Summary:
Cholesterol and bile salts are relevant modulators of Giardia encystation. Although several molecules within signaling cascades have been identified, and changes in their expression observed during giardial encystation, their underlying interactions leading to expression of cyst wall markers (CWPs and precursors of the GalNAc homopolymer) are not well defined. Recent experimental data and the completion of the Giardia Genome Project Database (GiardiaDB) allow us now to consider the role of bile salts as "natural stimuli" and the potential involvement of a Raf/MEK/ERK pathway mediating cholesterol-regulated expression of cyst-specific genes. These new findings may provide promising targets for diagnostics, drug design and prophylactic intervention against giardiasis.

KEY WORDS: Giardia duodenalis, encystation, cyst wall, cholesterol, bile salts, cholesterol receptor, SREBP, cell signaling, protist.

INTRODUCTION
Giardiasis is a leading diarrhoeal illness in humans and a common enteric infection in companion animals and livestock. WHO estimates an incidence of 2-7% in developed countries. In Asia, Africa and Latin America three billion people live at risk of the disease and this high prevalence results in one billion cases, contributing to some 2.5 million deaths annually from diarrheal disease. In 2004 giardiasis was included in the ‘Neglected Disease Initiative’. In addition, Giardia is considered a category B agent of bioterrorism by CDC and it is the most widespread protozoan diarrhoea with 200 million symptomatic individuals worldwide at any given time (WHO, 2006). The causal agent, Giardia duodenalis (syn. G. intestinalis, G. lamblia) has two stages: the pathogenic, vegetative trophozoite and the infective, quiescent cyst. The trophozoite-to-cyst conversion process (encystation) of this parasite represents a primitive response to cellular stress. Likewise Giardia is a relatively simple eukaryote with reduced/cryptic structures such as mitosomes, Golgi-like apparatus and nucleolus (Tovar et al., 2003; Luján & Touz, 2003; Jiménez-García et al., 2008) coupled to a compact genome encoding a limited proteomic repertoire (Morrison et al., 2007). Nevertheless Giardia is considered as the most highly evolved and adapted-to-parasitism diplomonad on the basis of morphogenetic criteria (Keeling & Brugerolle, 2006). The reproduction of the giardial life cycle in vitro (Gillin et al., 1987, 1989), the rodent models of giardiasis and the Giardia Genome Project Database (GiardiaDB at http://www.giardia.org/giardiaDB) are useful platforms from which to analyze Giardia en/encystation processes.

FACTORS INVOLVED IN ENCRYPTATION INDUCTION
Giardia encystation is a multifactorial process that requires four defined factors in the extracellular medium as defined by the following...
protocols using serum-supplemented TYI-S-33 medium for cyst production: a) deprivation of specific nutrients as cholesterol (Luján et al., 1997); b) inhibition of agonist-receptor interaction by antibodies directed against the cholesterol receptor (CR) with induction of a sterol-response element binding protein (SREBP) homolog (Kaul et al., 2001; Worgall et al., 2004); c) physico-chemical factors viz., gas infusion [N2/O2], (Sterling et al., 1988); and d) host-derived factors including bile or bile components (primary bile salts viz., micellar glycocholate and fatty acids viz., myristic acid) at slightly alkaline pH (Gillin et al., 1987, 1988). Of these protocols, a high concentration of bile (5 mg/ml) at pH 7.8 is widely used although encystation was also induced in the presence of extracellular cholesterol (Kaul et al., 2001; Sterling et al., 1988) and/or in the absence of bile components (Luján et al., 1997; Sterling et al., 1988). Indeed cholesterol and bile components are relevant modulators of giardial encystation and their role in this process is discussed herein.

**MOLECULAR AND STRUCTURAL MARKERS OF GIARDIAL ECYSTATION**

*Giardia* encystation entails a decrease in adhesion, reduced metabolic rate and multiplication of the pear-shaped trophozoite resulting in rounding-up, disassembly of the ventral disk, internalization of flagella, encasement in a protective envelope, the cyst wall (CW), and entering into cryptobiosis (a state still metabolically active but reduced to 20% of that of trophozoites). CW is a hallmark of encystation and plays a critical role in cysts since it resists a plethora of environmental stresses, excludes most small molecules and aids in sensing the stimuli for encystation. The CW matrix is composed of an inner two-layered membranous and an outer protofilamentous layer (CWF) 0.3-0.5 µm thick with filaments 7-15 nm in diameter. The filaments have two main components: 63% by weight of an unique β1,3-linked (GalNAC) homopolymer (Jarroll et al., 1986; Manning et al., 1992; Gerwig et al., 2002) and 37% by weight of at least four different embedded structural polypeptides: three are leucine-rich repeats (LRR)-containing proteins (CWPs 1-3) and the other is a cysteine-rich and variant surface protein (VSP)-like molecule named HCNCp (Mowatt et al., 1995; Luján et al., 1995; Sun et al., 2003; Davids et al., 2006).

