Structure of the TbBILBO1 Protein N-terminal Domain from Trypanosoma brucei Reveals an Essential Requirement for a Conserved Surface Patch*

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**Background:** TbBILBO1 is the only known component of the flagellar pocket collar, a cytoskeletal structure in the parasite Trypanosoma brucei.

**Results:** The TbBILBO1 N-terminal domain has a ubiquitin-like fold with a conserved surface patch; overexpression of constructs with a mutated patch is lethal.

**Conclusion:** The conserved surface patch is essential for TbBILBO1 function.

**Significance:** The surface patch is a potential therapeutic target.

TbBILBO1 is the only known component of the flagellar pocket collar, a cytoskeletal barrier element found in trypanosomes. The N-terminal domain (NTD) of TbBILBO1 was found to be dispensable for targeting of the protein in vivo. However, overexpression of constructs lacking the NTD caused complete growth inhibition, implying an essential requirement for this domain. A high resolution structure of the NTD of TbBILBO1 showed that it forms a ubiquitin-like fold with a conserved surface patch. Mutagenesis of this patch recapitulated the phenotypic effects of deleting the entire domain and was found to cause cell death. The surface patch on the NTD of TbBILBO1 is therefore a potential drug target.

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**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The rabbit anti-LdCentrin4 polyclonal antibody and mouse monoclonal anti-Ty1 (B82) antibody were gifts kindly received from Hira Nakhlas (FDA) and Cynthia He (National University of Singapore). The following antibodies were obtained from commercial sources: HRP-conjugated anti-mouse (Pierce), HRP-conjugated anti-rabbit (Dianova) mouse anti-α-tubulin (Roche Applied Science), anti-GFP (Roche Applied Science), Alexa Fluor 488 goat anti-mouse (Invitrogen), and Alexa Fluor 568 goat anti-rabbit (Invitrogen). An Annexin V-FITC apoptosis detection kit was purchased from eBioscience®. Ammonium chloride (15N, 99%) and d-Glu-

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4 The abbreviations used are: FP, flagellar pocket; aPKC, atypical protein kinase C; EGF, enhanced green fluorescent protein; FL, full-length; FPC, flagellar pocket collar; HSQC, heteronuclear single quantum coherence; NTD, N-terminal domain.
cose (13C, 99%) were purchased from Cambridge Isotope Laboratories, Inc. and Euriso-top, respectively.

Cloning and Site-directed Mutagenesis—The full-length TbBILBO1 (TbBILBO1-FL) open reading frame was amplified by PCR from genomic DNA and ligated into the custom vector HM15b. The vector provides an N-terminal His6 tag (cleavable by thrombin) and an N-terminal maltose-binding protein tag. All TbBILBO1 constructs were subcloned from TbBILBO1-FL.

TbBILBO1 localization constructs were generated by amplification of the relevant portions of the TbBILBO1 open reading frame by PCR, with addition of a BamHI restriction enzyme site and a 5′-end Ty1 epitope tag. The PCR products were ligated into the pXS2 expression vector using the BamHI site to generate the pXS2-TbBILBO1 plasmids.

The TbBILBO1-NTD (encoding residues 1–110) for NMR structure determination was cloned into pET15b (Novagen). For the generation of stable cell lines, the TbBILBO1-FL and TbBILBO1-ΔNTD (encoding residues 111–587) open reading frames were amplified from the HM15b-TbBILBO1-FL shuttle vector by PCR with the addition of HindIII and BamHI restriction enzyme sites and a 5′-end sequence encoding a Ty1 epitope tag. They were ligated into the pLEW100 expression vector using HindIII and BamHI sites to generate the pLEW100-TbBILBO1 plasmids.

Mutations in the NTD of TbBILBO1 were generated by site-directed mutagenesis on the pLEW100-TbBILBO1-FL plasmid using a QuikChange kit (Stratagene) according to the manufacturer’s instructions. Incorporation of mutations was confirmed by DNA sequencing. Two sets of mutations were generated, encoding Ty1-TbBILBO1-Mut1 (F12A, K15A, K60A, and K62A) and Ty1-TbBILBO1-Mut2 (W71A, Y87A, and F89A).

