The Giardia duodenalis 14-3-3 Protein Is Post-translationally Modified by Phosphorylation and Polyglycylation of the C-terminal Tail*

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The flagellated protozoan Giardia duodenalis (syn. lambia or intestinalis) has been chosen as a model parasite to further investigate the multifunctional 14-3-3s, a family of highly conserved eukaryotic proteins involved in many cellular processes, such as cell cycle, differentiation, apoptosis, and signal transduction pathways. We confirmed the presence of a single 14-3-3 homolog gene (g14-3-3) by an in silico screening of the complete genome of Giardia, and we demonstrated its constitutive transcription throughout the life stages of the parasite. We cloned and expressed the g14-3-3 in bacteria, and by protein-protein interaction assays we demonstrated that it is a functional 14-3-3. Using an anti-peptide antibody raised against a unique 18-aa amino acid sequence at the N terminus, we observed variations both in the intracellular localization and in the molecular size of the native g14-3-3 during the conversion of Giardia from trophozoites to the cyst stage. An affinity chromatography, based on the 14-3-3 binding to the polypeptide difopien, was set to purify the native g14-3-3. By matrix-assisted laser desorption ionization mass spectroscopy analysis, we showed that polyglycylation, an unusual post-translational modification described only for tubulin, occurred at the extreme C terminus of the native g14-3-3 on Glu246, Glu247, or both and that the Thr214, located in the loop between helices 8 and 9, is phosphorylated. We propose that the addition of the polyglycine chain can promote the binding of g14-3-3 to alternative ligands and that the differential rate of polyglycylation/deglycylation during the encystation process can act as a novel mechanism to regulate the intracellular localization of g14-3-3.

14-3-3s are a family of highly conserved dimeric proteins with an approximate molecular mass of 30 kDa. With the exception of prokaryotes, they have been found in all eukaryotes studied to date, including protozoa, yeasts, plants, and animals (1). Generally, unicellular organisms have either one or two 14-3-3s (e.g. the slime mold Dictyostelium discoideum has one, and the yeast Saccharomyces cerevisiae has two), whereas multicellular organisms contain several. Mammals, including Homo sapiens, contain seven 14-3-3 genes (i.e. β, γ, ε, σ, ζ, τ, η), and 15 genes coding for 14-3-3s have been identified in the plant Arabidopsis thaliana. 14-3-3 homologs have also been found in several protozoan and metazoan parasites (1, 2). The crystal structure of both animal and plant 14-3-3s has been shown to be conserved. The monomers, consisting of nine α-helices organized in a cup-like shape, are able to interact by their N-terminal portions to form U-shaped dimers (3–5).

The main property of 14-3-3s is their ability to bind other proteins containing a consensus Ser/Thr-phosphorylated binding sequence by the interaction with conserved residues located in the amphipathic groove of each monomer (6, 7). 14-3-3s were first described in mammals as activators of the brain enzymes tyrosine and tryptophan hydroxylases (8). To date, 14-3-3 interactors, identified in both animal and plant cells, have been shown to include enzymes, transcriptional factors, and structural proteins (9–11). The phosphorylation (or dephosphorylation) of the 14-3-3 ligands determines their intracellular localization, complex formation, enzyme activation, and conformational changes through the association or dissociation with 14-3-3s.

With regard to protozoan parasites, only limited information is available on 14-3-3s. Preliminary studies, which have mainly focused on expression and localization, have been conducted on Plasmodium falciparum, Plasmodium knowlesi, Toxoplasma gondii, and Eimeria tenella, and the results have suggested that 14-3-3s play a role in the transmission of the regulatory signals involved in a wide array of biological processes, including the proliferation and migration of the parasite during infection (2, 12, 13).

To date, no analyses of 14-3-3s have been performed for Giardia duodenalis. The flagellate protozoan G. duodenalis (syn. lambia or intestinalis), which parasitizes the upper part of the small intestine of mammals, including humans, is one of the major causes of non-bacterial diarrhea worldwide (14). This parasite belongs to the order Diplomonadidae, the earliest known branching eukaryotic lineage. Giardia is an “ancient” eukaryote, as inferred from the phylogenetic analysis of different genes and proteins (15, 16) and from the finding that it retains many prokaryotic characteristics, such as the size of the small subunit rRNA and the presence of bacterial-like metabolic enzymes. Moreover, it lacks typical eukaryotic structures (i.e. peroxisomes) and has a reduced Golgi apparatus and mitochondrial remnant organelles (known as “mitosomes”), probably as an adaptation to parasitism (17). The Giardia life cycle consists of two main stages, (i) the trophozoite, a teardrop-shaped binucleated cell that colonizes the host intestine and reproduces through binary fission, and (ii) the cyst, an infective stage that is able to survive in the external environment. Given that the life cycle can be reproduced in vitro and that the entire genome is available (18), Giardia is a suitable model for analyzing the functions of 14-3-3s in eukaryotes and the peculiar role of this class of protein in parasites.

In the present study we describe the cloning and expression of the
14-3-3 protein homolog of *G. duodenalis*. The recombinant 14-3-3 was biochemically characterized, and the expression and intracellular localization of the 14-3-3 protein were studied during the parasite life stages using specific antibodies. Moreover, mass spectroscopy analysis of the purified protein demonstrated that the *Giardia* 14-3-3 is phosphorylated and, for the first time, that it is a target for polyglycylation.

