The Crystal Structure of *Giardia duodenalis* 14-3-3 in the Apo Form: When Protein Post-Translational Modifications Make the Difference

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**Abstract**

The 14-3-3s are a family of dimeric evolutionarily conserved pSer/pThr binding proteins that play a key role in multiple biological processes by interacting with a plethora of client proteins. *Giardia duodenalis* is a flagellated protozoan that affects millions of people worldwide causing an acute and chronic diarrheal disease. The single giardial 14-3-3 isoform (g14-3-3), unique in the 14-3-3 family, needs the constitutive phosphorylation of Thr214 and the polyglycylation of its C-terminus to be fully functional in vivo. Alteration of the phosphorylation and polyglycylation status affects the parasite differentiation into the cyst stage. To further investigate the role of these post-translational modifications, the crystal structure of the g14-3-3 was solved in the unmodified apo form. Oligomers of g14-3-3 were observed due to domain swapping events at the protein C-terminus. The formation of filaments was supported by TEM. Mutational analysis, in combination with native PAGE and chemical cross-linking, proved that polyglycylation prevents oligomerization. In silico phosphorylation and molecular dynamics simulations supported a structural role for the phosphorylation of Thr214 in promoting target binding. Our findings highlight unique structural features of g14-3-3 opening novel perspectives on the evolutionary history of this protein family and envisaging the possibility to develop anti-giardial drugs targeting g14-3-3.

**Introduction**

Eukaryotic 14-3-3s are a family of dimeric highly conserved proteins (∼30 kDa) with pSer/pThr binding property. When 14-3-3s are present in multiple isoforms (e.g. seven in *Homo sapiens*), they can form either homo- and/or heterodimers. 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*. [1–2]. 14-3-3s bind to hundreds of client proteins thus affecting many cellular processes, including metabolism, signal transduction, cell cycle, differentiation, apoptosis, protein trafficking, transcription, stress responses and malignant transformation [3]. The overall 14-3-3 architecture is well conserved among the different isoforms and eukaryotic kingdoms. Each 14-3-3 monomer consists of nine antiparallel α-helices (A-I) organized in a cup-like shape with the dimerization domain located in the N-terminal half [4–8]. The 14-3-3-target interaction is mediated by conserved residues in an amphipathic groove of each monomer [formed by α-helices C, E, G, and I] and requires specific pSer/pThr containing motifs on the targets. The phosphate moiety of the target binding peptide directly contacts a cluster of positively charged residues in the 14-3-3 amphipathic groove including Lys49, Arg56, Arg127 and Tyr128 (human 14-3-3ζ numbering), whereas the phosphopeptide backbone and side chains establish interactions with a hydrophobic patch of 14-3-3 conserved residues. Three general 14-3-3 consensus binding motifs have been identified: RXX(ξpS/pT)XP, called mode-1, where pS/pT are phosphorylated serine or threonine residues and X stands for any type of residue; RX(Y/F)ξpS/pT(Xp)XP (mode-2) [9]; and XpS/T(Xp)ζ-COOH (mode-3), in which the phosphorylated residue occupies the penultimate C-terminal position in the target protein [10]. Nevertheless, 14-3-3s may also interact with some non-phosphorylated peptides, such as R18, containing the WLDLE sequence [11]. The overall rigid structure of the 14-3-3 dimer can induce, upon binding, conformational changes in the target protein resulting in the inter- and/or intra-compartmental sequestration of the target itself, activation/inactivation of the target enzymatic activity, and, in few cases, promotion/inhibition of the interaction between the target and other proteins [8]. From an evolutionary point of view, 14-3-3s can be subdivided into four major groups coinciding with the four kingdoms. The existence of an ancestral 14-3-3 that evolved independently into different
isoforms in each kingdom has been proposed. In particular, the independent clustering of Metazoaan epsilon isoforms strongly suggesting that the epsilon isoforms are relatively conserved and similar to the original animal 14-3-3 ancestor [1].

*Giardia duodenalis* (syn. *G. lamblia* or *G. intestinalis*, from now on referred to as *Giardia*) is a flagellated protozoan that parasitizes the upper part of the small intestine of mammals, including human, and causes giardiasis, the most common parasitic diarrheal disease worldwide [12]. *Giardia* is a deeply branched eukaryote, closer to fungi and animals than Euglenozoa [13]. The two life stages of *Giardia*, the binucleated trophozoite, which replicates and colonizes the host intestine, and the tetranucleated cyst, the infective and *Giardia* referred to as functional member of the family with a central role in multiple biological pathways of *Giardia* and its minimalistic genomic and cellular organization make this parasite a fascinating model to investigate basic cellular processes and different aspects of eukaryotes’ evolution [14].

*Giardia* possesses a single 14-3-3 isoform (g14-3-3) showing high sequence identity to the 14-3-3s of the epsilon subgroup. In previous works we have demonstrated that g14-3-3 is a fully functional member of the family with a central role in multiple biological pathways of *Giardia* [15–16]. Interestingly, two post-translational modifications (PTMs) make g14-3-3 unique in the 14-3-3 protein family: constitutive phosphorylation and polyglycyla-

motifs in vitro synthetic phosphopeptides reproducing multiple 14-3-3 binding bacterial recombinant protein, displayed an enhanced binding to fact, the g14-3-3 phospho-mimicking mutant T214E, expressed as role in promoting the binding of g14-3-3 to its targets. In binding to the targets [17]. The phosphorylation of Thr214 may helix G and at the extreme C-terminus, thus modulating the binding G14-3-3 protein family: constitutive phosphorylation and polyglycyla-

as for polyglycylation, this unusual PTM takes place at the penultimate g14-3-3 C-terminal residue, Glu246, and consists in the addition of up to 30 consecutive glycines per monomer. The length of the polyglycine chain is stage-dependent and decreases down to 10 residues during the cyst formation in parallel with a partial re-localization of g14-3-3 to the nuclei [15,18]. Both the in vivo expression of the E246A mutation, which disables the g14-3-3 polyglycyla-

phosphorylation of Thr214 promotes the binding to the target(s).

