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Mechanisms of adaptation in the intestinal parasite *Giardia lamblia*

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Abstract

Giardia lamblia, a parasite of humans, is a major source of waterborne diarrhoeal disease. *Giardia* is also an excellent system to study basic biochemical processes because it is a single-celled eukaryote with a small genome and its entire life cycle can be replicated *in vitro*. *Giardia* trophozoites undergo fundamental changes to survive outside the intestine of their host by differentiating into infective cysts. Encystation entails the synthesis, processing, transport, secretion and extracellular assembly of cyst wall components. To survive within the intestine, *Giardia* undergoes antigenic variation, a process by which the parasite continuously switches its major surface molecules, allowing the parasite to evade the host's immune response and produce chronic and recurrent infections. The objective of the present chapter is to provide a better understanding of the molecular mechanisms involved in adaptation and differentiation in *Giardia*, with a particular focus on the process of encystation and antigenic variation of this interesting micro-organism.

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Introduction

The ability of parasitic organisms to adapt to changes in the environment is an issue that has intrigued microbiologists for decades. Since most parasites occupy different niches during their journey through vectors and hosts, they have developed adaptive responses that allow them to survive under adverse conditions. The life cycles of protozoan and helminth parasites are, therefore, exceptional systems to study the mechanisms involved in cell adaptation and differentiation, such as developmentally regulated gene expression, specific signal transduction pathways and organelle biogenesis [1].

In the present chapter, I will focus on the intestinal parasite *Giardia lamblia* (synonymous with *Giardia intestinalis* and *Giardia duodenalis*). *Giardia* is a binucleated flagellated protist that inhabits the upper small intestine of its vertebrate hosts and is the most common cause of defined waterborne diarrhoea worldwide [2] (Figure 1A). Clinical manifestations of giardiasis vary from asymptomatic infection to acute or chronic disease associated with diarrhoea and malabsorption [2]. Phylogenetic studies of *Giardia* isolates allowed the identification of seven *G. lamblia* assemblages (A–G), assemblages A and B being maintained by direct transmission to humans and companion animals [3].

Besides its medical importance, *Giardia* has been of great biological interest because it was suggested to derive from one of the earliest branches of the eukaryotic line of descent, although previous reports presented contradictory interpretations regarding this issue [4].

Giardia trophozoites undergo essential biological changes to survive in hostile environments. To survive outside the host's intestine, *Giardia* trophozoites differentiate into cysts, which are released with the faeces and are responsible for the transmission of the disease (Figure 1B). Encystation comprises the synthesis of cyst wall components and the biogenesis of secretory organelles not present in non-encysting cells, such as ESVs (encystation-specific secretory vesicles),

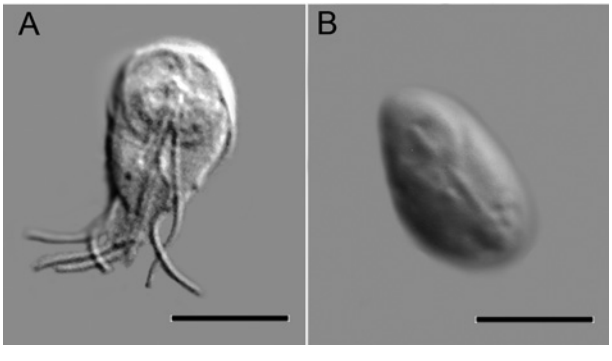

Figure 1. Trophozoite and cyst of *G. lamblia*
DIC (differential interference contrast microscopy) images of a trophozoite (**A**) and a cyst (**B**) showing the two characteristic stages of *Giardia*. In the disease-causing trophozoite, the four pairs of flagella, two nuclei and the medial body can be seen. In the cyst, the external protective cyst wall is visible. Scale bars, 10 μm.

necessary to transport cyst wall constituents for assembly into the extracellular cyst wall [5]. To colonize a new host, the cyst should then excyst in the upper small intestine, releasing the trophozoites, which proliferate attached to the epithelial cells of the gut by means of a particular organelle called a sucking or ventral disc [2]. Another adaptive mechanism developed by this parasite to survive within the host is antigenic variation [6]. This is a process by which *Giardia* continuously switches its major surface molecules, allowing the parasite to evade the host's immune response. These surface antigens belong to a family of VSPs (variant surface proteins), which are integral membrane molecules that cover the entire surface of trophozoites. Only one VSP, from a repertoire of ~200 VSP genes present in the parasite's genome, is expressed on *Giardia*'s surface at any point in time [6] (Figure 2).