**MATERIALS AND METHODS**

Chemicals—[γ-32P]ATP and [α-32P]dCTP (specific activities, 110 TBg/mmol) were from MP Biomedicals (Irvine, CA); PreScission protease was from Amersham Biosciences. The catalytic subunit of protein kinase A, dithiothreitol and iodoacetamide were from Sigma. The soluble Rail259p phosphopeptidase, LSQRQRST(pS)TPNVHVMV (pS, phosphoserine) was synthesized, and the anti-g14-3-3 polyclonal antibody was produced by NeoMPS (Strasbourg, France). Chemicals for gel electrophoresis were from Bio-Rad. All other reagents were of analytical grade.

**Cell Culture and Differentiation—** Trophozoites of the *G. duodenalis* WB-C6 strain were axenically grown for 72 h at 37 °C in TYI-S-33 medium supplemented with 10% bovine serum and bovine bile at pH 7.0. Encystation was induced essentially as described (19). Briefly, the cells were grown until confluency, and the medium was replaced with TYI-S-33 supplemented with 10% bovine serum and 10 mg/ml bovine bile at pH 7.8 and then incubated at 37 °C for the indicated time as shown in the figures.

**Nucleic Acid Isolation—** Genomic DNA was isolated from 107 cells of *G. duodenalis* using the phenol/chloroform extraction method. Total RNA was extracted from 105 trophozoites, or encysting parasites, using the RNAeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Plasmid DNA was isolated from bacteria using the QIAPrep kit (Qiagen).

**PCR Amplification—** The sequence encoding for the g14-3-3 protein without any intron was amplified directly from the genomic DNA of the WB-C6 isolate using the primers Gd14fors (5′-gagacgagctgcagggattcagctcgt-3′) and Gd14rev (5′-gaactcctacatctctggcattgcgtc-3′), designed to anneal with, respectively, the ATG and the stop codons of the coding sequence (the BamHI site is underlined, and the phosphorylation site coding sequence is in italics) and the recombinant 14-3-3 was phosphorylated, as described by Sambrook et al. (20), using specific probes previously labeled with [α-32P]dCTP using the RadPrime DNA labeling system kit (Invitrogen) following the manufacturer’s instructions.

**Vector Construction—** The g14-3-3 coding sequence was amplified by PCR as described above. For overlay experiments, a short sequence, coding for a protein kinase A phosphorylation site, was introduced by PCR at the 5′ end of the g14-3-3 coding sequence using the designed primers Gd14PKfors (5′-tgtctttcctctgcttgatgcggaccattgcgtc-3′) and Gd14rev to produce the modified gPK14-3-3 protein. The PCR conditions were the same as those described for g14-3-3 amplification. Both fragments were cloned in the BamHI and EcoRI sites of the pGEX6-P1 vector (Amersham Biosciences) in-frame with the glutathione S-transferase (GST) and introduced in *Escherichia coli* JM109 competent cells. The cloned fragments were verified by DNA sequencing. The plasmids were named pPK14-X and pPK14-3-3, respectively.

The mutants PK-K53E and T214A were obtained by site-directed mutagenesis of the g14-3-3 coding sequence using the QuickChange site-directed mutagenesis methods (Stratagen, La Jolla, CA). The plasmid pPK14-X was used as template to produce the PK-K53E mutant, and the plasmid pPK14-3-3 was used as a template for the T214A mutant. The primers used for mutagenesis were: K53Efors (5′-ctgctttcctctgcttgatgcggaccattgcgtc-3′) and K53Erev (5′-ggcgggcggacatctctggcattgcgtc-3′) (the mutated triplets are underlined). After 18 cycles of PCR (95 °C for 30 s, 53 °C for 1 min, and 68 °C for 8 min), 10 units of DpnI were added to the mixture to digest the templates, and the reactions were carried out at 37 °C for 2 h. Twenty μl of each reaction mixture was used to transform *E. coli* JM109 competent cells. The presence of the mutations was determined by DNA sequencing.

The plasmid pSCM138A, containing the difopein coding sequence fused to the enhanced green fluorescent protein (21), was kindly provided by Dr. Haian Fu. A 169-bp EcoRI difopein fragment was excised from the plasmid and cloned into the EcoRI-digested and dephosphorylated pGEX-6P1 vector in-frame with the GST coding sequence to produce the plasmid pDIF-X.

**Expression and Purification of the Recombinant Proteins—** E. coli-transformed cells were grown in SOB medium (2% bacto tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM MgCl2, pH 7.2) at 37 °C for 16 h following the manufacturer’s instructions.

**Protein Preparation—** Total proteins from soluble and membrane fractions were prepared according to the method of Moss et al. (22), with minor modifications. Briefly, 2 × 109 trophozoites or encysting trophozoites were collected by chilling on ice and washed 3 times with cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.2), and the cell pellet was frozen at −70 °C overnight. Cells were resuspended in 2 volumes of extraction buffer (30 mM Tris-HCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.5) at 4 °C for 16 h following the manufacturer’s instructions.
a protease-inhibitor mixture (P8340, Sigma) and a phosphatase-inhibitor mixture (P2850, Sigma), and then destroyed by sonication (5 times for 30 s at 60% power and 10% duty cycle) with a Sonopuls ultrasonic homogenizer (Bandelin electronic, Berlin, Germany). The lysate was centrifuged at 24,000 × g for 30 min at 4 °C, and the supernatant was collected and designated as soluble cytosolic fraction. The sediment containing the membranous material was washed twice with cold PBS and centrifuged both times for 30 min at 24,000 × g at 4 °C. The pellet was then resuspended in 3 ml of 8 M urea, 0.05 M Tris-HCl, 0.3 M KCl, and 0.002 M EDTA, pH 8.0, and constantly stirred at 4 °C overnight. Further solubilization was achieved by sonication (5 times for 30 s at 50% power and 20% duty cycle), and centrifugation was performed at 24,000 × g for 30 min at 4 °C. The supernatant was collected, and the urea was removed by diafiltration against TBE (40 mM Tris, 54 mM boric acid, 1 mM EDTA, pH 8.3) using a PM-5 membrane and then concentrated using Centricon 10 (Millipore Corp., Bedford, MA). The protein concentration was measured with the method of Bradford (52) (Bio-Rad), and the material was stored at −70 °C.