Materials and Methods

Vectors’ construction

Escherichia coli JM109 and XL1Blue competent cells were used for vector manipulation and recombinant proteins expression, respectively. Glutathione S-Transferase (GST)-difoipin, GST-g14-3-3 (plasmids p14-X) and GST-T214E expression vectors have been described elsewhere [15,18]. The R200K and the T208A mutants were obtained by site-directed mutagenesis using the p14-X vector15 as template, and the designed primers: R200Kfor 5’-AGGCTTGGACAGTTCGAGAAGGCTTGCAG-3’; R200Krev 5’-GGCGGTCGTAAGGCTTTCCTTGCGAAGCTCGCA-3’; T208Afor 5’-GCTTTCGAGGCGGCGACAGATCTGGAAG-3’; T208Arev 5’-GGCGTCAGGCTTGATTCGATCGGATCCTCGCT-3’ (mutated triplets are underlined). The reaction was performed as previously detailed [15] and according to manufacturer’s instruction. The obtained plasmids were designed as “pT208A-X” and “pR200K-X”. To obtain the polyG10 and polyG20 mutants in which the last two C-terminal residues were deleted and replaced with a stretch of 10 or 20 glycines, respectively, we took advantage of the presence of a KpnI site at position 404 of g14-3-3 coding sequence and a NotI site in the multicloning site of the pGEX-6P1 vector. A KpnI-NotI cassette was PCR amplified from the p14-X vector15 using the “g14KpnIfor” primer, 5’-CCCGGTCGTAAGGCTTTCCTTGCGAAGCTCGCA-3’ (BamHI restriction site is under-

in vitro the expression in *Giardia* of the non phosphorylatable T214A mutant behaved as a dominant negative leading to an impaired cyst development.

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Crystal Structure of *G. duodenalis* 14-3-3

First, the crystal structure of a g14-3-3 in the apo form is presented and discussed. The structure shows that the protein, in absence of both PTMs, oligomerizes through C-terminal domain swapping events. The formation of filaments was also confirmed by transmission electron microscopy. Second, the combination of various mutants with native PAGE and chemical cross-linking indicates that the oligomerization process does not occur when a polyglycine stretch is added to the protein C-terminus. Finally, molecular dynamic simulations support the hypothesis that the phosphorylation of Thr214 promotes the binding to the target(s).
KpnI/NotI-digested p14-X vector replacing the last 341 nucleotide of the g14-3-3 coding sequence, whereas the BamHI/NotI-digested 14-3-3 C was cloned in the BamHI/NotI-digested pGEX6P1 vector. The obtained plasmids were designated as “pPolyG10-X”, “pPolyG20-X” and “ph14-3-3 C-X”, respectively.

Proteins expression and purification

For protein expression, transformed E. coli were grown in SOB medium at OD_{600} = 0.6-0.8, and expression induced with 0.5 mM isopropyl thio-β-D-galactoside (IPTG) at 37 °C for 4 h. GST-fused proteins were purified by affinity chromatography on glutathione-sepharose 4B (GE Healthcare, Uppsala, Sweden) and released from GST by digestion with PreScission protease (GE Healthcare) at 4 °C for 16 h in digestion buffer according to manufacturer. Protein were dialyzed o.n. at 4 °C either in 50 mM Tris-HCl pH 7.5 (for crystallization) or in 10 mM Tris-HCl pH 7.0, for Circular Dicroism (CD) experiments, using a PM-5 membrane. Proteins were concentrated using Centricon 10 (Millipore Corporation, Bedford, MA, USA) and concentration was measured with Bradford’s method (BioRad, Hercules, CA, USA).

Circular dicroism

CD spectra were measured with a Jasco J-715 spectropolarimeter (Jasco Ltd, Hachioji City, Tokyo, Japan) in 1.0 cm quartz cuvettes between 260 and 195 nm. Samples were 0.1 μg/μl in 10 mM Tris/HCl buffer (pH 7.0).

Protein crystallization, data collection and processing

Crystals were grown by the hanging drop vapor diffusion method at 293 K. Protein sample was concentrated to 10.5 mg/ml. Crystallization drops consisted of 1 μl of protein solution mixed with an equal volume of the reservoir solution on a cover slip which was suspended over the reservoir containing 2 M ammonium sulfate, 5% (w/v) PEG400 and 0.1 M HEPES pH 7.5. Crystals, grown in 1–2 weeks, were cryo-protected with 20% glycerol, mounted in nylon loops and flash-cooled by submersion into liquid N2 for transport to the synchrotron-radiation source. Crystals are poorly reproducible and most of them diffract at low resolution. X-ray diffraction data were collected at 3.2 Å resolution as 0.5 ° oscillation frames at 100 K on the beam line ID14-1 at ESRF (Grenoble, France) using a CCD detector. The data were processed using the HKL2000 package [22] and the ID14-1 at ESRF (Grenoble, France) using a CCD detector. The resolution as 0.5 ° oscillation frames at 100 K on the beam line ID14-1 at ESRF (Grenoble, France) using a CCD detector.

Protein coordinates and structure factors have been deposited in the Protein Data Bank with accession number 4F7R. Protein coordinates and structure factors have been deposited in the Protein Data Bank with accession number 4F7R.

Purification of endogenous g14-3-3

Trophozoite of Giardia duodenalis WBC6 strain were grown as previously described [15]. Parasite’s protein lysate was prepared as previously described [18] and endogenous g14-3-3 was affinity purified on glutathione-Sepharose immobilized GST-difopein and g14-3-3 eluted with 100 μl of 5 mM “Raf1p” phosphopeptide, (LSQRQRST(pS)TPNVHMV), reproducing one of the 14-3-3 binding motifs of the human Raf-1 protein [13]. The excess of phosphopeptide was removed by dialysis against HT buffer (25 mM Hepes-KOH pH 7.6, 75 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.04% Tween 20) with Centricon 10 (Millipore Corporation, Bedford, MA, USA).

