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Saric, M; Vahrmann, A; Niebur, D; Kluempers, V; Hehl, A B; Scholze, H
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Abstract

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Dual Acylation Accounts for the Localization of α19-Giardin in the Ventral Flagellum Pair of *Giardia lamblia*

Mirela Šarić, Anke Vahrmann, Daniela Niebur, Verena Kluemper, Adrian B. Hehl, and Henning Scholze

Department of Biology/Chemistry, Biochemistry, University of Osnabrück, D-49069 Osnabrück, Germany, and Institute of Parasitology, University of Zürich, CH-8057 Zürich, Switzerland

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The human pathogenic parasite *Giardia lamblia* (syn. *Giardia intestinalis*), a phylogenetically basal eukaryote (41), is the causative agent of giardiasis, an intestinal disease most prevalent in developing countries (39). The protist has a simple life cycle consisting of two stages, a vegetative trophozoite dwelling in the host intestine and an infective cyst form. Proliferating trophozoites are distinguished by a complex cytoskeleton whose most striking feature are eight flagella and a ventral disk, by which the parasite attaches to the intestinal epithelium of the host (11). As a diplomonad protist, the parasite possesses four different pairs of flagella, of which only the ventral and posterolateral ones are replicated in the first round of the cell cycle; the other two pairs require two further cell divisions for their complete renewal (32).

Besides tubulin, the *Giardia*-specific giardins account for the major protein components of the giardial cytoskeleton (7, 36). From these 30- to 45-kDa proteins, the α-giardin have been recognized, based on sequence similarities, as annexin homologues (28). They represent a multiple set of all-helical protein structures protruding outside the cell body. Ventral Flagellum Pair of *Giardia lamblia*

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Department of Biology/Chemistry, Biochemistry, University of Osnabrück, D-49069 Osnabrück, Germany, and Institute of Parasitology, University of Zürich, CH-8057 Zürich, Switzerland

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Besides tubulin, the *Giardia*-specific giardins account for the major protein components of the giardial cytoskeleton (7, 36). From these 30- to 45-kDa proteins, the α-giardin have been recognized, based on sequence similarities, as annexin homologues (28). They represent a multiple set of all-helical protein structures distinguished by four annexin domains each. We have previously confirmed that some α-giardin genes revealed that 19 members of this family evolved from two widely ramified branches, whereby the genes coding for α14-giardin (annexin E1, according to the annexin nomenclature) and α19-giardin jointly form a single arm (47). α19-Giardin, the subject of the present study, carries a predicted N-terminal sequence extension with an MGCXXS motif known as a target for dual fatty acylation, i.e., myristoylation at the N-terminal glycine and palmitoylation at the cysteine residue (8). However, no experimental data for a lipid conjugation to this protein and any other giardin are currently available.

In the present study, we provide the first evidence that α19-giardin indeed can be both myristoylated and palmitoylated. In contrast to α14-giardin, which we found to be located in all giardial flagella as well as in the median body of the trophozoites (43, 46), α19-giardin appears exclusively localized to the ventral flagella of the trophozoites and is restricted to those portions protruding outside the cell body.

**MATERIALS AND METHODS**

**Cells.** Trophozoites of *G. lamblia* strain WBC6 (ATCC 50803) were cultured in Keister’s modified TYI-S-33 medium (20). Cells were harvested at the end of the logarithmic phase (after 3 to 4 days), washed twice in a buffer composed of 20 mM Tris–150 mM NaCl, pH 6.8 (TBS), and stored at −20°C in the presence of 10 μM (final concentration) trans-epoxyoctanoyl-L-lysylamide (4-
Expression constructs for analysis of the conditional expression and subcellular localization of wild-type and mutant α19-giardin. The complete open reading frame (GL-50803_4026) (27) was PCR amplified from genomic DNA using the oligonucleotide primers Alpha19Gi-HaPacl (antisense) CGT TAA TTA ATC AAG CGT AGT CGG CAT GTG ATG AAG CGC CGG GGG GAG TCG AGG ATTCC and Alpha19Gi-XbaI (sense) CGT CTA GAG GTG CGA CGA ACA CTT TTAG introducing a hemagglutinin (HA) epitope tag at the 3’ end. The −1.450-nucleotide fragment was cloned into the XbaI and PacI restriction sites of the pPacV-integ expression vector (19). The vector was linearized by restriction digestion with Swal and electroporated into trophozoites as described previously (14). Transgenic parasites were selected by resistance against puromycin. Selection was discontinued as soon as the resistant population emerged (5 to 7 days). Alternatively, to increase the signal in immunofluorescence of the substitutITagged (α19Gi-GFP) construct that had lost the ability to localize to the ventral flagellum, the circular plasmid was electroporated and maintained episomally under constant selection. Mutant variants (G2A and C3A) were generated by site-directed mutagenesis of the wild-type gene. A chimeric protein comprised of full-length α19-giardin fused to green fluorescent protein (α19-giardin:GFP) was constructed in the pPacV-integ expression vector.