**Western Blot Analysis**—Proteins were separated on SDS-PAGE (23) and transferred onto polyvinylidene difluoride membranes with 39 mM glycine, 48 mM Tris, 0.1% SDS, and 10% methanol membrane using a semidry apparatus (Bio-Rad). Membranes were blocked with 5% nonfat dried milk in T-TBS (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) for 1 h and then incubated with the primary antibody (Ab) in 3% non-fat dried milk/T-TBS. After incubation with an appropriate horseradish peroxidase-conjugated secondary Ab (1:2000–1:4000), the antibody/antigen interaction was revealed with the ECL system (Amer sham Biosciences). The anti-g14-3-3 polyclonal Ab was produced in rabbits against the g14-3-3 N-terminal peptide (EAFREDYVFMAQL-NENA), and the serum (N14) was used at a 1:4000 dilution, whereas the AXO49 mouse monoclonal Ab, kindly provided by Dr. M.H. Bré, was used at a 1:2000 dilution.

**Overlay Assay**—The overlay assay was carried out according to Lalle et al. (24). GST-gPK14-3-3 and the GST-PK-K53E mutant were labeled with [γ32P]ATP on a CAMP-dependent protein kinase phosphorylation site using the protein kinase A catalytic subunit and then released from the GST by cleavage with the PreScission protease. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane with 25 mM Tris, 192 mM glycine, and 0.01% SDS, pH 8.3, and blocked for 1 h with 5% nonfat dried milk in HT buffer (20 mM Hepes-KOH pH 7.6, 75 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, 0.04% Tween 20). The membrane was incubated at 4 °C overnight in HT buffer with 3% nonfat dried milk and with 1 μg/ml 32P-labeled gPK14-3-3 or PK-K53E (9 kBq/ml). Where indicated, 100 μM concentrations of the soluble phosphopeptide Raf259p (LSQRQRST(pS)TPPNVHM), reproducing one of the 14-3-3 binding motifs of the human Raf-1 protein, was added during the incubation with 32P-gPK14-3-3. After thorough washing with HT buffer, the interaction was revealed by autoradiography. For Pep-spot binding assay, kindly provided by Drs. G. Cesareni and S. Panni, 100 nmol/cm2 of phosphorylated and unphosphorylated forms of the Raf259 and Raf621 peptides were directly synthesized on cellulose-(3-amino-2-hydroxy-propyl) ether membranes, and after pre-washing the filter with 100% ethanol and HT buffer, the overlay assay was performed as described above.

**Immunofluorescence Microscopy**—Trophozoites or encysting cells were collected and fixed at 37 °C with 4% formaldehyde in PBS for 30 min. Fixed cells were stored in PBS at 4 °C until permeabilization, or they were immediately permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min and then spotted on slides. Slides were blocked with 1% gelatin in PBS for 30 min and then incubated at room temperature with polyclonal anti-g14-3-3 antiserum (1:100) for 1 h. After extensive washes with PBS, the parasites were incubated with a fluorescein isothiocyanate-conjugated anti-rabbit secondary Ab (Kirkegard and Perry Laboratories, Gaithersburg, MD) and the Texas Red-conjugated anti-CWP monoclonal Ab A300-TR at 1:30 dilution (Waterborne Inc., New Orleans, LA), and the nuclei were stained with 30 nM 4′,6-diamidino-2-phenylindole. After washes with PBS, the slides were mounted with an anti-fading agent (Vectorshied, Vector Laboratories, Burlingame, CA), and microscopy was performed using a Leica (Bensheim, Germany) confocal laser-scanning microscope with appropriate filter sets. A series of 20 optical sections with an approximate z distance of 0.3 μm was performed for each cell. The image processing was performed using the Huygen program (Scientific Volume Imaging BV, Hilversum, The Netherlands) and the Imaris program (Bitplane, Zurich, Switzerland).

**Pull-down Assay**—For pull-down assays, 15 μg of glutathione-Sepharose immobilized GST or GST-diopein were incubated with 3 mg of *Giardia* cystolic fractions from trophozoites or from trophozoites grown for 12 h in encystment medium (12 h encysting cells) in HT buffer at 4 °C for 2 h. After extensive washes with HT buffer, the 14-3-3 proteins bound to the beads were eluted with 100 μl of 5 mM soluble Raf259p phosphopeptide in HT buffer. An aliquot was run in SDS-PAGE and subjected to immunoblot with the N14 serum as described above.

