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# Differentiation-associated surface antigen variation in the ancient eukaryote *Giardia lamblia*

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## Summary

**Encystation of *Giardia lamblia* is required for survival outside the host, whereas excystation initiates infection. The dormant cyst was considered an adaptation to external survival and passage through the stomach. However, we found previously that trophozoites which had recovered after completion of the life cycle had switched their major variant surface protein (VSP), called TSA 417, but neither the timing nor the molecular mechanism of switching had been elucidated. Here we demonstrate that TSA 417 predominates in cysts, but is downregulated during the stage of excystation that models cyst arrival in the small intestine. Transcripts of new VSPs appear late in encystation, and during and after excystation. Trophozoites appear to prepare for switching during encystation, when the major VSP on the cell surface diminishes and is internalized in lysosome-like vacuoles. As short-range DNA rearrangements were not detected, giardial VSP switching during differentiation appears to resemble the *in situ* switching of surface glycoproteins in African trypanosomes. We also report a unique extended 15 nucleotide polyadenylation signal in all VSP transcripts, but not in other known giardial genes. Antigenic variation during encystation–excystation may be a novel form of immune evasion that could help explain the common occurrence of reinfection by Giardia and other parasites with similar life cycles.**

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## Introduction

In response to specific physiological signals, many prokaryotic and eukaryotic microbes differentiate into dormant cystic forms that are highly resistant to environmental stresses. Favourable conditions induce emergence from the cyst. *Giardia lamblia* is an important model for study of such differentiation because this amitochondriate protozoan belongs to the earliest diverging eukaryotic lineage (Sogin *et al*., 1989; Adam, 1991). Moreover, unlike many other parasites, the entire life cycle of *G. lamblia* can be reproduced *in vitro*, in response to specific physiological stimuli characteristic of its human host (Boucher and Gillin, 1990).

In addition to its central evolutionary position, *G. lamblia* is important as a leading cause of water-borne intestinal disease world-wide (Adam, 1991). As *Giardia* is non-invasive and secretes no known toxin or virulence factor, its adaptations to survival outside the host and evasion of immune responses are central to understanding its success as a pathogen. Transmission of the parasite occurs through ingestion of cysts followed by release of the disease-causing trophozoites in the upper small intestine in response to specific gastrointestinal stimuli (Adam, 1991). Disease manifestations are highly variable, ranging from asymptomatic carriage to severe diarrhoea and malabsorption (Adam, 1991; Farthing, 1994). Infected hosts may excrete large numbers of infectious cysts, leading to very high prevalence rates (Rendtorff, 1954; Adam, 1991).

Chronic infections are common (Farthing, 1994), and may be due, in part, to reinfection of the same host. In a hyperendemic area, 98% of drug-cured children were reinfected within 6 months (Gilman *et al*., 1988). Moreover, isolates of *G. lamblia* are very heterogeneous, with both heritable differences between genetic groups and surface antigenic variation (Nash, 1994; Ey *et al*., 1996). Antigenic variation is likely to be involved in determining the clinical spectrum of giardiasis and the ability to reinfect.

The flagellated trophozoite form that colonizes the human intestinal tract is covered by a dense coat composed of a single variant-specific surface protein (VSP) (Gillin *et al*., 1990; Mowatt *et al*., 1991; Nash, 1994). VSPs, which vary in size between ≈50 and 250 kDa, are unusual, highly cysteine rich (>11%) type I integral membrane proteins (Gillin *et al*., 1990; Adam, 1991; Nash and Mowatt, 1992; Papanastasiou *et al*., 1996). The N-terminal sequence is variable, but the C-terminal 27 amino acids, including the

membrane-spanning region and cytoplasmic anchor, are highly conserved (Mowatt *et al*., 1991). The gene encoding the major VSP expressed by our clone, called TSA 417, is very widespread among giardial isolates and defines the most common genetic groups (Nash, 1994; Ey *et al*., 1996), which include human and animal isolates from at least four continents. Moreover, important biochemical characteristics first found in TSA 417 have been found in the other VSPs investigated since (Gillin *et al*., 1990; Mowatt *et al*., 1991; Aley and Gillin, 1993; Nash, 1994).

VSPs can undergo spontaneous switching *in vitro* with high frequencies (Nash *et al*., 1991), but no defined sequence or order of VSP appearance has been discerned. The predominant VSP of a population can also change in response to selection by antibodies or physiological factors (Nash and Aggarwal, 1986; Nash *et al*., 1991). Antigenic variation has been documented in experimental human (Nash *et al*., 1990b) and animal infections (Gottstein and Nash, 1991). On the other hand, the predominant VSP can remain unchanged for months (Meng *et al*., 1993) to years (T. C. Meng, F. D. Gillin, S. G. Svard and J. M. McCaffery, unpublished) in culture. Moreover, *in vivo*, trophozoites infecting *scid* mice expressed the same VSP throughout the infection (Gottstein and Nash, 1991; Nash, 1994). The giardial VSP repertoire has been estimated as 30–150 genes per haploid genome (Nash and Mowatt, 1992), but expression of more than one VSP on the surface of a cell has not been detected. The molecular basis of antigenic variation in *Giardia* is not understood.

During excystation, *G. lamblia* takes elegant advantage of specific host signals encountered in its descent through the human gastrointestinal tract (Rice and Schaefer, 1981; Boucher and Gillin, 1990). Exposure of ingested cysts to gastric acid (stage I) initiates the excystation process, although for trophozoite survival, the cyst wall must not open until the parasite enters the small intestine. Emergence of the flagellated trophozoite is stimulated by exposure to intestinal fluid proteases (stage II) (Rice and Schaeffer, 1981). The same physiological stimuli induce the excystation of cysts *in vitro* (Boucher and Gillin, 1990).