Mass spectrometry analysis

Affinity purified g14-3-3 was separated on 1D-gel NuPAGE 4–12% (Novex, Invitrogen) run in morpholinepropanesulfonic acid (MOPS) buffer and stained with the Colloidal Blue Staining kit (Invitrogen). For the MS/MS identification of phosphorylated residue in g1433, in gel digestion and nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/ MS) analysis were performed, with minimal changes, as previously described [16] using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA U.S.A) on line connected with a linear Ion Trap (LTQ-XL, ThermoElectron, San Jose, CA). MS data were searched for matching with the Giardia protein database, downloaded from the web site http://www.giardiadb.org/ giardiadb, considering peptides specifically cleaved by trypsin on
and then subjected to cross-linking by the addition of 5 M triethanolammine pH 8.0 with or without 300 M buffer for 16 h at 4 °C either in presence or absence of 1 mM DTT. Reaction was stopped with a slow scan camera (Megaview III, Olympus). Electron micrographs were taken with a PHILIPS EM208S transmission electron microscope at an acceleration voltage of 80 kV. Electron micrographs were taken with a slow scan camera (Megaview III, Olympus).

Table 1. Crystal parameters, data collection statistics and refinement statistics of g14-3-3.

| PDB ID | 4F7R |
|--------|------|
| Space group | P31_2 |
| Unit cell parameters (Å) | |
| a | 100.9 |
| c | 140.5 |
| No. of molecules in the asymmetric unit | 4 |
| Resolution ranges (Å) | 54.5 |
| Unique reflections | 26426 |
| Completeness (%) | 100 (100) |
| Redundancy | 7.0 (7.0) |
| Rmerge (|)2 | 12.5 (76.2) |
| | |
| Rmerge (|)2 | 1.06 (1.2) |
| Rfree (%) | 16.3 (2.8) |
| Rfree (%) | 18.5 (29.4) |
| Rfree (%) | 23.1 (31.9) |
| rms (angles) (°) | 0.812 |
| rms (bonds) (Å) | 0.005 |
| Residues in core region of Ramachandran plot (%) | 930 (98.9) |
| Residues in generously allowed region of Ramachandran plot (%) | 10 (1.1) |
| Residues in disallowed region of Ramachandran plot (%) | 0 |

Values in parentheses are for the highest-resolution shell.

Crystal Structure of G. duodenalis 14-3-3

To achieve an amount of g14-3-3 suitable for structural analyses we used an N-terminally GST-fused g14-3-3 expressed in bacteria that was characterized in previous works for its ability to bind both to several phosphopeptides reproducing 14-3-3 binding motifs and to the phosphorylation-independent binding peptide difopein [15,18]. Although the g14-3-3-specific polyglycylase gTTLL3 (Tubulin Tyrosin Ligase-Like3) had already been identified and expressed [19], the low efficiency of the in vivo polyglycylation process did not allow us to obtain sufficient polyglycylated protein for crystallization studies. On the other hand, the g14-3-3-specific protein kinase is still unknown. Notice that the GST-
cleaved full-length unmodified g14-3-3 used for the crystallization experiments only differed from the native g14-3-3 for the presence of a GPLGS sequence upstream the initial methionine.

The structure of the g14-3-3 was determined at 3.2 Å resolution in the P32 space group and the asymmetric unit contained two functional dimers (Fig. 1A–1B). Each g14-3-3 monomer displayed Figure 1. Classical dimer of g14-3-3 assumes an “open” conformation. A) Two views of g14-3-3 N-terminal dimer (green, cyan). B) The superimposition of g14-3-3 and human 14-3-3ε (2BR9, violet) emphasizes the open conformation of g14-3-3. C) Close up of N-terminal dimerization interface. Residues are shown as lines if involved in hydrogen bonds, as sticks if involved in salt bridges. D) Detail of polar interactions between helices H and I. Residues not conserved in 14-3-3 family are represented as sticks. A conserved residue (Gln226 in g14-3-3 Gln222 in human 14-3-3ε) is shown as yellow sticks in B) and D) to highlight the different orientations of helix I.