**Mass Spectrometry Analysis**—Aliquots of the PreScission-cleaved recombinant g14-3-3, the T214A mutant or the purified native g14-3-3 were separated on a one-dimensional gel NuPAGE 4–12% (Novex, Invitrogen) run in MOPS buffer and stained with the Colloidal Blue staining kit (Invitrogen). Slices were excised, treated essentially as previously described (25), and digested with modified trypsin, sequencing grade (Promega Corp.). Peptide mixtures were desalted on a POROS-R2 (Applied Biosystems) handmade microcolumn or directly analyzed by MALDI-time of flight on a Voyager-DE STR mass spectrometer (Applera Corp.) in linear and/or reflector positive mode. As a matrix, we used re-crystallized α-cyano-4-hydroxycinnamic acid (α-cyano, Sigma) dissolved in 50% CH3CN, 0.1% trifluoroacetic acid (10 mg/ml) or 2,5-dihydroxybenzoic acid (DHB) (Sigma) in 50% CH3CN, and 1% orthophosphoric acid (30 mg/ml), which has been reported to enhance the efficiency of phosphopeptide ionization in MALDI by diminishing the loss of phosphoric acid (26). The presence of phosphopeptides in MALDI-MS spectra was confirmed by direct comparison between spectra acquired using 1% orthophosphoric DHB and α-cyano; the metastable decomposition product of the phosphopeptide is prominent with α-cyano-4-hydroxycinnamic acid, whereas it is present in lower quantities or not detectable in a 1% orthophosphoric DHB spectrum. Samples were loaded onto the instrument target using the dried droplet technique. Spectra were externally calibrated using a standard peptide mixture.

For the liquid chromatography-mass spectrometry analysis, LC/ESI-MS/MS and the LC/ESI-MS3 experiments were performed on an LCQ-DECA XP instrument equipped with a Surveyor MS pump (Thermo Electron Corp.). Peptide mixtures were analyzed on a capillary column, BioBasic C18, 100 × 0.180 mm, 5-μm particle size (Thermo Electron Corp.); the operating flow (2 μl/min) was obtained by the Surveyor MS pump through a homemade splitting device. The LCQ-ESI probe was equipped with a 34-gauge inner diameter metal needle (Thermo Electron Corp.). Peptides were eluted from the column in 50 min using a linear 5–60% acetonitrile gradient in 0.1% formic acid. The acquisition method for LC/ESI-MS/MS experiments was set to perform MS/MS data-dependent scanning on the three most abundant ions, enabling the
Giardia 14-3-3 Protein Post-translational Modifications

![Diagram of protein interactions and sequence alignments]

**RESULTS**

**Identification and Cloning of the 14-3-3 Coding Sequence from *G. duodenalis*—**A single putative 14-3-3 homolog gene has been annotated in the genome of the *G. duodenalis* WB-C6 clone (www.mbl.edu/Giardia). To verify the presence of other 14-3-3 homologs, we carried out an in silico screening of the *G. duodenalis* genome using the BLAST algorithm. As probes, we selected two amino acid sequences, RNLLS-P29305 and P29311, which are highly conserved across the 14-3-3 family (27). The search confirmed the presence of the single open reading frame of 744 bp (GenBank accession number AACB01000009), previously identified by the *Giardia* genome project (18). The gene lacks introns and codes for a protein of 248 amino acids with a predicted molecular mass of 28.5 kDa and a theoretical isoelectric point of 5.1 (data not shown). The sequence was confirmed by PCR amplification and sequencing of a 2.5-kilobase region spanning the ATG and the stop codon of the open reading frame (data not shown). This gene was named g14-3-3 (GenBank accession number DQ146480).

Using the ClustalW program, we performed a multiple alignment between the g14-3-3 protein and 117 sequences of different 14-3-3 isoforms from protozoa, fungi, plants, and animals, obtained from the GenBank data base. The g14-3-3 shows a degree of identity, ranging from 22 to 60%, with the other members of the 14-3-3 family (data not shown). In particular, we found 60% identity with the animal 14-3-3s (Fig. 1, panel B, sequence alignment of g14-3-3 protein). The g14-3-3 protein has been aligned with 14-3-3 sequences from other organisms, including protist parasites, yeasts, plants, and animals, using the ClustalX program. The g14-3-3 protein has been aligned with 14-3-3 sequences from other organisms, including protist parasites, yeasts, plants, and animals, using the ClustalX program.

**In Vitro Kinase Assays—**Approximately 20 μg of the glutathione-Sepharose immobilized recombinant GST-g14-3-3 or GST-T214A mutant protein was mixed with 50 μl of the reaction buffer containing 100 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 200 μM ATP, 2.5 μCi of [γ-³²P]-ATP, protease and phosphatase inhibitors, and 0.4 mg of the trophozoite cytoplasmic fraction. After incubation at 30 °C for 1 h, the reactions were stopped by adding 5 mM EDTA, and the beads were extensively washed with T-TBS. The recombinant wild type g14-3-3 and the T214A mutant protein were recovered by digestion with the trypsin protease and phosphorylation of the polypeptides were detected by autoradiography of dried gels.

**Densitometric Analysis—**The image was acquired using the GS-690 Imaging Densitometer (Bio-Rad) and analyzed with the Multi-Analyst 1.1 software (Bio-Rad).

**Sequence Analysis—**General homology searches with DNA and protein sequences were conducted on non-redundant GenBank databases using the BLAST algorithm, available at www.ncbi.nlm.nih.gov/BLAST. *Giardia* genome databases were analyzed on-line (www.mbl.edu/Giardia) by submitting the protein sequence on translated databases (TBLASTN). Sequence data were provided by the National Centre for Biotechnology Information. Multiple alignment programs were performed using the ClustalW program at www.ebi.ac.uk/clustalw.
motifs, a dimerization experiment was performed using the $^{32}$P-labeled gPK14-3-3 protein as bait and the g14-3-3 as prey. The g14-3-3 was able to dimerize with itself but not with GST alone, which was used as control (Fig. 2, panel A).

