosolic fraction) were used as enzyme in the CWS assay, there was no appreciable incorporation of any of these sugars in the ethanol precipitate.

What we know now. A summary of the current state of our knowledge relating to GalNAc synthesis and its regulation in *Giardia* during encystment is represented in Fig. 2. We believe that there are signal transduction events that trigger the changes that occur during encystment. Among these changes is the induction of glucosamine 6-phosphate isomerase the product of which appears to allosterically activate UDP-GlcNAc pyrophosphorylase and shift the dynamics of the equation in favor of GlcNAc and subsequently GalNAc synthesis. We are continuing to explore this pathway and to examine in depth the genes and the enzymes they encode to determine whether additional regulation occurs in the synthesis of GalNAc during *Giardia* encystment. In our studies, we have an eye towards the development of inhibitors of this process that may facilitate treatment of giardiasis.

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