The biosynthesis of the GalNAC glycopolymer is carried out by two processes: one consists of a cytosolic five-step enzymatic pathway that is developmentally induced/transcriptionally regulated (López et al., 2003) and converts fructuose-6-phosphate diverted from glycolysis into uridine diphosphate (UDP)-GalNAc, the saccharide precursor of the [GalNAC(β1→3)GalNAC(β1→3)]n homopolymer (Jarroll et al., 2001). The first enzyme of this pathway, glucosamine-6-phosphate-isomerase (G6PI), is reversible since it exhibits aminase/deaminase activities (Steimle et al., 1997) and the fourth enzyme (UDP-GlcNAc pyrophosphorylase) is a putative rate-limiting enzyme allosterically activated in the anabolic direction by glucosamine-6-phosphate levels (Bulik et al., 2000). The other process is the synthesis of the CW polysaccharide, where a particle-associated transferase activity tentatively referred to as cyst wall synthase (CWS) has been reported (Craig & Jarroll, 2004). Whether one or various enzymes are responsible for CWS activity is yet to be defined. All CWPs are developmentally and coordinately expressed, co-sorted to electron-dense secretory granules named encystation-specific vesicles (ESV), co-transported and co-localized exclusively to CWF (Fig. 1). HCNCp is detected in the nuclear envelope of trophozoites, ESV, CWF and the cell body within mature cysts. Each CWP is encoded by a single copy gene which share structural features: CWF-localized CWP1 and CWP2 are ≈ 26 kDa with five LRR tandem repeats while CWP3 is 27.3 kDa and has four and a possible fifth LRR tandem repeat. CWP2 is synthesized as a 39 kDa-sized precursor that is processed by a cysteine protease releasing the distinctive 13 kDa-basic C-terminal extension (TCWP2; Touz et al., 2002; DuBois et al., 2008). All CWPs have a peptide signal sequence at the N-terminus, possibly recognized by a cognate giardial receptor (Svärd et al., 1999) that targets CWP to endoplasmic reticulum (ER), and a cysteine-rich region after the LRR tandem repeats where all 14 cysteine residues are positionally conserved (Mowatt et al., 1995; Luján et al., 1995; Sun et al., 2003). Green fluorescent protein (GFP)-CWP1 chimeras have shown that the N-terminal domain may be needed for sorting CWP to secretory compartments and the LRR tandem repeats may help CWP to associate with CWF material (Hehl et al., 2000). The cysteine-rich domain may aid CWP in forming disulfide-bonded heterodimers (Luján et al., 1995; Sun et al., 2003) that are concentrated in the ER lumen potentially promoting ESV biogenesis (Luján & Touz, 2003). This is supported by the fact that dithiothreitol reduces CWP complexes to monomers, inhibits ESV formation and reversibly converts these secretory granules into ER-like flattened cisternae (Reiner et al., 2001). Likewise the TCWP2 moiety may confer on CWP a role as sorting factor for CWP complexes through a likely ligand activity with *Giardia*-specific ER membrane receptors. This is in turn consistent with the observation that non-encysting trophozoites transfected with haemagglutinin-tagged CWP1-TCWP2 chimera or the whole CWP2 display granules morphologically similar to ESV (Gottig et al., 2006) but these granules are not formed in similarly transfected mammalian cells (Elias et al., 2007).
CWP are used as markers to define the main phases of encystation: a) “induction”, in which encystation stimuli are transduced from surface to nucleus by signaling pathways that activate differential gene expression; (b) “intracellular”, involving biosynthesis and trafficking of cyst wall proteins (CWP) and precursors of the GalNAc homopolymer that will form the fibrillar wall matrix; and c) “extracellular”, hallmarked by the secretion and extracellular assembly of cyst wall material (Fig. 1).

Several studies have aimed at defining the transport pathway of CW components to the cell surface. *Giardia* lacks typical Golgi dictyosomes but possesses a basic framework of the vesicular transport system that includes coatomers (COP) I and II (Golgi- and ER-specific, respectively), clathrins, two adaptor protein (AP) complexes and key factors for vesicular targeting and membrane fusion processes such as members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and Rab families (Luján & Touz, 2003; Hehl & Marti, 2004). Encystation-specific vesicles (ESV) are specialized secretory granules that mature during their traffic from ER to the cell surface that act as the equivalents of late Golgi structures and are also a hallmark of encystation induction. ESV arise from: a) specialized regions of the ER, a trans-Golgi equivalent (Gottig *et al*., 2006); b) enlarged ER cisternae (Lanfredi-Rangel *et al*., 2003); or c) homotypic fusion of ER-derived COPII-coated transport vesicles into “pre-Golgi” vesicles (Marti *et al*., 2003). The modestly increased mRNA expression levels of structural and cargo processing molecules of ESV during encystation induction (Marti *et al*., 2003) suggest that these structures are derived from structural templates in the ER that differentially recruit components required for sorting and cargo maturation. ESV shape is thought to be changed from irregular to spherical as cargo matura-