Alignment of the g14-3-3 with the human 14-3-3ζ isoform showed that the residues involved in ligand binding and located in the amphipathic groove of each monomer (7) are also conserved in the G. duodenalis 14-3-3 (Fig. 1, panel A). In particular, it has been well documented that the K49E mutation of 14-3-3ζ, corresponding to Lys53 of g14-3-3, negatively affects the binding of 14-3-3 to different ligands (7, 28).

The binding properties of the recombinant g14-3-3 were tested by overlay assays using both phosphorylation-dependent and -independent binding motifs. To evaluate the specificity and the mechanism of the binding, we also produced the g14-3-3 point mutant K53E, in which the positively charged residue Lys53 was replaced by glutamic acid, a negatively charged residue. The gPK14-3-3 was able to bind to two synthetic phosphopeptides, reproducing Raf-1 14-3-3 binding motifs, when they were used as prey but not their unphosphorylated version (Fig. 2, panel B). On the contrary, the interaction of the K53E mutant with Raf257 phosphopeptides was abolished, and that with the Raf261 phosphopeptides was strongly reduced (Fig. 2, panel B).

At the same time we evaluated the interaction with a phosphorylation-independent motif using as target a GST-fused difopein (21), a polypeptide containing a tandem repeat of the WLDLE sequence and originally identified in the R18 peptide by phage display screening (7). The gPK14-3-3 interacted with GST-difopein but not with GST alone, whereas the K53E mutant did not interact at all (Fig. 2, panel C).

We examined the interaction of recombinant g14-3-3 with cytosolic and membrane protein fractions extracted from Giardia trophozoites. The recombinant gPK14-3-3 bound a large number of proteins on both fractions (Fig. 2, panel D). The specificity of the binding was confirmed by competition with soluble Raf259p synthetic phosphopeptide; in the presence of the phosphopeptide, the interaction with the g14-3-3 was completely abolished (Fig. 2, panel D).

Subcellular Distribution and Immunolocalization of g14-3-3 in the Giardia Trophozoite—The g14-3-3 was localized in cultured trophozoites. In permeabilized cells, the anti-g14-3-3 serum (N14) produced a spotted and intense labeling within the cytosol, whereas the nuclei were poorly stained. No signal was detected in the central area of the cell corresponding to the median body, and no labeling of the flagella was evident (Fig. 3, panel A).

Furthermore, the presence of g14-3-3 was assessed in different protein fractions obtained from trophozoites. The 14-3-3 protein was mainly detected in the soluble cytosolic fraction and, in a lower amount, in the membrane fraction (Fig. 3, panel B). A main band, corresponding to a molecular mass of ~32 kDa, was present in both fractions, whereas a second product of about 28 kDa was only detected in the membrane fraction. The difference between the theoretical molecular mass, 28.5 kDa, of the recombinant g14-3-3 protein and the 32 kDa of the observed main band could suggest that the native protein was subjected to one or more post-translational modifications.

The Subcellular Localization of the g14-3-3 Changes during Encystation—Two processes are essential in the life cycle of G. duodenalis; these are the development of the trophozoite into a cyst, or encystation, and the emerging of the infective trophozoites from the cyst, or excystation. We focused on encystation and investigated the expression levels of both mRNA and the g14-3-3 protein at different points in time during the process. For the Northern blot analysis, total RNA was probed with a 750-bp fragment encompassing the g14-3-3 coding sequence, and the expression of the g14-3-3 mRNA was compared with the mRNA levels of gap1, a constitutively expressed gene, and of cvp1, an encystations-induced gene (29). The g14-3-3 mRNA appeared to be constitutively expressed (Fig. 4 panel A).

The immunoblot analysis was performed using proteins extracted from trophozoites at different times during encystation. When the cytosolic soluble fractions were probed with the N14 serum, the 32-kDa band was immuno-decorated in all of the samples. A second band (of ~30 kDa) became progressively detectable in the samples obtained after 6 h of encystation and decreased after 24 h of induction, when almost 80% of the cells were developed into the cyst stage (Fig. 4, panel B). We did not obtain similar results for the membrane fraction, where the antiserum recognized a pattern of bands similar to that observed during the trophozoite stage (data not shown).
We also evaluated whether or not the intracellular localization of the protein during encystation was modified. The process was monitored using an anti-CWP2 monoclonal Ab (A300-TR), which immuno-decorates the encystation-specific vesicles in the encysting cells or the wall of the cysts. As in the case of the trophozoites, the encysting cells as well as the cysts showed the previously observed staining of the cytoplasm (Fig. 4, panel C). By contrast, an increasing nuclear localization of g14-3-3 was evident during the transition of cells from the early to the late encysting stage, yet the nuclei of the cysts were poorly stained (Fig. 4, panel C). The g14-3-3 was not present in the encystation-specific vesicles of the encysting cells, as demonstrated by the absence of co-localization signal with the CWP2 protein (Fig. 4, panel C).