Previously, we showed that trophozoites cultivated *in vitro* after encystation and excystation express different VSPs from the initial trophozoites (Meng *et al*., 1993). In this study, we questioned when in the life cycle this antigenic variation occurred and by what mechanism(s). We found that antigenic variation occurred at the transcript level, predominantly during stage II, which mimics the arrival of the cyst in the human small intestine. Expression of TSA 417, the initially predominant VSP transcript was downregulated and several new VSP transcripts were expressed. In contrast, TSA 417 protein had begun to disappear from the plasma membrane late in encystation and instead was found in lysosome-like vesicles, suggesting an endocytic pathway. Differences at the mRNA level suggest a mechanism based on regulation at the level of transcription and/or mRNA stability, similar to the *in situ* type of antigenic switching in African trypanosomes (Barry *et al*., 1990; Van der Ploeg *et al*., 1992; Borst and Rudenko, 1994; Horn and Cross, 1997). These studies implicate giardial differentiation in immune evasion and may help explain the common occurrence of repeated *G. lamblia* infections.

## Results

### *TSA 417 disappears from the plasma membrane during differentiation and localizes to the lysosomal compartment*

Completion of the life cycle of *G. lamblia* isolate WB clone C6 *in vitro* led to antigenic switching (Meng *et al*., 1993) from initial populations that expressed TSA 417 as the major VSP for >1 year (>85% TSA 417-positive, Fig. 1A) to populations that express different VSPs (Fig. 1B). The few TSA 417-positive cells in Fig. 1(B) are probably cross-reactive variants or TSA 417-negative cells that have 're-expressed' this epitope because of the frequency of switching (Nash and Aggarwal, 1986; Nash *et al*., 1990a; 1991; Meng *et al*., 1993; Nash, 1994). Excystation with normal human duodenal fluid instead of pure trypsin

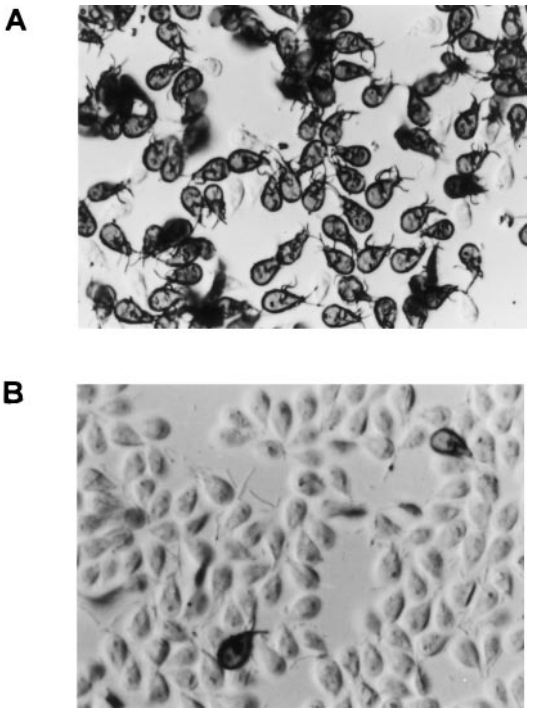


**Fig. 1.** Prevalence of the TSA417 epitope on intact *G. lamblia* trophozoites before encystation (A) and 48 h after (B) excystation. Glutaraldehyde-fixed, non-permeabilized *G. lamblia* WB clone C6 trophozoites were examined by immunocytochemistry using polyclonal antirecombinant TSA 417 antiserum and protein A–horseradish peroxidase. Magnification 600×.

after acid activation of cysts (Boucher and Gillin, 1990) gave identical results (data not shown). Moreover, excystation of cysts isolated from infected suckling mice also led to switching (Meng *et al*., 1993), showing that this was not an artefact of *in vitro* differentiation.

As VSPs can be released from trophozoites (Nash and Keister, 1985; Papanastasiou *et al*., 1996), it was important to determine the fate of TSA 417 protein during antigenic switching. Immunoblots with anti-rTSA 417 showed that large proportions of the TSA 417 antigen remained associated with the cells during and after excystation, suggesting that endocytosis may play a significant role in VSP switching at the protein level (Fig. 2A). Intact TSA 417 has a minor ≈85 kDa species that is a precursor of the major ≈66 kDa protein on the cell surface (Aley and Gillin, 1993). TSA 417 has a single site that is hypersensitive to proteolysis, yielding fragments that migrate at ≈45 and 30 kDa and are bound together and held to the cell by disulphide bonding in the native protein (Aley and Gillin, 1993). Interestingly, cysts contained much more of these proteolytic products than trophozoites and less of the 85 kDa precursor. In addition, both intact 66 and 85 kDa species largely disappeared after stage II of excystation,

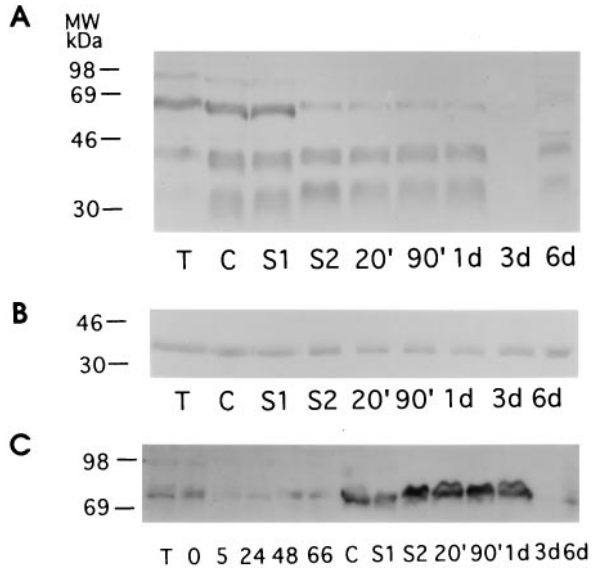


**Fig. 2.** Changes in VSP protein expression during excystation.
A. Western blot analysis of TSA 417 during excystation. Total protein from trophozoites (T), cysts (C), stage I (S1) and stage II (S2) treated cysts, 20 min (20′), 90 min (90′), 1 day (1d), 3 days (3d) and 6 days (6d) after excystation was separated by SDS–PAGE, transferred to nitrocellulose membrane and probed with anti-rTSA417 polyclonal antiserum.
B. Western blot analysis of the *G. lamblia* lectin Taglin control. Blots were prepared according to (A) and probed with anti-Taglin antibody (Ward *et al*., 1987).
C. Western blot analysis of VSP-rB2. Blot of total protein from the complete *G. lamblia* life cycle (trophozoites (T), 0, 5, 24, 48 and 66 h encysting cells, cysts and excysting cells as above) probed with antiserum against VSP-rB2, a VSP expressed by a variant clone of a sheep-derived *Giardia* isolate (Bruderer *et al*., 1993).

