

Antigenic variation in the intestinal parasite *Giardia lamblia*

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Giardia lamblia trophozoites undergo antigenic variation, where one member of the Variant-specific Surface Protein (VSP) family is expressed on the surface of proliferating trophozoites and periodically replaced by another one. Two main questions have challenged researchers since antigenic switching was discovered in *Giardia*: What are the mechanisms involved? How are they influenced by other cellular processes or by the environment? Two molecular mechanisms have been proposed, both involving small non-coding RNAs. Here we postulate that (a) chromatin remodeling, triggered by environmental factors, also plays an important role in selecting the VSP that will be expressed and (b) the particular VSP structure may not only protect the parasite in the small intestine but also signal the need to exchange the existing VSP for another.

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Introduction

Giardia lamblia is a non-invasive protozoan parasite that inhabits the lumen of the upper small intestine of humans and many other vertebrates [1]. This protozoan belongs to the earliest diverging branch of the eukaryotic line of descent [2], and has a small and compact genome of ~12 Mb containing nearly 9000 open reading frames (ORFs) [3]. Similar to other protozoa, *Giardia* lacks canonical transcription factors (TFs) and other gene regulatory elements [4]. The regulation of gene expression during

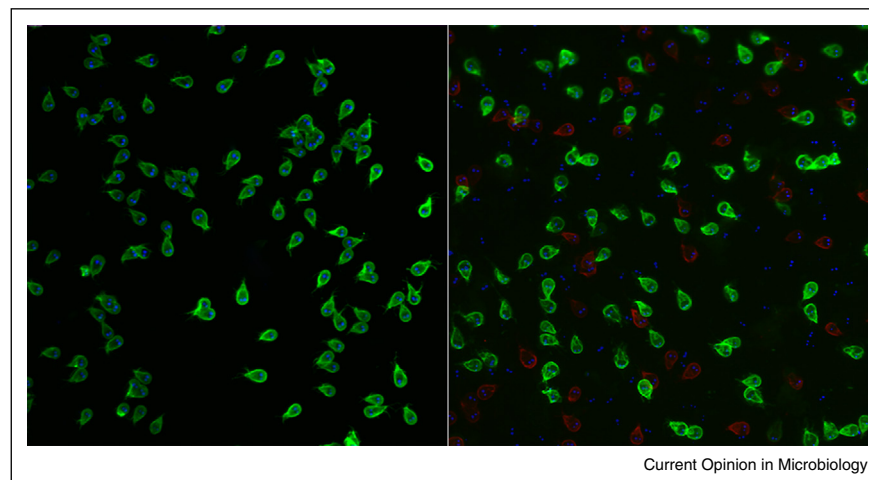
trophozoite differentiation (encystation/excystation) and the continuous switching of its surface antigens (antigenic variation) remain inadequately known or controversial.

Antigenic variation in unicellular microorganisms involves three essential features: (1) a large family of homologous genes encoding immunodominant surface antigens; (2) a mechanism allowing the expression of only one member of such family in individual cells; and (3) a molecular system for reversible expression, determining the switching in expression of one antigen by another [5]. In *Giardia*, a repertoire of ~200 VSP genes (*vsps*) is present in the parasite genome (feature 1). Here, we describe the proposed mechanisms of antigenic variation in *Giardia* (only related to feature 2) and attempt to establish a link between some potential stimuli and epigenetic factors likely influencing antigenic switching (feature 3).

Mechanisms for antigenic switching

The mechanisms proposed to explain antigenic variation in *Giardia* are based on the premise that this process must involve the expression of only one VSP on the surface of individual cells, selected from hundreds of *vsp* genes (Figure 1, left panel). The influence of genomic reorganization during this process has been discarded. The *vsps* are not located near the telomeres nor do they undergo gene movements during expression [6,7]. As in other *Giardia* genes, *vsps* have no introns, their 5' upstream regions are relatively short, without any obvious sequence or structural conservation, and the 3' untranslated region (3'-UTR) of their transcripts varies from 0 to 30 nt [1,8], with almost no possibility for regulation at this level. There is also discrepancy about the exact number of *vsps* present in the *Giardia* genome. Adam *et al.* [9] assigned 303, Morrison *et al.* [2] predicted and annotated 219 potential *vsp* genes, and Li *et al.* [10] identified only 73 putative *vsps*. This disagreement is attributed to different features regarded as necessary to define a VSP. For most researchers, VSPs are type 1 membrane proteins with an N-terminal, cysteine-rich extracellular region (with multiple CXXC motifs) and a conserved C-terminal domain comprising a unique transmembrane region and a short cytoplasmic tail of only five amino acids (CRGKA) [11]. In addition, Li *et al.* recently proposed a novel criterion for defining a VSP [10], which decreases the number of *vsp* genes to just 73 in the WB isolate. These authors suggested that the C-terminal 120-amino acid region of VSPs