Confocal laser scanning microscopy. Cells were harvested by cooling and centrifugation at 900 x g for 10 min. Fixation and preparation for fluorescence microscopy were done as described previously (24). Briefly, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 3% formaldehyde in PBS for 40 min at 20°C, followed by a 5-min incubation with 0.1 M glycine in PBS. Cells were permeabilized with 0.2% Triton X-100 for 20 min at room temperature and blocked overnight in 2% BSA in PBS. Incubations of G. lamblia cultures were done with a mouse monoclonal Alexa Fluor 488-conjugated anti-giardin antibody (dilution: 1:30; Roche Diagnostics, Mannheim, Germany) were done in 2% BSA-0.2% Triton X-100 in PBS for 1 h at 4°C. Washes between incubations were done with 0.5% BSA-0.05% Triton X-100 in PBS. Labeled cells were embedded with Vectashield (Vector Laboratories, Inc., Burlingame, CA) containing the DNA intercalating agent 4′,6-diamidino-2-phenylindole (DAPI) for detection of nuclear DNA. Immunofluorescence analysis was performed on a Leica SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with a glyoxeol objective (Leica HCX PL APO CS 63× objective/1.3 numerical aperture Corr). Image stacks were collected with a pinhole setting of 1 Airy unit and twofold oversampling. Image stacks of optical sections were further processed using the Imaris software suite (Bitplane, Zurich, Switzerland).

Live-cell microscopy. For live-cell microscopy, cells expressing the α19-giardin::GFP chimeric protein were harvested at 12 h postinduction and transferred to 24-well plates at a density of 6 x 10⁵/ml. After incubation on ice for 5 to 8 h, oxygenated cells were sealed between microscopy glass slides and warmed to 21°C or 37°C. For fluorescence recovery after photobleaching and time-lapse series, images were collected on a Leica SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) using a water immersion objective (Leica HCX PL APO CS 63× objective/1.2 numerical aperture W Corr). The pinhole was set to 2 Airy units in order to increase the thickness of the optical sections to accommodate the moving ventral flagella in the z-plane. Quantifiable criteria for cell viability were active attachment to substrate and continuous beating of the ventral and anterolateral flagellum pairs. Video and surface rendering of the raw time-lapse series were generated using the Imaris software suite (Bitplane, Zurich, Switzerland).

Membrane association of α19-giardin. Harvested and washed cells from 60-m1 cultures were disrupted by sonication, and the homogenate was centrifuged for 35 min at 100,000 x g and 4°C. The precipitated membrane fraction was resuspended in 100 μl of PBS, and from this 5-μl portions were incubated for 30 min on ice with the following solutions: (i) 100 μl of PBS containing 1% Triton X-100, (ii) 100 μl of the same buffer containing 23 mM EGTA, (iii) 500 μl of 0.2 mM Na₂CO₃, 1 mM EDTA, and 0.5 mg of multiamellar liposomes (brain extract; Sigma) was added to 10 μg of recombinant α19-giardin Δ1-16. The mixture was incubated for 40 min at room temperature under agitation and then centrifuged for 10 min at 15,000 x g. In a parallel experiment, the free Ca²⁺ concentration in the mixture was adjusted to 3.8 mM in control incubations the brain extract was omitted. The reversibility of Ca²⁺-dependent phospholipid binding of α19-giardin was examined via successive additions of EGTA (10 mM) to the pellet fraction. The supernatant and pellet fractions were subjected to SDS-PAGE and Western blotting using anti-α19-giardin antisera.

