The Release of the Variant Surface Protein of *Giardia* to Its Soluble Isoform Is Mediated by the Selective Cleavage of the Conserved Carboxy-Terminal Domain†

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ABSTRACT: The trophozoites of *Giardia duodenalis* are covered by a coat composed of an apparently single species of a group of novel, cysteine-rich proteins. These variant-specific surface proteins (VSPs) can be changed by sequential expression of different VSP genes, a process for which a gradual exchange of VSP molecules appears to be required. In the present study, we have examined the in vitro release of one of these VSPs (VSP4A1, formerly named CRISP-90) expressed by a sheep-derived variant *Giardia* clone. During in vitro incubation of the cloned trophozoites, the membrane-associated form of VSP4A1 (*m*VSP4A1) was specifically converted to a water-soluble isoform that was continuously released into the culture medium. The time required for *m*VSP4A1 to decline to half of the initial amount was 7.8 h. Analysis of the two purified protein species by mass spectrometry revealed molecular mass values of 68 991 Da for *m*VSP4A1 and of 65 425 Da for its soluble counterpart. Amino-terminal sequencing and metabolic labeling experiments indicated that the release of *m*VSP4A1 was associated with the cleavage of a carboxy-terminal peptide carrying the palmitic acid recently demonstrated to be attached to *m*VSP4A1. Calculations using the molecular mass and predicted amino acid sequence data indicated that fragmentation of the protein possibly occurs at a site located between the lysine and serine residues of the highly conserved NKSGLS motif directly preceding the hydrophobic sequence previously postulated to serve as a membrane-anchoring domain of other VSP molecules. The observed processing of the membrane-associated VSP to its soluble isoform is assumed to be an essential requirement for the ability of the parasite to undergo surface antigenic variation and thus for its establishment and survival within the vertebrate host.

Giardia duodenalis (syn. *G. lamblia, G. intestinalis*) is a flagellated protozoan parasite of humans and other vertebrates that is considered as one of the most ancient extant eukaryotes (Sogin et al., 1989). The trophozoites of these organisms express a major protein that covers the entire surface of the cell and can be altered by antigenic variation (Nash, 1992; Bruderer et al., 1993). These variant-specific surface proteins (VSP's)[1] share a number of notable structural features (Gillin et al., 1990; Mowatt et al., 1991; Nash, 1992; Ey et al., 1993). First, they are rich in cysteine (approximately 12 mol%), most of which appears in CXXC tetrapeptide motifs. Second, the sequence homologies that exist among different VSPs are found in a highly conserved, hydrophobic carboxy-terminal segment comprised of 26−27 amino acids which most likely serves as a membrane-anchoring domain. This fragment is followed by an invariant hydrophilic pentapeptide that possibly constitutes the cytoplasmic tail of the protein. Although the biological functions of *Giardia* VSPs have not yet been defined, the conservation of several structural features among these molecules suggests an important role within the host−parasite relationship. In earlier studies, it was observed that the VSPs expressed by two *Giardia* isolates were released from the trophozoite's surface membrane into the external milieu (Nash et al., 1983), but the mode of processing and nature of the removed products have not been further investigated.

Recently, we have cloned and sequenced a gene encoding a VSP (VSP4A1, formerly called CRISP-90; Bruderer et al., 1993) that is expressed by the variant *Giardia* clone O2-4A1 originally derived from sheep (T. Bruderer, unpublished experiments). The polypeptide predicted by the VSP4A1 gene revealed all the basic structural features typical of previously identified VSPs, but exhibited little sequence similarity to most other members of this protein family except for the highly conserved carboxy-terminal segment. In other work, we have shown that VSP4A1 is posttranslationally modified by the addition of a novel-type of glycans and of palmitic acid (Ph. Papanastasiou, unpublished experiments). Antigenic variation in *Giardia* and other infectious microorganisms is distinguished by the change from expression of one to another structurally and antigenically different surface antigen. In order that this antigen exchange can occur, the preceding surface molecules have to be removed from the cell membrane. In *Giardia*, but also in most other organisms capable of antigenic variation, little is known of how this process of antigen replacement is performed. Here, we report the specific in vitro release of a membrane-bound VSP and the biochemical characterization of its soluble isoform using the VSP4A1 expressing *Giardia* cells as a model system.