![Fig. 1. - Structural and molecular markers of *Giardia* encystation. A. Transmission electron microscopy of an encysting cell displaying multiple ESVs in the cytoplasm (arrows); B. Scanning electron microscopy of an encysting cell with the cyst wall filamentous material on surface; C. Confocal laser microscopy of an encysting cell stained with the CWP1-specific antibody 5-3C displaying reactivity at ESVs (asterisks) and cell surface (arrows); D. Detection of *cwp1* mRNA by semi-quantitative RT-PCR assay (upper panel) and of CWP1 polypeptide by Western blot with monoclonal antibody 5-3C (lower panel). The size of the amplified product and the molecular weight (Mr) of the reactive protein in trophozoites induced to encyst for different time periods are indicated at the left of each panel respectively.](image)
Membrane remodeling

There is an early recruitment of peripheral matrix proteins in membranes of immature ESV including βCOP, an homolog of yeast GTPase transporter Ypt1p-interacting protein (Yip), Rab11 and dynamin-like protein (DLP), and a late recruitment of others such as the clathrin heavy chain (CLH). Of these, CLH and DLP redistribute mostly to the ESV while the others redistribute to ESV at a lower extent upon encystation progression (Marti et al., 2003).

Quality control of cargo

Misfolded cargo molecules are most likely processed by proteasome and Sec61 translocon activities to allow ESV-to-ER retrograde transport of proteins within COPI-coated vesicles where chaperons such as BiP cycle between these compartments (Marti et al., 2003; Stefanic et al., 2006).

Post-translational modifications of cargo

A critical modification of CWP2 is its processing by a cathepsin B-like cysteine protease (CP2, orf EAA 41050), the most up-regulated of 25 clan CA proteases expressed by Giardia (DuBois et al., 2008), likely releasing the 26 kDa fragment (and hence CWP complexes) to the ESV lumen. CWP2 processing would be also performed by another ESV-contained cathepsin B-like cysteine protease (orf EAA 37074; DuBois et al., 2008) but not so for the peripheral vesicle (PV)-contained cathepsin C-like encystation-specific cysteine protease (ESCP, orf EAA 36907; Touz et al., 2002).

Near to the plasma membrane, ESV appears to carry out two processes: a) fusion with PV (Luján & Touz, 2003) that acidifies the cargo to limit CP2 activity and/or allow ESCP activity and favor other undefined processing mechanisms preceding the release of CW material on the cell surface; or b) fission (dispersal) into small secretory vesicles that eventually fuse with the plasma membrane to discharge its content (Hehl & Marti, 2004). Proposal ‘a’ is supported by the PV-to-ESV redistribution of CLH (Marti et al., 2003) while ‘b’ is consistent with microscope observations of the initial secretion of antigenic [glyco]polypeptide CW material as small protrusions on the surface of encysting cells (Erlandsen et al., 1990, 1996). CWP complexes are concentrated, stabilized, sorted and bud in ESV together with chaperons that allow their correct folding (by immunoglobulin heavy chain-binding protein (BiP)) and oligomerization (by protein disulfide isomerases (PDI) 1-3). Another ESV cargo protein (gGSP) helps to maintain low intra-ESV calcium levels to prevent premature assembly and to regulate exocytosis of CWP complexes (Luján & Touz, 2003).

The release of CW material from ESV is carried out by exocytosis and appears to involve incomplete fusion between plasma and ESV membranes (Benchimol, 2004). The release of ESV contents and CWF formation/assembly may occur stepwise in late encysting cells, i.e., CWP and HCNCP (glyco)polypeptide exposure on the cell surface precedes their co-polymerization with the [GalNac(β1→3)GalNac(β1→3)], complex (Argüello-García et al., 2002). This proposal is supported by independent estimations of the time for ESV content release (up to 1 min) (Hehl & Marti, 2004) compared with that required for complete CW assembly (5-6 h.), a process occurring by fibril tip growth (Erlandsen et al., 1996; Argüello-García et al., 2002) and by the presence of CW (glyco) polypeptides in 15-to-100 nm-sized protrusions laid on the surface of encysting cells (Erlandsen et al., 1996) which do not display typical CWF architecture. The cytologicalization of cyst wall synthase (CWS) activity in encysting cells will provide further insights into the process of CW formation. With respect to the induction phase of encystation, recent experimental and bioinformatics evidence provide new information that allow proposing a model discussed in the sections below.