Indirect immunofluorescence. Trophozoites of G. lamblia were attached to polylysinated coverslips at 37°C, fixed for 7 min with methanol, and permeabilized for 10 min with acetone, both at 20°C. In the following, the cells were rehydrated for 10 min with TBS and then incubated for 30 min with blocking buffer (3% bovine serum albumin [BSA] in TBS). After reacting with the rabbit anti-α19-giardin serum (1:100) or with the anti-giardin antibody (both at 1:500), the samples were washed three times with TBS and then incubated in the dark for 1 h with Cy3-conjugated anti-rabbit F(ab)₂ fragment from sheep (1:100; Sigma) or Cy2-conjugated anti-mouse IgG F(ab)₂ fragment (1:100; Dianova). After three washes with TBS again, the cells were analyzed using a fluorescence microscope (Leica DM 5500 B; Wetzlar, Germany).
and GTT CAC AAG CTT GTC GCC GCG GAG AGT CGA GGT TC (antisense); the amphoteric, after digestion with NdeI-HindIII, was cloned into the pET23a (+) vector encoding a C-terminal His tag (Novagen). The G2A mutant of α19-giardin was produced by site-directed mutagenesis. For this purpose, the construct pET23a(+)-ζi9wt was amplified using the following primers encoding the desired amino acid exchange (underlined): GTT TGT TAG CAT ATG GCC TGT GCC GCA TCA ACT CCC (sense) and GGG AGT TGA TGC GGC ACA GGC CAT ATG CAT ACAA (antisense). Primers used for the amplification of the gene encoding gNMT were designed based on sequence information obtained from the Giardia genome project, sequence CH991783, locus GLS0803_5772: GAA TAA AAA AGC CAG GCT AGC ATG CCT GAT CAC (sense) and GTG TTT TCT ATT CTG CAG TCA TAT CAA CAC GAC (antisense). The amplification product, after digestion with Nhel and Xho, was cloned into pET23d(+).

Coexpression of recombinant NMT and α19-giardin in E. coli. E. coli BL21(DE3)pLysS cells were cotransformed with pET23a(α19wt) and pET23a(α19G2A) and pET24d(gmut), either singly or in combinations. Cultures (2 ml) were grown to an optical density of 600 nm at 0.5 in LB medium containing the appropriate antibiotics, and expression of recombinant proteins was induced by the addition of 1 mM isopropyl-thio-galactoside (IPTG). Immediately afterwards, the cultures were subdivided into two 1-ml aliquots; one aliquot contained 100 μl of [9,10(-3H]myristate (GE Healthcare) in 6.6 μl of ethanol, and the other contained 6.6 μl of ethanol alone (23). The cells were then incubated at 37°C for a 3-h period, harvested (10,000 g for 5 min), and washed three times each with 1 ml of PBS. Bacteria were recovered by centrifugation, resuspended in 0.1 ml of cracking buffer (4% SDS–0.08 M Tris, pH 6.8), and lysed by ultrasonication for 5 min. Cellular debris were removed by spinning the lysate at 10,000 g for 5 min. The amount of protein in the supernatant was determined using a bichinonic acid kit (Pierce). Samples containing 100 μg of total protein were boiled after the addition of SDS sample buffer and separated by electrophoresis through a 12% SDS-polyacrylamide gel. The gels were treated with 2,5-diphenyloxazole (Fluka), dried in vacuo, and exposed to Konica-Minolta X-ray film at –80°C for 24 to 48 h.

Palmitoylation assays. The detection of palmitoylation of α19-giardin was performed using a yeast system containing the palmitoyl transferase gene PFA3 under the control of a galactose promoter as described by Hou et al. (18). For this purpose, the yeast strain CUY2171 (aα α leu2 trp1 ura3-52 prb1-122 pep4-3) was induced by the addition of 1 mM isopropyl-thio-galactoside (IPTG) and grown in SDC-Ura medium. Confocal fluorescence microscopy was performed using a Leica DM5500 fluorescence microscope (Leica AG, Solms, Germany). The biotinylation assay was conducted according to Hou et al. (17). Briefly, yeast cells were broken in a buffer containing 1% Triton X-100 and free thiol groups were quenched by the addition of 25 mM al. (17). Briefly, yeast cells were broken in a buffer containing 1% Triton X-100 and free thiol groups were quenched by the addition of 25 mM al. (17).

