Assembly Mechanism of Trypanosoma brucei BILBO1, a Multidomain Cytoskeletal Protein*

Keni Vidilaseris1, Ekaterina Shimanovskaya, Heather J. Esson2, Brooke Morriswood1, and Gang Dong4

From the Max F. Perutz Laboratories, University of Vienna and Medical University of Vienna, Dr. Bohr-Gasse 9, 1030 Vienna, Austria

Received for publication, January 30, 2014, and in revised form, July 14, 2014. Published, JBC Papers in Press, July 16, 2014, DOI 10.1074/jbc.M114.554659

Trypanosoma brucei BILBO1 (TbBILBO1) is an essential component of the flagellar pocket collar of trypanosomes. We recently reported the high resolution structure of the N-terminal domain of TbBILBO1. Here, we provide further structural dissections of its other three constituent domains: EF-hand, coiled coil, and leucine zipper. We found that the EF-hand changes its conformation upon calcium binding, the central coiled coil forms an antiparallel dimer, and the C-terminal leucine zipper appears to contain targeting information. Furthermore, interdimer interactions between adjacent leucine zippers allow TbBILBO1 to form extended filaments in vitro. These filaments were additionally found to condense into fibers through lateral interactions. Based on these experimental data, we propose a mechanism for TbBILBO1 assembly at the flagellar pocket collar.

Trypanosomes are unicellular protists belonging to the order Kinetoplastida, an early diverging eukaryotic lineage (1). They are obligate pathogens responsible for a number of serious diseases in both humans and livestock (2–4). Trypanosoma brucei, which is the best studied species, lives freely in the bloodstream of an infected mammalian host. Transmitted by tsetse flies which is the best studied species, lives freely in the bloodstream of an infected mammalian host. Transmitted by tsetse flies, trypanosomes unsurprisingly exhibit some of the highest rates of endocytic and secretory traffic of any eukaryote (6, 10).

At the neck of the FP is an electron-dense structure termed the flagellar pocket collar (FPC). The FPC encircles the top of the FP to clamp the neck to the flagellar membrane that encloses the axoneme. A recent electron tomography study of the region around the FP suggests that the electron-dense material comprising the FPC forms a horseshoe shape, thus leaving a gap for placement of a specialized microtubule quartet that extends from alongside the basal body down the longitudinal axis of the cell to the cell anterior (12).

Identified in a screen for novel cytoskeletal localizations using polyclonal mouse antisera, T. brucei BILBO1 (TbBILBO1) was the first characterized component of the FPC (13). Loss of TbBILBO1 is lethal in both the procyclic and bloodstream forms of T. brucei, suggesting an essential role in cell viability. Depletion of TbBILBO1 by RNAi causes a cell cycle defect, preventing FP biogenesis (13). The mechanistic explanation for this effect is not known, nor is it clear what function TbBILBO1 is performing in interphase cells.

Cloning of full-length TbBILBO1 (TbBILBO1-FL) and structure-based functional analysis of the N-terminal domain (NTD) have been described recently (14). Here, we report our follow-up structural dissection of the other domains of TbBILBO1 and their respective roles in protein oligomerization. The results provide insights into the self-assembly of TbBILBO1 and suggest a molecular mechanism for the filament-like assembly of the protein at the FPC.

5 The abbreviations used are: FP, flagellar pocket; FPC, flagellar pocket collar; TbBILBO1, Trypanosoma brucei BILBO1; CCD, coiled-coil domain; EFh, EF-hand; FL, full-length; LZ, leucine zipper; MBP, maltose-binding protein; NTD, N-terminal domain; SEC, size exclusion chromatography; SLS, static light scattering; NI-NTA, nickel-nitrotriacetic acid.

* This work was supported by funding from the Max F. Perutz Laboratories and by Grant P24383-B21 from the Austrian Science Fund (to G. D.).
1 Supported by an Österreichischer Austauschdienst graduate scholarship from 2009 to 2012.
2 Present address: Inst. of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, Braníčská 31 370 05, České Budějovice, Czech Republic.
3 To whom correspondence may be addressed: University of Vienna, Dr. Bohrgasse 9/3, 1030 Vienna, Austria. Tel.: 43-1-4277-2034; Fax: 43-1-4277-9616; E-mail: brooke.morriswood@univie.ac.at.
4 To whom correspondence may be addressed: Dept. of Medical Biochemistry, Medical University of Vienna, Dr. Bohrgasse 9/3, 1030 Vienna, Austria. Tel.: 43-1-4277-61625; Fax: 43-1-4277-9616; E-mail: gang.dong@meduniwien.ac.at.