The g14-3-3 Is Post-translationally Modified by Phosphorylation and Polyglycylation—To determine the possible presence and the nature of post-translational modifications, the native g14-3-3 was purified by affinity chromatography. For this purpose, we used the soluble proteins...
extracted from trophozoites and 12-h encysting cells, in which, according to the immunoblot analysis, the signal corresponding to the 30-kDa band was most evident. The complete set of immunolabeled bands, present in the soluble fraction from both trophozoites and 12-h encysting cells, was recovered in the purified material, as demonstrated by Western blot analysis with the N14 serum (Fig. 5, panel A). Furthermore, the calculated relative intensity of the 30- and 32-kDa bands at 12 h was comparable in the purified proteins and in the input soluble protein fraction (Fig. 5, panel B), suggesting that the modification(s) of g14-3-3 did not affect the affinity for either the difopein or the soluble Raf259 phosphopeptide.

The trophozoite-purified and the recombinant g14-3-3s were subjected to mass spectrometry analysis. The direct comparison of the peptide mass fingerprint of both proteins clearly showed largely comparable MALDI-MS spectra, confirming that the purified protein really was the g14-3-3. The spectra of the purified native sample lacked the internal tryptic peptide (202)AFDAITLDDLKTEESYK (202–219), expected at m/z 2030.00, and the peptide (230)DNLNWVTDSAGDDNAEEK (230–248), expected at m/z 2105.93 and corresponding to the extreme C-terminal peptide of the protein (Fig. 5, panel C).

In the MALDI-MS spectrum of the purified native sample (Fig. 5, panel A), acquired with 1% orthophosphoric acid DHB, the presence of the signal at m/z 2109.98, instead of the signal at m/z 2030.02, found in the recombinant protein, could be ascribed to a phosphorylation of the peptide (202–219). This hypothesis is supported by the presence in the MALDI-MS spectrum, acquired with α-cyano in reflector mode, of a peak at m/z 2102.12, not resolved, corresponding to the metastable decomposition product indicative of the loss of phosphoric acid (Δm = −98 Da).

To confirm this hypothesis, a tryptic digest of the trophozoite-purified protein was analyzed by LC-MS/MS. Fragmentation of the species at m/z 1054.6, corresponding to the doubly charged ion of the putative phosphorylated peptide (202–219), gave a strong signal at m/z 1006.4, with a neutral loss of 49 Da, indicating the presence of a phosphate moiety on the precursor peptide. The phosphorylation site was identified by LC-MS3 analysis of the tryptic mixture, in which the signal at m/z 1006.4, originating from the collision-induced loss of phosphoric acid from the putative phosphorylated peptide at m/z 1054.6, was selected and fragmented. An accurate inspection of the MS3 spectrum (Fig. 6, panel A) revealed that the phosphorylated residue was Thr214. Stoichiometry of Thr214 phosphorylation appears to be very high in trophozoite and 12-h encysting g14-3-3, as also confirmed by the presence in the MALDI/MS spectrum of detectable species corresponding to phosphorylated peptides (213–229) and (202–229), whereas their unphosphorylated counterparts are completely absent (data not shown).

To confirm the site of phosphorylation, a point mutant of g14-3-3 was generated, in which the threonine in position 214 was replaced with an alanine. The T214A mutant was expressed, and the presence of the mutation was verified by mass spectrometry (data not shown). Both the wild type and the T214A recombinant proteins were subjected to an in vitro phosphorylation assay in the presence of [γ32P]ATP and of the soluble protein fraction from trophozoites. A protein kinase activity was able to phosphorylate the recombinant wild type protein but not the T214A mutant (Fig. 6, panel B). However, no electrophoretic shift of the wild type recombinant protein occurred after the in vitro phosphorylation.

The absence in the trophozoite-purified protein of the C-terminal peptide (229–248), expected at m/z 2105.93, suggested that there was another post-translational modification(s) and/or a cleavage of this portion of the protein. Intriguingly, further inspection of MALDI-MS spectra at higher m/z values revealed the presence of peaks differing by multiples of 57 Da, which correspond to the expected mass increases due to successive additions of glycine residues (Fig. 7, panel A). This signal pattern can be assigned to polyglycylation of the C-terminal peptide. This post-translational modification, previously reported only for α- and β-tubulin (30), results from the addition of one or more glycine residues to the γ-carboxyl group of a glutamate. Two different glutamic acid residues, Glu246 and Glu247, are contained in this portion of the g14-3-3 protein, and both could be sites for the polyglycylation. Polyglycylation could completely explain the broadness and the slower migration of the band corresponding to the native form in comparison with the recombinant protein.

When the 14-3-3 doublet from the 12-h encysting cells was analyzed, we observed that both forms were phosphorylated on Thr214 (data not shown) and polyglycylated (Fig. 7, panel B). The extent of the polymodification differed between trophozoite- and 12-h encysting cell-purified proteins. As can be inferred by molecular mass calculations in the trophozoite-purified g14-3-3, glycine residues ranged from 10 to 31, with the greatest peak corresponding to 21 residues, and from 6 to 22 residues in the 12-h encysting lower band, with the main peak corresponding to the addition of 10 residues (Fig. 7, panel A and B).

The nature of this post-translational modification was assessed using the monoclonal antibody AXO49, previously developed against the polyglycylated tubulin and able to detect polyglycine chains of at least four residues (31). The antibody recognized g14-3-3 from both the trophozoites and the 12-h encysting cells both in the soluble fraction and in the purified g14-3-3, yet it failed to detect the unmodified recombinant protein expressed in bacteria (Fig. 7, panel C). However, when AXO49 was used to probe the membrane fraction from both the trophozoites and the 12-h encysting cells, it strongly recognized only a band with a molecular mass compatible with tubulin even though the identity of this band could not be confirmed (data not shown).