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; LPCM, low-protein-content medium; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS, mass spectrometry; PBS, phosphate-buffered saline; PVDF, poly(vinylidene difluoride); VSG, variant-specific surface glycoprotein; VSP, variant-specific surface protein; *m*VSP, membrane form of VSP; *s*VSP, soluble form of VSP.

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MATERIALS AND METHODS

Organisms and Cultivation. This study was conducted with trophozoites of the VSP4A1 expressing *Giardia* clone O2-4A1 which derived from an isolate (CH-O2) previously obtained from sheep in Switzerland (Strandén et al., 1990). Cultivation and harvesting of the trophozoites were performed as described elsewhere (Bruderer et al., 1993).

Biosynthetic Labeling Procedures and in Vitro Release of VSP41. Trophozoites of the variant clone O2-4A1 were biosynthetically labeled with L-[^{35}S]cysteine hydrochloride (20−150 mCi mmol^{-1}, Amersham). The procedure was performed essentially as described by Bruderer et al. (1993). The radiolabeled cells were washed 3 times with RPMI-1640, counted, and resuspended in ice-cold sterile low-protein-content medium (LPCM) to a concentration of 3.8×10^7 trophozoites mL^{-1}. The LPCM was composed of RPMI-1640 containing bovine bile salts, sodium iron(III) ammonium citrate, D-glucose, L-cysteine, ascorbic acid, and antibiotics at concentrations previously described for the *Giardia* culture medium (Bruderer et al., 1993), except that only two-fifths of the amount of yeast extract and casein digest were used and L-arginine was added to a final concentration of 2 g L^{-1}. Fetal calf serum was omitted. This medium allowed maintenance of the trophozoites for about 24 h with zero or minimal growth. One milliliter aliquots of the above-mentioned cell inoculum each were evenly dispersed into 60 mm polystyrene tissue culture dishes (Corning) containing 7 mL of LPCM prewarmed to 37 °C. The dishes, in triplicate, were placed in BBL GasPak Pouch bags (Becton Dickinson) and incubated for 1 h at 37 °C. Subsequently, the bags were opened, the supernatants were immediately aspirated and replaced with 8 mL of fresh LPCM preincubated at 37 °C, and the dishes were placed in new bags at 37 °C. This time point corresponded to time zero of the experiment. At various time points, including zero time, bags containing triplicates of the dishes were opened, and the dishes were placed on ice for 1 h to detach the adhered cells. The cells that did not detach during the 1 h incubation were retrieved after a further 1 h incubation and thorough washing with ice-cold phosphate-buffered saline (PBS). An aliquot was removed from the 14 mL final suspension (comprising the combined volumes of the previous incubations and washes) for cell counting and assessment of cell viability. The solution was centrifuged for 3 min at 4000*g* and 4 °C, and, to obtain a better separation, the retrieved supernatant was recentrifuged as above. An aliquot was removed from the supernatant to check for the possible presence of cells that could have leaked out from the pellet, and the remaining material was frozen at −20 °C. The time required for the detachment and centrifugations until the separation of the supernatant from the cell pellet was 3 h. The cell pellets were washed 3 times with PBS and, following centrifugation, stored at −20 °C. The supernatants were ultrafiltrated using Millipore 30-kDa-cutoff units as described by the manufacturers and then resuspended in 300 μL of distilled water, concentrated under vacuum centrifugation, and prepared for SDS−PAGE under nonreducing conditions. Cell pellets were lysed in 80 μL of SDS−PAGE sample buffer as described previously (Bruderer et al., 1993) and loaded onto polyacrylamide gels. After staining and incubation in the Amplify reagent, the gels were dried, and the radioactivity of the bands containing the VSP was quantitated by phosphorimaging (Molecular Dynamics). Fluorography of the gels was done as described before (Bruderer et al., 1993). For biosynthetic labeling with radioactive palmitate, trophozoites (1×10^8) were incubated for 2 h at 37 °C in 1 mL of RPMI-1640 containing 1 mCi of [9,10-^{3}H]palmitic acid in ethanol (47 Ci mmol^{-1}, NEN). One millicurie of the palmitate stock solution was dried under a nitrogen stream, resuspended in 10 μL of 100% ethanol, and mixed with 6 μL of 100 mg mL^{-1} fatty-acid-free BSA (a kind gift from Dr. A. Dieckmann-Schuppert) in RPMI-1640. After 1 h of shaking at room temperature, the palmitate−BSA complex was added to the cell suspension, and labeling was initiated. After the labeling procedure, the cells (1×10^8) were treated as described for the biosynthetic labeling with [^{35}S]cysteine and subsequently incubated for 15 h in LPCM. Incubation of the cells and processing of the released proteins were performed as described above.

