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Coexistence of sense and anti-sense mRNAs of variant surface protein in *Giardia lamblia* trophozoites

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ABSTRACT

A strategy of the parasitic protozoan *Giardia lamblia* to evade attack from the host immune system is periodic changes of its surface antigen, a member of the variant surface protein (VSP) family. A post-transcriptional gene silencing mechanism has been proposed to explain the presence of only one among many possible VSPs at any time. To investigate this phenomenon further, we extracted total RNA from cultured trophozoites of the *G. lamblia* C2 isolate, and cDNA was reverse-transcribed from the RNA. Sense and anti-sense VSPs were amplified from the total cDNA using nested PCR with primers designed from the 3′-conserved region and the known 5′ or 3′ end of the cDNA library. Sequence analyses of the amplified products revealed more than 34 full-length antisense VSPs and a smear of sense VSPs. Sequence alignments and comparisons revealed that these VSPs contained variable N-termini and conserved C-termini, and could be classified into 5 clades based on the sizes and variations of the N-terminal sequence. All antisense VSPs existed in the sense forms, but no corresponding antisense VSP existed for sense RNA (snsRNA) 16. The coexistence of sense and antisense VSP mRNAs in cultured *G. lamblia* supports the post-transcriptional regulation of VSP expression. We propose that VSPs transcribed simultaneously in the sense and antisense forms form double-stranded RNAs (dsRNAs) which are degraded by the Dicer endonuclease, while a VSP without an antisense transcription (e.g., snsRNA16) will be expressed on the surface of *Giardia*. In addition, in the course of this investigation VSPs were identified that were previously not known. PCR-based amplification of specific sense and antisense VSP cDNAs can be used to identify the specific VSP on *G. lamblia* trophozoites, which is easier than using specific monoclonal antibody approaches.

1. Introduction

Giardia lamblia is a unicellular flagellated protozoan. Worldwide, this parasite of the small intestine is a common cause of diarrheal and intestinal disease [1–5]. It was one of the first eukaryotes to diverge and is an interesting model system for studying early biological mechanisms [6]. *G. lamblia* has remarkable ability to adapt to changes in the environment [7], with sophisticated strategies to survive in various mammalian hosts and evade or neutralize the host's innate and adaptive immune defenses [8,9]. A major evasive mechanism allowing it to endure through chronic and secondary infections is antigenic variation [10–13], a process involving a family of variant surface proteins (VSPs) [14].

Species of *Giardia* are capable of expressing any of 303 VSP genes with VSP protein products that are cysteine-rich, variable in size (20–200 kDa), and contain conserved C-termini with transmembrane regions that end with the amino acid sequence CRGKA [13–16]. Interestingly, only one type of VSP is present on the surface of a vegetative trophozoite at any specific time [17], and switching among antigenically different VSPs occurs every 6–16 generations [18].

The N-terminal portions of the VSPs vary among the different members of the family with various numbers of CXXC motifs (where C represents cysteine and X represents any amino acid) [13]. The N-terminus is the extracellular domain, recognized by specific antibodies generated during the infection [14,16]. The C-terminus most likely remains in the cytoplasm with a nearly invariant CRGKA motif, and is shielded from the host [19,20]. VSPs exist as a thin coat spread across the entire surface of the trophozoite [16].

Many researchers have attempted to elucidate the regulatory mechanisms responsible for the changes in VSPs on the surface of *Giardia*. Results indicate that immunological pressure may be involved [21], and different host environments may dictate the

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selection of VSPs [22]. However, the regulation of VSP expression is not caused by gene mutation or by changes in the genes transcribed [13].

A recent report presented direct evidence for the involvement of post-transcriptional gene silencing (PTGS) in the regulation of VSP expression [23]. The process depends on RNA-dependent RNA polymerase (RdRP) and the formation of double-stranded RNA (dsRNA) complexes, which can be targeted by the action of the dsRNA endonuclease Dicer [23]. Argonaute (Ago) proteins are essential in gene-silencing pathways involving small RNAs [24]. The presence and activity of RdRP, dsRNAs, Dicer, and Ago suggest that an RNAi-like mechanism is involved in regulating the expression of VSPs in *Giardia* [23,25]. Indeed, silencing of Dicer or RdRP causes multiple VSPs to be present on an individual parasite [23]. A characteristic of RNAi is the degradation of dsRNA into 21–25 nucleotide siRNAs by Dicer [26]. The formation of dsRNAs is the key to this process. Typically, dsRNA is formed by the simultaneous syntheses of sense and anti-sense mRNAs catalyzed by RdRP [26]. However, the understanding of the synthesis and functions of sense and anti-sense mRNAs of VSPs in *G. lamblia* is limited.

Recent studies have identified putative microRNAs (miRNAs), derived from small nucleolar RNAs (snoRNAs), which may be involved in gene regulation in *G. lamblia* [27]. Five of the putative miRNAs, miR2, miR4, miR5, miR6, and miR10, have been identified by Northern blot, primer extension, 3′ rapid amplification of cDNA ends (RACE), and co-immunoprecipitation with Ago [27]. A partial anti-sense knockout experiment indicated that Dicer is needed for miRNA biogenesis, whereas Ago is required for miRNA-mediated translational repression [23,27]. Even though putative target sites were found in VSP genes, several other annotated and non-annotated open reading frames also carried binding sites for these miRNAs [27,28]. Whether the siRNAs are derived from snoRNAs or miRNAs specific for the repression of VSPs is unknown. There are still many uncertainties regarding the roles of miRNAs in VSP regulation in *Giardia*.