© 2014 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
Assembly Mechanism of Trypanosoma brucei BILBO1

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were obtained from commercial sources: HRP-conjugated anti-mouse (Pierce), anti-GFP (Roche Applied Science), Alexa Fluor 488 goat anti-mouse (Invitrogen), Alexa Fluor 568 goat anti-rabbit (Invitrogen). Sypro® Orange dye (5000× concentration in dimethyl sulfoxide) for thermal shift assays was purchased from Invitrogen. 5-nm Ni-NTA-Nanogold® was purchased from Nanoprobe.

Cloning—Cloning of TbBILBO1-FL has been described previously (14). Briefly, this involved amplification of the ORF from T. brucei genomic DNA by PCR and ligation into the HM15b vector. This vector provides an N-terminal His$_6$-maltose-binding protein (MBP) tag. The His$_6$ part of the tag can be removed using thrombin protease.

N-terminal YFP-tagged TbBILBO1 truncation constructs were subcloned from HM15b-TbBILBO1-FL into the pX52-YFP vector as described previously (14). All TbBILBO1 truncation constructs carrying an N-terminal His$_6$-MBP or MBP-His$_{10}$ tag were subcloned from HM15b-TbBILBO1-FL into either the HM15b or the custom expression vector MalpET. Construct sequences were verified by DNA sequencing.

For production of untagged or MBP-His$_{10}$-tagged recombinant protein of individual domains, PCR products of the TbBILBO1 EF-hand (EFh) (residues 178–250) and coiled-coil domain (CCD) (residues 263–533) were ligated into the MalpET vector. This vector provides an N-terminal MBP-His$_{10}$ tag, which is cleavable by the tobacco etch virus protease.

Cell Lines, Culture, and Generation—The procyclic T. brucei 427 Lister strain was used for localization tests of TbBILBO1 targeting constructs. Transfection of cells was carried out as described previously (14). Transfected cells were cultured overnight in SDM79 medium supplemented with 7.5 µg/ml hemin and 20% (w/v) heat-inactivated fetal calf serum (Sigma-Aldrich) at 27 °C.

Protein Expression and Purification—All recombinant proteins were expressed in Escherichia coli BL21(DE3) cells. Bacteria transformed with cloned constructs were grown at 37 °C to an A$_{600}$ of ~0.6–0.8 and then subjected to cold shock (on ice for 30 min). Protein expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside, and protein production was continued for 20–22 h at 16 °C. Cells were harvested and lysed as described previously for the TbBILBO1-NTD using lysis buffer containing 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole, and 5% (v/v) glycerol (14). Purification was carried out initially on a Ni-HiTrap column (GE Healthcare) as described previously for the TbBILBO1-NTD (14). For His$_6$-MBP-TbBILBO1-FL and truncation constructs, eluted proteins from the Ni-HiTrap column were either used directly for negative staining EM or further purified on a Sephacryl S-400 16/60 gel filtration column to remove the excess imidazole. Samples were incubated with 5-nm Ni-NTA-Nanogold (Nanoprobe) with a 1:50 molar ratio in the labeling buffer (30 min at room temperature) and loaded onto a Formvar-carbon-coated copper grids (30 s at room temperature) and stained in 2% (w/v) uranyl acetate (2 min at room temperature). The samples were viewed, and images were recorded either on an FEI Morgagni 268D transmission electron microscope operated at 80 kV and equipped with an Olympus-SIS charge-coupled device camera. Images were examined and analyzed using ImageJ software.

Circular Dichroism (CD)—CD measurements were performed using a Chirascan-plus CD spectrometer (Applied Photophysics). 150 µl of TbBILBO1-EFh (residues 178–250, 50 µg/ml) was incubated with each in duplicate between 190 and 260 nm, using a 0.5-cm path-length cuvette. A sampling time per point of 1 s.
and a bandwidth of 1 nm were used. CD signals of buffer alone were subtracted from all sample measurements.

Static Light Scattering (SLS)—SLS measurements were carried out on a mini-DAWN™ TREOS® instrument (Wyatt Technology Corp.) connected in-line to an AKTA purifier equipped with a Superdex S-200 10/300 GL size exclusion chromatography (SEC) column. For molecular mass determination of the TbBILBO1-EFh, the column was pre-equilibrated overnight with SLS buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM CaCl₂ or 2 mM EDTA). 100 μl of TbBILBO1-EFh (10 mg/ml) was incubated with either 2 mM CaCl₂ or 2 mM EDTA (1 h at room temperature) and then injected to the column and run with a flow rate 0.5 ml/min. All measurements were taken at room temperature. The results were analyzed using the ASTRA program. For molecular mass determination of the TbBILBO1-CCD, the column was equilibrated with SLS buffer without CaCl₂ or EDTA.