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the typical 14-3-3 structural organization [5–7] with nine antiparallel α-helices (A–I) arranged in a U-shaped fashion with a large central binding groove. Each monomer could be divided into two sub-domains: the N-terminal sub-domain (helices A–D), which consisted of the canonical dimerization interface as well as the floor of the binding groove, and the C-terminal sub-domain (helices E–H), which comprised the side walls and the roof of the binding groove (Fig. 1A–1B). The g14-3-3 buried N-terminal dimerization interface was 1240 Å² per monomer (8.5% of the total accessible surface area), scored 0.3 in Complexation Significance Score (CSS), indicating that the dimerization was not due to crystal packing and confirming, as expected, the relevance of the g14-3-3 dimerization surface. The g14-3-3 N-terminal dimerization relies on residues largely conserved among the 14-3-3 family members [6,25] involving, in this case, three salt bridges between Arg22 and Glu97, Asp9, and Lys82, and Glu25 and Lys93 (Fig. 1A and 1C). Arg22, Asp9, and Glu25 are located in g14-3-3 α-helices A and B of one monomer whereas Glu97, Lys82, and Lys93 reside in α-helix D of the other protomer. In the homodimeric human 14-3-3, as well as in the epsilon-like Cpl14e from the protozoan parasite Cryptosporidium parvum, dimerization involves only the first salt bridge (Arg19-Glu92 in h14-3-3e and Arg43-Glu119 in epsilon-like Cpl14e, respectively) [7,25]. The presence of a single salt bridge allows h14-3-3 to mainly heterodimerize with other human isoforms [25]. Interestingly, g14-3-3 dimerized through three salt bridges similar to 14-3-3 [4–41] despite the higher sequence identity of g14-3-3 with proteins of the 14-3-3 subgroup than with those of the 14-3-3 subgroup. This suggests that g14-3-3 only forms stable homodimers. In support of this, when g14-3-3 was expressed in Drosophila melanogaster or when fly isoforms were expressed in Giardia heterodimerization occurred in both cases only between g14-3-3 and D14-3-3 (LeoII) but never between g14-3-3 and D14-3-3e [20]. The three-dimensional superimposition of the g14-3-3 and h14-3-3e structures yielded a root mean square deviation (rmsd) of 0.95 Å. This low rmsd value indicates that helices A–H are largely super-imposable. The software automatically excluded helix I from the calculation as it was recognized as a largely dissimilar region. Indeed, helix I of g14-3-3, compared with the human isoforms, is rotated of about 180° around helix H (Fig. 1B and 1D) implying a substantial rearrangement of the loop connecting helices H and I (HI loop). The displacement of the HI loop causes the disruption of a “ST-motif” found in most 14-3-3s and including Ser210 (according to h14-3-3 numbering), which corresponds to Thr214 in g14-3-3. (Fig. 1D). This motif consists of 4–5 residues including a Ser or a Thr forming hydrogen bonds with two residues downstream in the sequence [43]. Instead, the displacement of the HI loop in g14-3-3 is favored by eight hydrogen bonds between helices H and I (Fig. 1D) that involve, among others, residues Arg200, Thr208, Thr214 and Asn233 which correspond, in the human isoforms, to Lys194, Ala202, Ser/Asn208 and Thr227, respectively (according to h14-3-3e numbering) (Fig. 2).

As consequence of the helix I rearrangement, the g14-3-3 structure showed a fully open “extended” conformation of the C-terminal region, supporting the notion that the g14-3-3 region encompassing helices G and I possesses a degree of flexibility allowing the binding groove to change from a “closed” to an “open” conformation and thus accommodating different types of peptides [25].

C-terminal dimer-dimer interface

The described g14-3-3 “extended” conformation entails the exposure of several hydrophobic residues located in helix G, H and I and usually buried in other 14-3-3s. A possible consequence of such exposure of hydrophobic residues is the observed domain swapping occurring between the C-terminus of two g14-3-3 monomers, each belonging to a different dimer, in which helix I from a monomer interacts with helices F and H from the monomer of the neighboring dimer (Fig. 3A; B and C). In contrast, in other available structures of 14-3-3 dimers, helix I is observed to interact with helices F and H from the same monomer [6]. Domain swapping entails a dimerization event at the C-terminal C-terminus with the formation of a four-helix bundle between two adjacent g14-3-3 dimers (Fig. 3B and 3C), leading to the assemblage of protein filaments. Domain swapping was observed in the structure of different proteins and it is defined as two identical protein chains exchanging a part of their structure to form an intertwined dimer or a higher-order oligomer [44]. The overall g14-3-3 crystal packing resulted in stacks of endless parallel filaments (Fig. 3D), each layer being rotated 60° with respect to the previous one. The calculated C-terminal dimer-dimer buried interface is 1576.9 Å² per monomer, i.e., about 11% of the total accessible surface area. The calculated solvation free energy gained upon formation of the interface (ΔG = -39.3 kcal/mol) suggests that the oligomer is highly stable. As previously stated, a stretch of hydrophobic residues is exposed at the C-terminus of each monomer so that the C-terminal dimer-dimer interface results predominantly hydrophobic and further stabilized by four intermolecular hydrogen bonds between Ser221 of helix I and Thr170 of loop FG’ (and Ser221 of helix I’ and Thr170 of loop FG), and Glu215 of loop HI and Leu213 of helix H’ (and Glu215 of loop HI’ and Leu213 of helix H). Nearly, all the described intermolecular interactions in g14-3-3 can be also observed in the available 14-3-3 structures in the form of intra-molecular interactions involving helix I, which perfectly replaces the swapped helix I’ (Fig. 3B). The only exception is represented by the hydrogen bond involving loop HI (residues Glu215-Leu213) that does not occur in the non-swapped dimer (Fig. 3B).

The g14-3-3 target binding site

The 14-3-3 binding to its target(s) primarily occurs through the interaction of conserved residues in the 14-3-3 binding groove and a phosphorylated peptide in the target(s). According to h14-3-3e numbering, the pSer/pThr phosphate moiety of the ligand binds to a highly conserved 14-3-3 triad formed by Arg57, Arg130 and Tyr131 whilst Asn176 and Asn227 interact with the phosphopeptide backbone [6,25].

In the g14-3-3 structure, despite helix I swapping, the conformation of the residues involved in the recognition and binding to phosphopeptide(s) resulted to be conserved (Fig. 4). Indeed, the visual inspection of the Fo-Fc electron density map revealed a peak that was assigned to a sulphate ion (Sup. Fig. S1), present in the crystallization solution, which lies in the phosphate binding site and interacts with the g14-3-3 Arg60-Arg135-Tyr136 triad (Fig. 4A and Sup. Fig. S1), thus mimicking the phosphate moiety of a pSer/pThr binding peptide. This was supported by the superimposition between g14-3-3 (in the apo form) and the human 14-3-3/RKQR(PS)AP phosphopeptide complex (PDB 2BR9). All the residues involved in the phosphopeptide binding (Arg60, Arg135, Asp132, Tyr136, Asn180, Asn231, according to g14-3-3 numbering) are conserved, although it is worth noting that Asn231 belongs to helix I’ in g14-3-3 (Fig. 4B–4C). The observed “extended” conformation of g14-3-3 with the large conformational change of helix I would disrupt the binding groove, which is indeed restored by domain swapping of helix I’, indicating that the protein assemblies can retain phosphopeptide binding properties even in the case of C-terminal dimerization (Fig. 4B–D).
Structural role of g14-3-3 phosphorylation