CHOLESTEROL REGULATION OF GENE TRANSCRIPTION AND GIARDIAL ENCYSTATION

Extracellular cholesterol promotes giardial growth and its depletion triggers encystation (Luján et al., 1996, 1997). This suggests a role for nutrient-cell surface receptor interactions promoting modulation of cell signaling cascades that lead to growth or differentiation. Cholesterol auxotrophy for Giardia growth is unambiguous in spite of the presence of gene transcripts of enzymes from initial steps of the mevalonate pathway. These include hydroxymethylglutaryl-CoA synthase (HMGCoA-syn) and the rate-limiting enzyme HMGCoA reductase (HMGCoA-red) (Hernández & Wasseman, 2006), the de novo synthesis of farnesyl and geranylgeranyl isoprenoids and ubiquinone (Luján et al., 1997) and the presence of sequences with low homology to squalene oxidase, squalene synthetase and lanosterol 14-α-demethylase in the annotated GiardiaDB. However, the cyclization of squalene to lanosterol and further conversion to cholesterol, demanding at least 11 O₂ molecules (Ikonen, 2008) are unlikely to occur in Giardia since it is microaerophilic. Instead trophozoites take up cholesterol by calcium-indepen- dent endocytosis (Luján et al., 1996, 1997) mediated by a 69-kDa cell surface glycoprotein receptor (CR) recognizing the cholesterol moiety of low density lipoproteins (LDL) (Kaul, 2003) which is antigenically similar in mammalian cells and Giardia (Kaul et al., 2001). In other eukaryotic models, upon interaction...
with cholesterol (i.e., during cell growth) CR displays intrinsic tyrosine kinase activity to generate phosphatidic acid (PA). PA stimulates phospholipase A (PLA₂) activity in a calcium-dependent manner to produce arachidonic acid (AA) and tromboxans (TxA₂) increasing intracellular cAMP and cGMP levels, respectively (Kaul, 2003). Protein kinase A (PKA) is activated by cAMP and phosphorylates the precursor form of the 125 kDa-sized sterol-responsive element binding protein (pSREBP; Fig. 2) which may be retained in the ER by the SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein (INSIG) (Ikonen, 2008; Kaul, 2003; Yellaturu et al., 2005). In Giardia specific Ca²⁺-dependent, membrane-associated and alkaline-soluble PLA₂ activities have been demonstrated (Vargas-Villarreal et al., 2007); AA is generated as an intermediate product in the giardial Lands cycle that allows the remodeling of uptaken phospholipids and generation of new ones (Das et al., 2001) and noteworthy endogenous cAMP promotes vegetative growth and the activation phase of encystation (Abel et al., 2001).

In the infected host, trophozoites carried down the colon are differentially exposed to major biliary lipids (bile salts, lecithin and cholesterol) at a slightly alkaline pH. Encystation is triggered at the lumen of the lower jejunum upon cholesterol depletion by epithelial absorption and a lower cholesterol uptake by trophozoites induced by micellar bile acids (Luján et al., 1997) released at the proximal duodenum and reabsorbed in the lower ileum. CR is not stimulated at this point, cAMP levels decrease and pSREBP is transported within COPII-coated vesicles to the Golgi apparatus and processed by proteolytic cleavage to generate the mature 47 kDa-sized form (mSREBP) (Kaul, 2003; Yellaturu et al., 2005) (Fig. 2). In good agreement with these notions cAMP levels in trophozoites are significantly lowered upon deprivation of growth factors (Abel et al., 2001). This partially explains why in vitro encystation can also be achieved using antibodies against CR which may be inactivated catalytically as well as cholesterol endocytosis (Kaul et al., 2001). Likewise infusion of N₂/CO₂ would have a similar effect as these gases are able to intercalate within membranes (Alberts et al., 2008) possibly altering CR activity. Moreover, the giardial mSREBP homolog is antigenically and functionally similar to its mammalian counterpart as reported in ovary cells transfected with CWP2 promoter-reporter constructs (Kaul et al., 2001; Worgall et al., 2004).

**Fig. 2.** – A. Western blot of extracts obtained from trophozoites cultured for 36 h under conditions inducing proliferation (T) and encystation (E) and incubated with monoclonal antibodies against human SREBP-1 (upper panel) and 5-3C antibodies (lower panel). Processing of SREBP from the precursor (pSREBP, 125 kDa-sized) to the mature form (mSREBP, 47 kDa-sized) is shown. B. Western blot of extracts from trophozoites incubated for different time periods in encystation medium and incubated with antibodies against the catalytic subunit of human PKCβ (upper panel) and with a phosho-ERK antibody generated against a 20-amino acids peptide antigen around the TXY motif of human p44 ERK1. The latter has 65 and 52 % identity with the same region of the giardial ERK1 and 2, respectively (lower panel). The levels of ERK1 phosphorylation are markedly decreased after the induction phase of encystation (3-5 h) has progressed. The relative mobility (Mr) is indicated in kDa at the left of each panel.