which models passage into the small intestine. The initial proteolytic fragments persisted within the cells for at least 24 h after excystation, but were not detected 3 days after excystation (Fig. 2A). This could be due to dilution by growth of TSA 417-negative cells, or to degradation or release into the medium. After 3 days, polyclonal anti-TSA 417 did not detect any VSP species, but after 6 days, several cross-reactive species of various molecular weights were observed. This suggests the continuing expansion of the VSP pool, rather than resetting of a specific sequence of variants after excystation. In contrast, the relatively constant levels of the giardial plasma membrane lectin, Taglin (Ward *et al*., 1987) (Fig. 2B), verified that the disappearance of TSA 417 was not due to general proteolysis by the trypsin in stage II of excystation.

Immunoelectron microscopy was used to determine the subcellular localization of TSA 417. In contrast to the abundant and even staining in >80% of the initial populations of trophozoites and early encysting cells (Fig. 3A) (Gillin *et al*., 1990; Meng *et al*., 1993; McCaffery *et al*., 1994), only small patches of TSA 417 remained on the outer leaflet of the plasma membrane beneath the wall of cysts (Fig. 3B) and of some trophozoites 20 min after emergence from the cyst (Fig. 3C).

In contrast, larger amounts of TSA 417 were localized to the lysosome-like peripheral vacuoles during and after excystation, suggesting an endocytic pathway (Fig. 3D). Importantly, this was also observed late in encystation and in cysts (Fig. 3B) (McCaffery *et al*., 1994), which suggests that this is the stage when the previously predominant VSP begins to be removed from the cell surface. The internal location of TSA 417, sequestering it from trypsin or duodenal fluid protease, supports the idea that the switch was not caused by proteolysis.

This agreed with the finding of faint or patchy reactivity in quantitative studies using immunofluorescence on the surface of ≈51% of live cells 1 h after excystation (data not shown). TSA 417 detectable on the surface of live cells decreased to ≈30.1% 2 h and 12.6% at 24 h after excystation. This epitope was present on fewer than 5% of the cells by 48 h (Fig. 1B). These low levels of TSA 417 re-expression were stable for several months after excystation (Meng *et al*., 1993).

### *New VSP transcripts are induced when TSA 417 is downregulated*

Previously, we showed approximately constant levels of the TSA 417 transcript during encystation by Northern analysis (Aley and Gillin, 1993). To determine whether there are changes during excystation, we probed Northern blots with a TSA 417-specific oligonucleotide. The TSA 417 transcript is present in 48 h encysting cells and in cysts treated with stage I solution, which mimics conditions in