The solved structure of g14-3-3 is not phosphorylated and Thr214 is buried at the C-terminal interface (Fig. 1D) and its side chain establishes a hydrogen bond with Thr222. Therefore, it seems likely that phosphorylation alters the Thr214 pattern of interactions (i.e. potentially affecting the local protein folding or energy) thus influencing target binding and/or domain swapping. In order to better understand how phosphorylation of Thr214 may affect the protein motion, the effects of phosphorylation on the dynamical behavior of g14-3-3 were analyzed by in silico phosphorylation of Thr214 in the 3D structure of the g14-3-3 monomer and MD simulations on both the unmodified (WT-g14-3-3) and the phosphorylated systems (Pho-g14-3-3). An MD simulation of 50 ns was run and the first 20 ns were discarded to take into account the system equilibration so that all the analyses were carried out on the last 30 ns (Fig. 5A). The effects of the phosphate group on the protein motion are clearly visible (Fig. 5B). In the WT-g14-3-3 monomer, helices H and I fluctuated much more than in Pho-g14-3-3. The calculated per residue Root Mean Square Fluctuation (RMSF) showed that residues 1-170 fluctuated similarly in WT-g14-3-3 and Pho-g14-3-3, whereas differences could be observed in the helices upstream and downstream Thr214 (Fig. 5B). Interestingly, fluctuations of the loop HI linking helices H and I were similar in the two systems. The behavior of the C-terminal of the protein upon phosphorylation was even more evident in the principal component analysis (PCA) results (Fig. 5C, 5D and 5E). The first 10 eigenvectors (Fig. 5B) described about the 70% of the fluctuations in the essential space and represented most of the protein motion in both phosphorylated and non-phosphorylated proteins (each dot in the plot represents an eigenvector). However, the weight of the first two eigenvector represents about the 35% of the global fluctuations in the WT-g14-3-3 monomer (Fig. 5C), and only the 25% in Pho-g14-3-3 (Fig. 5C). The first two eigenvectors described predominantly the motion of helices H and I, which were subjected to larger movements in WT-g14-3-3 (Fig. 5E) than in Pho-g14-3-3 (Fig. 5D), where they were also closer to each other. The results of the PCA were consistent with the MD snapshots taken at 20, 30, 40, and 50 ns (Fig. 6A). Overall, the computational study showed that the phosphorylated residue acts as a “lock” in the elbow between helix H and I. The negatively charged phosphate, indeed, could still allow the interaction with the polar side chain of Thr224, as the

Figure 2. Sequence alignment of g14-3-3 protein. The g14-3-3 protein has been aligned with two Ordeum vulgaris isoforms, one from epsilon and one from non-epsilon plant 14-3-3 subgroups (b14-3-3A, P29305.1; b14-3-3B, Q43470.1, respectively), the two Saccharomyces cerevisiae yeast isoforms (BMH1, P29311.4; BMH2, P34730.3) and the seven human 14-3-3 isoforms (h14-3-3, accession number NP_647539.1; h14-3-3c, NP_008681.1; h14-3-3γ, NP_003396.1; h14-3-3ζ, NP_003397.1; h14-3-3γ, NP_036611.2; h14-3-3σ, NP_006133.1; h14-3-3c, NP_006752.1). Alignment has been generated using ClustalW2 software and edited with BOXSHADE 3.21. Identical residues are black boxed, similar residues are gray boxed, divergent ones are left unboxed. Dashes indicate gaps. A grey line above the alignment indicates the α-helices, the thin line indicates a region of helix310, observed also in h14-3-3 γ, η and σ. Residues involved in N-terminal dimerization are indicated by stars; the triad of residues contacting the phosphate moiety in the target phosphopeptide are indicated by empty triangles. Dots indicate other residues taking contact with the target phosphopeptide backbone and lateral chains. Black triangles indicate Thr214 and Glu246 of g14-3-3. White squares indicate the residues Arg200, Thr208, and Asn233 forming intermolecular hydrogen bond in the C-terminal dimer interface.

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Structural role of g14-3-3 phosphorylation

The solved structure of g14-3-3 is not phosphorylated and Thr214 is buried at the C-terminal interface (Fig. 1D) and its side chain establishes a hydrogen bond with Thr224. Therefore, it seems likely that phosphorylation alters the Thr214 pattern of interactions (i.e. potentially affecting the local protein folding or energy) thus influencing target binding and/or domain swapping. In order to better understand how phosphorylation of Thr214 may affect the protein motion, the effects of phosphorylation on the dynamical behavior of g14-3-3 were analyzed by in silico phosphorylation of Thr214 in the 3D structure of the g14-3-3 monomer and MD simulations on both the unmodified (WT-g14-3-3) and the phosphorylated systems (Pho-g14-3-3). An MD simulation of 50 ns was run and the first 20 ns were discarded to take into account the system equilibration so that all the analyses were carried out on the last 30 ns (Fig. 5A). The effects of the phosphate group on the protein motion are clearly visible (Fig. 5B). In the WT-g14-3-3 monomer, helices H and I fluctuated much more than in Pho-g14-3-3. The calculated per residue Root Mean Square Fluctuation (RMSF) showed that residues 1-170 fluctuated similarly in WT-g14-3-3 and Pho-g14-3-3, whereas differences
non-phosphorylated Thr214 did, and additionally formed a network of interactions with the polar side chain of Tyr218 and with the positively charged side chain of Lys219 (Fig. 6B), by establishing several hydrogen bonds and a salt bridge. The stiffening of the hinge between helices H and I in presence of the phosphate group forced the two helices to keep a much closed conformation during the simulation and oscillated much less than in the non-phosphorylated protein. These interactions were much weaker, or even missed, in WT-g14-3-3 where the negatively charged phosphate group on Thr214 is absent. The lack of such interaction network in WT-g14-3-3 made helix I more flexible and capable of moving away from helix H, thus permitting the observed "extended" conformation of the protein C-terminal.