RESULTS

Evidence for the expression of α19-giardin in trophozoites of G. lamblia. As a first approach to provide evidence for the existence of a transcription product of the α19-giardin gene in trophozoites of G. lamblia, we performed RT-PCR on first-strand cDNA using primers constructed according to sequence information from the Giardia genome project (27). This way, we got a clear signal with the expected size of ~1.3 kb in an agarose gel (Fig. 1A). Control sequencing of the amplification product revealed the predicted N-terminal sequence, MGC AAS, which serves as a signature sequence for dual acylation (2). As derived from its complete nucleotide sequence, α19-giardin consists of 438 amino acid residues with a calculated molecular mass of 47.7 kDa and a weakly acidic isoelectric point of 5.5. We cloned a gene fragment encoding an N-terminally truncated α19-giardin that included the complete four annexin repeats but lacked the potential acylation motif (see below). We heterologously overexpressed this gene in E. coli strain BL21 and used the affinity chromatographically purified recombinant protein as an antigen for raising polyclonal antibodies in rabbits. In a Western blot assay employing a trophozoite extract, the antiserum reacted with a protein of about 47 kDa mainly from the pellet fraction (Fig. 1B), suggesting that native α19-giardin is probably membrane associated in the trophozoites.

α19-Giardia resides in the ventral flagella. As reported previously, the most closely related α14-giardin localizes to all flagella and, to a smaller extent, is found in the median body of the trophozoites (43, 46, 47). To find out whether α19-giardin is also immunologically detectable in these organelles, we mechanically detached all the flagella from the cell body and separated them from cellular debris by density gradient centrifugation in a Percoll gradient (46). In immunoblots of this flagellar protein fraction, strong signals emerged when antibodies were directed against the transaldolase Tali as a marker for a soluble protein (40) and the ATPase subunit Vmat6 as a marker for the vacuolar fraction (kindly provided by C. Ungermann) were employed.
indicated that the α19-giardin protein emerged solely from the ventral flagella, whereas the antitubulin antibody recognized all the flagella (46). Yellow signals indicating colocalization in merged images conspicuously occurred solely at the distal part of the ventral flagella, suggesting that α19-giardin is located only in that part of the axonema protruding outside the cell body. Arrest of the cell cycle in G1 by subsequent treatment with nocodazole and aphidicolin according to the protocol of Poxleitner et al. (37) revealed that α19-giardin permanently remains in the ventral flagella during cell division (Fig. 2B).

**Membrane association of α19-giardin.** Of all the sequences of the α-giardin in the Giardia genome data base, α19-giardin is the only one containing an N-terminal signature sequence for dual lipid conjugation with myristate and palmitate (30). Correspondingly, α19-giardin appeared in the membrane fraction after centrifugation of a trophozoite homogenate (Fig. 1B). To analyze the strength and mode of the membrane association, we subjected the pellet fraction of a crude cell extract to extraction experiments with sodium carbonate, sodium chloride, urea, and Triton X-100. This method serves as a standard protocol to assess the membrane association of a protein via a lipid anchor (45). Analyses of the samples by SDS-PAGE as shown in Fig. 3A revealed that only the non-ionic detergent Triton X-100 was able to release α19-giardin into the soluble fraction in significant amounts. This observation suggests that α19-giardin behaves like an integral membrane protein because neither unspecific adsorption nor precipitation of unfolded protein is apparently responsible for its membrane association. Moreover, in a partition experiment with Triton X-114, we recovered the native protein in the detergent phase although a portion with lower molecular mass emerged in the aqueous phase as well (Fig. 3A). This may be due to peptidolytic splitting off of the N terminus containing the attached fatty acid moiety through a temperature shift to 30°C that provokes a phase separation during this experiment. A typical behavior of annexins from higher eukaryotes is their capability to associate with negatively charged phospholipids in the presence of Ca\(^{2+}\) ions. In order to assess whether α19-
giardin likewise possesses this characteristic property of annexins, we performed phospholipid-binding assays using a bovine brain extract according to the method of Boustead et al. (3). Because the full-length recombinant α19-giardin was insoluble in detergent-free aqueous solutions, we employed the N-terminally truncated recombinant protein mentioned above in these experiments. Western blot analyses showed that the protein associated with the phospholipids in the presence of Ca\(^{2+}\) ions (Fig. 3B). In the absence of free Ca\(^{2+}\), the protein remained in the supernatant fraction, in both the presence and absence of phospholipids. After addition of the Ca\(^{2+}\)-chelator EGTA, the bound protein detached from the phospholipids, which demonstrates that this mode of phospholipid association, like that of annexins, is reversible.