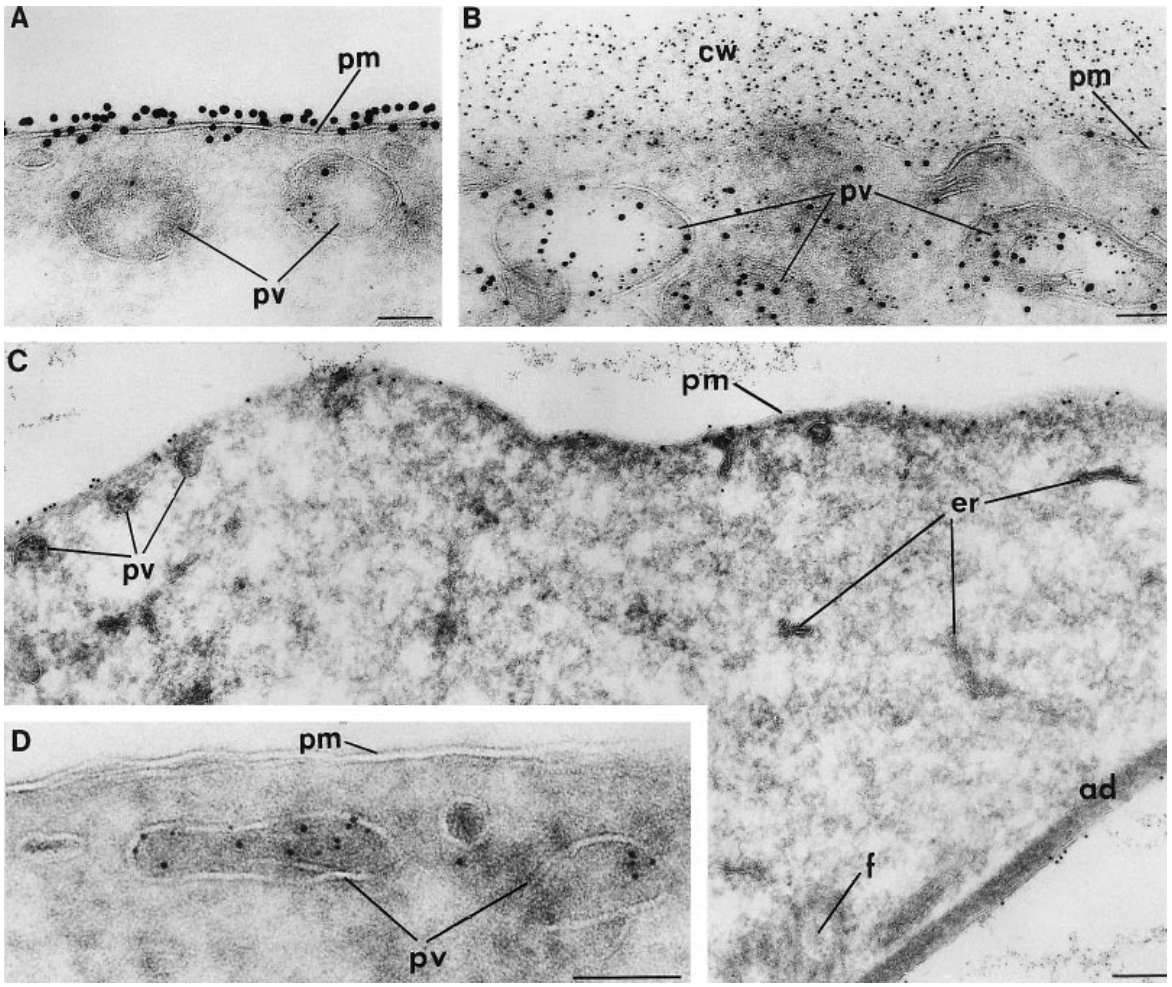


**Fig. 3.** Ultrastructural localization of TSA 417 during the *Giardia* life cycle. Bar 0.1 μm; 10 nm gold, rabbit anti-rTSA 417; 5 nm gold, mAb 8C5; anti-cyst wall protein; pm, plasma membrane; cw, cyst wall; pv, peripheral vacuole (lysosomal compartment); er, endoplasmic reticulum; f, flagella and ad, adhesive disc.
A. Ultrathin cryosection of 15 h encysting cells showing large amounts of TSA 417 mainly on the surface of the plasma membrane.
B. Cyst from 48 h encysting culture with large amounts of TSA 417, mainly in the lysosome-like peripheral vacuoles (PV) and little on the plasma membrane beneath the cyst wall.
C. Newly excysted trophozoite, 20 min after excystation with reduced amounts of TSA 417 on the surface and larger amounts in PVs.
D. Enlargement of field in (C) demonstrating TSA 417 in PVs.

the stomach (Fig. 4). However, the TSA 417 transcript was greatly decreased 90 min after excystation, and remained at very low levels for 6 days after excystation, compared with the constitutively expressed gene PDI-1 (Fig. 4). Thus, TSA 417 is downregulated at the RNA level after exposure of cysts to conditions that mimic human intestinal fluid.

To address the question of whether there was a stage when no VSP transcripts were present, and to analyse overall VSP expression during excystation, we used a probe directed against the conserved membrane-spanning region of VSPs (Mowatt *et al.*, 1991). The major transcript in trophozoites, 48 h encysting cells and after treatment of cysts with stage I solution was of the same size as the TSA 417 transcript. This indicates that TSA 417 is the major expressed VSP in these stages (Fig. 4). Additional bands were seen on longer exposures (data not shown). The $<20\%$ of TSA 417-negative parasites probably comprise a heterogeneous population expressing a number of VSPs. However, the transcript pattern changed completely by 90 min after excystation, and continued to change during 6 days of growth (Fig. 4). Our hypothesis is that the period after excystation continues to be a time of VSP heterogeneity because of selection for certain VSPs *in vitro. In vivo*, this may allow trophozoites to colonize diverse intestinal niches.

Analysis of VSP expression throughout encystation and excystation using RT-PCR with a degenerate oligonucleotide directed against the conserved membrane-spanning region (Fig. 5) showed that VSP transcripts are always present. To analyse the composition of VSP transcripts

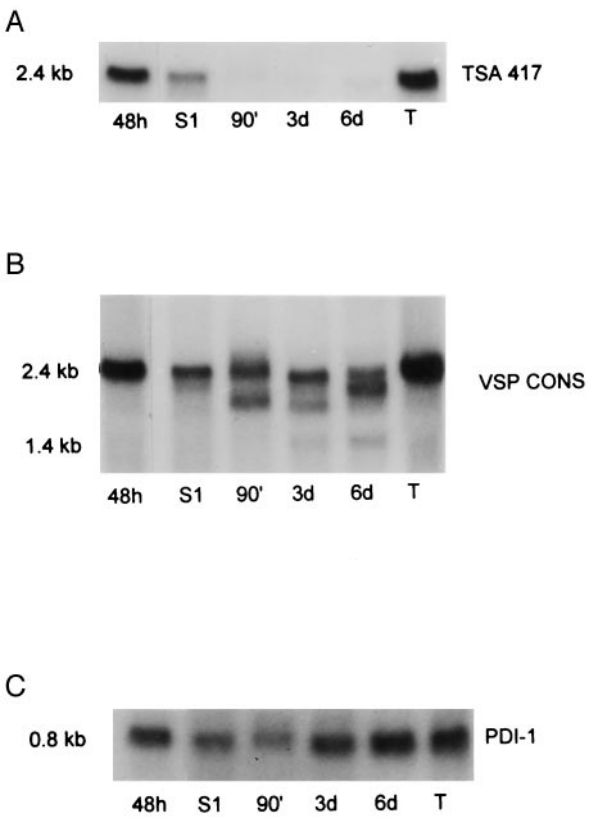


**Fig. 4.** Northern analysis of VSP expression during excystation.
A. A 10 μg sample of total RNA extracted from trophozoites (T), 48 h encysting cells (48 h), stage I-treated cysts (S1), trophozoites 90 min, 3 days and 6 days after excystation separated on an agarose–formaldehyde gel, blotted onto nylon membrane and probed with a specific oligonucleotide directed against TSA 417.
B. Northern blot from (A) probed with an oligonucleotide directed against the conserved membrane-spanning region of VSPs.
C. Northern blot from (A) probed with a random primer labelled PCR fragment from the constitutively expressed gene PDI-1.

in the cell during and after stage II, we cloned and sequenced VSP RT-PCR products from Fig. 5. Eight of 10 clones from trophozoites and encysting cells (not shown), as well as cysts, corresponded to the TSA 417 3′ end (Fig. 6). In contrast, after stage II, only 1 of 10 clones corresponded to TSA 417 and another VSP (VSP-1EX) became predominant in the VSP pool. The VSP pool continued to change for at least 5 days after excystation (Fig. 6). This is in agreement with the results of the Northern analysis in Fig. 4, and it shows the dynamic nature of the antigenic variation during and after excystation. Similar switching was observed in a smaller sampling of another clone called 1F. Before excystation, three clones of five expressed the 1F VSP, whereas none of five expressed 1F after excystation (data not shown).