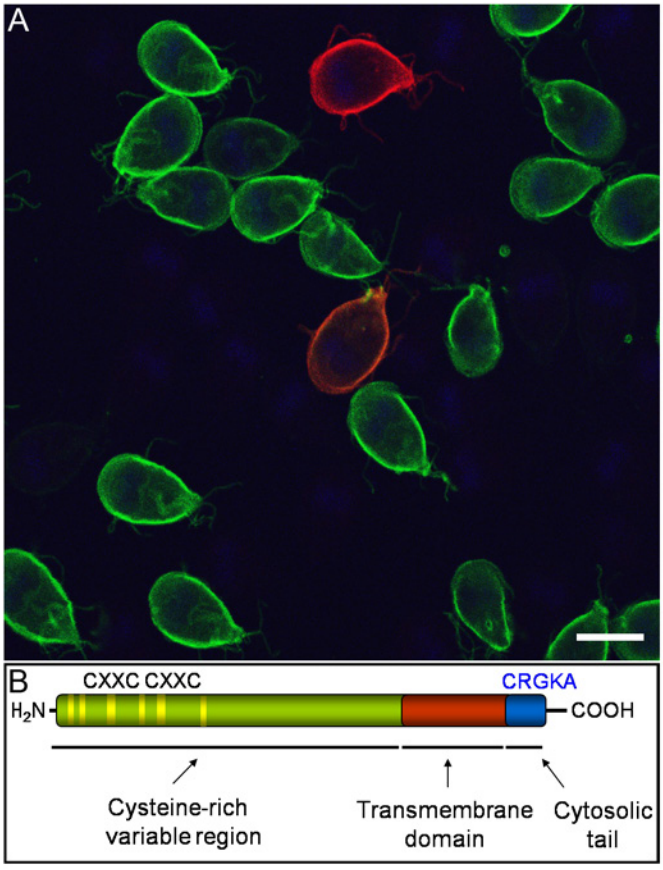

Figure 2. Antigenic variation in *G. lamblia*

(**A**)Trophozoites in culture were labelled with two monoclonal antibodies specific against cells expressing a particular VSP. Some trophozoites in the population are expressing VSP9B10 on their surface (green), whereas others are expressing VSP1267 (red). Others remain unlabelled because they are expressing variant antigens not detected by these monoclonal antibodies. Nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole; blue). Scale bar, 10 μm. (**B**) Schematic representation of a *Giardia* VSP.

Although once considered the 'missing link' between prokaryotes and eukaryotes [7], *Giardia* is, in fact, a typical eukaryotic organism since it possesses two nuclei, an endomembranous system consisting of the ER (endoplasmic reticulum) and lysosome-like peripheral vacuoles, and a complex cytoskeleton [8]. Nevertheless, *Giardia* lacks other organelles characteristic of higher eukaryotes, such as typical mitochondria, peroxisomes and the distinctive sorting organelle named the Golgi apparatus [8]. Some of its particular cellular characteristics are probably the result of secondary loss of complex cell structures and metabolic pathways as a consequence of its parasitic life style rather than the primitive simplicity believed for early diverging protists [9].

During recent years, many novel (and surprising) aspects of the biology of *Giardia* have been deciphered. Nevertheless, many others are still unknown and puzzling. In the following sections, I will describe the biology of *Giardia* regarding adaptation to different environments within the context of cellular differentiation (encystation and antigenic variation), and identify the questions that need to be answered in the future not only to better understand this fascinating micro-organism, but also to provide insights for the development of new strategies for diagnosis and prevention of *Giardia* infections.

Giardia differentiation into cysts

Encystation of *G. lamblia* can be divided into three main stages: (i) the reception of the stimulus for encystation, transmission of this signal to the nuclei and the expression of encystation-specific genes; (ii) the synthesis of precursors and cyst wall molecules, the biogenesis of secretory organelles absent in non-encysting trophozoites and the intracellular trafficking of cyst wall components; and finally (iii) the exocytosis of ESVs and assembly of the extracellular cyst wall [5].