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Transcription factor mSREBP translocates to the nucleus where it interacts with genomic SRE at gene promoters of either the cholesterol biosynthesis pathway (HMGCoA-syn and HMGCoA-red) and fatty acid biosynthesis (LDLr) and CWPs resulting in their up-regulation (Worgall et al., 2004; Ellis et al., 2003) (Fig. 3). However, mSREBP is readily degraded within the mammalian nucleus after a rise in sterol concentration. Of interest, Giardia exhibits increased levels of both HMGCoA-syn and HMGCoA-red transcripts up to 12 h into encystation progression. These then decline by 24 h post-induction due to gene down regulation by de novo synthesis of mevalonate-derived metabolites but CWP transcripts remain up-regulated (Hernández & Wasser-

![Diagram](attachment://image.png)

**Fig. 3.** – Proposed signaling cascades of cholesterol-mediated gene transcription in *G. duodenalis* and their relation to encystation induction. The giardial CR (that in mammalian cells consists of a 32 kDa-sized α subunit and two 19 kDa-sized β subunits) upon interaction with the cholesterol moiety from LDL may display both cholesterol endocytosis and RTK activity promoting a rise in cAMP levels that is associated with trophozoite motility and replication. PKA activation may keep the 125 kDa-sized form (pSREBP) phosphorylated which will be retained at the endoplasmic reticulum. In addition to pSREBP, Raf1 may be phosphorylated by PKA and/or dephosphorylated by PP2a rendering Raf1 in an inactive state. If CR activity is not induced or blocked (e.g., by low levels of extracellular cholesterol or binding by micelles of primary bile salts or CR-specific antibodies) cAMP levels decrease, pSREBP is proteolytically processed to the 47 kDa form (mSREBP) and Raf1 may be activated and translocated to the cell membrane to be phosphorylated by activated PKCβ. Further MEK1 may be phosphorylated by Raf1 and ERK1 re-localizes to the membrane and after phosphorylation by MEK1 migrates to the nucleus where mSREBP is activated. This molecule together with other transcription factors (GLP1, ARID1, Myb2 and WRKY-like) may interact with promoters activating transcription of genes encoding encystation-related markers and enzymes of the mevalonate pathway. Green ovals represent signaling molecules experimentally identified and red ovals are those deduced by bioinformatics data from the GiardiaDB (see Table I for details).

**Abbreviations:** AA: arachidonic acid; CR: cholesterol receptor; ERK1: extracellular signal-activated kinase 1; HMGCoA syn/red: hydroxymethylglutaryl coenzyme A synthase/reductase; LDL: low density lipoproteins; PA: phosphatidic acid; PKA/C: protein kinase A/C; PLA2/D: phospholipase A2/D; p/mSREBP: precursor/mature sterol-response element binding protein; PP2a: protein phosphatase 2a; Raf1: mitogen-activated protein kinase 1; TxA2: thromboxan A2.
ARE BILE SALTS RELEVANT FOR ENCYSTATION INDUCTION?

It has been proposed that bile salt micelles incorporate ionized fatty acids at slightly alkaline pH reducing their giardial cytotoxicity (Gillin et al., 1989) and that these micelles stimulate giardial encystation by an indirect, inhibitory effect on cholesterol uptake by trophozoites (Luján et al., 1997). Nevertheless, bile or bile acid deprivation at the duodenum of infected CF-1 mice by surgical cholestasis or by diets containing a bile acid-sequester resin (cholestyramine) dramatically reduced Giardia cyst shedding without affecting trophozoite establishment and replication (Erlandsen, 2005). This strongly supports the view that high bile concentrations and a slightly alkaline pH in TYI-S-33 medium efficiently mimics conditions found in the small intestine environment (Gillin et al., 1987) and that bile acids are a “natural stimulus” for giardial encystation. Moreover glycine and taurine N-acyl conjugates of primary bile salts (cholate and chenodeoxycholate) are far more efficient at inducing Giardia encystation than secondary salts (deoxycholate) (Gillin et al., 1987). Indeed taurodeoxycholate at micellar concentrations (≥ 5 mM) inhibited the transfer of cholesterol from medium to trophozoites (Luján et al., 1997) although this secondary bile salt stimulated encystation with very low efficiency (Gillin et al., 1987). Inhibition of cholesterol uptake by bile salts is an event not sufficient in itself to complete the encystation process. Thus an interaction between chenodeoxycholate-rich micelles and cell surface components is likely to occur in agreement with the known selectivity of CR for the cholesterol moiety of LDLs taking into account that primary bile acids are the closest resembling compounds to cholesterol while secondary bile acids are derived from primary ones by bacterial dehydroxlylation (Alberts et al., 2008). Further, the chenodeoxycholate moiety is more similar to cholesterol than cholate and regardless of whether chenodeoxycholate is conjugated to glycine or taurine, the cyst yields are comparable to those obtained with glycodeoxycholate (see Fig. 3 of Gillin et al., 1987). However the less voluminous glycine group might have a lesser hindrance effect than the more voluminous taurine group favoring CR inactivation as glycodeoxycholate is more effective than taurodeoxycholate inducing encystation (Gillin et al., 1987).