Thermal Shift Assay—The assay was carried out based on a previously published protocol (15). Solutions of 7.5 ml of 17× Sypro Orange (Molecular Probes) and 12.5 ml of protein buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% (v/v) glycerol) containing either 5 mM CaCl₂ or 5 mM EDTA were used. 5 ml of TbBILBO1-EFh (2.5 mg/ml) was added onto a 96-well thin-wall PCR plate (Bio-Rad), which was then sealed with optical-quality sealing tape (Bio-Rad) and heated in an iCycler iQ real-time PCR detection system (Bio-Rad) from 20 to 95 °C with increments of 0.5 °C.

Chemical Cross-linking by Glutaraldehyde—The TbBILBO1-CCD (10 μl, 0.1 mg/ml) was incubated in cross-linking buffer (20 mM HEPES-NaOH, pH 8.0, and 100 mM NaCl) in the absence or presence of 0.01, 0.001, or 0.0001% (v/v) glutaraldehyde (room temperature for 10 min). Reactions were terminated by adding 1 μl of 1 M Tris-HCl, pH 8.0, to the 10-μl reaction solution. Results were analyzed by SDS-PAGE.

RESULTS

Protein Domain Organization—TbBILBO1 is a modular protein with three structured domains, comprising the NTD (residues 1–110), two tandem EFh motifs (residues 183–249), and the coiled-coil region (residues 263–578). Regularly spaced repetitive leucines in a predicted coiled coil are the characteristic feature of a leucine zipper (LZ) (16). Closer examination of the coiled-coil region by the 2ZIP-Server (17) revealed a putative LZ (residues 534–578) at its C-terminal end (Fig. 1A). The primary sequences of these structured domains are all highly conserved in the Trypanosomatida, with the exception of a linker region, which is not found in Leishmania species. Sequence conservation in the linker region is also lower than in the structured domains (Fig. 1B).

Targeting of TbBILBO1 to the FPC—We recently reported that the NTD is not required for TbBILBO1 targeting to the FPC (14). To determine whether the putative LZ played a role in targeting, we tested three more YFP-tagged truncation constructs (Fig. 2A). These constructs were transiently expressed in T. brucei and their localization analyzed by immunofluorescence microscopy. YFP-TbBILBO1-FL localized to the FPC region (Fig. 2B, arrowhead), which matches the distribution seen for the endogenous protein (13). Similar to what was seen for the NTD alone (14), a construct containing the NTD and EFh domains localized to the cytoplasm and not to the FPC (Fig. 2C). Furthermore, a second, longer construct encoding the NTD, EFh, and CCD but not the LZ still did not target correctly. This construct (YFP-TbBILBO1-ΔLZ) instead formed numerous cytoplasmic punctae. Although there was a concentration of these punctae in the region of the FPC, they did not correctly localize to the FPC (Fig. 2D, arrow). This suggested that the LZ was required for correct targeting. The LZ by itself was cytoplasmic (Fig. 2E). It was concluded that the LZ is necessary but not sufficient for targeting to the FPC. Expression of the truncation constructs was confirmed by immunoblots of whole-cell lysates from transfected cells using anti-GFP antibodies (Fig. 2F, left and middle panels). All proteins were expressed, although the expression levels of the two short constructs, YFP-TbBILBO1-NTD-EFh and -LZ, were considerably lower than the others. Blotting with anti-TbBILBO1 antibodies showed that the total amount of any given truncation construct was significantly less than the total amount of endogenous TbBILBO1 in the population (Fig. 2F, right panel).

The EFh Motifs Change Their Conformation upon Binding Calcium—Previously, we had determined the structure of the NTD and its essential role in cell viability but found that it is not required for TbBILBO1 localization (14). Given their predicted structural properties and/or role in FPC targeting (Figs. 1 and 2), it was hypothesized that the other domains of TbBILBO1 are important for its assembly at the FPC.

TbBILBO1 has two predicted tandem EFh motifs between residues 183 and 249. EFh motifs have been found in a large number of protein families, with most of them possessing the ability to bind calcium (18). The TbBILBO1-EFh is homologous to a human calmodulin-like protein, hCLP, with a sequence identity of 29.4% (19). Taking the hCLP structure as the template, we carried out homology modeling of the TbBILBO1-EFh using the (PS)2-v2 protein structure prediction server (20). The results showed that the other domains of TbBILBO1 are important for its assembly at the FPC.