Figure 1



Antigenic variation in *Giardia lamblia*. Left panel: A fresh culture of *Giardia* WB trophozoites expressing the same VSP on the surface after selection by limiting dilution. Right panel: The same culture, after several days of proliferation *in vitro*, shows the 'spontaneous' switching in VSP expression. Only some trophozoites were labeled with two specific anti-VSP monoclonal antibodies; they are surrounded by several cells expressing undetermined VSPs, with only the nuclei being stained with DAPI (blue). VSP expression was detected by immunofluorescence assays using two specific mAbs against VSP-9B10 (green) and VSP-1267 (red).

can be divided into motif 1 (45 amino acids containing two CXXC motifs separated by 12–15 amino acids located near the plasma membrane) and motif 2 (the transmembrane region plus the 5-amino cytoplasmic acid tail), and concluded that the presence of both motives is necessary for VSP membrane surface localization. In our opinion, however, these authors' interpretations were incorrect because surface localization of different episomally-expressed VSPs (with or without motifs 1 and 2) contained Myc tags at the intracellular C-terminus of the proteins was analyzed by using non-permeabilized cells to localize these VSP variants. Therefore, although the number of *vsp* genes may vary among different strains and even species of *Giardia*, we consider it is close to 200. Although this issue may appear irrelevant at this point, the number of *vsp* genes plays an important role when considering the proposed mechanisms for controlling antigenic variation in this parasite.

Since *Giardia* is a binucleate organism, with both nuclei being equally active and functional, any molecular mechanism controlling antigenic variation must coordinate *vsp* expression between both nuclei of the parasite. In this scenario, a post-transcriptional control of gene expression in *Giardia* appears to be advantageous. Over the last few years, two cytoplasmic mechanisms involving small RNAs (sRNAs) have been described [12[•],13]. Small RNAs make up a family of regulatory non-coding RNAs of 19–28 nt in length, which are derived from double-stranded RNAs (dsRNAs) through processing mediated by RNase III type enzymes [14]. Two major classes of sRNAs are involved in RNA silencing: microRNAs (miRNAs) and small interfering RNAs (siRNAs). Both

were described as regulating important biological processes in eukaryotes via post-transcriptional gene silencing (PTGS).

miRNA-mediated translational repression model: Over the last eight years, different research groups have been focused on detecting miRNAs through analysis of homology searching, *in silico* prediction or deep sequencing of *Giardia* trophozoites [12[•],15,16]; some authors have proposed miRNA participation in repressing VSP expression at the translational level [17]. To date, 166 putative miRNAs have been identified, ranging between 24 and 28 nt in length, with a peak at 26 nt [12[•]]. Most of these putative miRNAs were obtained via the analysis of next-generation sequencing libraries from *Giardia* Argonaute (GIAGO)-associated small RNAs.