Cell Lines, Culture, and Generation—The procyclic T. brucei 29.13 strain was used to generate cells inducibly expressing Ty1-TbBILBO1 constructs (TbBILBO1-FL, -ΔNTD, -Mut1, and -Mut2) (13). The pLEW100-TbBILBO1 constructs were linearized by NotI digestion, and 30 μg was used to transfect 5 × 10^7 29.13 cells by electroporation. Electroporation was carried out using a Gene Pulser XCell (Bio-Rad) with the following parameters: 1500 V, 25 μF, 2 pulses with a 10-s interval. Putative clones were screened for growth in SDM79 medium supplemented with 5 μg/ml phleomycin by limiting dilution. Putative clones were screened for integration of the targeting construct in the genome by PCR using genomic DNA as a template and primers that annealed within the Ty1 epitope and the 3′ site of TbBILBO1 open reading frame. Genomic DNA was isolated using DNeasy genomic DNA isolation kits (Qiagen). The presence of the engineered point mutations in the mutagenesis constructs was further confirmed by sequencing PCR products obtained from amplification of the integrated transgenes. Inducible expression of Ty1-TbBILBO1 constructs was confirmed by immunoblot and immunofluorescence analysis using anti-Ty1 antibodies.

The procyclic T. brucei 427 Lister strain was used for localization of TbBILBO1 targeting constructs. Cells were cultured in SDM79 medium supplemented with 7.5 μg/ml hemin, 20% (w/v) heat-inactivated fetal calf serum (Sigma-Aldrich) at 27 °C. 29.13 cells additionally required 15 μg/ml neomycin and 50 μg/ml hygromycin to maintain T7 polymerase and tetracycline repressor transgenes.

Protein Expression and Purification—For NMR structure determination, Escherichia coli BL21 (DE3) bacteria were transformed with TbBILBO1-NTD (pET15b) and grown in LB medium (37 °C, overnight). The starter culture was then diluted 1:1000 (v/v) in M9 minimal medium containing 1 g/liter NaH4Cl as the sole nitrogen source and 1 g/liter D-[13C]glucose as the sole carbon source. Bacteria were grown at 37 °C to an A600nm ~0.6–0.8 and then subjected to cold shock (ice, 30 min). Recombinant protein expression was induced by addition of 0.5 mM isopropyl 1-thio-β-d-galactopyranoside, and protein production was continued (16 °C, overnight). Cells were harvested by centrifugation in a Sorvall GS3 rotor (6,000 × g, 12 min, 4 °C) and resuspended in 25 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 5% (v/v) glycerol) per liter of expression culture.

Bacteria were lysed in an EmulsiFlex-C3 homogenizer (Avestin) and then centrifuged (40,000 × g, 40 min, 4 °C) to remove cell debris. The supernatant was filtered (0.45-μm pore size) and loaded onto a Ni-HiTrap column (GE Healthcare) pre-equilibrated with the same lysis buffer. The column was washed with 5× column volume of lysis buffer, and bound protein was eluted by a linear gradient concentration of imidazole (20–600 mM, 10× column volume) in the lysis buffer. The N-terminal His6 tag was removed by incubation with 2% (w/w) of thrombin (4 °C, overnight). The protein was concentrated to 2 ml by centrifugation in an Ultra-15 Centrifugal Filter Unit (Amicon) with a 3-kDa molecular mass cut-off and further purified on a Superdex-200 16/60 column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.8) and 100 mM NaCl. The protein was then concentrated to ~1 ml with a volume of 0.5 ml for NMR measurements. For rotary metal shadowing EM experiments, the starter cultures of the TbBILBO1 truncation constructs were diluted in LB medium and grown and lysed as described above. The filtered supernatants were loaded onto a Ni-HiTrap column as described above.