### *Some VSPs appeared transiently in excystation*

To address the question of when new VSP transcripts are induced, we made a specific probe against one of the 3′ end clones detected in the post stage II transcript pool (VSP-4EX, Fig. 6). Figure 5 shows that expression of VSP-4EX was first evident in cysts, although a weak signal was detected at 48 h of encystation after overexposure of the gel (data not shown). Expression of this VSP was greatly increased after stage II, the same time that TSA 417 expression decreases. Expression of VSP-4EX was transient, as no expression was detected 3 days post-excystation. In contrast, the gene encoding the ER protein BiP (Soltys *et al*., 1996) was expressed fairly constantly throughout the whole life cycle (Fig. 5).

Metabolic labelling with [$^{35}$S]-cysteine, which mainly labels VSPs, because of their high cysteine content, showed that several new VSPs are expressed after excystation (Meng *et al*., 1993). To study the protein expression of a specific VSP other than TSA 417 during the *G. lamblia* life cycle, we analysed the expression of VSP-rB2 (Bruderer *et al*., 1993), which is expressed by a variant clone of a sheep-derived *G. lamblia* isolate (O2-4A1-rB2). Polyclonal antiserum against this isolate cross-reacted with members of known *Giardia* genetic groups (Ey *et al*., 1996), yielding a characteristic 73–75 kDa double band. Figure 2C shows that our isolate reacts with anti-rB2, and that the expression is low in trophozoites and throughout encystation. Expression of rB2 was high in cysts and remained high for 24 h after excystation. Interestingly, it decreased 3 days after excystation, like the TSA 417 protein and the VSP-4EX transcript. This shows that new VSPs are induced when TSA 417 expression is down-regulated, and that the time during and after excystation is a period of active antigenic flux. Switching of the rB2 epitope could not be measured at the molecular level because it has not been cloned (P. Köhler, personal communication).

### *Antigenic variation without detectable short range DNA rearrangements*

Antigenic variation in microorganisms is often dependent on DNA rearrangements (Van der Ploeg *et al*., 1992; Borst and Rudenko, 1994; Horn and Cross, 1997). Expression of a new VSP starts late in encystation, and we investigated whether this was due to detectable DNA rearrangements. DNA extracted from trophozoites and cysts was digested with four restriction enzymes, and a Southern blot was analysed with a probe against the 3′ end of the induced VSP-4EX gene. No changes were detected in the restriction pattern (0.5–10 kb) around this gene (data not shown). Probing the same Southern blot with the conserved VSP and TSA 417-specific probes used in Northern blots also failed to detect changes in the restriction pattern (data not shown). Earlier studies of antigenic variation in *G. lamblia* have also failed to detect

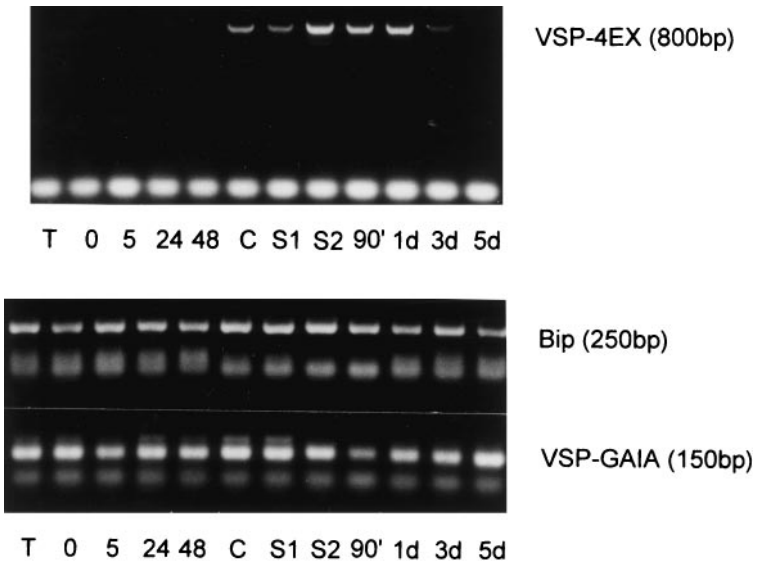


**Fig. 5.** RT-PCR analysis of VSP expression during the complete *G. lamblia* life cycle. 3′ RACE RT-PCR was performed with cDNA generated with a poly-T-oligonucleotide using 5 μg of total RNA extracted from trophozoites (T), 0, 5, 24 and 48 h encysting cells, cysts, stage I-treated cysts (S1), stage II-treated cysts (S2), trophozoites 90 min (90′), 1 day (1d), 3 days (3d), and 5 days (5d) after excystation. In the PCR reaction, the poly-T-oligonucleotide was combined with a gene-specific oligonucleotide directed against VSP-4EX (Fig. 6), BiP, or a degenerate oligonucleotide directed against VSPs (VSP-GAIA). The small invariant bands correspond to primer.

DNA rearrangements (Yang and Adam, 1994; Ey *et al.*, 1996), which indicates that stage-specific antigenic variation is not dependent on DNA rearrangements close to the VSP genes. However, we cannot rule out longer distance DNA rearrangements (Upcroft *et al.*, 1997).

## Discussion

Transmission of *G. lamblia* occurs world-wide and infected hosts can excrete large numbers of cysts, leading to very high prevalence rates (Adam, 1991). Both the symptoms