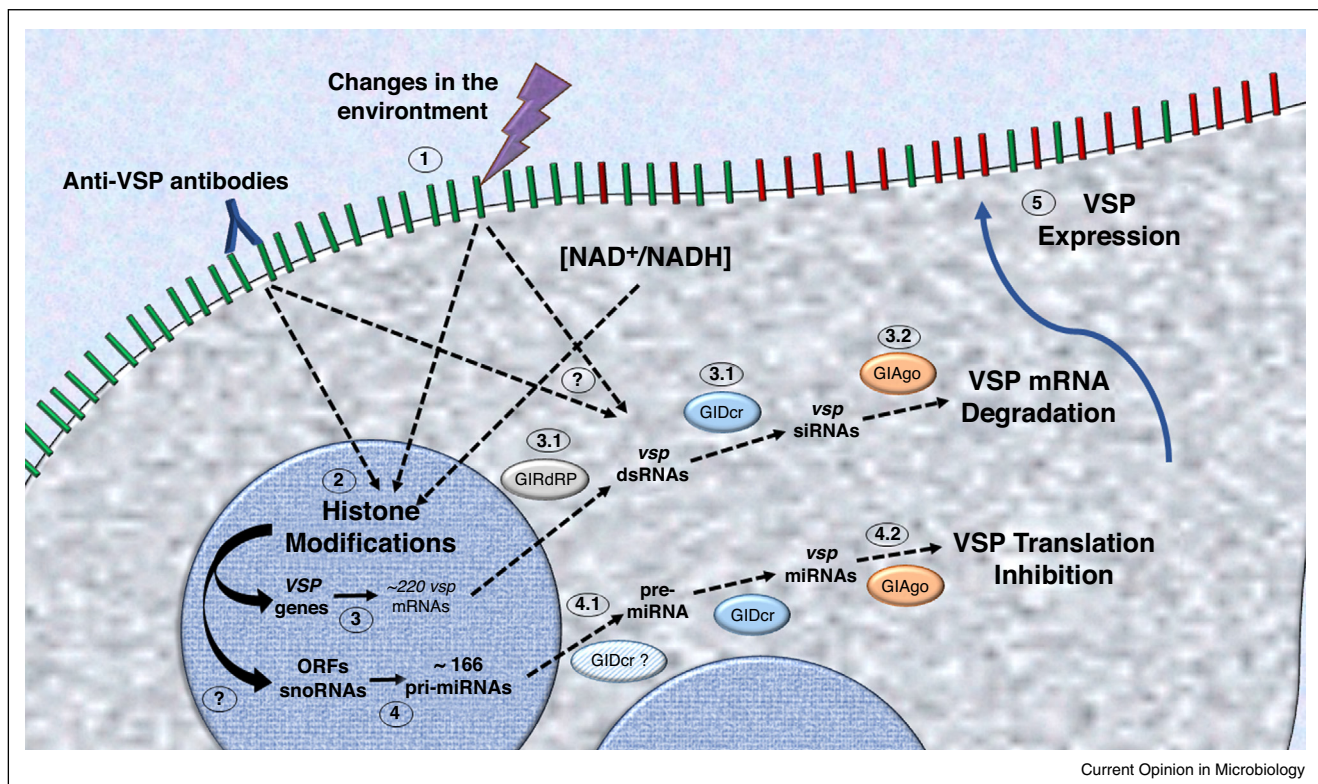
Of the only 105 putative miRNAs described in 2014 by Wang and coworkers, 55.2% are derived from ORFs, 40% from the other genomic regions, and only 4.8% from small nucleolar RNAs (snoRNAs) [12[•]]. A comparison of these miRNAs from different *Giardia* isolates showed that only 4 are conserved between isolates WB and GS, 35 are conserved between isolates WB and P15, 25 are conserved among all 3 isolates, and 41 are specific to the WB isolate. Wang and coworkers studied the action of putative miRNAs over four episomally-expressed tagged VSPs, each one having different number and position of potential miRNA target sites (two overlapping target sites, two separated target sites and multiple overlapping and separated target sites), all of them within the ORF region. The authors were unable to locate any potential miRNA target site within the *vsp* 3'UTR regions, which is

common in higher eukaryotes transcripts [18]. Their findings suggest that multiple miRNAs could be distributed covering the entire *vsp* ORF, and that when they do not overlap to each other their repression is enhanced. In addition, functional differences were detected when a particular miRNA was located at different target positions of a *vsp* mRNA, suggesting differences in mRNA secondary structure and miRNAs binding thermodynamics [12*]. Based on these results, Wang's group proposed a mechanism of miRNA-mediated translational repression of the entire coding region of the VSP mRNAs targeted by multiple miRNAs. Interestingly, since Drosha/Pasha and Exportin 5 are absent in *Giardia*, the authors suggested that a primary miRNA must be exported to the cytoplasm by the Exportin CRM1 complex and processed

by the *Giardia* endonuclease Dicer (GIDcr) to generate pre-miRNAs, and then digested again by GIDcr to produce the double-stranded miRNA [19] (Figure 2), which is a highly uncommon way of processing.