**Myristoylation of α19-giardin.** Both the N-terminal motif and the biochemical properties of α19-giardin argue for the existence of a lipid anchor on α19-giardin. To test directly the acylation of α19-giardin with a myristoyl moiety, we employed a dual expression system in which we cotransformed an appropriate expression strain of *E. coli* with different expression vectors, with one encoding gNMT and the other one encoding wild-type α19-giardin and the G2A mutant. Protein expression was induced by isopropyl-β-D-thiogalactopyranoside in the presence of [9,10(n)-\(^3\)H]myristate (4), and the products were analyzed by both immunoblotting and fluorography. As demonstrated by Western blotting, the expression rates of the α19-giardin genes used in all samples were stable and comparable in amounts. However, in the fluorographs the incorporation of the α19-giardin genes used in all samples were stable and comparable in amounts. However, in the fluorographs the incorporation of the α19-giardin gene (Fig. 4). Neither omission of the gNMT nor the use of the G2A mutant as a reaction partner of the gNMT resulted in a signal. This clearly proves the N terminus of α19-giardin to be a signature sequence for an acylation with a myristoyl moiety. To test whether the post-translational modification of the glycine in position 2 with a myristoyl group is necessary for the characteristic localization of α19-giardin, we mutated this position to alanine (G2A) in the construct α19-giardin-HA. Transgenic parasites expressing a single integrated copy of the α19-giardin-HA (G2A) gene by the pPacV-integ expression vector did not show a detectable signal in immunofluorescence microscopy (data not shown), whereas the C-terminally HA-tagged wild-type α19-giardin showed the expected exclusive localization in the ventral pair of flagella (Fig. 5A). Additional localization to other flagellum types could be observed only in transgenic parasites with strong overexpression due to multiple copies if the circular plasmid was maintained episomally (data not shown). In the same way, we overexpressed the mutant. These cells showed a completely cytoplasmic localization of α19-giardin-HA mutated in this single position, consistent with abrogation of the specific targeting of this variant (Fig. 5B). To test whether localization of a tagged copy of α19-giardin impaired the function of the ventral flagella, we expressed an α19-giardin::GFP chimera in transgenic trophozoites. The cells showed a flagellar localiza-

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**FIG. 4.** Evidence of a myristoylation of α19-giardin. Western blot (A) and autoradiograms (B) of *E. coli* extracts after heterologous production of wild type α19-giardin (α19WT) and α19-giardin (G2A) (α19G2A), with or without gNMT in the presence of [\(^3\)H]myristate. Western blots were immunodecorated with α19-giardin antiserum.

**FIG. 5.** Expression analysis of α19-giardin in transgenic trophozoites. (A) Expression of a single integrated copy of the α19-giardin-HA-tagged gene (wild type) product in transgenic cells with a chromosomally integrated copy of the pPacV-integ expression vector. Note the highly restricted localization of the protein (green) to the ventral flagellum pair. A total projection of the entire image stack (62 optical sections) is shown. (B) Overexpression of the α19-giardin-HA (G2A) mutant variant (green) in transgenic cells reveals its cytoplasmic distribution. A total projection of the entire image stack (46 optical sections) is shown. Nuclear DNA is stained blue with DAPI. DIC, bright-field (BF) differential interference contrast image. (C) Single-frame of a time-lapse series of an actively attached cell expressing the α19-giardin::GFP reporter (green) localized in the ventral flagella. Note the typical wave form of the beating flagellum pair. The corresponding bright-field image is shown in the right panel. Scale bar, 10 μm. (See also supplemental material.)
tion identical to that of the HA-tagged variant (Fig. 5C), and beating of the ventral flagellum pair was unimpaired.