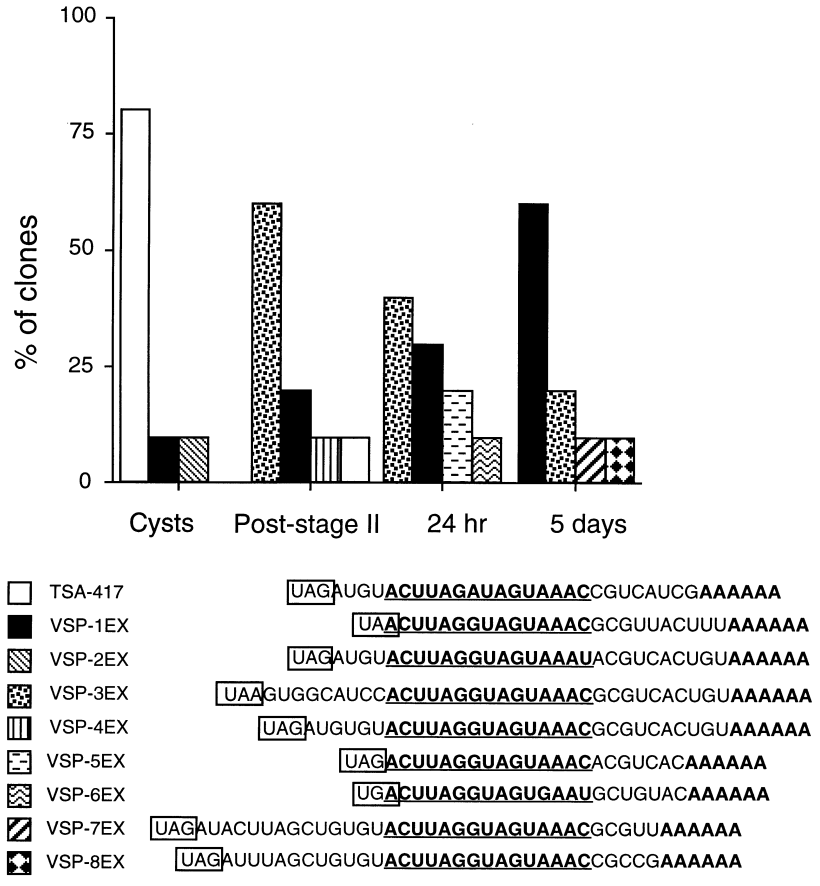


**Fig. 6.** Dynamics of VSP expression during and after excystation. 3′ RACE RT-PCR products were generated with a poly-T and a degenerate VSP oligonucleotide using cDNA from four time points in excystation (cysts, post-stage II, 1 and 5 days after excystation) (Fig. 5). The products were cloned, and 10 clones from each time point were sequenced. The 3′ UTR after the conserved C-terminal pentapeptide CRGKA is shown, the stop codon is boxed and the putative extended VSP polyadenylation signal is underlined.

and the duration of infection are highly variable, and reinfection after treatment is common (Gilman *et al*., 1988). Variation of the major surface antigens (VSPs) of *Giardia* is one likely explanation for some of the important clinical manifestations of giardiasis. Cytotoxic antibody responses to VSPs have been documented in experimental infections of humans and animals (Nash *et al*., 1990b; Gottstein and Nash, 1991), suggesting that the parasite can evade antibodies raised in previous infections when reinfecting the same host. In this study, we have shown that *Giardia* can alter its major surface antigen when the life cycle is completed. During encystation, *G. lamblia* trophozoites undergo a complex morphological and molecular differentiation into a cyst in response to specific signals from the host small intestine (Adam, 1991; McCaffery *et al*., 1994). Differentiation-associated switching of surface antigens could be one reason for the common occurrence of repeated giardial infections (Gilman *et al*., 1988), because of the downregulation of the major VSP and appearance of new VSPs after excystation. Reinfection may occur because the previously infected host, or others in the same community, can be infected by their own cysts, because new variants that are not recognized by existing antibodies can colonize the intestine.

The ability of *G. lamblia* to diversify its surface antigens during differentiation may also help explain why the cysts are so highly infectious (Rendtorff, 1954). As the intestinal lumen is such a varied environment, and individual VSPs probably differ in the degree to which they can protect the parasite or facilitate colonization of a specific host or niche, the expansion of the VSP pool that occurs during and after excystation may permit parasites to colonize multiple microenvironments and to infect a larger number of hosts. VSP genes occupy a large proportion of the small giardial genome, supporting their importance to the biology of the organism. Fifty-three of 205 gene homologues identified by random sequencing of ≈9% of the *G. lamblia* genome corresponded to VSPs (Smith *et al*., 1998). Although the biological function of these unusual proteins is not known, it is thought that they protect the parasite from intestinal factors, such as digestive enzymes and bile salts that inhibit most other microbes from colonizing the normal human small intestine (Mowatt *et al*., 1991; Nash *et al*., 1991; Nash and Mowatt, 1992; Aley and Gillin, 1993; Nash, 1994; Upcroft *et al*., 1997).

*Giardia lamblia* that infects humans is heterogenous (Nash, 1994; Ey *et al*., 1996), because several biochemical and genetic differences have been detected between groups of isolates (Nash and Mowatt, 1992; Nash, 1994; Ey *et al*., 1996). To see whether antigenic variation is isolate or group-specific, we investigated the GS/M isolate that belongs to the most distant and heterogeneous Group 3 of human isolates, and was estimated to have diverged from the WB isolate millions of years ago (Nash and Mowatt, 1992; Nash, 1994). Interestingly, the predominant VSP transcript in the GS/M isolate did not disappear after excystation, even although new transcripts were induced (data not shown). This suggests the need for *both* changes in VSP transcripts and disappearance of the old VSP. This is in agreement with previous results in which cysts excreted from humans experimentally infected with GS/M were excysted *in vitro* without the disappearance of the major VSP (Nash *et al*., 1990b). In contrast, we detected antigenic variation in the RB isolate, a third, unrelated *G. lamblia* strain (Meng *et al*., 1993), which, like WB, belongs to genetic Group 1 (Nash, 1994). Thus, three VSPs from human isolates (TSA 417, 1F and RB) switched after excystation. Moreover, the rB2 epitope (identified in *Giardia* from a sheep Bruderer *et al*., 1993; Ey *et al*., 1996), which was a minor VSP in the WB C6 population, also varied during differentiation. Thus, all four VSPs tested in Group 1 *Giardia* switched, whereas one in Group 3 did not. These results indicate that differentiation-associated antigenic variation may differ between genetic groups, which may help to explain the observed differences in infectivity and immunity between giardial isolates (Adam, 1991; Farthing, 1994; Nash, 1994).