The stimulus for encystation and the regulation of encystation-specific gene expression

Giardia trophozoites are easy to cultivate in the laboratory in a complex medium containing casein digest, yeast extract, bile, serum and high concentrations of reducing agents, such as cysteine and ascorbic acid, at pH 6.8–7.0 (Figure 3A). Gillin et al. [10] reproduced *Giardia* encystation *in vitro* for the first time by increasing the bile concentration and raising the pH of the medium to 7.8 (Figure 3B). With these tools, non-encysting trophozoites, encysting cells and cysts could be collected and analysed separately, and therefore several molecular and cell biological aspects of this process of differentiation have been revealed. Nevertheless, the molecular basis for the induction of encystation remained undefined, primarily because several different conditions, in addition to high bile concentrations, were reported to trigger trophozoite differentiation *in vitro* [5].

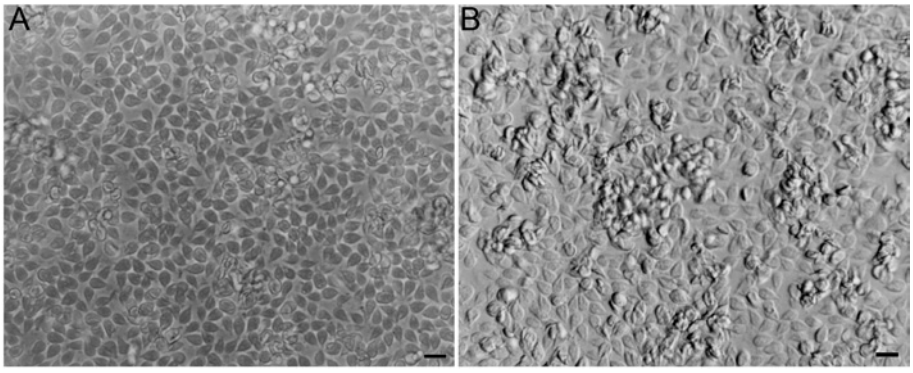

Figure 3. Life cycle stages of *Giardia* cultivated *in vitro*
Giardia can be grown in TYS-S-33 medium (pH 7.0) supplemented with bile and adult bovine serum at pH 7.0 (**A**). Cells attach to the wall of the tube forming a monolayer of pear-shaped trophozoites. Encystation can be induced by using the same medium but supplemented with lipoprotein-free serum or high concentrations of porcine bile at pH 7.8 (**B**). Cysts (round and oval forms), which detach from the culture tube, can be collected from the supernatant medium, whereas encysting trophozoites containing ESVs remain attached until exocytosis of ESVs occurs. Scale bars, 10 μm.

Giardia trophozoites, like most eukaryotic cells, require lipids for proliferation because of their inability to synthesize cholesterol and phospholipids *de novo* [2]. To multiply and colonize their hosts, trophozoites might depend on preformed biliary lipids present in the upper small intestine. Since the absorption of lipids in humans occurs almost completely in the jejunum, it was reasoned that as trophozoites travel down the intestine they might confront a lipid-poor environment, which could trigger their differentiation into cysts. Then, the induction of cyst formation *in vitro* when trophozoites are starved of cholesterol was clearly demonstrated [11]. A secondary role for bile salts in *Giardia* encystation, inhibiting cholesterol uptake from the trophozoites, was also shown. These results indicated that cholesterol starvation is necessary and sufficient for the stimulation of *Giardia* encystation *in vitro* and, possibly, in the intestine of mammalian hosts [11]. Although these findings represented a significant step forward in the understanding of this process, important questions remain undefined. For example, how does a *Giardia* parasite sense low levels of cholesterol in the environment? How does the sensing mechanism correlate with the regulation of specific gene expression during encystation?

To date, gene regulation during encystation is poorly understood. Several encystation-specific genes have been identified and characterized during the last decade, and shown to be up-regulated with similar kinetics during encystation, suggesting that their regulation is at the transcriptional level [5]. The genome of *Giardia* is compact in structure and contains 11.7 Mb divided into five chromosomes [4]. DNA synthesis and transcription seem to be simple when compared with other eukaryotes and many characteristics of transcription are uncommon [4]. Several genes overlap and others are within

100 nt of an adjacent ORF (open reading frame). *Giardia* has a highly divergent TATA-binding protein, lacks eight out of 12 general transcription factors, and neither its TATA nor CCAAT boxes have been described. Apparently, AT-rich sequences can act as initiators of transcription for many genes. Recently, several publications described putative transcription factors that regulate the expression of encystation-specific genes, but, unfortunately, the results obtained so far have not solved the issue of how specific gene regulation during encystation is regulated (reviewed in [12]). Therefore is there a common transcription factor for all encystation-regulated genes? Do the potential transcription factors act as enhancers or repressors of transcription, since this process seems to be very relaxed in *Giardia*?