Ultracentrifugation. Culture plates (Corning) containing cells labeled with [^{35}S]cysteine were left for 15 h in GasPak Pouch bags. The incubation medium was then transferred into Corning tubes and separated from the cells as described above. The contents of the medium were transferred to polyallomer Quick seal centrifuge tubes (Beckman), filled up to the top with PBS to a volume of 12 mL, and centrifuged for 1 h at 130000*g* and 4 °C in an L8-60 M Beckman ultracentrifuge. The upper 11.5 mL of the solution, constituting the soluble fraction, was transferred to a separate tube; the remaining 0.5 mL contained the sedimented particles and associated protein. Both fractions were ultrafiltrated as described above and analyzed by SDS−PAGE. Samples loaded onto the gels derived from 5×10^6 cells.

Protein Determination and Amino Acid Sequence Analysis. Protein was determined by the Bio-Rad protein assay based on the method of Bradford (1976). For amino-terminal sequencing of the VSP, the procedure described by Matsudaira (1987) was used with some modifications. The transfer buffer contained 25 mM Tris-HCl/50 mM glycine in an aqueous solution of 20% methanol. A poly(vinylidene difluoride) (PVDF) membrane (Millipore) was soaked for 4 min in 100% methanol and laid onto the gel, and then a nitrocellulose membrane (Schleicher & Schüll) was placed on the PVDF membrane facing the anode (Aley & Gillin, 1993). Electroblotting was carried out in a mini transfer cell (Bio-Rad) set at 250 mA for 1.5 h. The protein was visualized by staining of the PVDF membrane for 5 min with 0.1% Coomassie brilliant blue R-250 in 50% methanol. Destaining was performed with three washes in 50% methanol, 10% acetic acid. The membrane was washed 3 times with water and dried, the band of interest excised, and the protein sequenced on an Applied Biosystems 477AS protein sequencer (Applied Biosystems).

Immunoprecipitation. Trophozoites radiolabeled with [^{35}S]cysteine were incubated for 15 h in LPCM, and the culture supernatant was washed by ultrafiltration and dried under vacuum centrifugation as described above. The proteins were resuspended in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 0.5% Triton X-100 and proteinase inhibitors. Further experimental details of this procedure and the immunoprecipitation protocol have been described previously (Bruderer et al., 1993).

Analytical and Preparative SDS−PAGE. Polyacrylamide gel electrophoresis was performed as previously described (Bruderer et al., 1993). For molecular weight determinations

by mass spectrometry, cell-associated VSP4A1 was purified by Triton X-114 phase separation and DEAE-cellulose chromatography (Ph. Papanastasiou, unpublished experiments). Purified VSP4A1 and fractions containing the released VSP isoform were subjected to preparative SDS−PAGE. The bands of interest were excised from the gel and incubated with shaking for 1 h at room temperature in a solution containing 10 mM potassium phosphate, 140 mM NaCl, and 50 mM EDTA, pH 8.3. The solution was removed and the protein elution procedure repeated twice. The pooled eluates were ultrafiltrated using Micropure-Microcon 30-kDa-cutoff units (Amicon) as described by the manufacturers.

Mass Spectrometry. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was carried out using a Voyager Elite workstation (Perspective Biosystems). The samples (0.5 μL of about 1 pmol μL^{-1} protein) were mixed with an equal volume of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 50% (by volume) aqueous acetonitrile and allowed to crystallize on the target at room temperature. Sixteen laser shots from a 337 nm nitrogen laser were accumulated and averaged. For calibration of the mass spectra, bovine serum albumin and horse heart myoglobin (both from Sigma) were used as external standards. For ionization electrospray MS, a Finnigan MAT Model TSQ-710 triple quadrupole mass spectrometer was used. Prior to analysis, 10 μg (about 0.1 nmol) of the protein was desalted by reversed-phase HPLC on a Poros IIR/H column (0.32 × 250 mm, LC Packings) coupled directly to the mass spectrometer. The instrument was calibrated using horse heart myoglobin (Sigma), setting the resolution to 30 mass units (half-height) at 16951. The protein was eluted from the column at a flow rate of 3 μL min^{-1} with a linear gradient of 0−100% solvent B in solvent A over 20 min (solvent A, 0.1% TFA in water; solvent B, 80% acetonitrile and 0.085% TFA in water) with a coaxial flow of 3 μL min^{-1} methoxyethanol as a sheath liquid.