To elucidate the mechanisms of RNAi in the regulation of VSP expression, sense and anti-sense VSP mRNAs were generated from *G. lamblia* trophozoites and the sequence variations and mechanisms of expression of *Giardia* VSPs were analyzed.

2. Materials and methods

2.1. G. lamblia culture

Trophozoites of *G. lamblia* isolate C2, collected from a patient in Southwest China [29], were axenically cultured in modified trypticase yeast extract iron-serum-33 medium (TYI-S-33) [29], pH 7.0, supplemented with 10% heat-inactivated bovine serum (Sijiqing Biological, China) and 0.05% bovine bile (Sigma, USA) in borosilicate glass screw-cap culture tubes, as described previously [29].

The culture was started with 4×10^3 trophozoites per 4 mL per tube at 37 °C without shaking, and subcultured 3 times a week. 1 μL with only one *Giardia* trophozoites was innobated in one well of six well microplant to the clonal population.

To collect the parasites, the cultures were chilled on ice for 20 min to detach the trophozoites from the walls of the tubes. The parasites were pelleted by centrifuging at 367*g* at room temperature for 10 min, and then washed with 1× phosphate buffered saline pH 7.4. The freshly collected trophozoites were snap-frozen at −80 °C.

2.2. RNA isolation

Total RNA was isolated from freshly thawed trophozoites using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. The concentration of isolated total RNA was measured at 260 nm and 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). A 4-μL aliquot of the RNA was run on a 1% agarose–formaldehyde gel to assess RNA quality.

2.3. Primer design and synthesis

Sixteen primers were used in this study (Table 1). The SMART Oligo IV oligonucleotide, the CDSIII/3′ PCR primer, and the 5′ PCR primer were supplied in the SMART cDNA library construction kit (Clontech Laboratories, USA). Primer VSP-C1 was a degenerate primer designed based on the 3′ conserved region of the VSPs. The VSP-C2 primer was the nested primer designed to sequence prior to the 5′ end of VSP-C1 primer for specific amplification of sense or anti-sense VSP cDNAs (Fig. 1). Specific primers (SP 1–8) were designed based on specific VSP sequences obtained from the VSP cDNA amplifications using the VSP-C1 and VSP-C2 primer and the 5′ PCR primer or the 3′ PCR primer.

2.4. cDNA synthesis

Full-length cDNAs from *G. lamblia* trophozoites were synthesized using the SMART cDNA library construction kit and modified long-distance PCR (both: Clontech, USA). First strand cDNA was transcribed from total RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase and the modified oligo(dT) CDS III/3′ PCR primer. The 5′ end of the cDNA was extended with the SMART IV Oligo, which contains an oligo(G) sequence at its 3′ end to complement the oligo(C) at the 5′ end of the cDNA created by the MMLV. The full-length double-stranded cDNAs were amplified with the CDS III/3′ PCR primer and the 5′ PCR primer using the Advantage 2 Polymerase provided in the Advantage 2 PCR Kit (Clontech, USA). The PCR was performed using an initial denaturation at 95 °C for 1 min; and 18 cycles of 95 °C for 15 s and 62 °C for 6 min.

2.5. Amplification of sense and anti-sense VSP cDNAs

As shown in Fig. 1, the sense VSP cDNAs were amplified from the total cDNA by nested PCR using VSP-specific primers VSP-C1 and VSP-C2 and the 5′ PCR primer provided by the SMART cDNA library construction kit. The anti-sense cDNAs were amplified

Table 1
Primers used in this study.*

	Primer	Sequence
1	VSP-C1	5′-CCYCKRCACATGAACCACCA-3′
2	VSP-C2	5′-AGGAASCCNACRAGRCCMCC-3′
3	SMART IV**	5′-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3′
4	CDSIII/3′**	5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30N-1N-3′
5	5′ PCR**	5′-AAGCAGTGGTATCAACGCAGAGT-3′
6	SP 1F	5′-GTGGGCACAGACGTCGATGGATTC-3′
7	SP 2F	5′-TGCTCCGTAGACGGATGTA-3′
8	SP 3F	5′-AACCATTGACAATAAATGCGTGAAGTGC3′
9	SP 4F	5′-CATTAATGGAAAATGCACAA-3′
10	SP 5F	5′-GTAGGCACTGATAGTACCTC-3′
11	SP 6F	5′-AGCAACTGATGGCAACTGT-3′
12	SP 6R	5′-TCTAAGCATATGACTGAACTACC-3′
13	SP 7F	5′-AACATGTAATGGAGCAGCTACA-3′
14	SP 7R	5′-GACGATGGAGGAGCGCAGTTTAG-3′
15	SP 8F	5′- ATGCTACTGGGGGATTCTTCAA-3′
16	SP 8R	5′-TGGAGCGCATTTCTTACAGTCT-3′

Y = C/T, K = G/T, R = A/G, N = A/C or G/T.
* F and R indicate the forward and reverse primers, respectively.
** Supplied by the SMART cDNA library construction kit (Clontech Laboratories, USA).