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Cysteine-Dependent Zinc Binding by Membrane Proteins of *Giardia lamblia*

YU-YING ZHANG,[1] STEPHEN B. ALEY,[2] SAMUEL L. STANLEY, JR.,[1] AND FRANCES D. GILLIN[2,3]*

Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110,[1] and Department of Pathology, School of Medicine,[2], and Center for Molecular Genetics,[3] University of California at San Diego, San Diego, California 92103-8416

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The abundant, highly variable surface proteins (VSPs) which cover the surface of *Giardia lamblia* trophozoites compose a group of extremely cysteine (C)-rich proteins in which more than half of the cysteines are in the motif CXXC. Because of the constancy of these features among the known VSPs and the prominence of cysteine and particularly CXXC in proteins that bind zinc and other metals, we asked whether *G. lamblia* VSPs bind zinc in vitro. VSPs are the major protein component of Triton X-114 detergent-phase extracts of *G. lamblia* trophozoites and can be readily identified by surface iodination of intact cells. The partitioning of ^{65}Zn binding into the Triton X-114 detergent phase and the correspondence between surface iodination and zinc binding patterns of four *G. lamblia* strains or sublines with different VSPs support the idea that VSPs bind zinc. The requirement for renaturation of blots with a reducing agent indicates that Zn^{2+} is coordinated by cysteine residues, rather than by other amino acids. Binding did not appear to be specific to zinc since it was inhibited by competition with other divalent metal ions. The abundance of the VSPs and the prevalence of metal binding motifs among all known variants suggest that they may play an important role in trophozoite survival and colonization in the host.

The trophozoite form of *Giardia lamblia* colonizes the human small intestine, where it is exposed to complex and changing concentrations of bile, digestive enzymes, and hydrogen ions, as well as secretory antibodies. The predominant protein on the trophozoite surface is one of a unique group of extremely (~12%) cysteine-rich proteins called variant surface proteins (VSPs) (1, 9, 12, 15). VSPs can vary spontaneously or in response to antibody (1) or environmental (17) selection both in vitro and in vivo (16), and loss of a VSP leads to replacement with another cysteine-rich variant which is usually immunologically distinct (1, 15). Antigenic variation is thought to contribute to the frequent chronicity of giardiasis (14).

Despite the frequency of VSP switching (estimated at ~1/6.5 to 13 cell doublings [15]), certain features are common to all known VSPs. In our analysis of the first complete VSP gene sequence, TSA 417, we noted the striking occurrence of the tetrapeptide CXXC, where X is any amino acid, which occurred 29 times throughout the 61.3-kDa external domain of this integral membrane protein (9). To date, five independent VSP gene sequences have been published, and all contain a high proportion of cysteine (11 to 12%), more than half of which is in the motif CXXC (15). The constancy of this motif in the VSP group of proteins prompted us to ask whether it may reflect a conserved biologic function that may help trophozoites survive in such a hostile environment.

Cysteine residues, especially the CXXC motif, have been shown to coordinate metal atoms, particularly zinc (22). Recently, alignment of VSP sequences showed that four of five had an arrangement of nine cysteines and a histidine residue (15) that is reported to be involved in zinc binding by a number of proteins (10). The ability to bind zinc is prominent in many nucleic acid binding proteins, as well as metalloenzymes (5). Because all the VSP CXXC motifs appear to be exposed on the outer face of the trophozoite plasma membrane (9), we do not expect that the VSPs function mainly as nucleic acid binding proteins. In this report, we show the results of experiments to determine whether TSA 417 and other VSPs of *G. lamblia* trophozoites bind zinc and, if so, whether binding is specific.

MATERIALS AND METHODS

Parasites and cultivation. In these studies, we used three sublines of *G. lamblia* WB (from a patient infected in Afghanistan) that express different major VSPs, but presumably all have the same genetic repertoire (15). Recently cloned populations are heterogeneous since immunostaining shows negative cells in a positive clone and cells that re-express a given epitope appear in negative clones (15). Of recently cloned populations of subline C6, 80 to 95% express the TSA 417 epitope, while clone 1F, which was selected from C6 with antiserum against TSA 417 and complement, is mainly TSA 417 negative. Of cells in recent 1F subclones, approximately 5 to 20% react with antiserum against recombinant TSA 417. Labeling with [^{35}S]cysteine or surface iodination suggested that in 1F, TSA 417 is replaced by a VSP of similar molecular weight that does not react with anti-TSA 417. Subline A6 (1) of strain WB has been maintained separately from C6 for >8 years, and fewer than 1% react with anti-TSA 417 (11). We also used strain GS/M, an unrelated isolate from an infection acquired in Alaska. GS/M and WB have distinct VSP repertoires and differ in a number of other antigens and an rRNA gene sequence (13, 15). WB subline A6 and strain GS/M were the generous gifts from T. Nash.