Synthesis and transport of cyst wall proteins

Once initiated, encystation progresses with the co-ordinated synthesis, processing and transport of cyst wall constituents for subsequent assembly into a protective cyst wall. In *Giardia,* the mechanisms that control protein transport are not well understood.

Giardia has been reported to lack a stack of flattened cisternae that is the hallmark of the Golgi apparatus, a fundamental organelle essential for protein secretion in eukaryotes [13]. It was reported that during encystation, *Giardia* trophozoites undergo the induction of Golgi enzyme activities that correlates with the appearance of a morphologically identifiable Golgi complex [14]; however, it seemed to be an artefact since later, by analysis of higher eukaryote Golgi-specific molecules, a typical Golgi apparatus was not found in this organism [15]. Nevertheless, the induction of carbohydrate-processing enzymatic activities (Golgi function) associate with the appearance of ESVs, which transport cyst wall components to the plasma membrane of the encysting cell and release their content to the cell exterior during cyst wall formation.

Among the molecules induced during encystation, some are protein components of the cyst wall (CWP1–3, where CWP is cyst wall protein), others are enzymes that form the precursors of the carbohydrate polymer present on the cyst wall, whereas others facilitate protein folding and transport. The CWP genes predict acidic and leucine-rich proteins targeted to the secretory pathway by N-terminal signal peptides. Interestingly, these three proteins share several characteristics: CWP1, 2 and 3 possess ~60% identity of the amino acid sequence in a 26 kDa overlapping region, contain a cysteine-rich region and five tandem copies of a leucine-rich repeat, and all colocalize within ESVs in encysting trophozoites and in the cyst wall of mature cysts (Figure 4). Besides being structurally similar, the kinetics of induction during encystation is identical for these cyst wall proteins [5].

In higher eukaryotes, proteins destined for secretion are folded, assembled and glycosylated as they are transported from the ER through the Golgi to the cell surface, and secretory granules form in the *trans*-side of the Golgi apparatus [13]. The ability to regulate the formation of these granules in *Giardia*

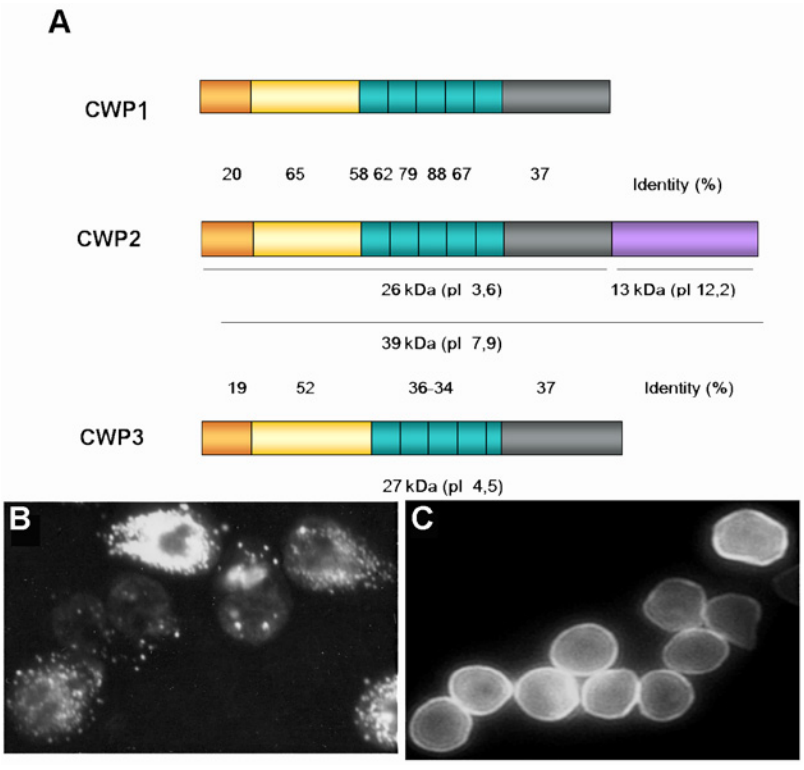

Figure 4. Structure of cyst wall proteins and their localization in encysting *Giardia* trophozoites and cysts

Schematic diagram of the three CWPs of *Giardia* showing their domains and degree of identity among them (**A**). A monoclonal antibody directed to CWP1 shows its presence within ESVs in encysting trophozoites (**B**) and in the cell wall of mature cysts (**C**). pI, isoelectric point.

just by changing the culture medium makes this parasite an excellent model to study granule biogenesis in general.