It is known that EFh motifs often undergo structural rearrangements upon binding calcium (18, 21). To verify whether the putative EFh motifs in TbBILBO1 indeed bind calcium and whether calcium binding induces any conformational change to the protein, we performed biophysical analysis using CD, SLS, and temperature-based ThermoFluor methods. The experiments were carried out on untagged recombinant TbBILBO1-EFh (residues 178–250). The far-UV CD analysis showed that removal of calcium by 2 mM EDTA (apo-form) resulted in a classical random coil structure with a maximum below 0 millidegrees and a minimum at ~200 nm. In contrast, the results showed that the TbBILBO1-EFh has several conserved acidic residues in the loops, which are likely to coordinate calcium binding (Fig. 3A) (20).
Assembly Mechanism of Trypanosoma brucei BILBO1

**Figure 1. TbBILBO1 domain organization and sequence alignment.** A, schematic depicting the arrangement of the four domains of TbBILBO1: NTD, EF, CCD, and LZ. Amino acid numbers are indicated above the schematic. B, sequence alignment of TbBILBO1 and homologs from related trypanosomatids. Identical (black) and similar (gray) residues are shaded. Domains are indicated by bars under the aligned sequences using the color scheme detailed at the bottom. Conserved leucines in the C-terminal LZ are highlighted in gold. Tb, T. brucei; Tc, T. cruzi; Lb, Leishmania braziliensis; Li, Leishmania infantum; Lm, Leishmania major.

more loosely packed, allowing them to be eluted earlier from the gel filtration column. Furthermore, ThermoFluor assays showed that the melting temperatures for the calcium-bound and the apo forms were 57.0 and 38.5 °C, respectively (Fig. 3D). This further demonstrated that binding to calcium makes the EF motifs become well folded, with a more compact structure that resists induced unfolding at high temperatures.

The TbBILBO1 Coiled-coil Domain Forms an Antiparallel Homodimer—Given that the CCD (residues 263–533) represents nearly half of the full-length protein, it is necessary to know the structural arrangement of this domain in order to find out how TbBILBO1 assembles. Using SLS it was found that untagged recombinant CCD forms a dimer in solution (Fig. 4A). The dimeric conformation of the CCD was further confirmed by glutaraldehyde-mediated cross-linking, which showed the formation of homodimers after incubation of the protein with 0.001–0.01% (v/v) glutaraldehyde (Fig. 4B). Initial crystallization experiments using the CCD did not yield any crystals that diffracted well, so low resolution EM methods were employed instead to elucidate the relative orientation of the two coiled coils in the dimer. For these experiments, recombinant TbBILBO1-CCD was expressed with an N-terminal MBP-His\textsubscript{10} tag. The 42-kDa MBP has a globular structure of ~5 nm in diameter, which is significantly larger than the ~1.5-nm width of a typical two-stranded coiled coil. If the CCD forms an antiparallel dimer, one would expect to see a dumbbell-like structure with two globules at opposite ends of the coiled-coil rod. Conversely, a parallel dimer would show a hammer-like structure with both MBP moieties spatially close to each other (Fig. 4C). Using rotary metal-shadowing EM, the fusion protein was found to appear in a dumbbell-like configuration, suggesting that the TbBILBO1-CCD forms an antiparallel dimer (Fig. 4D).
The TbBILBO1 Leucine Zipper Mediates Protein Oligomerization—Recombinant His6-TbBILBO1-FL was expressed in bacteria, but no soluble protein could be purified by affinity chromatography.6 His6-MBP-TbBILBO1-FL was however sol-

uable when expressed and could be purified by affinity chromatography. The purified protein was checked by SEC (Fig. 5). Curiously, a large fraction of His6-MBP-TbBILBO1-FL failed to pass through the column. The intact protein that did pass through was almost exclusively present in the void volume ($V_0$) of the column, suggesting that the protein either aggregated or formed very large oligomers (Fig. 5, A and B). Deletion of the

---

6 K. Vidilaseris, E. Shimanovskaya, H. J. Esson, B. Morriswood, and G. Dong, unpublished data.

---

**FIGURE 2.** The TbBILBO1-LZ is necessary but not sufficient for targeting to the FPC. A, TbBILBO1 truncation constructs used for the experiment. B–E, localization of YFP-tagged TbBILBO1 truncations in transiently transfected T. brucei cells. Fluorescence microscopy was used to visualize protein localizations. For YFP-TbBILBO1-NTD-EFh and -LZ, low expression levels precluded observation of YFP directly, and anti-GFP antibodies were used to visualize the protein instead. DAPI (blue) was used to stain DNA. The insets in B and D, indicated by white-outlined boxes, are enlargements of the smaller boxed areas. Scale bars: 5 μm. F, immunoblots of whole-cell lysates from transiently transfected T. brucei cells probed using anti-GFP antibodies or anti-TbBILBO1-NTD. The YFP-tagged proteins are indicated by arrows. Longer exposures were used for YFP-TbBILBO1-NTD-EFh and -LZ to better visualize the target proteins. Endogenous TbBILBO1 proteins are marked by an empty arrowhead. The YFP-TbBILBO1-LZ construct is not detectable with the anti-TbBILBO1-NTD antibodies, as it does not contain the NTD.