NMR Spectroscopy and Structure Calculations—All NMR experiments were performed at 298 K on Varian Inova 500 MHz and 800 MHz and Varian Direct Drive 600 MHz spectrometers equipped with 5-mm triple resonance probes and pulsed field gradients. NMR spectra were processed with NMRPipe and analyzed with Sparky software (15). NMR samples had a concentration of ~1 mM for triple resonance experiments or structure determination. In addition, the sample contained 5–10% (v/v) D2O for field/frequency lock as well as 0.1–0.2% (w/v) NaN3 to inhibit bacterial growth.

Backbone signal assignment for the TbBILBO1-NTD was obtained by pulsed field gradients. NMR spectra were processed with NMRPipe and analyzed with Sparky software (15). NMR spectra were obtained on three-dimensional TOCSY-15N HSQC, HNHA, HCCH-TOCSY, and NOE identification and assignment was obtained from three-dimensional NOE spectroscopy, three-dimensional NOE SYN, 15N HSQC,
and three-dimensional NOESY–\(^{13}\)C HSQC. Backbone chemical shifts were analyzed using TALOS software (16).

To determine a detailed solution structure of the TbBILBO1-NTD, a simulated annealing procedure using the Xplor-NIH software package (version 2.17 and above) was used for structure calculations (17). A set of structures was calculated with a root mean square deviation values were performed using MOLMOL (18).

**TABLE 1**

Summary of conformationally restricting experimental constraints

| Constraint Type                   | Total | S.D. | Z-score |
|-----------------------------------|-------|------|---------|
| NOE-based distance constraints    | 859   | 111  | 0.55    |
| Intraresidue \([i = j]\)           | 111   |      |         |
| Sequential \([i - j = 1]\)         | 341   |      | -0.22   |
| Medium range \([1 < |i - j| < 5]\) | 151   |      | -0.45   |
| Long range \([|i - j| \geq 5]\)   | 256   |      | 0.13    |
| NOE constraints per restrained residue\(^a\) | 8.2   |      |         |
| Hydrogen bond constraints         | 86    | 51   | 2.8 Å   |
| Total number of restraints\(^b\)   | 1,085 |      |         |
| Total number of restraints per restrained residue\(^c\) | 10.3  |      |         |
| Restricting long range constraints per restrained residue\(^d\) | 2.9   |      |         |
| Total structures computed/selected | 500/21|      |         |
| Residual constraint violations\(^e\) | 25    |      |         |
| Distance violations per structure \( (>1\) Å) | 0.409 Å |      |         |
| Maximum distance violation\(^f\) | 2.8 Å |      |         |
| Dihedral angle violations per structure \( (>10°)\) | 0     |      |         |
| Root mean square of dihedral angle violation\(^g\) | 0.868° |      |         |
| Maximum dihedral angle violation\(^h\) | 3.2°  |      |         |
| Root mean square deviation values  | All   | 1.9 Å| 1.3 Å   |
| All backbone atoms                 | 2.6 Å | 1.8 Å| 1.8 Å   |
| All heavy atoms                    | 0.45  |      |         |
| Structure quality factors, overall statistics | Mean score | 0.22 | NA\(^a\) |
| Procheck G-factor\(^i\) \((\phi/\psi\) only) | 0.22  |      | NA\(^b\) |
| Procheck G-factor\(^i\) \(all dihedral angles\) | -0.45 |      | NA\(^b\) |
| Verify three-dimensional           | 0.13  |      | 0.0374  |
| MolProbity clashscore             | 2.18  |      | 1.2211  |
| Ramachandran Plot Summary from Procheck\(^j\) | 94.10% |      | 1.15    |
| Most favored regions               | 94.10%|      |         |
| Additionally allowed regions       | 4.10% |      |         |
| Generously allowed regions         | 1.70% |      |         |
| Disallowed regions                 | 0.00% |      |         |

\(^a\) There are 105 out of 110 residues with conformationally restricting constraints.

\(^b\) Analyzed for the TbBILBO1-NTD (residues 1–110).

\(^c\) Using average \(r^2\) averaging.