Despite the more closed conformation imposed by the phosphorylation of Thr214, the structural superimposition of Pho-g14-3-3 (at 20 ns of MD), WT-g14-3-3 and the h14-3-3e/RRQRpSAP complex (Fig. 6C) shows that the Pho-g14-3-3 helix I does not overlap with helix I of h14-3-3e, as indeed the swapped helix I' of g14-3-3 does (Fig. 4), leading to the possibility that phosphorylation alone might not be sufficient to both hide the patch of

**Figure 3. C-terminal domain swapping leads to fibrils formation in the crystal.** A) Two adjacent dimers within the crystal (green, orange), corresponding to one asymmetric unit, give rise to non-canonical C-terminal dimerization through the swapping of helix I. B) Detail of the interface showing the residues involved in hydrogen bonds. C) The superimposition of g14-3-3 and human 14-3-3 (violet) emphasizes the swapping of helix I (g14-3-3 αI' vs h14-3-3e αI) as indicated by the arrow. D) Crystal packing (36 unit cells, 6x6x1). The crystal is formed by layers (blue, red, yellow) of endless parallel filaments.

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**Figure 4. Phosphopeptide binding site is preserved upon helix I swapping.** A) A sulfate ion bound in correspondence of the phosphate binding site in g14-3-3. 2mFo-dFc electron density (contoured at 1σ) is shown for sulfate ion and the residues involved in the interaction. B) Superimposition of g14-3-3 (green, orange) and 14-3-3e-phosphopeptide (2BR9, violet-pink) complex showing that the swapped helix I' can participate to peptide binding. C) Detail of the peptide-binding site. The peptide (from the 2BR9 structure) and the residues involved in the interaction, all conserved in g14-3-3, are represented as sticks. D) Orientation of the phosphopeptides modeled into a g14-3-3 filament. The phosphopeptides in the binding cleft are represented by arrows blue to red (N-terminus to C-terminus).

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hydrophobic residues that are in the C-terminal dimer interface and impair the C-terminal domain swapping.

Effect of amino acid substitutions and PTMs on oligomerization

In order to confirm the tendency of recombinant g14-3-3 to form multimers and shed light on the mechanisms leading to the observed “extended” conformation and domain swapping, different g14-3-3 mutants were constructed. Mutants were analyzed by native PAGE and chemical cross-linking, two well established methods already used to assess h14-3-3 dimmerization properties [42]. As previously observed, residues Arg200, Thr208, and Asn233, which are unique to g14-3-3, may contribute to promote/stabilize the “extended” conformation and the co-occurring C-terminal domain swapping. For this reason, two of them were mutated, Arg200 into lysine (R200K) and Thr208 into alanine (T208A). Lysine and alanine are the residues found in the corresponding positions in all human 14-3-3 isoforms (Fig. 2). Furthermore, due to the relevance of polyglycilation, two mutants were constructed in which the last two C-terminal residues of g14-3-3 were replaced by a stretch of 10 or 20 glycines (polyG10 and polyG20). These mutants could potentially mimic both the presence and the variation in length of the polyglycine chain occurring during Giardia encystation [15]. In addition, the previously described T214E mutant was also included [18]. As controls, the well-characterized h14-3-3 and the affinity-purified endogenous g14-3-3 from Giardia trophozoites, which are physiologically phosphorylated at Thr214 (Sup. Fig. S2), and with different length of polyglycine chain at Glu246 [15], were also analyzed. No variation in the secondary structure could be evidenced by circular dicroism analyses between the unmodified recombinant g14-3-3 and the mutant proteins (Fig. 7A).

As a result of the chemical cross-linking reactions using DMP (9.2 Å spacer arm), bands compatible with the formation of covalent dimer products were visible for both the endogenous g14-3-3 and, as expected, for the h14-3-3 [42], as consequence of the N-terminal dimerization (Fig. 7B, left panel). In agreement with the solved crystal structure, dimeric, trimeric and tetrameric forms were clearly visible in the recombinant g14-3-3, and each of the T214E and R200K mutants, likely as combination of both N-terminal and C-terminal dimerization. Moreover, faint bands at molecular weight compatible with pentameric forms were also

Figure 5. Molecular Dynamic simulation. A) The evolution of the RMSD (Root Mean Square Deviation) of WT-g14-3-3 (in red) and Pho-g14-3-3 (in black) during the simulation. In order to allow the system to equilibrate, the first 20 ns of simulation were discarded from the analyses (region shaded in grey). B) The per residue RMSF (Root Mean Square Fluctuation). Helices H and I are enclosed in dashed boxes. The position of Thr214 is indicated with an arrow. The bottom plot (grey) shows the per residue RMSF difference between WT-g14-3-3 and Pho-g14-3-3. A schematic secondary structure diagram of the g14-3-3 is reported on the top. C) Eigenvectors cumulative weight on total motion for WT-g14-3-3 (in red) and Pho-g14-3-3 (in black). Projection of motion along the first eigenvector for Pho-g14-3-3 D) and the WT-g14-3-3 E) protein structures. The amplitude of motion follows the color scale from red to blue. The phosphorylated residue is represented in grey and indicated with an arrow.

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visible suggesting the occurrence of higher oligomers that, although, could not be resolved in the gel matrix. In contrast, the formation of trimers and tetramers was disfavored in the T208A mutant, as well as in polyG10 mutant and, even more, in polyG20 mutant (Fig. 7B, left panel), thus suggesting that in these mutants C-terminal dimerization could be hampered. Furthermore, when the incubation and the cross-linking were performed in presence of DTT no differences in oligomerization could be observed (Fig. 7B, right panel), thus indicating that disulfide bridges, if any, are not involved in g14-3-3 oligomerization.