Trophozoites were grown to the late log phase in modified TYI-S-33 medium (6) with bile but without added iron, antibiotics, or vitamins (8). For harvest, trophozoites were detached by chilling culture flasks on ice, concentrated by centrifugation (10 min at 833 × *g*), and washed twice with

* Corresponding author.

cold phosphate-buffered saline (PBS). Triton X-114 phase separation followed the method of Bordier (4), and unless otherwise specified, detergent phase extract equivalent to $\sim 2 \times 10^6$ cells per lane was used since VSPs partition into and are the major proteins of the detergent-rich phase (9).

Zinc binding. Zinc blotting was performed as described by Barbosa et al. (3), with a few modifications. In all experiments, alcohol dehydrogenase (ADH) was included in a side lane as a positive control. In brief, samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes as described previously (21). After transfer, the blots were incubated in a renaturing buffer (100 mM Tris HCl [pH 6.8], 50 mM NaCl) with 20 mM β-mercaptoethanol (except where specified otherwise) for 1 h with three changes of buffer. All buffers were flushed with N_2 (3). Proteins were labeled by incubation for 2 h at room temperature in 20 ml of renaturing buffer containing 10 μCi of $^{65}ZnCl_2$ per sample lane (16). Blots were rinsed twice in renaturing buffer, washed three times for 20 min in renaturing buffer, and autoradiographed. For inhibition studies, the protocol was the same, but cold $ZnCl_2$ (0.01 mM), $CdCl_2$ (0.1 mM), $MgCl_2$ (10 mM), or $CaCl_2$ (10 mM) was added to the labeling mixture containing $^{65}ZnCl_2$. At these concentrations, Zn and Cd have been shown to inhibit binding by known zinc binding proteins, while Mg and Ca did not (3). In some cases, blots were reprobed with rabbit serum against TSA 417 expressed in *Escherichia coli* (9). This serum reacted with several species of a single protein encoded by the TSA 417 gene and with no other giardial proteins (9, 19).

Surface labeling. For surface iodination, 2×10^8 live trophozoites in 200 μl of PBS were incubated for 10 min on ice with 0.5 mCi of carrier-free $Na^{125}I$ in glass tubes coated with 100 μg of Iodogen (Pierce Chemical Co., Rockford, Ill.). Trophozoites were then washed four to five times with cold PBS. Total proteins from radiolabeled trophozoites were solubilized in reducing SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and autoradiographed on Kodak XAR-5 (9).

RESULTS

Schiff et al. (20) described a method for identifying zinc binding proteins after electrophoretic separation and transfer to nitrocellulose. More recently, Barbosa et al. (3) found that renaturation in the presence of a reducing agent after transfer permitted zinc binding by proteins that coordinate the metal through cysteine residues, rather than other amino acids. To identify possible zinc binding proteins, we compared transfers of trophozoite proteins which had or had not been renatured in the presence of β-mercaptoethanol. After reduction, binding of ^{65}Zn to trophozoite proteins, as well as to the ADH control, was readily detected (Fig. 1A). In contrast, without reduction (Fig. 1B), no binding to any trophozoite protein or to the ADH control was detected, supporting the importance of cysteine in coordinating the zinc. Comparison of proteins after Triton X-114 phase separation (4) (Fig. 1A, lanes 2 and 3) showed that >90% of the ^{65}Zn binding was to proteins in the detergent-rich phase, suggesting the importance of membrane-associated proteins. We showed earlier that VSPs partition into and are the major proteins of the Triton X-114 detergent-rich phase, probably because of their hydrophobic C-terminal membrane-spanning sequence (9), which is extremely conserved (12).