\(^d\) Largest constraint violation among all the reported structures.

\(^e\) Residues with sum of \(\phi\) and \(\psi\) order parameters \(>1.8\). Ordered residue ranges: 2A–10D, 13N–23P, 27I–45L, 55K–72V, 74L–76R, 78D–80L, 84C–91P, 97K–99S, 104P–108K.

\(^f\) Residues selected based on dihedral angle order parameter, with \(S(\phi) + S(\psi) \geq 1.8\). Selected residue ranges: 2A–10D, 13N–23P, 27I–45L, 55K–72V, 74L–76R, 78D–80L, 84C–91P, 97K–99S, 104P–108K.

\(^g\) With respect to mean \(\pm\) S.D. for a set of 252 x-ray structures <500 residues, of resolution \(\leq 1.80\) Å, \(R\text{-factor} \leq 0.25\), and \(R\text{-free} \leq 0.28\); a positive value indicates a "better" score.

\(^h\) NA, not available.

and a given tetracycline concentration and \(x_0\) is the cell concentration in the control (uninduced) population. The results were then plotted against tetracycline concentration using Sigma-Plot software. Tetracycline concentration required to reduce 50% of cells growth (IC\(_{50}\)) was determined from experimental cells growth versus drug concentrations by fitting the rectangular hyperbolic function: \(I = I_{max}C/(IC_{50} + C)\), where \(I\) is percentage of inhibition, \(I_{max}\) is 100% inhibition, and \(C\) is tetracycline concentration (19).

**Propidium Iodide/Annexin V Viability Assay**—Cells (5 × 10⁶) were centrifuged (800 × g, 5 min) and washed with PBS. The cell pellet was resuspended in 500 μl of Annexin V-binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂), and Annexin V-FITC and propidium iodide were added to a final concentration of 5 μg/ml. The suspension was incubated in the dark for 10 min, and the fluorescence intensity was measured on FACScan Calibur flow cytometer (BD Biosciences) and analyzed by FlowJo software (version 8.8.7).

**Immunofluorescence Microscopy**—Cells were washed with PBS, attached to coverslips by centrifugation (3,000 × g, 5 s), and fixed in 4% (w/v) paraformaldehyde (20 min, room temperature). Fixed cells were washed with PBS, permeabilized with 0.25% Triton X-100 in PBS (v/v) (5 min, room temperature), washed again with PBS, and blocked with 3% BSA in PBS (w/v)

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**Structure of the TbBILBO1 N-terminal Domain**

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**Growth Inhibition Assay**—Cells (seeded at 2 × 10⁶ cells/ml) were grown in 10 ml of SDM79 medium with the addition of 0, 5, 10, 20, 40, and 50 ng/ml tetracycline each day to induce and maintain protein expression. A 4-day time course was followed. Culture densities (cells/ml) were measured with a Z2 coulter® (Beckman Coulter) every day, and 2 ml of each was taken for immunoblotting analysis. Growth inhibition due to expression of a TbBILBO1 construct at a given tetracycline concentration was calculated after 4 days with the following formula: Inhibition (%) = 100 − \(x^*100/x_0\), where \(x\) is the cell concentration at
The coverslips were incubated in a humidified chamber with primary antibodies diluted in PBS (60 min, room temperature), washed three times with PBS, and then incubated with secondary antibodies diluted in PBS (60 min, room temperature). After three washes with PBS, the coverslips were rinsed with Milli-Q water and mounted on glass slides using Fluoromount G/DAPI (Southern Biotech). For preparation of detergent-extracted cytoskeletons, the cells were incubated in PEME buffer (0.1M PIPES-NaOH (pH 6.9), 2 mM EGTA, 1 mM MgSO4, 0.1 mM EDTA)/0.5% (v/v) Nonidet P-40) (5 min, room temperature), washed three times with PBS, and then treated as described previously for intact cells. Cells were imaged using an inverted microscope (Axio Observer Z1, Carl Zeiss Microimaging) equipped with a PCO 1600 camera (PCO) and using manufacturer’s drivers in a custom C program. A 100×/1.46 Plan-APoCHROMAT oil immersion lens and Immersol 518F (Zeiss) immersion oil were used. Image processing was carried out using ImageJ and Adobe Photoshop CS3 software.