RESULTS

Release of VSP4A1 in Vitro. The ability of *Giardia* to release its VSP into the culture medium was assessed by biosynthetic labeling of VSP4A1 expressing *Giardia* trophozoites with [^{35}S]cysteine and subsequent incubation of the labeled organisms at 37 °C in a low-protein-content medium. The distribution of protein-associated radioactivity in the cells and culture medium supernatants was analyzed by SDS−PAGE, and the labeled VSP bands were quantified by phosphorimaging. The major cysteine-rich protein detected in the trophozoite lysate (Figure 1, lane L) was membrane-associated VSP4A1 (designated *m*VSP4A1) that appeared in its nonreduced form of 62 kDa (Bruderer et al., 1993). In addition, a 156-kDa component recently identified as a conformational variant of VSP4A1 (Ph. Papanastasiou, unpublished experiments) could be visualized on the gel. As also shown in Figure 1, a most prominent cysteine-rich protein of around 88 kDa (nonreducing conditions) continuously accumulated in the in vitro culture medium. Various experimental data described below indicated that this protein is a soluble, released isoform of *m*VSP4A1 and hence was denoted *s*VSP4A1. Since lysed or dead cells were not detected during the 25 h incubation period, it can be assumed that the released VSP resulted from living cells, rather than from cell injury or lysis that could have occurred during the culturing procedure or sample processing. The small quantities of the 62-kDa *m*VSP4A1 consistently present in the supernatant fractions have possibly originated from partial cell lysis during the 4 °C processing. Careful monitoring of the cell culture incubations showed that negligible amounts of cells (0−0.5% of the total cell population) were found to remain in the supernatants following centrifugation of the culture medium.

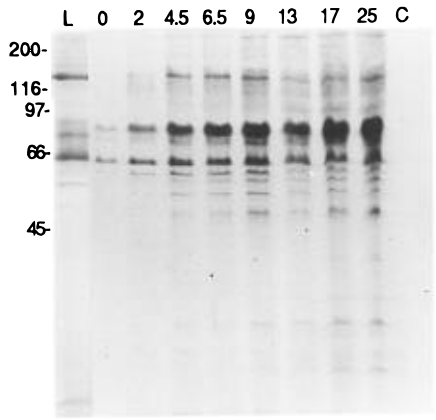

FIGURE 1: Release of *m*VSP4A1 upon in vitro incubation of trophozoites. Trophozoites of the *Giardia* clone O2-4A1 were metabolically labeled with [^{35}S]cysteine and maintained for the times indicated in the LPCM culture medium as described under Materials and Methods. Cell lysates and culture supernatants were subjected to SDS−PAGE under nonreducing conditions and the proteins visualized by fluorography. Lane L, total protein of the cell lysate at time 0. Lanes 2−9, total protein contained in the supernatants at the times indicated. Lane C, controls for cell presence in the supernatant after sample processing, performed as described under Materials and Methods. The latter sample represents a total lysate derived from 3.3×10^4 trophozoites (average cell number present in the supernatant). Molecular mass markers (in kDa) are indicated on the left.

The kinetics of the release of labeled VSP were established using the relative amounts of cell-associated *m*VSP4A1 (62 kDa and 156 kDa) and the released *s*VSP4A1 (88 kDa) forms following their separation by SDS−PAGE and subsequent quantitation by phosphorimaging. With these data, a logarithmic correlation was obtained between incubation time and the rate of ^{35}S-labeled VSP release (Figure 2). In this curve, all time points, including the zero value, were extended by 3 h because control experiments had shown that the labeled protein recovered in the medium at time zero (Figure 1, lane 2) was released from the trophozoites during the 3 h period of their detachment from the culture dish walls and processing at 4 °C. Based on additional control experiments, the values obtained for the 3 h cell processing at 4 °C were corrected for the rate of protein release at 37 °C. The kinetics clearly indicate that *m*VSP4A1 was rapidly and continuously liberated from the trophozoites into the surrounding medium. The time required for the labeled *m*VSP4A1to decline to half of its initial amount was 7.8 h.