Because nothing is known about polyglycylating enzymes to explain how g14-3-3 can be a target for this enzymatic activity, we attempted to define a putative polyglycylation target sequence common to tubulins and g14-3-3. The last 13 C-terminal amino acids of the g14-3-3 protein, (230)VTDSAGDDNAEEK, are unique to this isoform when compared with the other 14-3-3s and contain the polyglycylated glutamic acid(s). We aligned this portion of the g14-3-3 with the last 16 C-terminal residues surrounding the polyglycylation sites of the α- and β-tubulin from G. duodenalis, Tetrahymena thermophila, and Paramecium tetraurelia, in which polyglycylation has been described and the polyglycylated residues have been identified (32–34). As result of the alignment, we observed that conserved residues were present in the tubulins as well as in the g14-3-3 (Fig. 7, panel D). These conserved amino acids defined a motif (T/G)X0–1(D/E)X1–2G(D/E)X1–2(gE)2–4, where X is a polar or a negative charged amino acid, and gE is a polyglycylated glutamic acid.

**DISCUSSION**

We report on the cloning and characterization of a new member of the 14-3-3 protein family isolated from the parasite protozoa G. duodenalis. Our investigation of the G. duodenalis genome confirmed the presence of a single 14-3-3-homolog gene, previously annotated by the *Giardia* genome project (18). The presence of a single 14-3-3 coding gene is also consistent with data on other unicellular organisms, including parasites such as *T. gondii*, Neospora caninum, and *Plasmodium* spp. (12, 35, 36), although the ciliate *Entodinium caudatum* and the parasite protozoa *Entamoeba histolytica* harbor, respectively, four and three different 14-3-3 genes, probably as the result of gene duplication (1).
FIGURE 5. Mass spectrometry analysis of g14-3-3. A, pull-down of g14-3-3. Soluble proteins from trophozoites (T) or 12-h encysting cells (12 h) were incubated with GST or GST-difopein and eluted with 2 mM Raf259p-soluble phosphopeptide. An aliquot (1/20) was analyzed in duplicate on 4–12% gradient SDS-PAGE and compared with 500 ng of PreScission-cleaved recombinant g14-3-3 and with 1/100 of the input cytosolic fraction. Upper panel, Coomassie-stained gel; lower panel, immunoblotting with the N14 serum (g14-3-3). The arrows on the left indicate the molecular mass (kDa) of different g14-3-3 bands.

B, densitometric analysis. The relative intensity of the g14-3-3 doublet present in the soluble proteins (input 12 h) and in the affinity-purified sample (pull-down 12 h) from 12 h encysting cells, detected in the Western blot of panel A, was calculated by Quantity One™ Image analysis software. In the graphs, the area of each peak is reported as a percentage of the total area of the two peaks.

C, detailed view of the MALDI-MS spectra (from m/z 1997 to m/z 2120) acquired in reflector mode, with 1% phosphoric acid DHB of native g14-3-3 from trophozoites (a) and of the recombinant g14-3-3 (b) purified from SDS-PAGE and digested with trypsin. Monoisotopic masses are indicated. In the trophozoite, sample signals at m/z 2030.02 and 2105.93, found in the recombinant protein and corresponding to theoretical g14-3-3 tryptic peptides, are absent, whereas a strong signal at m/z 2109.98 appears. In the g14-3-3 sequence on the bottom, the position of the discussed peptides (202–219 and 230–248) is in bold.
The comparison of the amino acid sequences revealed a high degree of identity of the g14-3-3 protein with the H9280 subgroup of Metazoan 14-3-3s. This is particularly important when considering that the H9280 isoform is the most closely related to the hypothetical ancestral animal 14-3-3 protein (27). However, a phylogenetic tree based on the alignment of 14-3-3 proteins cannot be used to argue the evolutionary position of G. duodenalis given that, as previously proposed, the protozoan 14-3-3 isoforms are highly divergent from the other 14-3-3s, and it is difficult to select one of them to root a phylogenetic tree (1).

With regard to the properties of the g14-3-3 protein, in vitro interaction experiments using both its recombinant and native form have clearly demonstrated that it is a functional member of the 14-3-3 family with the ability to form homodimers and bind both phosphorylation-dependent and -independent binding motifs. The specificity of these interactions is also demonstrated by the use of the K53E mutant in which, as for the well characterized K49E mutant of the mammalian 14-3-3 isoform (7, 28), the charge inversion at that particular position hampers the binding ability of g14-3-3. This experimental evidence strongly suggests that the general mechanism regulating the 14-3-3-target interaction is well conserved in the g14-3-3.

The involvement of the g14-3-3 in a complex network of interactions in vivo is supported in vitro by the specific binding to a large number of proteins and by the presence of g14-3-3 not only in the cytosol but also in association with membrane proteins. The localization of 14-3-3 proteins in different subcellular compartments and in association with membrane proteins has been widely demonstrated (37, 38), and a lipid raft-associated form of the T. gondii 14-3-3 homolog has been recently described (13).

In other parasites, such as P. falciparum, the expression of the 14-3-3s during the erythrocyte stage of the parasite has been suggested to depend on both the formation of a specific membranous structure and the inhibition of the translocation of the protein kinase C to the membrane of the infected cell (12). It has been suggested that 14-3-3s could be involved in the stage-specific development of T. gondii (35).