Giardia encystation: induction and signaling

Cholesterol-mediated regulation of gene transcription is associated with encystation induction in Giardia and mSREBP production appears to be a critical step for biosynthesis and accumulation of CWPs. However, a signaling cascade is needed to up-regulate SRE-containing gene promoters by mSREBP. The transcriptional activity of mSREBP is decreased by its ubiquitylation and/or sumoylation while the effect of its phosphorylation depends on the kinase involved: extracellular-signal regulated kinase (ERK)-mediated phosphorylation increases transcription by mSREBP while it decreases by PKA or glycogen synthase kinase (GSK)-mediated phosphorylation (Arito et al., 2008). Giardial homologues of the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 have been identified (Ellis et al., 2005). Endogenous ERK1 exhibited increased activity, slightly higher phosphorylation and a noticeable partial relocalization to the cell membrane at an early time post-encystation induction (= 2 hrs; see Figs. 6A and 6B of Ellis et al., 2003) while ERK2 at this stage relocates from the membrane to the cytosol concomitantly with a decrease in its activity (Ellis et al., 2003). Interestingly, Giardia and Dictyostelium discoideum ERK2s are closely related at the amino acid (aa) level (66 % identity and 84 % homology, expectation value of 8e-130) (Table I) with a co-localized cAMP-dependent PKA phosphorylation site (KRIT) that in the slime mold is involved in activation and translocation of ERK2 to the nucleus during the multicellular development response to extracellular factors that increase intracellular cAMP levels. These observations suggest the involvement of ERK1 in giardial mSREBP phosphorylation.

Considering MAPK activation by the dual specificity MAPK kinases 1 and 2 (MAPKKs = MEK1/2; McCubrey et al., 2007), there is one giardial homolog of MEK1 (STE20 type) in the GiardiaDB (orf 22165) (Table I). The sequences of human (393 aa long) and giardial (357 aa-long) MEK1s show at most 39 % identity and 56 % homology (e.v. 1.8e-40) over a 209 aa-stretch. Giardia MEK1 displays the ATP-binding and serine/threonine signature domains of bona fide MEK1s. Likewise one homolog to MAPKKK1 (syn. Raf1, MEK kinase 1), a MEK-phosphorylating small GTPase, is present in the annotated GiardiaDB (orf 15868) (Table I). Its aa sequence displays at most 26 % identity and 46 % homology to MEK kinase 1 from Gallus gallus with an e.v. 9e-17 over a 206 aa-stretch. The giardial homolog exhibits the two zinc Finger profile domains (SWIN and RING) near the N-terminus present in Raf-related
kinases but the kinase domain at the C-terminus in Raf prototypes (e.g. human v-Raf-1, 648 aa-long) is atypical or truncated since the putative giardial homolog is shorter than the Raf-related G. gallus sequence displaying this domain (606 vs 1346 aa, respectively). In addition Raf-related sequences, including giardial Raf1-like, lack a Ras-binding domain (RBD) that allows Ras-mediated Raf translocation to cell membrane (McCubrey et al., 2007). To date one putative RBD has been described in the phosphatidylinositol-3-kinase-1 (PI3K) sequence of this protist (Gox et al., 2006) and a Ras-like GTPase sequence was reported in the annotated GiardiaDB (orf 9718) suggesting that Ras-like is preferentially involved in the partially characterized PI3K signaling pathway of G. duodenalis (Gox et al., 2006; Morrison et al., 2002). Therefore giardial Raf1-like may be recruited to the cell membrane during encystation by another small GTPase such as a Rab protein, of which at least nine members are present in the GiardiaDB. Together the bioinformatics evidence suggests a pivotal role for the Raf1/MEK1/ERK1 pathway in the induction phase of giardial encystation (Fig. 3). As an upstream activator of this pathway, Raf1 activity is positively regulated by protein kinase C and negatively regulated by PKA and protein phosphatase 2A (PP2A) (McCubrey et al., 2007). The catalytic subunit of giardial PKA (PKAc) retains the ability of protein expression at 2 h post-encystation induction without relocating from cytoskeletal structures to the cell membrane (Gibson et al., 2006). In the case of giardial PP2A, it has been observed that the catalytic subunit (PP2A-C) is associated to basal bodies/centrosomes, ventral disk and paraflagellar dense rods (PDRs) in trophozoites. Shortly after encystation induction (up to 4 h) it was no longer found in the anterior PDRs, its mRNA and protein levels decreased, no redistribution occurred and it was localized only in the CW of encysting cells. Moreover, trophozoites transfected to stably express antisense PP2A-C mRNA showed decreased levels of PP2A-C and CWP1 proteins, less ESV formation and lower encystation (Lauwaet et al., 2007). Based on a similar localization of PKAc, ERK1, centrin, calmodulin and PP2A-C in trophozoites and changes of their cyto-localization upon encystation/excystation induction, it was proposed that PP2A-C acts as a dual regulator: initially for pathways leading to loss of adhesion, motility and cell division during ongoing encystation and then in pathways leading to reactivation of these functions during excystation (Lauwaet et al., 2007). This conclusion supports the absence of interactions between PP2A and Raf1-like at trophozoite membrane during encystation induction. On the other hand, protein kinase C (PKC) homologs including one conventional (βII-like), three novel (δ, ε, θ) and one atypical (ζ) were identified in Giardia (Bazán-Tejeda et al., 2007). Of these, endogenous PKCβII-like required Ca²⁺, phosphatidylerine and diolen/diacylglycerol for full kinase activity; this protein was redistributed from cytoplasm to cell membrane during the first 10-30 min after encystation induction and by 2 h post-encystation induction this kinase relocated again to the cytoplasm. General or β-specific PKC inhibitors affected encystation but not cell growth (Bazán-Tejeda et al., 2007). These data suggest the activation of PKCβ as it occurs for ERK1 but at an earlier time post-encystation induction (10-30 min. vs 2 h, respectively) as expected from the signaling cascade (Raf1/MEK1/ERK1) proposed to be involved in this process (Figs 2 and 3). As per other models, PKC activity remains inhibited when the CR is stimulated to down-regulate the expression of some transcription factors such as c-myc by mechanisms that may inhibit the accumulation of PKC cofactors as diacylglycerol (Sikand et al., 2006). This observation together with possible SREBP maturation when the CR-dependent cAMP/PKA pathway is switched off suggest the existence of regulation points at the interphase of the CR-dependent and the MAPK-dependent pathways in this protist. As far as the signaling regulation of growth and differentiation in Giardia is concerned, encystation commit-