Characterization of sVSP4A1. Previous analyses have demonstrated a large difference in electrophoretic mobility on SDS−polyacrylamide gels between alkylated (99 kDa) and nonreduced (62 kDa) forms of *m*VSP4A1 (Bruderer et al., 1993). A similar, but less pronounced, mobility shift was also observed with *s*VSP4A1 that migrated under the same conditions at 93 and 88 kDa, respectively (Figures 1 and 3). The close structural relationship of the ^{35}S-labeled *s*VSP4A1 with *m*VSP4A1 was demonstrated by the observa-

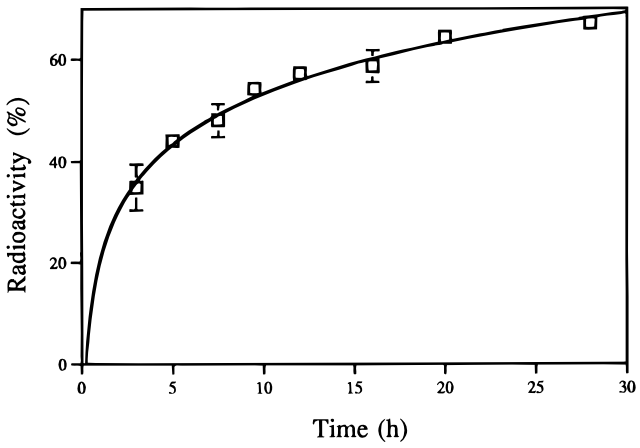

FIGURE 2: Kinetics of *m*VSP4A1 release. Values on the ordinate represent the radioactivity of VSP released into the medium as a percentage of radioactivity associated with total VSP4A1. VSP radioactivity was quantitated by phosphorimaging. The amounts of *s*VSP4A1 radioactivity released during the 3 h processing at 4 °C were corrected for 37 °C using a factor of 1.67 that was calculated from appropriate control experiments. The amounts of *s*VSP4A1 radioactivity were corrected for the loss observed during ultrafiltration of the culture supernatants (recovery 85%). This was done for samples from every individual tissue culture dish, and the averages of the triplicates expressing the kinetics of the ^{35}S-labeled VSP during the course of the experiment are represented in the figure. Further experimental details are described under Materials and Methods. Error bars represent standard deviations of triplicate experiments.

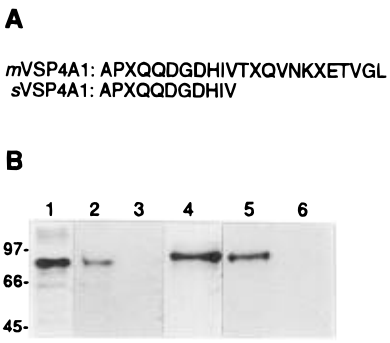

FIGURE 3: Common structural and immunological features of cell-associated *m*VSP4A1 and its soluble *s*VSP4A1 isoform. Panel A: amino-terminal sequence comparison of 62-kDa *m*VSP4A1 and its 88-kDa released isoform. X = unidentified amino acid. Panel B: fluorograph following SDS−PAGE of 88-kDa *s*VSP4A1 immunoprecipitated with anti-VSP4A1 antiserum. Samples are from proteins collected in culture medium (LPCM) supernatants following incubation of ^{35}S-labeled trophozoites for 15 h. Lanes 1 and 4, protein contained in the culture medium prior to treatment with the sera. Lanes 2 and 5, *s*VSP4A1 immunoprecipitated from culture medium with the anti-VSP4A1 antiserum. Lanes 3 and 6, normal rabbit serum controls of lanes 2 and 5. Lanes 1−3, nonreduced samples; lanes 4−6, alkylated samples. Molecular mass markers (in kDa) are indicated on the left.

tions that both proteins are rich in cysteine (Figure 1) and immunoprecipitable with the anti-VSP4A1 antiserum (Figure 3B). In addition, both proteins possessed the identical amino-terminal sequence, APXQQDGDHIV (Figure 3A). Major differences between the two VSP forms were found to exist in their electrophoretic mobilities and hydrophilicity characteristics. On polyacrylamide gels under nonreducing conditions, the released protein migrated more slowly (88 kDa) than its 62-kDa cell-associated counterpart (Figure 1). Sedimentation studies revealed that *s*VSP4A1 (Figure 4A, lane 2) was retained in the soluble fraction following high-