---

23874 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 289 • NUMBER 34 • AUGUST 22, 2014
EFh motifs resulted in more protein being eluted, but the majority remained at $V_0$ (Fig. 5, A and C). Interestingly, however, deletion of the C-terminal LZ not only allowed almost all protein to pass through the column but also shifted the elution peak to a position corresponding to a molecular mass of 300 kDa (Fig. 5, A and D). The gel filtration column separates proteins based on their Stokes radius, which is determined by both the molecular weight and the shape (23). An elongated molecule typically elutes much faster than a globular protein on SEC (24). Therefore, the 300-kDa peak is consistent with the ∼200-kDa dimeric His$_6$-MBP-TbBILBO1-ΔLZ containing an extended coiled coil. This implied that the LZ mediates oligomerization of TbBILBO1.

**The TbBILBO1 Leucine Zipper Mediates Filament Assembly**—The behavior of His$_6$-MBP-TbBILBO1-FL on SEC suggested that it might form large oligomers. To examine this possibility, rotary metal-shadowing EM experiments were carried out using the purified His$_6$-MBP-TbBILBO1-FL eluted in the $V_0$ of the SEC column. The EM images revealed a filament-like structure decorated with paired globules along the filament (Fig. 6A). The lengths of the interval rods were measured at 40–45 nm, in good agreement with the length of the TbBILBO1-CCD rod (Fig. 4D). The globular structures, 5–7 nm in diameter, matched the size of the MBP moiety together with the fused TbBILBO1-NTD. This assembly mode suggests that the oligomerization of TbBILBO1 is mediated by one or more of the...
structural elements flanking the CCD: the NTD and the EFh on the N-terminal side and the C-terminal LZ (Fig. 1A). This hypothesis was systematically tested by removing each domain in turn and then examining the purified protein by rotary metal-shadowing EM.

Removal of the NTD did not significantly affect filament formation, although it led to a reduction in the average filament length (Fig. 6B). Deletion of the EFh motifs produced a similar effect (Fig. 6C). However, deletion of the LZ completely abolished filament assembly, resulting in only dumbbell-like
structures as seen for the MBP-His10-tagged CCD alone (compare Fig. 6D with Fig. 4D). The frequency of the dumbbell-like structures was quantified for the TbBILBO1-CCD (Fig. 4D) and compared with that seen for TbBILBO1-H9004/LZ (Fig. 6D). For both constructs, the frequency of occurrence in the rotary metal-shadowing micrographs was around 95% (Fig. 6E). This emphasizes that in the absence of the LZ, TbBILBO1 preferentially forms antiparallel dimers. These results are consistent with the SEC data, which also suggested that deleting the LZ favored dimer formation (Fig. 5, A and D).

TbBILBO1 Assembles into Fibrous Bundles—Given that the His6-MBP-TbBILBO1-FL forms filament-like oligomers (Fig. 6A) and the majority of it was unable to pass through the SEC column (Fig. 5, A and B), it seemed likely that TbBILBO1 might form even larger and higher order assemblies. Therefore, His6-MBP-TbBILBO1-FL was examined directly after affinity purification without doing SEC. It was found that the linear filaments were capable of forming much larger assemblies. Negative staining EM images showed that two TbBILBO1 filaments could laterally interact with each other (Fig. 7, A and B). Furthermore, multiple filaments were frequently observed to interact laterally to assemble into a highly ordered fiber (Fig. 7, C and D). These bundled fibers could condense and extend for a few micrometers, suggesting that it is a specific interaction-mediated higher order assembly. Furthermore, the examination of ~100 of these fibrous structures in our collected EM images indicated that, although the single and double filaments appeared in similar frequencies, the highly ordered fibrous bundle was much more abundant (Fig. 7E). This sug-
gested that the bundled structure could be stably maintained after formation.