The model based on the action of miRNAs on antigenic variation is highly influenced by the number of *vsp* genes considered. When the 220 annotated *vsp* genes were considered, 5 miRNAs analyzed targeted 178 of them [16], whereas in a later estimation of only 73 *vsp* genes in *Giardia* WB isolate, all of them carried multiple miRNA target sites [12*]. It is unlikely that only a fraction of the entire repertoire of *vsp* genes is controlled by this proposed mechanism. Furthermore, the mechanism implies a redundancy of miRNA target sites, where almost all the described putative

Figure 2



Molecular mechanisms controlling antigenic variation in *Giardia*. The mechanisms proposed for regulating antigenic switching in *Giardia* include siRNAs-mediated RNAi silencing of all but one VSP mRNAs, miRNA translation inhibition, and epigenetic factors (histone tail modification at a particular *vsp* locus). (1) External factors (anti-VSP antibodies or environmental changes) or the nutritional state of the cell ([NAD⁺/NADH]) can trigger different signals. Conformational changes in the expressed surface antigen (green), due to either antibody binding or rearrangement of the CXXC motifs, could start a still undetermined signal transduction cascade that modifies the transcription rate of different VSP genes by epigenetic factors or by activating the PTGS pathway. (2) Chromatin remodeling could play a critical role in regulating *vsp* transcription. A variation of histone acetylation state in the immediate upstream region of the expressed VSPs is regulated by histone deacetylases, whose activity can be modified by the redox state of the cell. (3) Most, if not all, *vsps* are simultaneously transcribed, although with different efficiency, and transported to the cytoplasm. (3.1) GIRDp detects multiple homologous VSP mRNAs and generates antisense RNAs targeting all but one VSP transcripts. VSP dsRNAs are processed into siRNAs by GIDicer. Silencing of GIRDp or GIDicer leads to a change from a single to multiple VSP expression on the trophozoite surface. (3.2) Silencing of GIAGO did not produce viable cells. (4) miRNAs of different origin (ORFs, snoRNAs) may also be involved. (4.1) In the absence of Drosha, the pri-mRNAs would be processed in the cytoplasm by GIDicer. How the mRNAs reach the cytoplasm in the absence of Exportin 5 is unknown. (4.2) Different miRNAs have target sites in the same *vsp* mRNA, which could lead to inhibition of translation. (5) Only one transcript is translated into the VSP that is expressed on the surface of the trophozoites (red), bypassing the described silencing pathway (blue arrow).

miRNAs target these *vsp* genes at multiple sites. If that is true, it is speculative to imagine that a similar mechanism occurs with all other *Giardia* genes potentially regulated by miRNAs. Conversely, deep sequencing sRNAs transcriptome of four different stages of *Giardia* differentiation [20^{*}] reflected that most sRNAs are endo-siRNAs, and that of the 166 miRNAs reported so far by different laboratories, only 5 were found to be produced from known ncRNAs. These authors concluded that although canonical miRNAs can be encoded in the *Giardia* genome, their abundance is too low to play any important function [20^{*}]. Then, if miRNAs are involved in the control of antigenic variation, how is their expression regulated? Many aspects of this model remain highly speculative, although easily testable.