**Accession Code**—Coordinates and structure factors of the TbBILBO1-NTD NMR structure have been deposited in the Protein Data Bank (PDB) under accession code 2MEK.

**RESULTS**

**Domain Organization and Targeting of TbBILBO1**—
TbBILBO1 is a modular protein with three structural domains. In order, they are (i) an NTD (residues 1–110), (ii) two tandem EF-hand motifs (residues 183–249), and (iii) a coiled coil domain (residues 263–578) (Fig. 1A). The three domains pack closely together in the primary sequence, with the exception of a linker region that lies between the NTD and the EF-hand motifs. In a protein BLAST search of the Protein Data Bank at the NCBI website using the primary sequence of TbBILBO1, no known structures were found to be homologous to the TbBILBO1-NTD. This implied that the NTD might have a novel fold.

To determine whether the NTD was responsible for TbBILBO1 targeting to the FPC, YFP-tagged truncation constructs were tested (Fig. 1B). These constructs were transiently expressed in *T. brucei* cells, and their localization was analyzed by immunofluorescence microscopy. Anti-GFP antibodies were used to visualize YFP-TbBILBO1-NTD as low expression levels precluded direct observation of YFP. DAPI was used to stain DNA (blue). Regions enlarged in insets are indicated with white boxes. Scale bars, 5 µm. F, immunoblot of whole cell lysates from transiently transfected *T. brucei* cells, probed using anti-GFP antibodies. Arrows indicate the expected size of the YFP-tagged TbBILBO1 constructs.

(30 min, room temperature). The coverslips were incubated in a humidified chamber with primary antibodies diluted in PBS (60 min, room temperature), washed three times with PBS, and then incubated with secondary antibodies diluted in PBS (60 min, room temperature). After three washes with PBS, the coverslips were rinsed with Milli-Q water and mounted on glass slides using Fluoromount G + DAPI (Southern Biotech). For preparation of detergent-extracted cytoskeletons, the cells were incubated in PEME buffer (0.1M PIPES-NaOH (pH 6.9), 2 mM EGTA, 1 mM MgSO4, 0.1 mM EDTA) + 0.5% (v/v) Nonidet P-40) (5 min, room temperature), washed three times with PBS, and then treated as described previously for intact cells. Cells were imaged using an inverted microscope (Axio Observer Z1, Carl Zeiss Microimaging) equipped with a PCO 1600 camera (PCO) and using manufacturer’s drivers in a custom C++ program. A 100×/1.46 Plan-APoCHROMAT oil immersion lens and Immersol 518F (Zeiss) immersion oil were used. Image processing was carried out using ImageJ and Adobe Photoshop CS3 software.

**FIGURE 1. The TbBILBO1-NTD is not required for localization.** A, schematic depicting the arrangement of the three domains of TbBILBO1: N-terminal domain (NTD), EF hands (EF), and coiled coil domain. Amino acid numbers are indicated above the schematic. B, TbBILBO1 truncation constructs used for the localization experiments. Abbreviations are defined in the text. C–E, localizations of YFP-tagged TbBILBO1 full-length and truncation constructs in transiently transfected *T. brucei* cells. Intact cells were analyzed by immunofluorescence microscopy. Anti-GFP antibodies were used to visualize YFP-TbBILBO1-NTD as low expression levels precluded direct observation of YFP. DAPI was used to stain DNA (blue). Regions enlarged in insets are indicated with white boxes. Scale bars, 5 µm. F, immunoblot of whole cell lysates from transiently transfected *T. brucei* cells, probed using anti-GFP antibodies. Arrows indicate the expected size of the YFP-tagged TbBILBO1 constructs.