In this regard, it was suggested that the structural characteristics of CWP2 may play an important role in granule formation. CWP2 has a 121 amino acid terminal extension rich in basic amino acids [16]. It was proposed that oligomerization or aggregation of CWPs could result in ESV budding. The formation of ESVs will thus be a direct consequence of the synthesis of the CWPs, especially CWP2. A report has provided new insights regarding secretory granule biogenesis during encystation of this parasite [16]: although CWP1, 2 and 3 are structurally similar in their 26 kDa leucine-rich overlapping region, CWP2 is distinguished by the presence of a 13 kDa basic extension. Expression in non-encysting trophozoites of different CWP chimaeras indicate that the CWP2 basic extension is necessary for biogenesis of ESVs, which occurs in a compartment derived from the ER. Nevertheless, this extension itself is insufficient to trigger ESV formation, indicating that other domains

in CWPs are also required. Additional results demonstrated that CWP2 is a key regulator of ESV formation by acting as an aggregation factor for CWP1 and CWP3 throughout interactions mediated by its conserved region, as well as a ligand for sorting via its C-terminal basic extension [16]. These findings show that granule biogenesis requires complex interactions among granule components and membrane receptors at the most early steps in the secretory pathway and not in the *trans*-Golgi region [16].

Intracellular protein transport in eukaryotic cells utilizes transport vesicles and tubulovesicular structures to deliver cargo proteins and lipids from one internal compartment to the next [13]. The highly specific nature of transport between compartments appears to require the use of distinct combinations of mutually recognizable factors. The factors that facilitate fusion between donor and acceptor compartments include the SNAREs (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors). SNAREs comprise a family of membrane-associated proteins that confer the tight docking and subsequent fusion of membrane bilayers. We identified and characterized genes for seventeen SNAREs in *Giardia* and defined the minimal set of subcellular organelles present during growth and encystation [15]. Expression and localization of all *Giardia* SNAREs demonstrated their presence in distinct subcellular compartments, which may represent the extent of the endomembrane system in eukaryotes, namely the nuclear envelope, the ER, the lysosome-like peripheral vacuoles, the ESV, the plasma membrane and unidentified vesicles that might represent transport intermediates or ER exit sites. Remarkably, *Giardia* SNAREs homologous to Golgi SNAREs from other organisms do not allow the detection of a typical Golgi apparatus in either proliferating or differentiating trophozoites. However, some features of the Golgi, such as packaging and sorting function, seem to be performed by the ER and/or the nuclear envelope.

Nevertheless, Stefanic et al. [17] define a different scenario. They suggest that Golgi functions in *Giardia* are carried out by the ESVs on the basis of the facts that these vesicles acquire clathrin during maturation and possibly divide into small ESVs before exocytosis.

Cyst wall assembly

Answers to the following questions may facilitate our understanding of cyst wall morphogenesis. Which other molecules in addition to the CWPs are present in the cyst wall? How do these molecules interact to form the final cyst wall architecture? What is the function of the individual components in the cyst wall?

Regarding the carbohydrate portion of the cyst wall, galactosamine was identified as the predominant sugar associated with the cyst wall filaments, and the entire pathway for *N*-acetylgalactosamine formation has been described [18]. Moreover, a novel β1,3-*N*-acetylgalactosamine polymer has been found

to be the major component of the cyst wall [19]. Nevertheless, where and how this polymer is formed remains elusive.

Antigenic variation in *Giardia*

Antigenic variation is a clonal phenotypic variation, and sequential expression of surface-exposed antigenic determinants is used by several microbial pathogens to evade host immune responses and maintain chronic infections under the continuous immune pressure generated by their hosts [20].