Similarly, in native electrophoresis both h14-3-3 and endogenous g14-3-3 existed exclusively as N-terminal dimers (Fig. 7C). Compatibly with the gel resolution limits, for the recombinant g14-3-3, and each of the T214E and R200K mutants, a tetrameric form was favored over the dimeric one, with the R200K mutant nearly completely tetrameric, as supported by densitometric analysis of the protein bands (Fig. 7C–7D). On the opposite, in the polyG20, polyG10 and T208A mutants, the dimeric form was prevalent, with polyG20~polyG10~T208A (Fig. 7C–7D), further supporting that C-terminal dimerization was disfavored. For all the tested proteins, binding to target did not affect neither the dimerization nor the oligomerization as it resulted by the incubation of the proteins alternatively with the Raf1p phosphopeptide, reproducing a g14-3-3 consensus mode-1 binding sequence of the Raf-1 protein kinase [15], or the corresponding non phosphorylated peptide (Raf1) (Fig.7B-7C). However, this observation cannot be extended to the endogenous g14-3-3 that, due to the purification procedure, was already in complex with the Raf1p (Fig. 7B–7C).

These results strongly indicate that: i) the addition of a C-terminal polyglycine stretch, but not the presence of a negative charge at position T214, primarily impairs oligomerization of recombinant g14-3-3; ii) mutations R200K and T208A indeed affect oligomerization but in opposite directions, i.e. R200K favors it whereas T208A does not.

Finally, to further prove that the observed g14-3-3 filaments were not a crystallographic artifact and that recombinant g14-3-3 can form multimers in solution, the protein was studied with transmission electron microscopy. As shown (Fig. 8A), filaments of approximately 6 nm of thickness and ranging in length from 0.8 up to 1.6 μm were visible in the g14-3-3 samples. Intersecting filaments of greater length (up to 4 μm) were observed when the
protein concentration was increased from 0.1 to 1 \( \mu \text{g}/\text{ml} \) (Fig. 8C, 8D and 8E). In agreement with native gel and cross-linking data, no filament could be observed in the polyG20 sample (Fig. 8B). The size of the observed g14-3-3 filaments was also in good agreement with measurement obtained from the solved crystal structure (Fig. 8F). Altogether, these data are in favor of the notion that oligomerization of g14-3-3 end up in filamentous structures, and non in globular structures, that according to the crystallographic data likely depends on domain swapping-mediated C-terminal dimerization.

**Discussion**

We report the X-ray structure of *Giardia duodenalis* 14-3-3 (g14-3-3). A structural analysis confirmed that the protein has many of the features common to the other 14-3-3 family members and also highlights functional/structural roles for two peculiar g14-3-3 post-translational modifications, i.e. phosphorylation of Thr214 and polyglycation of Glu246.

Despite the quite large number of 14-3-3 crystal structures available, we provide the first evidence that a *Giardia* member of the 14-3-3 family, deprived of post-translational modifications, forms oligomers *in vitro*. Analyses of the crystal structure revealed that recombinant unmodified g14-3-3 forms filaments likely by concatenation of the functional dimer. Such process, which was further confirmed by transmission electron microscopy, is enabled by the combination of N-terminal dimerization and the swapping of two helices between two adjacent g14-3-3 monomers with the formation of a four-helix bundle. Interestingly, this assembly retains the peptide-binding groove, but requires the rearrangement of the HI-loop with respect to other known 14-3-3 structures.

Domain swapping is dependent on the so called “hinge loop”, defined as a region of the protein that folds back on itself in the monomer but adopts an extended conformation in the domain-swapped form. Mutations in this region can in principle modulate
the propensity of the protein to domain-swap (44). Consistently
with this notion and our MD simulations, the g14-3-3 HI region
seems to represent the hinge loop, since it hosts residues
responsible for the “extended” C-terminal conformation, which
are unique among 14-3-3 family members, such as Thr208 in the
beginning of loop HI, and Arg200, in helix H. Indeed, mutation of
Thr208 to an evolutionary conserved alanine and Arg200 to an
evolutionary conserved lysine affects oligomerization but in
opposite directions. As expected, T208A slightly decreases
oligomerization likely due to the inability of alanine to form
hydrogen bonds with Gln226 and Arg229 thus weakening the
interactions between helices H and I. In contrast, the increased
oligomerization due to the R200K mutation can be explained by
the ability of the arginine to establish an intra-helix interaction
with Asn233 and an inter-helix interaction with Asp204 (in helix
H), thus favoring an equilibrium, whereas the lysine can only
interact with Asn233 on the same helix (Fig. 3D).

The exclusively dimeric nature of the in vivo polyglycylated g14-
3-3 and the hampered oligomerization and filament formation in
both polyG10 and polyG20 mutants strongly suggest that the
physiological role of polyglycylation is to prevent oligomerization
of the protein in vivo. This is consistent with the observation that
g14-3-3 is always polyglycylated in vivo. In fact, a very short
polyglycine chain (less than ten residues) can be detectable at the
g14-3-3 C-terminus even in parasite lines over-expressing the two
g14-3-3-specific deglycylases [19], supporting a detrimental effect
of the lack of the polyglycine chain in vivo. This role of
polyglycylation is in addition to the previously described regulation
of stage-dependent re-distribution of g14-3-3 between nuclei and
cytoplasm already assigned to this PTM [15,18]. The mechanism
by which polyglycylation impairs oligomerization may be due to
steric hindrance and/or disorder promotion. In this regard, the C-
terminal tail of all known 14-3-3s is enriched in disorder-
 promoted residues (i.e. glycine). In fact, deletion of the last 15
C-terminal residues of the h14-3-3 induces aggregation of the
protein in solution at physiological temperature, even if the protein
retains its ability to bind phosphorylated ligands [45]. Since the
g14-3-3 displays a shorter C-terminal tail, polyglycylation may
introduce disorder in the protein [46–47]. Since there is evidence
that polyglycine stretches can form extended structures both in
crystals and in solution, we can speculate that an extended
polyglycine chain could lay between helices H and I of each g14-3-
3 monomer and act as a barrier hindering domain swapping.
Intriguingly, in the C-terminal part of both the S. cerevisiae 14-3-3