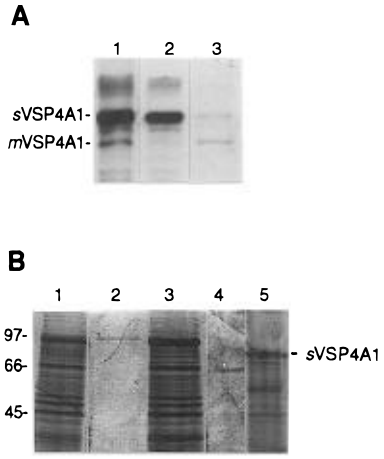

FIGURE 4: Fractionation of *m*VSP4A1 and its soluble isoform by ultracentrifugation and detergent phase separation. Panel A: fluorograph following SDS−PAGE analysis of soluble and sedimentable protein fractions of culture medium in which ^{35}S-labeled trophozoites had been incubated for 15 h. Lane 1, medium fraction prior to ultracentrifugation; lane 2, supernatant following ultracentrifugation at 130000*g*; lane 3, pellet fraction following ultracentrifugation at 130000*g*. All samples were nonreduced. Panel B: SDS−PAGE analysis of the protein fractions obtained from Triton X-114 phase separation of medium in which trophozoites had been incubated for 15 h. Lane 1, sample prior to the detergent treatment; lanes 2 and 4, detergent phase; lanes 3 and 5, aqueous phase. Lanes 1−3, alkylated samples; lanes 4 and 5, nonreduced samples. Proteins were visualized with Coomassie brilliant blue. Molecular mass markers (in kDa) are indicated on the left.

speed centrifugation (130000*g*) of cell culture supernatants. In contrast, under similar experimental conditions, *m*VSP4A1 that was present in minimal amounts in the same fraction due to partial cell lysis during the 4 °C processing was found to be sedimentable (Figure 4A, lane 3). The particulate nature of *m*VSP4A1 observed in this experiment was possibly due to its association with membrane fragments or to the formation of larger multimeric protein complexes. Following phase separation with Triton X-114, *s*VSP4A1 was retrieved in the aqueous phase (Figure 4B, lanes 3 at 93 kDa and 5 at 88 kDa; see also Figure 3B) while its membrane-associated counterpart was found to remain in the detergent fraction (Figure 4B, lanes 2 at 99 kDa and 4 at 62 kDa; Bruderer et al., 1993). When both VSP forms were subjected to DEAE-cellulose chromatography, *s*VSP4A1 did not bind to the ion exchange material, whereas *m*VSP4A1 was retained on the column (data not shown). The latter protein could be released from the column with high salt concentrations (150 mM NaCl; Ph. Papanastasiou, unpublished experiments) in the eluting buffer, suggesting that the two VSP isoforms are distinguished by different net charges and/or charge distributions.

In further experiments, the two isoforms of the VSP purified by preparative SDS−PAGE were used for molecular weight determination by MALDI-TOF MS. As shown in the spectra of Figure 5 (panels A and B), the molecular weights for *m*VSP4A1 and *s*4VSPA1 were determined to be 68 991 and 65 425, respectively, indicating that the release of the membrane-bound VSP into the culture supernatant is associated with a reduction in its molecular weight by about 3500. The molecular weight of the soluble isoform determined by electrospray ionization MS (Figure 5, panel C) resulted in a slightly higher value than that derived from MALDI-TOF MS analysis. Determination of the mass of