To determine whether the NTD was responsible for TbBILBO1 targeting to the FPC, YFP-tagged truncation constructs were tested (Fig. 1B). These constructs were transiently expressed in *T. brucei* and their localization analyzed by immunofluorescence microscopy. Full-length (FL) TbBILBO1 localized to a region between the nucleus and the kinetoplast (mitochondrial genome) and close to the point of flagellum entry into the cell (Fig. 1C, arrowhead). This is consistent with an FPC localization and matches the distribution seen for the endogenous protein (12). TbBILBO1-NTD displayed a cytoplasmic distribution, suggesting that it was not required for targeting (Fig. 1D). Consistent with this, TbBILBO1-ΔNTD exhibited an identical localization to TbBILBO1-FL (Fig. 1E, arrowhead). It was concluded that the NTD does not appear to be required for targeting of TbBILBO1 to the FPC. The expression of all truncation constructs was verified by immunoblots of whole cell lysates taken from transfected cell cultures (Fig. 1F).
**Structure of the TbBILBO1 N-terminal Domain**

**Figure 2. The TbBILBO1-NTD is required for normal cellular growth.** A, PCR amplification of genomic DNA confirms the presence of the indicated TbBILBO1 transgenes. Primers annealing to sequences encoding the Ty1 epitope tag (5′-end) and ORF (3′-end) were used. B, immunoblots of whole cell lysates from cells inducibly expressed the indicated Ty1-tagged TbBILBO1 transgenes in the presence or absence of 20 ng/ml tetracycline. Probing with anti-Ty1 antibodies confirmed that both transgenes were inducibly expressed and localized correctly to the region of the cell corresponding to the flagellum entry point. Arrows indicate the point of flagellum entry into the cell. Boxed areas are enlarged in insets. C, growth inhibition curves from stably transfected cells inducibly expressed Ty1-tagged TbBILBO1-FL or TbBILBO1-ΔNTD at different concentrations of tetracycline after a 4-day time course. Values were calculated from the mean of at least three independent experiments. Error bars show S.E.

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**Overexpression of TbBILBO1-ΔNTD Has a Dominant Negative Effect on Cell Growth**—As the localization data indicated that the TbBILBO1-NTD was not required for targeting, it was hypothesized that it performed a functional role. If so, then the TbBILBO1-ΔNTD construct lacking the NTD should have a dominant negative effect when overexpressed. To test this, stable cell lines inducibly expressing either Ty1-tagged TbBILBO1-FL or TbBILBO1-ΔNTD were generated. PCR amplification of genomic DNA confirmed that the transgenes were present (Fig. 2A). Tight and tetracycline-inducible control over protein expression was confirmed by immunoblotting with anti-Ty1 antibodies (Fig. 2B). As expected, both constructs localized correctly to the region of the cell corresponding to the flagellum. Fibrinopeptide C was used to stain DNA. DAPI was used to stain DNA. Scale bars, 5 μm. C, no labeling was seen in the absence of tetracycline. D and E, both Ty1-tagged TbBILBO1 constructs localized correctly. Arrows indicate the point of flagellum entry into the cell. Boxed areas are enlarged in insets. F, growth inhibition curves from stably transfected cells inducibly expressed Ty1-tagged TbBILBO1-FL or TbBILBO1-ΔNTD at different concentrations of tetracycline after a 4-day time course. Values were calculated from the mean of at least three independent experiments. Error bars show S.E.

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**The NTD Has a Ubiquitin-like Fold**—Given that in silico analysis had suggested the TbBILBO1-NTD had a novel fold and in vivo data supported the notion that it might perform an essential function, structural studies on the NTD were carried out.