**Palmitoylation of α19-giardin.** Palmitoylation of proteins is a reversible process, which may be why we were unable to detect this modification directly in *Giardia* lysates. To test whether the α19-giardin molecule is a substrate of a palmitoyl transferase, we transfected the yeast strain CUY2171 with a GFP construct encoding the wild-type and a C3A mutant of α19-giardin. This yeast strain overexpresses the palmitoyl transferase Pfa3, which is known to add palmitoyl residues to its target proteins with a broad specificity and independently of previous myristoylation (18). As shown in the fluorescence micrographs of Fig. 6A, the wild-type α19-giardin associated with the membrane of the main vacuole in the presence of Pfa3, whereas in the absence of Pfa3 it distributed over the entire cytosol. This result is supported by cell fractionation: α19-giardin in the presence of Pfa3 occurred in the vacuolar fraction, whereas in the absence of Pfa3 it remained in the supernatant after centrifugation (Fig. 6B). The C3A mutant also delocalized throughout the whole cytosol in both the presence and absence of Pfa3. This finding is consistent with the results of a biotin switch experiment. Here, all free thiol groups of the yeast lysate were quenched with N-ethylmaleimide, and the remaining thioesters were cleaved by hydroxylamine. The liberated thiol groups were coupled to biotin, and the biotinylated protein was captured by a neutravidin pull-down assay (17). As shown in Fig. 6C, wild-type α19-giardin was palmitoylated in the presence of Pfa3. These findings clearly verify that α19-giardin is a substrate for this rather unspecific palmitoyl transferase activity.

**DISCUSSION**

In this study, we report on a novel annexin-homologous protein, α19-giardin, one of 21 α-giardins of the human pathogenic parasite *G. lamblia*. Although the overall sequence identities among the α-giardins (varying from 15.3% to 19.6%) are rather low, the presence of four homologous annexin-like domains supports their common evolutionary ancestry (29). α19-Giardin differs from most other giardins in carrying an N-terminal sequence extension that comprises 34 residues prior to the start of the first annexin repeat (see protein sequence in the NCBI GenBank, accession number AY781315). This is the largest sequence extension within α-giardins, such as α1-giardin and α2-giardin (2 residues) or α14-giardin (23 residues), and it is larger than that of human annexin A5 (15 residues). Furthermore, the α19-giardin molecule contains a C-terminal stretch that extends the fourth annexin repeat for about 70 residues; the importance of this part of the molecule is still unclear. However, both an integrin binding motif (RGD motif)
and a glycosylation site (NDT) in this part of the molecule may argue for an extracellular function; whether and how the protein may arrive at the exterior of the cell has not yet been discovered, however. Recently published tertiary structure determinations of α11-giardin and α14-giardin by X-ray crystallography support the notion that the α-giardins fold to all-helical structures typical for annexins of other eukaryotes (9, 34, 35).

The α19-giardin molecule starts with the N terminus MG CXXS that in many proteins serves as a signature sequence for dual lipid conjugation with a myristoyl group at the N-terminal glycine (after cleavage of the methionine residue) and a palmitoyl moiety at the following cysteine residue (38). According to our database searches, α19-giardin is the only annexin, besides human annexin A13, that contains a myristoyl moiety and, as far as we know, the only annexin homolog at all that contains a dual lipid conjugation motif. Other examples of proteins with dual lipid conjugations near their N termini are the Src protein kinases and the guanine nucleotide-binding protein-α (Gα) subunits (2). The attachment of a myristoyl residue to the N-terminal glycine occurs cotranslationally and is generally catalyzed by an NMT. By database searches, we found a single gene in the Giardia genome encoding a putative NMT. We amplified and cloned this gene and showed by heterologous coexpression with the α19-giardin gene in the presence of [9,10(n)3H]myristate that this giardin indeed can be acylated by gNMT. The same result was obtained when the gene encoding human NMT1 was employed (data not shown), supporting the notion that the first six amino acid residues are most important because their fitting into the enzyme’s substrate binding pocket is a prerequisite for the acylation reaction (26).

Palmitoylation of proteins is a posttranslational, enzymatic process catalyzed by the so-called DHHC-cysteine rich-domain family of proteins, which apparently are involved in trafficking events within a cell (2). The reversibility of this reaction could be the reason why we were unable to detect this modification in Giardia extracts. However, in an experimental approach in which we coexpressed the α19-giardin gene together with the Pfa3 gene from yeast, a gene encoding a palmitoyl transferase with relatively broad substrate specificity (18), α19-giardin changed its localization and associated with vacuolar membranes, which suggests palmitoylation under particular physiological conditions. According to our database searches, the genome of Giardia contains nine genes encoding putative palmitoyl acyl transferases (PATs). One of them, designated gPAT, catalyzes the palmitoylation of the variant surface protein VSPH7 in the trophozoites (33, 44). The substrates of the other giardial PATs, including those named gDHHC2 and gDHHC3, are still unknown. One of their target proteins could be α19-giardin.