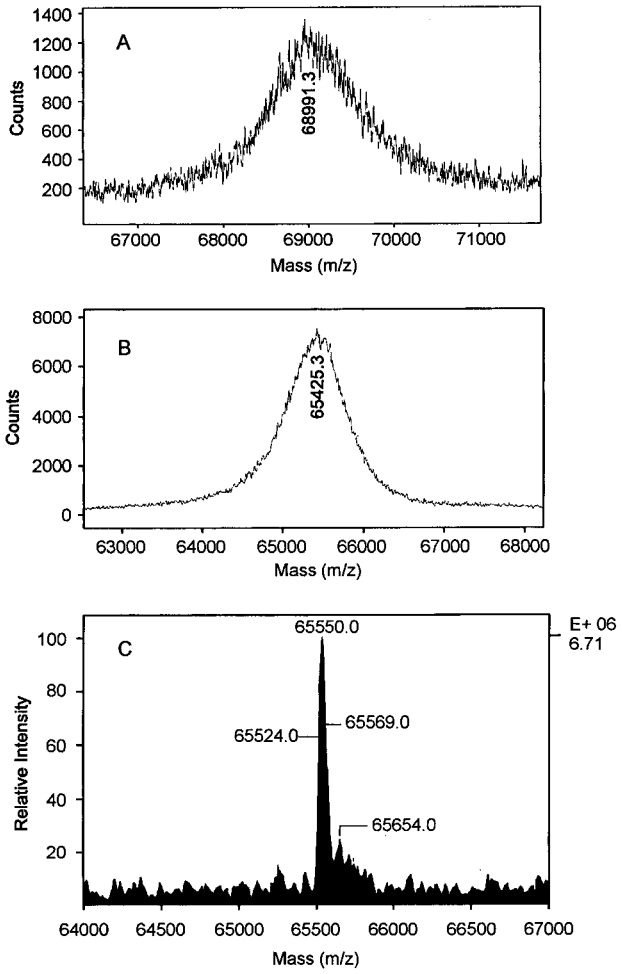

FIGURE 5: Analysis of purified *m*VSP4A1 (A) and *s*VSP4A1 (B) by MALDI-TOF MS and of *s*VSP4A1 (C) by electrospray ionization MS.

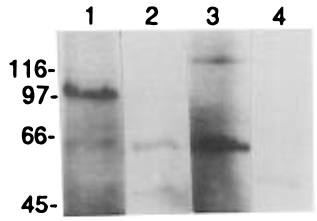

FIGURE 6: Loss of [^{3}H]palmitic acid from *m*VSP4A1 during processing to its soluble isoform. Fluorograph following SDS−PAGE analysis of trophozoites labeled with [^{3}H]palmitic acid and of medium fractions derived from incubations of labeled trophozoites for 15 h. Lanes 1 and 3, trophozoite lysate containing palmitic acid labeled *m*VSP4A1 in alkylated (99 kDa) and nonreduced form (62 kDa), respectively. Lanes 2 and 4, alkylated and nonreduced medium fractions containing *s*VSP4A1 lacking palmitic acid derived radiolabel. Molecular mass markers (in kDa) are indicated on the left.

*m*VSP4A1 by electrospray MS was not possible, because attempts to purify the protein by reversed-phase HPLC, a necessary requirement for this type of MS analysis, were unsuccessful. In additional experiments using metabolically labeled cells, it was shown that the palmitic acid recently demonstrated to be covalently attached to *m*VSP4A1 (Ph. Papanastasiou, unpublished experiments) was found absent from *s*VSP4A1 (Figure 6, lanes 2 and 4), indicating that the lipid moiety is located within the cleavable carboxy-terminal peptide sequence of the VSP molecule.

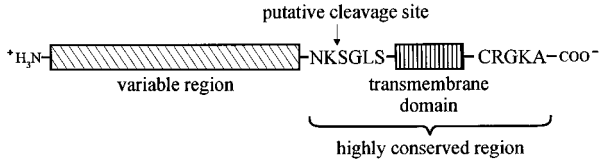

FIGURE 7: Schematic representation of the structure of *m*VSP4A1 with the putative proteolytic cleavage site for processing the *s*VSP4A1 isoform. The putative cleavage site is located in close proximity to the highly conserved membrane-spanning domain.

DISCUSSION

In the present work, we have analyzed the kinetics of the in vitro release of the membrane form of a VSP (*m*VSP4A1) that is expressed by a cloned *Giardia* isolate and have partially characterized the soluble VSP isoform. The initial rate of radiolabeled *m*VSP4A1 release was very rapid with 70% of these molecules being removed within 32 h. Since injury or lysis of trophozoites has not been detected during the cultivation procedure, the removal of the cell-associated VSP is likely to be a specific physiological process. Earlier experiments have shown that in cultures of the human-derived *Giardia* WB isolate, 70% of the major surface-labeled antigen was released from the trophozoites within 24 h into the external medium (Nash et al., 1983). This component was subsequently identified as a 170-kDa VSP (VSPA6 or CRP170) expressed by a variant progeny of the WB isolate (Nash et al., 1988). However, the released protein was not further characterized by the authors. Although the incubation media and the methods used for cell labeling and quantification of the released proteins differed remarkably between both studies, the loss of both proteins from the surface membrane occurred with similar kinetics.