To reveal the substructures of the lateral associations, we further labeled the N-terminal polyhistidine tag with 5-nm Ni-NTA-Nanogold (Fig. 8A). The labeled sample was then examined by negative staining EM (Fig. 8, B–E). The micrographs showed similar linear structures marked by gold particles ~45 nm apart (Fig. 8B). These filaments were found to interact bilaterally with each other (Fig. 8C) and form two-stranded structures (Fig. 8D). Frequently, bundled structures formed by multiple filaments were observed (Fig. 8E). Furthermore, we found that the Nanogold particles were clustered regularly along the assembled bundles with intercluster distances of ~45 nm (Fig. 8E, white arrowheads). It suggests that the TbBILBO1-NTDs in the fibrous bundles are in register and spatially close to one another.

**DISCUSSION**

We recently reported the high resolution structure of the TbBILBO1-NTD and demonstrated its essential requirement for cell viability (14). Here, we report the results of our investigation on the assembly mechanism of TbBILBO1. Bioinformatic analysis suggested that TbBILBO1 consists of four structural domains and not three as originally annotated (Fig. 1). The C-terminal end of the CCD was found to encode a putative LZ element. Immunofluorescence analysis using various truncations of the protein revealed that this C-terminal LZ is necessary but not sufficient for targeting (Fig. 2). The EFh motifs were found to bind calcium, which induced dramatic conformational changes to the protein (Fig. 3). To understand the architecture of the CCD, we employed rotary metal-shadowing EM to demonstrate that it forms an antiparallel dimer (Fig. 4).
SEC results showed that purified recombinant TbBILBO1 forms large assemblies (Fig. 5). Further experiments were then carried out on the purified recombinant full-length protein and various truncations. Results from rotary metal-shadowing EM demonstrated that TbBILBO1-FL forms linear oligomers and that deletion of either the NTD or EFh domains does not significantly impair the oligomerization capacity. Removal of the C-terminal LZ, however, prevented the formation of oligomers and led to a predominance of dimeric forms (Fig. 6). When purified recombinant full-length TbBILBO1 was analyzed by EM without first separating the preparations by SEC, it was found that the protein formed large fibrous bundles with multiple strands associated together laterally (Fig. 7). Both the linear and the lateral assemblies of TbBILBO1 were also confirmed by Nanogold labeling EM studies (Fig. 8).

Drawing from all these observations, the following model for the structure and assembly of TbBILBO1 is proposed (Fig. 9). TbBILBO1 forms an antiparallel dimer via its central CCD, with the NTD and EFh domains projecting outward from the dimer in opposing directions. During purification of the various constructs of TbBILBO1, it was observed that the variable loop region between the NTD and EFh domains appears to be more prone to degradation than the rest of the protein. The formation of interdimer contacts via the projecting domain of TbBILBO1, lamin proteins form a parallel hammer-like homodimer, which further assembles linearly by head-to-tail interactions of neighboring dimers (33).

Most leucine zippers form parallel dimers (35–40). Nevertheless, both naturally occurring and designed antiparallel leucine zippers have been reported (11, 25, 41). Observation of the antiparallel homodimer of the LZ deletion construct (Fig. 6D) and the long filament of full-length TbBILBO1 (Fig. 6A) strongly suggests that the LZ is in an antiparallel configuration so as to mediate the interaction of two neighboring homodimers (Fig. 9).

Therefore, to the best of our knowledge, TbBILBO1 appears to be the first protein that not only contains a stably assembled long antiparallel coiled coil but is also interconnected by antiparallel LZs at the C termini of the helices to form a filament of potentially infinite length. The filament of TbBILBO1 is decorated with calcium-binding EF-hand motifs and functional globular domains (the NTDs), which together make TbBILBO1 a highly unusual protein oligomer. The positioning of the NTDs and EFh motifs in the filaments makes it possible that these domains have a role in coordinating the oligomeric interactions (Fig. 9, inset). This is partially supported by the observation that although deletion of these two regions does not affect filament assembly, it does appear to shorten the total length of the oligomers, implying a stabilizing function (Fig. 6, B).
Assembly Mechanism of Trypanosoma brucei BILBO1

...and C). In the case of the EFh deletion, non-specific lateral interactions seemed to occur, as demonstrated by multiple clustered bead-like structures along the filament (Fig. 6C). This offers further support for the hypothesis that the EFh might regulate the lateral associations of neighboring filaments. In the future, further examination of the EFh-regulated fibrous assembly of TbBILBO1 should be carried out in vitro and/or in vivo.

Previous studies showed that although overexpression of untagged and epitope-tagged TbBILBO1-FL is nontoxic, overexpression of either N- or C-terminally EGFP-tagged TbBILBO1 results in a growth arrest and a dominant negative phenotype similar to that seen following RNAi depletion of the protein (13). Our proposed assembly model of TbBILBO1 could provide a mechanistic explanation for these phenomena. In the model, both the N and C termini of TbBILBO1 are adjacent to the filament junction (Fig. 9, inset). It is tempting to speculate that the large size of the EGFP tag (37 kDa) might either sterically hinder the stable interaction at the junction or interfere with the lateral interactions between neighboring filaments (Fig. 9, inset).