The expression of a vast number of α-giardins in trophozoites of G. lamblia and their putative subcellular localizations have already been reported by Weiland et al. (47). However, statements concerning their specific functions are rather speculative. As annexin homologs, these proteins may establish a connection between the plasma membrane and the cytoskeleton. In the case of α19-giardin, its exclusive location in the plasma membrane covering the ventral flagella argues for an involvement of this protein in the specific tasks of these flageullum types. Particular functions of the ventral flagella are supposed to be the anchoring of the parasite to the intestinal epithelium of the host and providing it with nutrients from the intestinal lumen (7). For instance, during attachment of the parasite to the intestinal wall, only the ventral pair, which emerges from the posterior end of the disk, continuously beats in an approximately sinusoidal swinging movement in the plane of the disk. This action may generate negative pressure below the contact zone of the ventral disk at the intestinal epithelium and, like a suction cup, fix the trophozoites to the intestinal wall (16). The fluid transport generated by the ventral flagella may then sweep matter under the body of the parasite, providing it with nutrients (10). These particular functions of the ventral flagella may be directly concerned with the local occurrence of the α19-giardin.

Generally, the components of flagella are synthesized in the cytoplasm and transported as large proteinaceous particles to the distal areas of the flagella. Assembly and maintenance of these continuous movements from the cytosol to the flagella involves proteins that mediate the transport of transport vesicles and their fusion with the apical membrane (22). Likewise, the flagellar calcium binding protein, FCaBP, from Trypanosoma cruzi, the trigger of Chagas disease, possesses two fatty acid modifications, with myristoyl and palmitoyl residues, in an N-terminal stretch. Interestingly, the specific targeting of this protein to the flagellum is a calcium-dependent process (12). We could not yet prove a direct influence of calcium on the localization of α19-giardin in the ventral flagella, but our finding that the N-terminally truncated protein reversibly binds to phospholipids in a Ca2+-dependent manner may implicate Ca2+ in the specific targeting of α19-giardin to the ventral flagella prior to its palmitoylation. This reversible binding may ensure that the α19-giardin molecule, despite the complete reorganization of the flagella during cell division, remains in the ventral flagella throughout the cell cycle, as indicated in our cell synchronization experiments (32, 37).

Taken together, our data suggest that the annexin-homologous α19-giardin from trophozoites of G. lamblia is dually acylated. These nonproteinaceous modifications may help to fulfill two functions in this intestinal parasite: (i) anchoring of cytoskeletal proteins to distinct sites at the plasma membrane within the ventral flagella and (ii) involvement of α19-giardin in intracellular transport processes of proteins to their functional sites. By this means, the α19-giardin protein may take on a role as a specific mediator between the cytoskeleton and plasma membrane, particularly in the ventral flagella. A deeper insight into the biochemical properties of α19-giardin...
and its dynamic within the cells should shed light on its actual intracellular function.