Antigenic variation has been detected in a number of pathogenic microorganisms, but switching of variant surface glycoproteins (VSGs) in African trypanosomes is the best-characterized example in protozoa (e.g. Borst and Rudenko, 1994; Pays *et al*., 1994). Two major mechanisms of antigenic variation have been demonstrated in African trypanosomes: one is associated with DNA rearrangements where a silent VSG gene replaces the active gene at a certain, frequently telomeric active expression site through DNA recombination. The other mechanism, called *in situ* switching, involves activation of transcription from a new VSG and the concomitant inactivation of the previously active one without detectable DNA rearrangements (Pays *et al*., 1994; Horn and Cross, 1997). Differentiation-driven switching of surface antigens has been detected in *Trypanosoma brucei* (Pays *et al*., 1994). One example is the switch of the surface glycoprotein PARP of the procyclic form to the VSG coat of the metacyclic form in the tsetse fly salivary gland (Graham and Barry, 1995). All metacyclic variable antigen types (≈25) are activated simultaneously by an *in situ* mechanism, although usually only one is expressed per cell, yielding an antigenically mixed metacyclic trypanosome population that later infects the host (Tetley *et al*., 1987). It has been suggested that this mechanism enhances the probability of this population being transmitted to reservoir animals already partially immune to the VSG repertoires of local trypanosomes (Barry *et al*., 1990). Simultaneous activation of several variable surface protein (var) genes has recently been detected in malaria parasites after invasion of red blood cells (Chen *et al*., 1998). The similarity to what

we have found in an intestinal parasite is striking, even though the life cycles of the parasites are very different, which suggests that this mechanism for infection and reinfection could be commonly used in protozoan parasites.

One poorly understood question in antigenic variation is what happens to the 'old' surface protein after antigenic variation. The VSG coat of bloodstream trypanosomes is removed by a developmentally regulated protease when the parasites transform into the procyclic form in the fly mid-gut (Ziegelbauer *et al.*, 1993). The soluble, proteolytic product is released into the culture medium and synthesis of the procyclin surface protein PARP is induced. The fate of the 'old' VSP during giardial antigenic switching is not known, but processing of membrane-associated VSPs to a soluble isoform has been suggested to be important in the spontaneous antigenic switching of VSPs on trophozoites (Papanastasiou *et al.*, 1996). Our results suggest that endocytosis is used in differentiation-associated antigenic variation in *Giardia*. Much of the TSA 417 in cysts has already been cleaved at the protease-hypersensitive site and taken into the lysosomal compartment. The specific cleavage of the native forms of TSA 417 may occur within this compartment during both encystation and excystation and could be catalysed by the cathepsin B-like giardial cysteine proteinase(s) that function in excystation (Ward *et al.*, 1997). The cleaved TSA 417 fragments persisted in that compartment until > 24 h after excystation, but were not detected after 3 days. The diminution may be due to dilution by TSA 417-negative cells, but may also entail release into the medium, possibly as a decoy to the immune system. Secretion of VSPs after a proteolytic process on the surface would be prevented by the cyst wall, which may explain why the endocytic pathway is used during antigenic variation in encystation.

We have shown that stage-specific antigenic variation in *Giardia* is due to changes in the steady-state levels of VSP mRNAs. No detectable DNA rearrangements have been linked to VSP antigenic variation in this or previous studies (Yang and Adam, 1994), which suggests that it is an *in situ* mechanism. In general, VSPs are not telomere linked (Adam, 1991; Nash, 1994; Upcroft *et al.*, 1997), and the mechanism of switching is unknown. Chromatin structure affects transcriptional activation and inactivation (Edmonson and Roth, 1996) and has been suggested to be important for *in situ* activation in *T. brucei* (Pays *et al.*, 1994) and *G. lamblia* antigenic variation (Yang and Adam, 1994). *Giardia* has two nuclei that are both transcriptionally active (Kabnick and Peattie, 1990). The cyst form of *Giardia* contains four nuclei, and the DNA appears more tightly packed (Hetsko *et al.*, 1998). We have detected an overall increase of transcripts after stage II of excystation of *in vitro* cysts (Hetsko *et al.*, 1998). This change may be due to alterations in chromatin structure and transcription when the trophozoite emerges.

Another possibility is differences in RNA stability. Antigenic variation of cysteine-rich surface proteins, i-antigens, has been described in ciliates (Caron and Meyer, 1989). Temperature-dependent antigenic switching in *Tetrahymena thermophila* is due to changes in RNA stability (Love *et al.*, 1988), and changes in RNA stability after stage II of excystation may be another mechanism for antigenic switching in *Giardia*. AGUAAA has been suggested to be a polyadenylation signal in *Giardia* (Adam, 1991). We found this sequence in the 3′ UTR of every VSP transcript. Moreover, we found that the sequence around the AGUAAA is highly conserved. When all known VSP gene and transcript sequences were compared, an extended 15 nt polyadenylation signal with the sequence 5′-ACTYAGRTAGTRAAY-3′ could be detected (Fig. 6). This sequence was not found in any other *Giardia* gene in GenBank (April 1998), but is in all reported expressed and non-expressed VSPs. The function of this conserved VSP signature sequence is not known, but the 3′UTR is important for RNA stability and stage-specific regulation of the surface protein PARP of procyclic-form trypanosomes (Pays *et al.*, 1994).

Encystation is a form of immune evasion, as the trophozoite membrane is covered by the cyst wall, making it resistant to both antibody and non-immune secretory defences. A new facet of this immune evasion during differentiation is the loss of the old VSP from the parasite plasma membrane. New variant transcripts appear late in encystation, suggesting that preparation for antigenic switching is an integral aspect of encystation (Que *et al.*, 1996). Moreover, the major changes in transcript levels during excystation occur in response to distinct host signals that model cyst arrival in the small intestine (Hetsko *et al.*, 1998). These studies suggest that in addition to rapid and co-ordinated emergence from a dormant form and cell division, excystation entails expression of new VSP transcripts, which can lead to antigenic switching.

Several other medically important parasites, including *Cryptosporidium* and *Entamoeba histolytica*, start infection of the host by excystation of cyst or oocyst forms in the small intestine. Therefore, our work could have broader relevance to other intestinal pathogens that may use similar mechanisms of surface antigen variation.