Initially, we compared the binding patterns of two subclones of strain WB, C6 (Fig. 1A, lane 1, VSP TSA 417 positive) and 1F (Fig. 1A, lanes 2 and 3, a TSA 417-negative subclone of C6). Proteins at ~97, 80, 63, and 49 kDa which strongly bound ^{65}Zn were observed in both sublines, although their relative intensities differed. We have shown that the TSA 417 VSP has multiple electrophoretic species, typically of 85 and 66 kDa, and smaller proteolytic products (9). However, the VSPs tend to exhibit electrophoretic variability, probably because of their high cysteine content (14). Therefore, to determine whether some or all of the binding activity was due to the VSPs, we developed the same protein blot used for ^{65}Zn binding with a monospecific rabbit antiserum against recombinant TSA 417 (9). Anti-TSA 417 reacted with three proteins of clone C6 (Fig. 1C, lane 1), of apparent molecular masses of 97, 80, and 49 kDa each of which clearly comigrated with one of the ^{65}Zn binding proteins (Fig. 1A), while the ~63-kDa zinc binding band did not react with the antiserum, suggesting that three of the four zinc binding bands were TSA 417 species. In contrast, this antiserum reacted only faintly with proteins of similar size in the TSA 417-negative clone 1F (Fig. 1C, lanes 2 and 3), probably because of the 5 to 20% of clone 1F trophozoites that re-express a TSA 417-like epitope. This is consistent with our observations that in clone 1F, the major VSP species have a size similar to that of TSA 417, but are immunologically distinct (11).

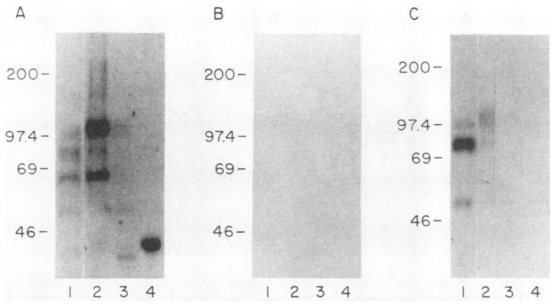

FIG. 1. Requirement of reduction for zinc binding by proteins from two subclones of *G. lamblia* with different VSPs. (A) Binding of ^{65}Zn to proteins after renaturation in β-mercaptoethanol. (B) Absence of zinc binding to a replicate blot which had been renatured without mercaptoethanol. (C) Western blot (immunoblot) of panel A reacted with rabbit antiserum against the VSP called TSA 417, which had been expressed in *E. coli*. Lanes: 1, strain WB, clone C6 (TSA 417 positive), Triton X-114 detergent-phase extract; 2, strain WB, subclone 1F (TSA 417 negative), Triton X-114 detergent phase; 3, subclone 1F, Triton X-114 aqueous-phase extract; 4, ADH (5 μg). Numbers on left show size in kilodaltons.

The idea that at least some of the ^{65}Zn binding bands are VSPs was supported by the involvement of cysteine, the partition of the binding activity into the Triton X-114 detergent-rich phase, and the differences in binding profile between subclones with different VSPs. Because of the similarities in electrophoretic migration of zinc binding and VSPs of these two sublines, we tested this correlation further by comparing patterns of surface labeling, which predominantly labels VSPs (1, 9), and ^{65}Zn binding of four strains or sublines with different VSPs. Because of the demonstrated frequency of antigenic switching (15), it was necessary to perform surface labeling and zinc binding studies on samples of the same cells. Cell harvests were divided, and a portion of the intact cells was labeled with ^{125}I, while the remainder was not labeled. Total cell extracts were then subjected to identical SDS-PAGE (on long-format gels for better resolu-

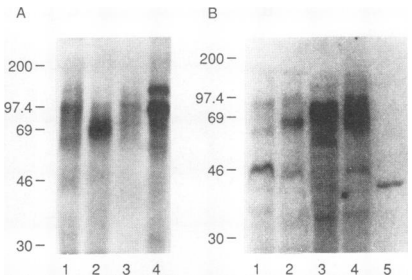

FIG. 2. Correspondence between surface-labeled and zinc binding proteins of four stabilates with different VSPs. (A) Autoradiogram of whole extracts of intact cells that had been surface labeled with ^{125}I. (B) Binding of ^{65}Zn by replicate samples of trophozoites that had not been iodinated and were separated by identical SDS-PAGE. Lanes: 1, strain WB, clone 1F; 2, strain WB, clone C6; 3, strain WB, clone A6; 4, strain GS/M; 5, ADH. Numbers on left show size in kilodaltons.

tion) and electrotransfer, and the unlabeled blot was renatured and incubated with ^{65}Zn under standard conditions.