Our data demonstrate that the g14-3-3 mRNA and the g14-3-3 protein are constitutively expressed both in the Giardia trophozoite and during the encystation stage, and transcription and translation levels are stably maintained. However, encystation signals affect both the rate of post-translational modifications and the intracellular localization of the encoded protein.

To the best of our knowledge this is the first study to show that polyglycylation, previously detected only in tubulins, occurs in the g14-3-3 protein and that the amount of glycines bound to the protein is high in the trophozoite yet drastically reduced during encystation. Polyglycylation, differently from other post-translational modifications such as phosphorylation and tyrosinylation, dramatically influences the protein structure through the addition of a polyglycine side chain covalently attached by an isopeptide bond to the γ-carboxylate group of a glutamate residue. Polyglycylation has been described as a post-translational modification of the C-terminal portion of the tubulin isolated from the...
**FIGURE 7. Polyglycylation of g14-3-3.** MALDI-MS spectra, acquired in reflector mode using α-cyano as the matrix, of the trophozoite-purified g14-3-3 (A) and 12h-encysting g14-3-3 (B) recovered from SDS-PAGE and digested with trypsin is shown. Three panels were selected from the spectra to better compare the distribution of glycylated species in the two proteins. An asterisk indicates polyglycylated peptides; the numbers of glycine residues are reported. C, Western blot analysis with the anti-polyglycylated tubulin-specific antibody (AXO49) (upper panel) and the N14 serum (g14-3-3) (lower panel). An aliquot (1/100) of the input soluble protein fraction from trophozoites (T) and encysting cells (12 h), an aliquot (1/20) of pull-down-purified g14-3-3 from trophozoites (T) and 12-h encysting cells (12 h), and 0.5 μg of recombinant g14-3-3 (R) were loaded on 4–20% SDS-PAGE. The immunodetection was visualized using horseradish peroxidase-conjugate secondary antibody and a chemiluminescent assay. Arrows indicate the different g14-3-3 forms. D, alignment of g14-3-3 with α- and β-tubulin of G. duodenalis (GenBankTM/EBI accession numbers AAN46106 and P05304), α- and β-tubulin of P. tetraurelia (GenBankTM/EBI accession numbers CAA67848 and CAE75646), and α- and β-tubulin of T. thermophila (GenBankTM/EBI accession numbers P41351 and P41352). The alignment was performed with the ClustalW program and manually refined. The amino acids in gray define the hypothetical polyglycylation sequence (T/G)X0–1[D/E]X1–2G(D/E)0–4. The asterisks indicate the two glutamic acid residues of g14-3-3 possibly glycylated. Putative or experimentally defined (in bold) polyglycylated glutamic acid residues are underlined.
cilia of Paramecium (30) and for both α- and β-tubulin of the ciliate and flagellate cells (39). Polyglycylation has already been demonstrated in Giardia, where it is the main post-translational modification of tubulins (32). We show that the polyglycylation occurs at the extreme C-terminal portion of the g14-3-3 protein, as in the case of tubulins. However, we are not able to define whether one or both glutamic acid residues, Glu246 and Glu247, are subjected to polyglycylation. Polyglycylase and deglycylase activities have not been previously described even if the presence of the complete set of glycylases in Giardia has been inferred (40), yet in our study the sequence homology between the C-terminal tails of g14-3-3 and tubulins from Giardia, Tetrahymena, and Paramecium, where polyglycylation is predominant, suggests that the motif (T/G)Xn_1(D/E)Xn_2(G/D)Xn_3(E/F)n+, which we identified in all of these proteins, could be a possible target sequence for polyglycylation.

Despite the generally high level of sequence homology among 14-3-3s, the C termini of different isoforms are hypervariable and differ in length; they are shorter in mammalian 14-3-3s (excluding the ε isoform) and longer in yeast and plant isoforms (27). The g14-3-3 protein belongs to the group of short isoforms, as evident in the multiple alignment (Fig. 1).

In view of the general high flexibility of the 14-3-3 C-terminal tails (3–5), we propose that the addition of polyglycine chain(s) to the extreme C terminus of the g14-3-3 could act as a removable tail affecting the intracellular localization of the protein and/or allowing the g14-3-3 dimer to interact at the same time with canonical ligands and with other molecules by means of a further mechanism mediated by the glycine chain(s).

It has recently been demonstrated that, in barley (Hordeum distichum), proteolytic cleavage of the C-terminal tail occurs for the 14-3-3A during seed germination, yet this has not been reported for other barley isoforms (1). The truncation does not seem to alter the binding property, yet it excludes the protein from the nucleus (41).

In a similar way our data suggest that the addition of polyglycines does not affect the binding properties of g14-3-3. The affinity of both the high and low glycylated g14-3-3 for the GST-difopein is comparable, since the relative amount of the two forms was constant before and after the affinity purification. This result is also in agreement with the observation that the binding affinity of human 14-3-3C tagged with green fluorescent protein is not altered (41), although a quantitative assay that includes a non-polyglycylated g14-3-3 would be necessary to fully support our result.

By contrast, we observed that the appearance of a less polyglycylated g14-3-3 during encystation coincided with the translocation of the protein inside the nuclei. In plants and animals the nuclear localization of 14-3-3s and their association with transcription factors have been already reported (36), and longer in yeast and plant isoforms (27), the g14-3-3 protein belongs to the group of short isoforms, as evident in the multiple alignment (Fig. 1).

In vivo studies, conducted in Tetrahymena with various polyglycyla-

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