Recombinant TbBILBO1-NTD was expressed in *E. coli* and purified to homogeneity. The purified protein was monomeric, soluble, and properly folded as indicated by the well dispersed resonances in a HSQC spectrum (Fig. 3A). The three-dimensional structure was subsequently determined by multidimensional NMR spectroscopy. Distance constraints were derived from observed NOE intensities; torsion angle and hydrogen bond constraints were derived for the regions with regular β strand or α helical secondary structure (Table 1). Superposition of 10 final models with minimal total energy and minimal restraint violations showed an overall well folded structure with a relatively flexible C-terminal tail (residues 95–110) (Fig. 3B). The overall structure had an elongated shape and consisted of five β strands (β1–β5) and one α helix (α1). The α helix was packed diagonally across one side of the five-stranded β sheet (Fig. 3C). The flexible C-terminal tail was loosely twisted around one end of the elongated structure.

The solved NMR structure allowed a search using the three-dimensional structure-based protein comparison program DaliLite v3 (20). Unexpectedly, given the lack of any homology...
at a primary structural level, the top hit of the searches was the PB1 domain of Par6, which has a ubiquitin-like fold. The root mean square deviation was 2.4 Å \((Z \approx 5.2)\) for backbone atoms in the 70 aligned residues, suggesting a high similarity between the two structures (Fig. 3D). Although the topological arrangement of the five-stranded \(\beta\) sheet and the \(\alpha\) helix in the two structures is very similar, \(\beta 1\), \(\beta 2\), and \(\alpha 1\) are all significantly longer in the TbBILBO1-NTD, leading to a more elongated structure for the TbBILBO1-NTD as a whole (Fig. 3E).

The NTD Has a Conserved Surface Patch Essential for TbBILBO1 Function—The three-dimensional structure of the NTD revealed the presence of an extensive conserved patch. This patch is on the solvent-exposed side of the \(\beta\) sheet and contains four aromatic residues (Phe-12, Trp-71, Tyr-87, and...
Essential requirement for a conserved surface patch on the TbBILBO1-Nterminal Domain.

A. ribbon diagram of the TbBILBO1-NTD with the seven conserved residues shown as sticks. The three aromatic residues at the bottom of the conserved crater are colored in red; the four flanking residues at the rim are shown in blue. B. surface plot with the same orientation and color scheme as in A. C. primary sequence alignment of the NTDs of TbBILBO1 and homologs. Residues altered by site-directed mutagenesis in the Mut1 (rim residues) and Mut2 (aromatic residues at the crater bottom) constructs are indicated. Tb, T. brucei; Tc, T. cruzi; Lb, Leishmania braziliensis; Li, Leishmania infantum; Lm, Leishmania major. D. PCR amplification of genomic DNA to confirm the presence of the indicated TbBILBO1 transgenes. E. anti-Ty1 immunoblots of whole cell lysates from cells inducibly expressing Ty1-tagged TbBILBO1-Mut1 or -Mut2 transgenes in the presence or absence of 20 ng/ml tetracycline. F and G, Ty1-tagged TbBILBO1-Mut1 and -Mut2 constructs localizing correctly to the FPC. Arrows indicate the point of flagellum entry into the cell. DNA is labeled with DAPI. Scale bars, 5 μm. H, mutagenesis of the TbBILBO1-NTD causing cell growth inhibition. Growth inhibition curves are from stably transfected cells inducibly expressing Ty1-tagged TbBILBO1 constructs at different concentrations of tetracycline after 4 days of induction. I, immunoblots of whole cell lysates from the four cell lines taken at various concentrations of tetracycline and probed using anti-Ty1 antibodies (upper panels). The expression of all four constructs was approximately the same at each tetracycline concentration and saturated at around 20 ng/ml. Immunoblotting with anti-tubulin antibodies was used as a loading control (lower panels).

Phe-89) and three basic ones (Lys-15, Lys-60, and Lys-62) (Fig. 4A). These residues together generate a crater-like structure on an otherwise flat surface, with three of the four aromatic residues (Trp-71, Tyr-87, and Phe-89) lying at the bottom of the crater and four other conserved residues forming the surrounding rim (Fig. 4B). A primary sequence alignment of the NTD from several trypanosomatids showed that this patch is highly conserved (Fig. 4C).