The data obtained from molecular weight determinations and radiolabeling experiments suggest that the release of *m*VSP4A1 is associated with the cleavage of a carboxy-terminal peptide of about 3500 Da with a palmitic acid residue attached to this sequence and that fragmentation of this peptide probably occurs in close proximity to the hydrophobic sequence previously postulated to serve as a membrane-anchoring domain (Gillin et al., 1990; Mowatt et al., 1991; Ey et al., 1993). The known predominance of hydrophobic amino acids and the presence of a lipid residue within the cleavable sequence of *m*VSP4A1 would also explain the differences in solubility characteristics observed between the two VSP isoforms. The precise proteolytic cleavage site has not been identified, but calculations using the molecular weight and deduced amino acid sequence data of *m*VSP4A1 (T. Bruderer, unpublished experiments) suggest that it is between the lysine and serine residues located within the highly conserved NKSGLS motif directly preceding the hydrophobic carboxy-terminal stretch of the molecule (Figure 7). Limited information is available regarding the location of the palmitic acid, but examination of the derived carboxy-terminal sequence of *m*VSP4A1 indicates that the conserved two serine and cysteine residues located within the cleavable peptide sequence could be candidates for possible *O*- or *S*-ester acylation (T. Bruderer, unpublished experiments). The observations that the release of *m*VSP4A1 results in the formation of a distinct cleavage product and also occurs in a protein-free culture medium (data not shown) indicate that the hypothetical proteolytic activity responsible for the processing is an endogenous parasite enzyme of high

specificity. However, the nature of the putative membrane protein-solubilizing proteinase and its site of action within the parasite are completely unknown.

The specific release of the extracellular domains of surface-associated proteins to generate soluble isoforms is widespread in nature and caused, as in the case of *m*VSP4A1, by hydrolytic cleavage of the polypeptide's membrane anchor (Ehlers & Riordan, 1991). Among such proteins are cytokine receptors, growth factors, leukocyte antigens and enzymes of mammalian cells, and the amyloid precursor component of patients with Alzheimer's disease. The formation of soluble counterparts of membrane proteins was also shown for other protozoan parasites. In differentiating bloodstream forms of *Trypanosoma brucei*, the membrane-associated variant surface glycoprotein (VSG) is known to be rapidly released into the culture medium following proteolytic cleavage of a carboxy-terminal peptide fragment (Bülow et al., 1989; Ziegelbauer et al., 1993). The putative proteinase responsible for the VSG release has not been identified but is suggested to be a unique hydrolytic enzyme activated after triggering trypanosome transformation. In contrast, bloodstream-form trypanosomes growing in vitro were found to release their VSG rather slowly (Bülow et al., 1989; Seyfang et al., 1990), a process known to be initiated by cleavage of the glycosylphosphatidylinositol (GPI) membrane anchor possibly via the action of endogenous GPI-specific phospholipase C.

In many instances, the specific proteolytic processing of membrane-associated proteins to soluble isoforms serves specialized functions (Ehlers & Riordan, 1991). For *Giardia*, the specific hydrolytic cleavage of VSPs may constitute an important step in the continuous process of surface antigen replacement and variation. It appears possible that following removal of the greater part of the VSP molecule the remaining plasma membrane-embedded short peptide segment is internalized through the endocytotic machinery of the cell. Commensurate with this plasma membrane expenditure, membrane material carrying newly synthesized VSP that is either identical or, after a VSP gene switch, different from that of the existing variant type will be cycled back to the cell surface. Membrane elements necessary for protein sorting and transport to the plasma membrane have previously been identified in *Giardia* (McCaffery & Gillin, 1994). The possible biological role of the soluble VSP isoforms released into the external milieu is still obscure, but it can be assumed that they serve as a decoy for the host's immune system. Supposing that VSP release occurs also *in vivo*, these considerations suggest that processing of membrane-associated VSPs to soluble isoforms provides part of a mechanism important for the establishment and survival of the parasite within the hostile environmental conditions of the vertebrate small intestine.

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