Antigenic variation in *G. lamblia* was initially discovered *in vitro* [5] (Figure 2) and later confirmed in both laboratory mammals and human infections [21]. In *Giardia*, antigenic variation is supposed to account for the variable and/or persistent course of some infections as well as the propensity for multiple reinfections, and it involves VSPs that cover the entire surface of the trophozoites and are the major antigens recognized by the host immune system. There is a repertoire of about 200 VSP genes (*vsp*s) in the parasite's genome, but only one VSP is expressed at any given time in each trophozoite. Switching to the expression of another VSP occurs even in the absence of any immune pressure, as can be observed in culture.

The mechanism controlling antigenic variation

Over the last two decades, several groups have made great efforts to elucidate the mechanism controlling antigenic variation in *G. lamblia*. Since the discovery of this phenomenon, different approaches have been undertaken, but neither DNA sequence alterations nor DNA rearrangements have been associated with the regulation of VSP expression [22]. Transcriptional control of VSP expression was initially proposed, since in clones expressing a particular VSP, only transcripts for its gene were detected. After VSP switching occurs, the initial transcript disappears and is replaced by distinct VSP mRNA [5,21].

Constitutive and regulated genes in *Giardia* are controlled by small promoter sequences identified upstream of the translational start site [2,4]. Studies of these sequences indicate that the presence of AT-rich regions is able to direct transcription. However, no specific VSP promoter sequences have been identified and, in contrast with the genes developmentally regulated during encystation, the upstream regions of *vsp*s do not have any evident homology. Early information suggested that some sort of transcriptional control exists for the regulation of VSP expression. However, two reports have proposed a post-transcriptional mechanism for the regulation of *vsp* expression, as discussed below.

In the first study, it was found that one snoRNA (small nucleolar RNA) has similarities to the precursors of miRNAs (microRNAs) [23]. In this report, the authors identified many putative miRNA recognition sequences and evaluated the involvement of key enzymes in the RNA-mediated silencing mechanism. Their results indicated that miRNAs products are involved in translational repression on the basis of sequence similarity to the 3′ end of

hundreds of *Giardia* genes, including 22 *vsp*s. The authors suggested that a post-transcriptional mechanism exists by which miRNAs control the expression of VSPs. Nevertheless, it is unlikely that only a fraction of the entire repertoire of VSP genes is controlled by this proposed mechanism.

In the second study study, to determine whether more than one *vsp* is transcribed at the same time, RT (reverse transcription)–PCR and nuclear run-on experiments using trophozoites expressing only one VSP were performed. Results indicated that many VSP genes were transcribed simultaneously. Interestingly, clonal trophozoite populations only exhibit accumulation of a single VSP transcript, suggesting that the regulation of VSP expression was controlled post-transcriptionally [24]. These results raise the question of how a single *vsp* mRNA can reach a steady-state level required for translation into the mature VSP, given that many VSP genes are transcribed simultaneously. One possibility would be to silence all but one among the pool of transcribed surface antigen mRNAs (Figure 5).

In recent years, important advances in understanding the mechanisms leading to gene silencing have been reported. An RNA-based silencing system that results in gene repression by sequence-specific RNA-targeting was described in nematodes and was found to share mechanistic components with other PTGS (post-transcriptional gene silencing) phenomena previously demonstrated in plants, fungi and animals [25]. A key feature of PTGS is the production of dsRNAs (double-stranded RNAs) sharing sequence homology with the silenced gene. It was also demonstrated that the artificial introduction of these molecules into protozoan, insect and mammalian cells specifically suppressed the expression of their homologous endogenous genes [a process termed RNAi (RNA interference)]. The molecular machinery essential for RNAi involves, at a mimimum, endonucleases of the Dicer and Ago (Argonaute) families, and in some cases RdRPs (RNA-dependent RNA polymerases) in charge of generating dsRNAs using aberrant transcripts as template [25].

To look for direct evidence supporting the involvement of the RNAi machinery during antigenic variation in *Giardia*, the expression of gRdRP, gAgo, and gDicer (where g is *Giardia*) was knocked down by constitutive expression in trophozoites of antisense transcripts corresponding to these molecules [24]. Results indicated that silencing of gRdRP or gDicer in trophozoites generated cells expressing more than one VSP (most probably the entire VSP repertoire), as determined by immunofluorescence analysis using monoclonal antibodies directed against several different VSPs (Figure 6). Silencing of gAgo did not generated any viable clone, indicating that this enzyme may play a more complex role in the biology of the parasite, perhaps controlling the integrity of the genome by silencing the activity of transposons.