The creation of the observed fibrous bundles of TbBILBO1 (Fig. 7) seems likely to occur by means of lateral interactions between filaments. The data obtained using Nanogold labeling of the filaments offer some support for the idea that the filaments are in register, with the NTDs in alignment (Fig. 8). This is in contrast to the spontaneous half-staggered lateral associations of vertebrate intermediate filament proteins (22).

In summary, our studies provide a structural view and shed light on the assembly mechanism for TbBILBO1 at the T. brucei FPC. Future studies will aim to provide a high resolution structural view of the interdimer junction of the filament and to investigate how TbBILBO1 regulates FPC biogenesis in vivo.

Acknowledgments—We are grateful to G. Warren for insightful input and constructive criticism during the course of the project. We thank I. Lesigang for technical assistance in the laboratory and G. Resch and M. Brandstetter for helping with our EM analysis.

REFERENCES

1. Simpson, A. G., Stevens, J. R., and Lukes, J. (2006) The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22, 168–174

2. Welburn, S. C., and Maudlin, I. (2012) Priorities for the elimination of sleeping sickness. Adv. Parasitol. 79, 299–337

3. Schmich, G. A., and Yaden, Z. E. (2010) Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop. 115, 14–21

4. Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjoux, P., Cano, J., Jannin, J., den Boer, M., and WHO Leishmaniasis Control Team (2012) Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7, e53671

5. Gull, K. (1999) The cytoskeleton of trypanosomatid parasites. Annu. Rev. Microbiol. 53, 629–655

6. Field, M. C., and Carrington, M. (2009) The trypanosome flagellar pocket. Nat. Rev. Microbiol. 7, 775–786

7. Engstler, M., Pfohl, T., Herminghaus, S., Boshart, M., Wiegertjes, G., Hedergott, N., and Overath, P. (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131, 505–515

8. Barry, J. D. (1979) Capping of variable antigen on Trypanosoma brucei and its immunological and biological significance. J. Cell Sci. 37, 287–302

9. Pal, A., Hall, B. S., Jeffries, T. R., and Field, M. C. (2003) Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in Trypanosoma brucei. Biochem. J. 374, 443–451

10. Engstler, M., Thilo, L., Weise, F., Grünfelder, C. G., Schwarz, H., Boshart, M., and Overath, P. (2004) Kinetics of endocytosis and recycling of the GPI-anchored variant surface glycoprotein in Trypanosoma brucei. J. Cell Sci. 117, 1105–1115

11. Bryson, J. W., Betz, S. F., Lu, H. S., Suich, D. J., Zhou, H. X., O’Neil, K. T., and DeGrado, W. F. (1995) Protein design: a hierarchic approach. Science 270, 935–941

12. Lacombe, S., Vaughan, S., Gadelha, C., Morphew, M. K., Shaw, M. K., McIntosh, J. R., and Gull, K. (2009) Three-dimensional cellular architecture of the flagellar pocket and associated cytoskeleton in trypanosomes revealed by electron microscopy tomography. J. Cell Sci. 122, 1081–1090

13. Bonhivers, M., Nowacki, S., Landrein, N., and Robinson, D. R. (2008) Bio-genesis of the trypanosome endo-exocytotic organelle is cytoskeleton mediated. PLoS Biol. 6, e105

14. Vidalisea, K., Morriswood, B., Kontaxis, G., and Dong, G. (2014) Structure of the TbBILBO1 N-terminal domain from Trypanosoma brucei reveals an essential requirement for a conserved surface patch. J. Biol. Chem. 289, 3724–3735

15. Ericsson, U. B., Hallberg, B. M., Dettita, G. T., Dekker, N., and Nordlund, P. (2006) Thermofluor-based high-throughput stability optimization of proteins for structural studies. Anal. Biochem. 357, 289–298

16. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA-binding proteins. Science 240, 1759–1764

17. Bornberg-Bauer, E., Rivals, E., and Vingron, M. (1998) Computational approaches to identify leucine zippers. Nucleic Acids Res. 26, 2740–2746

18. Lewit-Bentley, A., and Réty, S. (2000) EF-hand calcium–binding proteins. Curr. Opin. Struct. Biol. 10, 637–643

19. Han, B. G., Han, M., Sui, H., Yaswen, P., Walian, P. J., and Jap, B. K. (2002) Crystal structure of human calmodulin-like protein: insights into its functional role. FEBS Lett. 521, 24–30