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**Table I. – Molecules proposed in the MAPK/ERK signalling pathway leading to SREBP activation during induction of G. duodenalis encystation.** (Fig. 3). Codes: Orf: open reading frame; c: catalytic subunit; r: regulatory subunit.

<table>
<thead>
<tr>
<th>Signalling protein</th>
<th>Orf in GiardiaDB (<a href="http://www.giardiaDB.org/giardiaDB/">http://www.giardiaDB.org/giardiaDB/</a>)</th>
<th>GenBank/NCBI Accession No.</th>
<th>Maximal identity value and homolog species (full length/partial)</th>
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<td>ERK1</td>
<td>GL50803_17563</td>
<td>AAN73429.1*</td>
<td>51 %, Dictostelium discoideum (f)</td>
<td>Ellis et al. (2003)</td>
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<tr>
<td>ERK2</td>
<td>GL50803_22850</td>
<td>AAN73430.1*</td>
<td>66 %, D. discoideum (f)</td>
<td>Ellis et al. (2003)</td>
</tr>
<tr>
<td>MEK1-like</td>
<td>GL50803_22165</td>
<td>XP_001704049.1*</td>
<td>39 %, Arabidopsis thaliana (p)</td>
<td>This review</td>
</tr>
<tr>
<td>RAF1-like</td>
<td>GL50803_15868</td>
<td>XP_001705158.1*</td>
<td>26 %, Gallus gallus (p)</td>
<td>This review</td>
</tr>
<tr>
<td>cPKCβ-like</td>
<td>GL50803_86444</td>
<td>XP_001704119.1*</td>
<td>58 %, Drosophila melanogaster (p)</td>
<td>Bazán-Tejeda et al. (2007)</td>
</tr>
<tr>
<td>rPKCβ-like</td>
<td>GL50803_137754</td>
<td>EDO76444.1*</td>
<td>36 %, Tribolium castanum (p)</td>
<td>Bazán-Tejeda et al. (2007)</td>
</tr>
<tr>
<td>cPKA</td>
<td>GL50803_11214</td>
<td>AGA90429.1*</td>
<td>48 %, D. discoideum (f)</td>
<td>Abel et al. (2001)</td>
</tr>
<tr>
<td>rPKA</td>
<td>GL50803_9117</td>
<td>AAV71055.1*</td>
<td>37 %, D. discoideum (f)</td>
<td>Gibson et al. (2006)</td>
</tr>
<tr>
<td>cPP2A</td>
<td>GL50803_5010</td>
<td>XP_767901b</td>
<td>63 %, Homo sapiens (f)</td>
<td>Lauwaet et al. (2007)</td>
</tr>
</tbody>
</table>