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REFERENCES

1. Bauer, B., S. Engelbrecht, T. Bakker-Grunwald, and H. Scholze. 1999. Functional identification of alpha 1-giardins as an annexin of Giardia lamblia. FEMS Microbiol. Lett. 173:147–153.
2. Bijlmakers, M. J., and M. Marsh. 2003. The on-off story of protein palmitoylation. Trends Cell Biol. 13:32–42.
3. Bousted, C. M., R. Brown, and J. H. Walker. 1993. Isolation, characterisation and localization of annexin V from chicken liver. Biochem. J. 291:601–608.
4. Breurer, S., H. Gerlach, B. Kolaric, C. Urbanke, N. Opitz, and M. Geyer. 2000. Biochemical identification of functional palmitoyl-dependent acyltransferase changes in HIV-1 Nef. Biochemistry 45:2339–2349.
5. Clos, J., and S. Brandau. 1994. pIC20 and pIC40—two high-copy-number vectors for T7 RNA polymerase-dependent expression of recombinant genes in E. coli. Ph.D. thesis, Freiburg. 513 pp.
6. Douglas, M., D. Finkelstein, and R. A. Butow. 1974. Attachment of Giardia lamblia to hamster intestinal epithelium by a calcium-myristoyl/palmitoyl switch mechanism. EMBO J. 18:2097–2065.
7. Hou, H., D. S. Reiner, and J. M. McCaffrey. 1996. Cell biology of the eukaryotic flagellum. Annu. Rev. Microbiol. 50:679–705.
8. Godsel, L. M., and D. M. Engman. 1999. Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism. EMBO J. 18:3231–3237.
9. Ghosh, S., M. Frisardi, R. Rogers, and J. Samuelson. 1999. How Giardia swim and divide. Infect. Immun. 69:7866–7872.
10. Gillin, F. D., D. S. Reiner, and J. M. McCaffrey. 1996. Cell biology of the eukaryotic flagellum. Annu. Rev. Microbiol. 50:679–705.
11. Hou, H., K. Subramanian, T. J. LaGrassa, D. Markgraf, L. E. Dietrich, J. Urban, N. Decker, and C. Ungermann. 2005. The DHHC protein Pfa3 affects vacuole-associated palmitoylation of the fusion factor Vac8. Proc. Natl. Acad. Sci. USA 102:17366–17371.
12. Hou, H., A. T. Peter, C. Meiringer, K. Subramanian, and C. Ungermann. 2009. Analysis of DHHC acyltransferases implies overlapping substrate specificity and a two-step reaction mechanism. Traffic 10:1061–1073.
13. Jimenez-Garcia, L. F., G. Zavaleta, B. Chavez-Munguia, P. Ramos-Godinez, S. S. Aley, R. D. Adam, F. D. Gillin, and M. L. Sogin. 2000. The Giardia genome project database. FEMS Microbiol. Lett. 189:271–273.
14. Morgan, R. O., and M. P. Fernandez. 1995. Molecular phylogeny of annexins and identification of a primitive homologue in Giardia lamblia. J. Mol. Evol. 12:967–979.
15. Morgan, R. O., and M. P. Fernandez. 1997. Distinct annexin subfamilies in plants and protists diverged prior to animal annexins and from a common ancestor. J. Mol. Evol. 44:178–188.
16. Morrison, H. G., A. G. McArthur, M. D. Gillin, S. B. Aley, R. D. Adam, G. J. Olsen, et al. 2007. Genomic minimalism in the early diverging intestinal parasite Giardia lamblia (Giardia intestinalis). Mol. Biol. Evol. 24:1921–1926.
17. Neufeld, V., N. Arold, D. Taube, and W. Ehrhardt. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis 9:255–262.
18. Nohynkova, E., P. Tumova, and J. Kulda. 2006. Cell division of Giardia intestinalis: flagellar developmental cycle involves transformation and Exchange of flagella between mastergins of a diplomonal cell. Eukaryot. Cell 5:373–376.
19. Papanastassiou, P., M. J. McConville, J. Ralton, and P. Kohler. 1997. The variant-specific surface protein of Giardia, VSP4A1, is a glycosylated and palmitoylated protein. Biochem. J. 322:49–56.
20. Pathuri, P., E. T. Nguyen, S. G. Svárd, and H. Lagee. 2007. Apo and calcium-bound crystal structures of alpha-11 giardin, an unusual annexin from pathogenic fungi: Crytococcus neoformans, Histoplasma capsulatum, and Candida albicans. J. Biol. Chem. 282:2996–3009.
21. Marti, M., Y. Li, F. M. Schranner, P. Wild, P. Kohler, and A. B. Hehl. 2003. The secretory apparatus of an ancient eukaryote: protein sorting to separate export pathways occurs before formation of transient Golgi-like compartments. Mol. Biol. Cell 14:1433–1447.
22. Mascaró, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035–10038.
23. McArthur, A. G., H. G. Morrison, J. E. Nixon, N. Q. Passamanack, U. Kim, G. Hinkle, M. K. Crocker, M. E. Holder, R. Farr, C. I. Reich, G. E. Olsen, S. B. Aley, R. D. Adam, F. D. Gillin, and M. L. Sogin. 2000. The Giardia genome project database. FEMS Microbiol. Lett. 189:271–273.
24. Morgan, R. O., and M. P. Fernandez. 1997. Distinct annexin subfamilies in plants and protists diverged prior to animal annexins and from a common ancestor. J. Mol. Evol. 44:178–188.
25. Morrison, H. G., A. G. McArthur, M. D. Gillin, S. B. Aley, R. D. Adam, G. J. Olsen, et al. 2007. Genomic minimalism in the early diverging intestinal parasite Giardia lamblia (Giardia intestinalis). Mol. Biol. Evol. 24:1921–1926.