## Experimental procedures

### *Cultivation and differentiation of parasites*

*Giardia lamblia* isolate WB belongs to genetic Group 1, the most common and homogeneous group of giardial isolates, defined by their molecular resemblance to WB (Nash, 1994; Ey *et al.*, 1996). Isolate GS/M belongs to Group 3, consisting of the most distant and heterogeneous human isolates (Nash, 1994). *G. lamblia* trophozoites (strain WB, ATCC30957, clone C6) were routinely cultivated, encysted and excysted as

described (Meng *et al*., 1996). Excystation was carried out by a two-step method modelling passage of cysts from fresh cold water into the host stomach and then into the small intestine (Rice and Schaeffer, 1981; Meng *et al*., 1996). Briefly, 66 h encysting cells were first water-treated at 4°C to lyse trophozoites and any incompletely formed cysts. The cysts were suspended in a low-pH excystation solution (pH 4.0) containing reduced glutathione and L-cysteine in Hanks' buffer and incubated for 20 min at 37°C (stage I). After centrifugation, the cyst pellet was resuspended in excystation stage II solution (pH 8.0) containing 1 mg ml$^{-1}$ of trypsin (Type 2, from porcine pancreas, Sigma Chemicals) in Tyrode's salt solution and incubated for 1 h at 37°C. The cyst pellet was collected and inoculated into prewarmed TYI-S-33 growth medium with bovine bile, which mimics the complex nutrients of the small intestine, and incubated at 37°C for 30 min, if not otherwise specified, and the motile excysted trophozoites were enumerated on a haemacytometer. Total cultures were harvested at the stages indicated for each experiment. The results in each figure are from a single experiment that is representative of several repeats. The times used in each study were chosen to reveal the major differences at the protein or transcript level.

### *Light and frozen-section immunoelectron microscopy*

Immunoperoxidase staining of trophozoites and excysted cells was performed as described (Meng *et al*., 1993). Cells were processed for cryosection immunoelectron microscopy (McCaffery *et al*., 1994) and double-labelled with polyclonal rabbit antibodies against TSA 417 (Gillin *et al*., 1990) and monoclonal antibodies (8C5) against cyst-wall protein (Campbell and Faubert, 1994). Detection was with gold-labelled goat anti-rabbit (10 nm) or goat anti-mouse antibodies (5 nm) (McCaffery *et al*., 1994).

### *Western blot analyses*

Cells harvested at each stage of encystation or excystation were washed three times in cold PBS with 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM *trans*-epoxysuccinyl-L-leucyl(4-guanidino)-butane (E64) added to the final wash. Cell pellets were resuspended in cold 6% trichloroacetic acid and incubated on ice for 10 min, then pelleted at 13 000 r.p.m. for 3 min. The supernatants were discarded and pellets were quick frozen and stored at −70°C until use. After thawing, the pellets were neutralized with NaOH and resuspended in SDS–PAGE sample buffer at $3.75 \times 10^7$ cells ml$^{-1}$ and boiled for 6 min. Equivalent amounts of protein (6 μg of protein per lane, determined by the Bradford method) were analysed. SDS–PAGE, transfer and processing of blots were as described (McCaffery *et al*., 1994). Blots were reacted with rabbit anti-TSA 417 (1:200) (Gillin *et al*., 1990) or anti-rB2-4AO1 (1:1000, a generous gift from Dr P. Köhler, University of Zurich; Bruderer *et al*., 1993) and probed with protein A–alkaline phosphatase. Controls for equal loading were reacted with antibodies to the giardial lectin, Taglin (1:250, a generous gift from Dr H. Ward, Tufts University; Ward *et al*., 1987.

### *Northern and Southern analysis*

Total RNA was isolated from *G. lamblia* at the indicated stages of differentiation by extraction with RNAzol B according to the manufacturer's directions (Tel-Test). Genomic DNA was isolated using the QIAGEN Blood and Culture DNA Kit protocol for tissue DNA isolation. For Northern hybridization, samples of total RNA (10 μg) were fractionated in 1.5% formaldehyde–agarose gels, capillary blotted in 20× SSC (standard saline citrate) and immobilized onto nylon membranes (Zeta-Probe, Bio-Rad) by baking in a vacuum for 1 h at 80°C. Blots were prehybridized in 6× SSC, 5× Denhardt's solution, 0.5% SDS and 100 μg ml$^{-1}$ salmon sperm DNA for 2 h at 65°C. Hybridization at 40°C was continued overnight in the presence of kinase-labelled oligonucleotides: NVSP, 5′-GAACCACCAG-CAGAGGAAGCC-3′; and TSASpec-1, 5′-AACTGTAAGGG-TACCTGAATCCAA-3′. The membrane was washed twice in 6× SSC/0.1% SDS at room temperature for 15 min, and then once at 50°C for 15 min in 0.5× SSC/0.1% SDS. The washed membrane was autoradiographed overnight. Hybridization at 65°C overnight with a random-primed 700 bp fragment from a constitutively expressed gene (PDI-1) was used as a control for equal loading. Southern blots were probed with kinase-labelled oligonucleotides TSASpec-1, NVSP and VSP4EX-1.

### *RT-PCR*

3′ RACE RT-PCR was used to detect transcribed VSP species during the life cycle. It was performed on cDNA generated with 5 μg of total RNA from each time point in the life cycle using Superscript II (Gibco-BRL), according to the manufacturers' instructions using poly-T-oligonucleotide SGS-10 (5′-CGAGCTGCGTCGACAGGC(T)17-3′). VSP4EX-1 (5′-TTG-AGCATGATGGTGATCTGA-3′) and BIPRA (5′-GGCTCAG-GACTGGCTCCGTGA-3′) were used as gene-specific primers. VSP-SAS (5′-TGAG(CGT)GC(CG)AT(AC)GC(GATC)-GG(CGT)AT-3′), which binds to the conserved membrane-spanning motif, was used to detect expression of VSPs. PCR was run with gene-specific primers and SGS-10 under conditions that were optimized to the linear range of amplification for each set of oligonucleotides using *Taq* polymerase and buffer from QIAGEN. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using the Sequenase 2.0 Kit, Amersham.

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