These results demonstrate that an endogenous RNAi system is involved in regulating the expression of antigenic variants in *G. lamblia*.

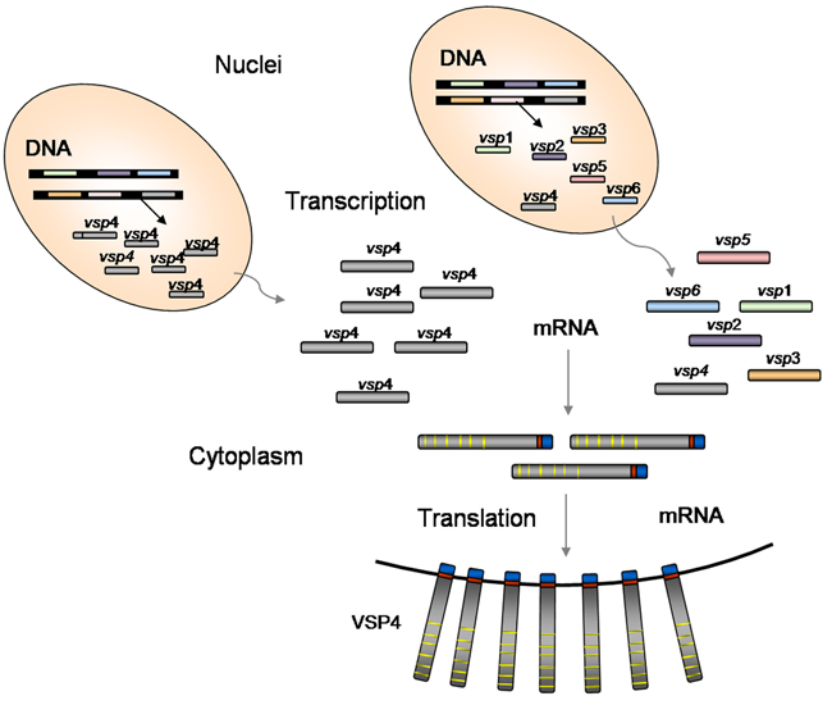

Figure 5. Diagram of the mechanism controlling antigenic variation in *Giardia*
Scheme showing that VSP genes are simultaneously transcribed at both nuclei of the parasite. Once in the cytoplasm, only one VSP transcript accumulates and is translated into the protein that will be expressed at the parasite surface. Other VSP RNAs are silenced by a mechanism similar to RNAi. How only one VSP RNA bypasses the silencing process remains unknown.

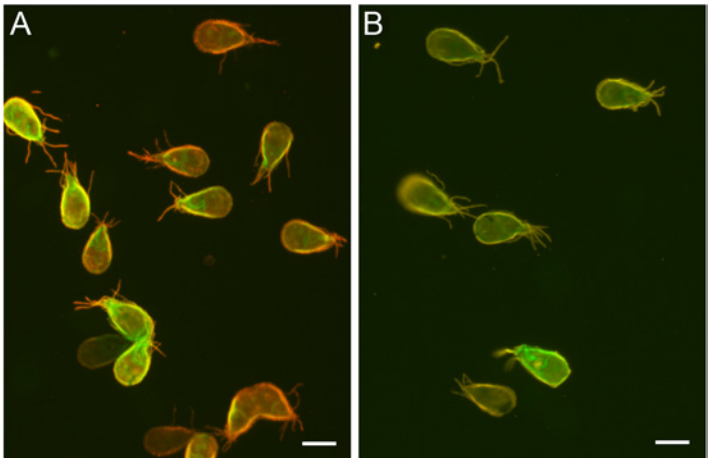

Figure 6. Disruption of antigenic variation in *Giardia*
When components of the RNAi pathway are knocked down, *Giardia* trophozoites express more than one VSP on their surface, as detected by monoclonal antibodies specific for different surface antigens. Merged images show VSP9B10 (green) and VSP1267 (red). Yellow represents colocalization of both VSPs on the surface of individual trophozoites. (**A**) Silencing of Dicer. (**B**) Silencing of RdRP. Scale bars, 10 μm.

Disruption of antigenic variation in an animal model of giardiasis

The biological significance of antigenic variation in *Giardia* was speculative, although escape from the host's immunological response is frequently mentioned. However, although the chronicity of some infections could be due to antigenic variation, there was no experimental data directly supporting this hypothesis since antigenic variation was never disrupted in any organism [26].