Comparison of autoradiograms shows close correspondence between the major surface-labeled proteins (Fig. 2A) and the ^{65}Zn binding species (Fig. 2B) of each. In lanes 1 (WB subline 1F), the major surface-labeled species of ~97, 66, and 46 kDa each correspond to a zinc-binding protein, although the intensities differ. In lanes 2 (WB subline C6), the major iodinated and zinc binding bands are ~69 kDa, with minor bands at ~46 kDa. In lanes 3 (WB subline A6), both labels were heterodisperse, from ~60 to 97 kDa; and in lanes 4 (strain GS/M), the major surface iodinated bands were ~68 to 98 and 135 kDa. Zinc binding overlapped with the former, but did not appear to correspond to the 135-kDa surface labeled band. The latter may be due to differences in migration of the same species or to a non-zinc binding surface protein. The heterodisperse bands are typical of these cysteine-rich proteins, especially under reducing conditions (16). The lower-molecular-mass bands of ~49 and 32 kDa appear to be additional zinc binding proteins in the total cell extracts. These data support the idea that the VSPs do bind Zn^{2+}, although they likely are not the only *Giardia* proteins that do so.

To determine whether metal binding is specific to Zn^{2+}, we did binding in the presence of other divalent metal ions (Fig. 3), using Triton X-114 detergent-phase extracts of the four isolates. Binding of ^{65}Zn by the VSPs was reduced below detectable levels by competition with unlabeled Zn^{2+} (Fig. 3B) and by Cd^{2+} (Fig. 3C), as well as by Mg^{2+} (Fig. 3D), or Ca^{2+} (Fig. 3E). Iron also appeared to compete (data not shown). In contrast, binding of zinc by ADH was >90% inhibited only by Zn^{2+} or Cd^{2+}, as reported previously for

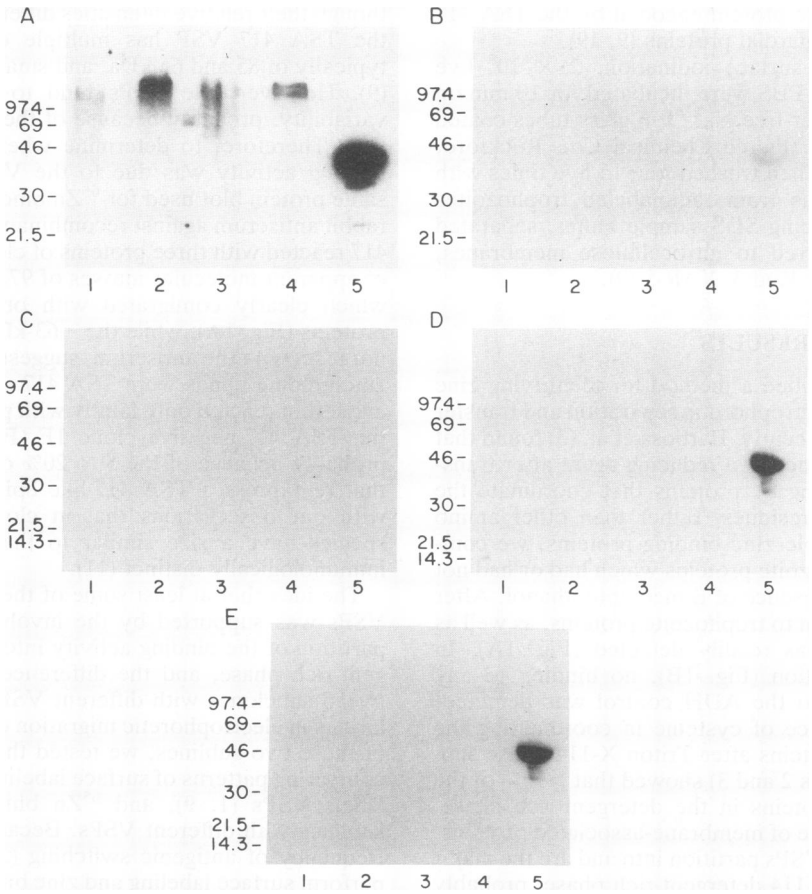

FIG. 3. Ablation of zinc binding by VSPs in Triton X-114 detergent-phase extracts from the four stabilates by competition with other divalent metal ions. (A) No competing metal; (B) competition with 0.01 mM $ZnCl_2$; (C) competition with 0.1 mM $CdCl_2$; (D) competition with 10 mM $MgCl_2$; (E) competition with 10 mM $CaCl_2$. Lanes: 1, strain WB, clone C6; 2, strain WB, clone A6; 3, strain WB, clone 1F; 4, strain GS/M; 5, ADH. Numbers on left show size in kilodaltons.

this zinc binding enzyme (3), although Mg^{2+} and Ca^{2+} appeared to compete to a lesser extent. This experiment suggests that metal binding by trophozoite proteins may not be specific to zinc.