Figure 8. Recombinant g14-3-3 form filaments in a concentration-dependent manner. A) Recombinant g14-3-3 (0.1 µg/µl) forms
numerous filaments with moderate length (between 0.8–1.6 µm) and an average diameter of 5.2±0.7 nm, as measured by iTEM soft imaging system
(OLYMPUS). B) At the same concentration (0.1 µg/µl), the presence of the polyglycine stretch in the polyG20 mutant prevents filaments formation
and the protein is found only as amorphous aggregates. C) Longer filaments (more than 3–4 µm) are formed by recombinant g14-3-3 at higher
concentration (1 µg/µl). D) and E) are sections of panel C at high magnification (100.000X). Representative fields on the grids are depicted. F)
Structure-based views of a g14-3-3 filament of concatenated 10 dimers. Dimension (nm) of the filaments are reported.

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primary role of the constitutive g14-3-3 phosphorylation in vivo. This suggests that the glutamic acid mimics the phosphate moiety of the phosphorylated threonine, is still able to oligomerize. This supports that the primary role of the constitutive g14-3-3 phosphorylation in vivo is rather target binding. In MD simulations, upon phosphorylation of Thr214, helix I moves closer to helix H and causes Trp235, Asn231 and Leu223 to steadily face the phosphopeptide binding groove. Interestingly, these three residues correspond to Thr228, Asn231 and Leu216 (h14-3-3e numbering), which in plant and mammalian 14-3-3s are directly involved in the binding to target peptides both in phosphorylation-dependent and –independent manners [8]. In contrast, in the non phosphorylated g14-3-3, the interaction of Thr235, Asn231 and Leu223 with the target proteins would first require a large conformational change repositioning helix I closer to helix H or would need domain swapping of helix I from another g14-3-3 dimer. This would likely entail a weaker initial binding of g14-3-3 to its target. Thus, in vivo phosphorylation might promote a faster and/or much more stable interaction with at least a pool of client proteins. This may also explain the defect in encystation observed when the non-phosphorylatable T214E mutant is overexpressed in G. duodenalis [18].

The determination of the g14-3-3 structure raises new interesting questions. Due to the early evolutionary branching of Giardia and the long evolutionary history of this parasite, why have divergent residues been fixed in the g14-3-3 leading to conformational rearrangement and C-terminal domain swapping? Why have PTMs been selected over amino acid mutations to overcome the risk of oligomerization? Completely deglycylated g14-3-3 has not been observed in vivo so far, however, it cannot be excluded that C-terminal domain swapping and limited oligomerization may occur in vivo possibly to mediate multiprotein complex formation. In this regard, the presence of 14-3-3s in protein aggregates have been observed in various human neurodegenerative diseases [49], although the biological significance of 14-3-3s in this context is still far to be clarified. Furthermore, arachidonic acid, known to favor tau and synuclein polymerization, also promotes in vitro oligomerization of h14-3-3S [50]. Intriguingly, sequence similarity at the C-terminal region, in particular in the HI loop, has also been observed between 14-3-3s [31–52], including g14-3-3 (data not shown) and z-synuclein, which form fibrils in Parkinson’s diseases and co-localizes with perinuclear inclusions of huntingtin, and, thus strengthening the structural relevance of this region. It is tempting to speculate that ancestral 14-3-3s underwent C-terminal-mediated oligomerization and that this ability has been overcame during evolution in different ways, i.e. by post-translational glycosylation, C-terminal polyglutamine stretch insertion, or engagement of the C-terminus tail into the binding groove.

In summary, we demonstrated that, despite the highly structural conservation of the 14-3-3 family members, unexpected features can be highlighted by the analysis of a 14-3-3 from the early branching caryophylline Giardia duodenalis. Since inhibition and/or stabilization of 14-3-3-protein interactions has gained substantial interest in pharmaceutical research and provided novel opportunities for the treatment of several diseases [53], the structural peculiarity of g14-3-3 may concretely support the possibility to design antigiardial drugs acting selectively as inhibitor or stabilizer of g14-3-3/target interaction, but ineffective on the human counterparts. The determination of the structure of phosphorylated and/or polyglycylated g14-3-3 in complex with binding peptides or protein targets would help answer several of the still open questions.

Supporting Information

Figure S1 Detailed view of the electron density in the g14-3-3 phosphopeptide binding site. A) mFo-dFc omit map computed without sulfate ion, contoured at 3σ (green) or -3σ (red) in a radius of 4Å around the residues shown as sticks. B) mFo-dFc omit map as in A plus 2mFo-dFc omit map (blue) contoured at 1σ. C) 2mFo- dFc (grey, 1σ) mFo-dFc (green, 3σ; red, -3σ) computed after sulfate ion modeling. (TIF)

Figure S2 Tandem MS analysis of the g14-3-3 phosphorylated peptide (202-219). The MS/MS spectrum of the phosphopeptide A593FDAAITDLDPTEESYK219 (precursor ion (MH)2+ 1055.5) is shown. Detected peaks corresponding to the ions of the b and y series are labeled and indicated in red and blue respectively. The ion at m/z 1006.6 is due to 49 Da neutral loss, corresponding to a phosphoric acid molecule, and demonstrates the presence of a phosphorylated peptide. The distance between y3 and y9 definitively localizes the phosphorylation on the threonine 214. No peak corresponding to the unphosphorylated peptide could be observed. (TIF)

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Author Contributions

Conceived and designed the experiments: ML AI AF AV. Performed the experiments: AF ML AI DdM LB SC. Analyzed the data: AF AI ML AV DdM LB SC. Contributed reagents/materials/analysis tools: ML AI DdM LB SC. Wrote the paper: ML AF AI EP DdM LB SC.

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