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ment has to be achieved at the expense of cell growth in which the transducing activity of some receptors for growth factors or nutrients localized on the cell surface is crucial. Most of these receptors have intrinsic tyrosine kinase activity and are included in the so called receptor tyrosine kinases (RTKs) family. In spite that no tyrosine-specific kinases have been predicted from the GiardiaDB (Morrison et al., 2007) protein tyrosine phosphorylation has been present in this parasite (Parsons et al., 1993; Luján et al., 1994). Besides CR, *Giardia* has at least a RTK for insulin-like growth factor (ILGFR) that promotes its growth and cysteine uptake (Luján et al., 1994). These ILGFRs are linked to the Ras/PI3K/PTEN/Akt/TOR pathway (Alberts et al., 2008) which has been partially characterized and proposed to be functional in this protist on the basis of transcriptional analyses and bioinformatics data mined from GiardiaDB and related sources (Morrison et al., 2002; Cox et al., 2006; Hernández et al., 2007). In addition a significant inhibitory effect of PI3K inhibitors on trophozoite growth was observed (Cox et al., 2006; Hernández et al., 2007) strongly arguing against its involvement in differentiation induction whenever giardial Akt (or protein kinase B) was reported as up-regulated at the transcriptional level by 17 h post-induction of encystation (Kim et al., 2005), a time much longer than that proposed herein for the induction phase (3-5 h). In this context other signal-recruiting molecules such as the protein 14-3-3 identified in *G. duodenalis* exhibits intracellular changes of localization during encystation (from cytosolic to partially intranuclear) by 12 h post-encystation induction, a time at which ESVs have already been formed (Lalle et al., 2006). These latter observations lead to hypothesize that other signaling pathways involving mediators as PKB, PI3K2 and protein 14-3-3 have a role in encystation but at later stages than the induction one.

**SREBP, ANOTHER FACTOR IN THE SCENARIO**

Early encystation markers including CWPs 1-3 and G6P1-B have similar biosynthetic kinetics (Luján et al., 1997; Steimle et al., 1997; Mowatt et al., 1995; Sun et al., 2003) suggesting that their expression is regulated by similar signaling pathways although other transcriptional factors are also involved. In spite of lacking 8 of 12 general eukaryotic transcription factors (Best et al., 2004), *Giardia* possesses at least four other transcription factors that are over-expressed in encystation and bind to and transactivate gene promoters of encystation markers: GARP-like protein 1 (GLP1) and AT-rich interaction domain protein 1 (ARID1) for *cwp1* promoter; Myb2 for promoters of *cwp 1-3, g6pi-b* and *myb2* itself and a WRKY protein homolog for promoters of *cwp 1-2, myb2* and *wrky* itself (Sun et al., 2002; Sun et al., 2006; Wang et al., 2007; Pan et al., 2009). The manipulated over-expression of some of these transcription factors provided further insights: for instance, the constitutive over-expression of Myb2 moderately increased *cwp1* expression (= 3.7-fold) as compared with levels observed during bile-induced encystation (= 47-fold; Huang et al., 2008). This suggests that all these transcription factors interact to reach expression levels beyond a threshold to allow encysting cells to progress to ESV biogenesis and biosynthesis of precursors of the [GalNAc(β1→3)GalNAc(β1→3)]₅ polymer. Of note, when ERK1 was constitutively over-expressed, transfected trophozoites showed increased levels of *cwp 1-2, wrky* and *myb2* mRNAs (Pan et al., 2009), which provides additional evidence that these encystation-regulated markers may be downstream elements of the MAPK/ERK pathway described herein (Fig. 3).

To date, there is some evidence of involvement of the MAPK/ERK signaling pathway in processing and regulation of SREBP activity, while for GLP1, ARID1, Myb2 and WRKY-like factors the accumulated evidence mostly relies on characterization of their DNA-binding capacities and regulation of *cwp* expression.

**CONCLUSION**

Giardial encystation is a multifactorial process where cholesterol starvation or bile acid micelles are necessary to accomplish the induction phase but not sufficient to complete cyst biogenesis. This phase includes transcription regulation of genes encoding early encystation markers (CWPs 1-3 and G6P1-B) by a cholesterol-mediated process which requires inactivation of the CR-coupled signaling that may be accomplished by primary bile acid-micelles and even by gas infusion or antibody binding. Experimental and bioinformatics data suggest that this inactivation is concomitant with the activation of a Raf1/MEK1/ERK1 pathway leading to SREBP maturation and activation. It is necessary to define whether the other well characterized encystation-induced transcription factors are regulated by similar or distinct signaling pathways. Characterizing these processes at a functional level will reveal new targets for diagnosis, drug design and prophylactic intervention similarly to the cyst wall polysaccharide-targeting drugs and nucleotide-based cyst wall biosynthesis inhibitors (Jarroll & Sener, 2003; Suk et al., 2007) and the engineered expression of CWP2 in bacterial vectors for transmission-blocking vaccines (Lee & Faubert, 2006; Abdul-Wahid & Faubert, 2007).

Parasite, 2009, 16, 247-258
ACKNOWLEDGEMENTS

The authors are grateful to Jacqui Upcroft and Edward Jarroll for critically reviewing the manuscript. This work was supported by Conacyt-Mexico grant No. 49724 and ECOS-ANUIES grant No. MO6SO3 France-Mexico.

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Reçu le 20 juin 2009
Accepté le 10 septembre 2009