20. Chen, C. C., Hwang, J. K., and Yang, J. M. (2009) (PS)2-v2: template-based protein structure prediction server. BMC Bioinformatics 10, 366

21. Zheng, C., Liu, H. H., Zhou, J., and Zhang, B. (2010) EF-hand domains of MCFD2 mediate interactions with both LMAN1 and coagulation factor V or VIII. Blood 115, 1081–1087

22. Herrmann, H., and Aebl, U. (2004) Intermediate filaments: molecular structure, assembly mechanism, and integration into functionally distinct intracellular scaffolds. Annu. Rev. Biochem. 73, 749–789

23. Erickson, H. P. (2009) Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. Biol. Proced. Online 11, 32–51

24. Dubin, P. L., and Principi, J. M. (1989) Optimization of size-exclusion separation of proteins on a Superose column. J. Chromatogr. 479, 159–164

25. Oakley, M. G., and Hollemen, J. J. (2001) The design of antiparallel coiled coils. Curr. Opin. Struct. Biol. 11, 450–457

26. Biou, V., Yaremchuk, A., Tukalo, M., and Casu, L. (1998) The 2.9 Å crystal structure of T. thermophila seryl-tRNA synthetase complexed with tRNA(Ser). Science 263, 1404–1410

27. Stebbins, C. E., Borukhov, S., Orlova, M., Polyakov, A., Goldfarb, A., and Darst, S. A. (1995) Crystal structure of the GreA transcript cleavage factor from Escherichia coli. Nature 373, 636–640

28. Melby, T. E., Ciampaglio, C. N., Briscoe, G., and Erickson, H. P. (1998) The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. J. Cell Biol. 142, 1595–1604

29. Lassu, D. M., Lee, M., Rackham, O., Stanley, W. A., Sadowska, A., Filipska, A., Fox, A. H., and Bond, C. S. (2012) Structure of the heterodimer of human NONO and paraspeckle protein component 1 and analysis of its role in subnuclear body formation. Proc. Natl. Acad. Sci. USA 109, 4846–4850

30. Zuccola, H. J., Rozelle, J. E., Lemon, S. M., Erickson, B. W., and Hogle, J. M. (1998) Structural basis of the oligomerization of hepatitis delta antigen. Structure 6, 821–830

31. Lu, Q., Ye, F., Wei, Z., Wen, Z., and Zhang, M. (2012) Antiparallel coiled-coil-mediated dimerization of myosin X. Proc. Natl. Acad. Sci. USA 109, 17388–17393
32. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **101**, 789–800

33. Dittmer, T. A., and Misteli, T. (2011) The lamin protein family. *Genome Biol.* **12**, 222

34. Gerace, L., and Huber, M. D. (2012) Nuclear lamina at the crossroads of the cytoplasm and nucleus. *J. Struct. Biol.* **177**, 24–31

35. Diao, J. (2010) Crystal structure of a super leucine zipper, an extended two-stranded super long coiled coil. *Protein Sci.* **19**, 319–326

36. Lavigne, P., Crump, M. P., Gagné, S. M., Hodges, R. S., Kay, C. M., and Sykes, B. D. (1998) Insights into the mechanism of heterodimerization from the 1H-NMR solution structure of the c-Myc-Max heterodimeric leucine zipper. *J. Mol. Biol.* **281**, 165–181

37. Junius, F. K., O’Donoghue, S. I., Nilges, M., Weiss, A. S., and King, G. F. (1996) High resolution NMR solution structure of the leucine zipper domain of the c-Jun homodimer. *J. Biol. Chem.* **271**, 13663–13667

38. Alber, T. (1992) Structure of the leucine zipper. *Curr. Opin. Genet. Dev.* **2**, 205–210

39. O’Shea, E. K., Klemm, J. D., Kim, P. S., and Alber, T. (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* **254**, 539–544

40. Song, X., Li, B., Xiao, Y., Chen, C., Wang, Q., Liu, Y., Berezov, A., Xu, C., Gao, Y., Li, Z., Wu, S. L., Cai, Z., Zhang, H., Karger, B. L., Hancock, W. W., Wells, A. D., Zhou, Z., and Greene, M. I. (2012) Structural and biological features of FOXP3 dimerization relevant to regulatory T cell function. *Cell Rep.* **1**, 665–675

41. Oakley, M. G., and Kim, P. S. (1997) Protein dissection of the antiparallel coiled coil from *Escherichia coli* seryl tRNA synthetase. *Biochemistry* **36**, 2544–2549