RNA interference (RNAi) model: Another post-transcriptional mechanism controlling VSP expression involving small RNAs but of different origin (siRNAs) was also described [13]. The proposed RNA interference (RNAi) model involves the action of a cytoplasmic *Giardia* RNA-dependent RNA polymerase (GIRdRP), which generates antisense RNAs complementary to VSP mRNAs. It was demonstrated that most of the *vsp*s are simultaneously transcribed and that the produced *vsp* dsRNAs trigger the action of an endonuclease complex that includes GIDcr and GIago. This complex processes these molecules into short ~26-nt-long dsRNAs in an ATP-dependent fashion, a typical feature of RNAi-silencing systems [13]. This model includes the cytoplasmic recognition by GIRdRP of the highly homologous VSP mRNA by scanning the entire *vsp* transcriptome and acting as a concentration-dependent sensor of aberrant mRNAs [13]. Therefore, the differences in the level of individual transcripts might serve as the exclusion factor that allows the *vsp* mRNA with the highest concentration to evade the action of GIRdRP, avoiding degradation by the silencing machinery. The direct involvement of GIRdRP and GIDcr was demonstrated via experiments in which these enzymes were knocked down, leading to a change from expression of a single VSP to expression of multiple VSPs in individual trophozoites [13] (Figure 2). *Giardia* was the first parasite in which the mechanism of antigenic variation was disrupted, and these altered cells expressing the entire repertoire of variable surface antigens were crucial to confirm that antigenic variation in parasites is essential for evasion of the host immune response. Immunization with the whole repertoire of expressed VSPs was necessary and sufficient to generate a strong, long-lasting protection against subsequent infections [21].

Despite the discrepancies between the two proposed mechanisms, it is clear that control of antigenic variation in *Giardia* occurs post-transcriptionally and that disruption of the RNAi machinery allows the expression of the entire repertoire of VSPs on the parasite surface. How do wild type parasites select the VSP that will be expressed on the parasite surface? How does the exchange of a VSP

for another occur? These questions remain unresolved but, hopefully, recent research findings may help to elucidate the molecular basis of this process.

Completing the puzzle

Antigenic variation in *Giardia* is often reported to occur ‘spontaneously’ *in vitro* [22] (Figure 1, right panel). This observation could reflect an ancient, evolutionarily-acquired mechanism whereby an internal clock would trigger switching of a particular VSP. Nevertheless, a closer look at early neglected publications as well as recent results from our group raises many questions regarding generally accepted concepts. For example, studies conducted in the 1980s and 1990s showed that: (i) VSP switching rates differ significantly among different isolates [23,24]; (ii) the switching rate of the same clone expressing a particular VSP varies remarkably when compared during culture proliferation or during experimental infections [25,26]; (iii) antigenic variation starts very early during animal infections (since day 4 in mice and gerbils) [26,27], indicating that *in vivo* antigenic variation in *G. lamblia* would be mediated by an antibody-independent mechanism, which is able to induce a positive or negative VSP selection, depending on the particular host and VSP; (iv) clones expressing a particular VSP are capable of infecting certain hosts better than others [28], suggesting that some VSPs are more suited to the conditions of the upper small intestine of different hosts.

In light of these observations, we have endeavored to reproduce and complement these experiments in recent years and to analyze them in the context of the proposed mechanisms of antigenic variation. Our results reveal that: (i) the VSP switching rate in trophozoites varies with the time in culture, suggesting that the nutritional status of the cell might have an influence on antigenic variation (Rios *et al.*, unpublished); (ii) at a given point in time, the switching rate varies during experimental infection in different animals, being faster in small animals (gerbils) than in larger ones (dog and cats), which suggests that environmental conditions may favor or limit VSP switching (Serradell *et al.*, unpublished); (iii) remarkably, we found that high concentrations of antibodies against VSPs agglutinate the trophozoites without cytotoxic effects, but low antibody concentrations trigger immediate VSP switching (Tenaglia *et al.*, unpublished). In addition to these features, which are related to the causes of VSP switching (antibody-mediated or environmentally triggered), it is clear that external factors are playing a role in the selection of the particular VSP that will be translated and expressed on the parasite surface. Therefore, spontaneous switching may not occur.

In other parasites that undergo antigenic variation, chromatin remodeling plays a critical role in regulating surface antigen expression [29^{*},30^{*}]. *Giardia* has two copies of histones H2A, H2B, and H3, and three of H4, but no

putative homologue of the linker histone H1 has ever been found [31,32], which could be consistent with the hypothesis that H1 was recruited during eukaryotic evolution after the acquisition of the core histones to further refine the chromatin structure [33].