To analyse the hypothesis that trophozoites expressing the entire repertoire of VSPs on their surface are able to confer protection to subsequent infections, experiments were performed using the WB isolate of *Giardia* (because monoclonal antibodies to many different VSPs of this strain were available) and in the gerbil model of giardiasis (because it better resembles the human infections). Results showed that initial infection with cells expressing all VSPs encoded in their genome fully protected the animals to subsequent infection by *Giardia* clones expressing a unique VSP on their surface. Additionally, immunization of gerbils with membrane preparations isolated from these cells or affinity-purified VSPs using a monoclonal antibody targeting the 5-amino-acid cytoplasmic tail conserved in all VSPs also conferred complete protection to secondary infections. Additional results showed that serum and intestinal content obtained from infected or VSP-immunized animals were able to agglutinate trophozoites of any clone. These results showed, for the first time in any parasite: (i) direct experimental evidence that antigenic surface variation is an essential mechanism responsible for evasion of the host immune response; (ii) that variable surface antigens are crucial for the establishment of the infection in a host; and (iii) immunization with the whole repertoire of variable surface antigens protect the host against secondary infections by generating a strong mucosal and systemic immune response [26]. These last results pave the way to generate vaccines not only to *Giardia*, but also to other parasites that also undergo antigenic variation.

Conclusions

Giardia is an excellent system to study the molecular mechanism of parasite adaptation and differentiation due to its simplicity [2], its minimalistic genome [4] and the capability to reproduce its entire life cycle in the laboratory, including antigenic variation (Figure 7). During recent years, an extensive amount of information has been generated regarding the biology of this parasite. This has contributed to the development of novel and more efficient diagnostic tools and a potential vaccine. Nevertheless, we are far from fully understanding many fundamental biological processes that this parasite uses to survive within and outside its hosts. The development of innovative tools and approaches will not only contribute to deciphering the particular molecular pathways of this fascinating cell, but also provide new insights into related pathogenic organisms that show similar behaviour.

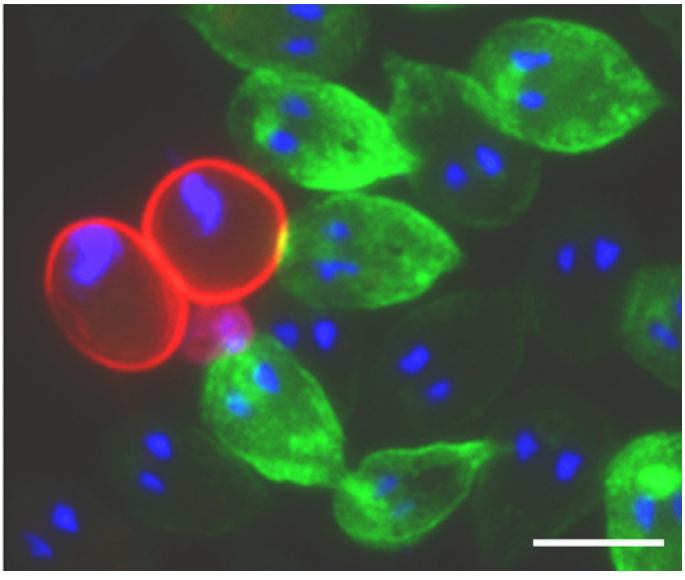

Figure 7. Encystation and antigenic variation as seen in a culture of *Giardia lamblia*
A population of *Giardia* cells in culture showing simultaneously the process of antigenic variation [some cells are labelled with a monoclonal antibody specific to VSP9B10 (green) whereas others are not; nuclei are in blue] and the process of encystation, as detected by the formation of round or oval forms labelled with a monoclonal antibody specific to CWP1 (red). Scale bar, 10 μm.

Summary

- Giardia *has a simple life cycle that can be use to study the mechanisms of cellular adaptation and differentiation.*
- *To survive outside its hosts,* Giardia *trophozoites differentiate into cysts.*
- *During encystation, synthesis, transport and extracellular deposition of a cyst wall occurs.*
- *To survive within its hosts,* Giardia *undergoes antigenic variation.*
- *Antigenic variation in* Giardia *is regulated by a mechanism similar to RNAi.*
- *Disruption of the RNAi pathway has been demonstrated* in vivo *to be essential for a protective immune response.*

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