DISCUSSION

Since the VSPs cover the surface of the *G. lamblia* trophozoite (9, 18), this group of proteins forms the major component of its interface with the host. The frequency of variation (15) may help the parasite evade host defenses (14). On the other hand, although the WB and GS/M isolates used in this study are estimated to have diverged millions of years ago (13), striking features of their VSPs have been maintained. Therefore, we asked whether the conserved prevalence of cysteine and the CXXC motif, as well as the type I membrane protein conformation (9, 15), may reflect a key physiologic function of the VSPs.

These studies showed that the VSPs of trophozoites are capable of binding ^{65}Zn in vitro. The partitioning of zinc binding and VSPs into the Triton X-114 detergent phase and the correspondence between surface labeling and ^{65}Zn binding patterns of four stabilates with different VSPs support the idea that the VSPs bind zinc. The requirement for renaturation of blots with a reducing agent indicates that coordination of Zn^{2+} is due to cysteine residues, rather than to other amino acids, notably histidine (3), which is not abundant in VSPs (15). Binding appeared not to be specific to zinc since it was ablated by competition with other divalent metal ions under conditions in which specific zinc binding was not completely inhibited (3). Since these studies were done with large excesses of competing metal ion, we do not know whether binding is totally nonspecific or whether there may be a preference for one metal.

Most zinc binding proteins are enzymes, transport-storage proteins, or proteins that interact with nucleic acids (5, 10, 22). Because of the localization of the VSPs on the external surface of the trophozoite (9, 18), it is not likely that they interact with nucleic acids. In enzymes, zinc may be at the active site or may serve a structural role. To date, no enzymatic function has been suggested for a VSP, based on either sequence analyses or biochemical studies.

The best-studied zinc storage-transport proteins are the metallothioneins, a group of very small, highly conserved proteins with multiple CXC and CC sequences (5). Interestingly, despite the high prevalence of cysteine in the VSPs, CXC and CC sequences are strikingly rare (1, 9, 12, 15). Nonetheless, VSPs could function in the binding and/or transport of zinc or other metal ions. If the main function of VSPs is in metal transport or storage, their abundance suggests that trophozoites require large amounts of the metal. VSPs may function in uptake of iron required by iron-sulfur electron transport chain proteins (23), although this is speculative.

On the basis of occurrence of zinc binding motifs in VSPs and some similarity in symptoms of clinical giardiasis and zinc deficiency, Nash (15) proposed that zinc binding by VSPs could lead to inhibition of a number of important intestinal enzymes. Our study showed that the VSPs can bind zinc, but whether this sequestration could affect the patient is not clear, especially in view of the nonspecificity of metal binding by the VSPs and the fact that the average requirement for dietary zinc is ~15 to 20 mg/day. On the other hand, severe chronic diarrhea caused by giardial infection could lead to metal malabsorption (7).

Since a single zinc ion is bound by multiple cysteine residues and a single cysteine can bind to two zinc ions, a prominent function is to act as bridges, maintaining secondary (intramolecular) or tertiary (intermolecular) structure in proteins, both of which would be disrupted by reduction and removal of the metal (5). Comparison of the migration of VSPs on reducing and nonreducing SDS-PAGE, however, revealed few, if any, dimers or higher multimers, indicating that metal binding by VSPs does not promote dimerization. Because of the tetrahedral geometry, metal bridges could lead to more compact structures (5). Neither we (2) nor others (15) have detected free sulfhydryl groups in VSPs on intact cells, supporting the idea that the cysteine residues are ligated to metal ions and/or in disulfide bonds.

The intestinal lumen can be considered a hostile environment, where trophozoites are exposed to high concentrations of bile salts, which are detergents, as well as to proteases and lipases. Thus, it is possible that metal binding by VSPs may maintain the surface protein in a more compact conformation, shielding potential substrates from proteolysis and protecting the plasmalemma from disruption by bile salts and lipases. We have found trophozoites to be very tolerant of bile salts (8). Nash et al. (17) have shown that *Giardia* strains with different VSPs differ in their susceptibility to trypsin and chymotrypsin. Differences in susceptibility to proteolysis could be related to differences in the location of metal bridges among VSPs. We have observed that native TSA 417 molecules are held together after extensive proteolytic cleavage by bonds that are sensitive to reduction (2), but we do not yet know whether these are disulfide bonds, metal bridges, or both.

We showed that VSPs of *G. lamblia* bind zinc in vitro. Since metal binding requires reduction, it is important to ask whether it can occur in vivo. Future studies will address this question, as well as the specificity and stoichiometry of metal binding and its function for the parasite. The abundance of the VSPs and the prevalence of metal binding motifs among all known variants suggests that they may play an important role in trophozoite survival and therefore in colonization of the human small intestine.

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