The lack of allele-specific expression of *vsp* genes and the absence of DNA rearrangements associated with antigenic variation suggest the presence of an epigenetic form of control for VSP gene expression in *Giardia* [1,6,34]. This was initially suggested in 2006 [35], when Nash's team linked antigenic variation with histone acetylation in the immediate upstream sequences of the expressed VSPs. An epigenetic mechanism during parasite differentiation into cysts was also proposed by Hehl's group [36]. Nonetheless, they found only ~1% of the VSP family modulated by a general histone hyperacetylation process using a histone deacetylase (HDAC) inhibitor. This group described the presence of several histone modifying enzymes in *Giardia*: 5 histone acetylases, 6 histone deacetylases and 6 histone methylases, whereas demethylases seem to be absent [36].

Our group also found evidence that histone deacetylases are involved in the regulation of antigenic variation, since the presence of HDAC inhibitors increases the switching rate of a VSP clonal population (Gargantini *et al.*, unpublished). Accordingly, the crosstalk between histone modifications and the RNAi pathway has been clearly documented [37,38^{*}]. In particular, epigenetic transcriptional repression is not only due to heterochromatin formation, but also to the fact that RNAi of nascent transcripts can guide epigenetic silencing and associated histone modifications [38^{*}]. This confirms our previous hypothesis that one VSP transcript evades degradation by RNAi possibly due to differences in VSP transcription efficiency [13]. Remarkably, a major role for NAD⁺-dependent deacetylases of the Sirtuin family was determined. Since these enzymes are dependent on the nutritional state of the cells, they seem to be a potential link between the environment and the transcription rate of different *vsp* genes.

Are the CXXC motifs in VSPs involved in antigenic switching?

Giardia is protected by a tight coat comprising the VSPs, which cover the entire surface of the parasite, with the extracellular cysteine-rich N-terminal region playing an important role in the interplay between host and environment. This abundance of cysteine residues, besides promoting resistance to intestinal proteases and bile salts, may be involved in the detection and/or transduction of external switching signals.

Interestingly, multiple CXXC motifs in surface proteins are considered key elements against redox variations, acting as a rheostat that prevents protein destruction

[39], and/or triggering signal transduction pathways under detrimental oxygen concentrations in the environment [40]. In this context, the SH groups exposed on the outer surface may protect trophozoites from oxygen or free radicals, and be also involved in the interaction with host intestinal epithelial cells. Recently, in a proteomic study performed on *Giardia*, Emery *et al.* [41] described a significant increase in the expression of several VSPs during the interaction of trophozoites with enterocytes and soluble factors released by these host cells. The up-regulated VSPs constituted over a third of all the up-regulated proteins, suggesting that these changes in VSP expression likely represent selection of favorable variants for host pathogenesis or virulence [41].

Moreover, the conserved CXXC motif in the stalk region of the protein CD3 plays a key role in the TCR signaling. Many potential functions of these motifs have been proposed, such as their involvement in CD3 dimerization and in the correct orientation of the CD3 cytoplasmic tails during aggregation [40].

Thus, conformational changes in the expressed surface antigen simply due to either antibody binding or rearrangement of the CXXC motifs in response to changes in the environment might start a still undetermined signal transduction cascade that modifies the transcription rate of different VSP genes before control of the RNAi pathway.

Conclusions

Over the last decades, several groups have made great efforts to elucidate the mechanism controlling antigenic variation in *Giardia*. Since no specific VSP promoter sequences have been identified and the upstream regions of *vsps* do not have any evident homology, the two mechanisms described above proposed a post-transcriptional system for the regulation of VSP expression by the action of sRNAs of different origin (miRNA and siRNA). Both mechanisms are based on common molecular machinery, Dicer and Argonaute, which are both present and functional in *Giardia*.

Nevertheless, independently of the considered model, VSP expression is likely to be regulated by small RNAs and a crosstalk with epigenetic factors may establish a response network to changes in the environment [42]. Although important advances have been made, there is still a long way to completely elucidate the process that drives the expression of a single VSP on the surface of the trophozoites and how environmental changes are associated with the general process of parasite evasion from the host immune response.

Additional information regarding the molecular basis of how *Giardia* senses a stimulus that triggers antigenic variation, what signal transduction mechanisms produce the epigenetic changes required to fine-tune *vsp* gene

transcription, and how a particular VSP is selected during antigenic switching will provide a framework not only to better understand antigenic variation in this intestinal parasite, but also to provide new insights regarding this process in other protozoa.

Competing interests

The authors declare no competing interests.

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