To find out whether this surface patch is essential for the function of the TbBILBO1-NTD, two site-directed mutagenesis constructs were derived from TbBILBO1-FL (Fig. 4C). Mut1 (F12A, K15A, K60A, and K62A) substituted the “rim” residues with alanines; Mut2 (W71A, Y87A and F89A) substituted the aromatic “bottom” residues with alanines. Stable cell lines inducibly expressing the respective mutagenized constructs with N-terminal Ty1 epitope tags were generated. As before, integration of the transgene into the genomic DNA was confirmed by PCR, and tight and inducible expression was verified by immunoblotting with anti-Ty1 antibodies (Fig. 4, D and E).

Correct localization of the two mutagenesis constructs was confirmed by immunofluorescence using anti-Ty1 antibodies (Fig. 4, F and G). The effects of overexpression of the two mutagenesis constructs were then compared with overexpression of Ty1-TbBILBO1-FL and Ty1-TbBILBO1-ΔNTD. Approximately equivalent expression levels of the four constructs at each tetracycline concentration used for induction were verified by immunoblotting (Fig. 4D). Remarkably, both mutagenesis constructs recapitulated the level of growth inhibition seen upon deletion of the entire NTD (Fig. 4H). The tetracycline concentrations required to reduce cell growth by 50% (IC50) for Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2 were calculated to be 4.5, 6.3, and 5.8 ng/ml, respectively. This is >1
order of magnitude lower than the value calculated for Ty1-TbBILBO1-FL (77.9 ng/ml). Quantitative immunoblotting estimated the amount of overexpression of the inhibitory constructs to be more than five times over the levels of the endogenous TbBILBO1 protein.5

To observe the effect of deletion of the NTD and the two mutations in vivo, cells overexpressing these Ty1-tagged TbBILBO1 constructs were analyzed by immunofluorescence microscopy. In dividing cells, Ty1-TbBILBO1-FL was associated with both the old and new flagellum (Fig. 5A, arrows). B–D, overexpression of Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2 causes gross morphological effects, notably a detached new flagellum in replicating cells (arrowheads). Note the absence of TbBILBO1 labeling at the detached new flagella. Scale bars, 5 μm.

5 K. Vidilaseris, B. Morriswood, G. Kontaxis, and G. Dong, unpublished data.

FIGURE 5. TbBILBO1-NTD deletion or mutagenesis causes dominant negative effects. All cells were assayed by immunofluorescence microscopy after a 2-day induction using 20 ng/ml tetracycline. Cells were labeled using anti-Ty1 antibodies; DAPI was used to stain DNA. Boxed regions are enlarged in insets. A, Ty1-TbBILBO1-FL does not cause gross morphological abnormalities. In replicating cells, Ty1-TbBILBO1-FL was associated with both old and new flagella (arrows). B–D, overexpression of Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2 causes gross morphological effects, notably a detached new flagellum in replicating cells (arrowheads). Note the absence of TbBILBO1 labeling at the detached new flagella. Scale bars, 5 μm.
kinetoplast was near this new flagellum, but Ty1-TbBILBO1-ΔNTD, -Mut1, or -Mut2 labeling was associated only with the old but not the new flagellum (Fig. 5, B–D, arrows). The TbBILBO1-Mut2 construct was consistently found to form thread-like morphologies, whereas the others exhibited a more canonical punctate morphology (Fig. 5D).

These morphological alterations of the cells overexpressing Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2 were strikingly similar to those described upon depletion of TbBILBO1 by RNAi. In TbBILBO1 RNAi cells, a feature associated with the detached posterior flagellum is the presence of the nucleating basal body at the plasma membrane (12). To analyze whether the detached flagella were proximally associated with a basal body, the cells were co-labeled with TbCentrin4. TbCentrin4 localizes to both the basal body and a cytoskeletal structure called the bilobe (21, 22). In duplicating cells overexpressing Ty1-TbBILBO1-FL, TbCentrin4-labeled basal bodies were observed at the base of both old and new flagella (Fig. 6A). In replicating cells overexpressing Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2, TbCentrin4-positive basal bodies were observed at the base of both old and new flagella (Fig. 6, B–D, arrowheads). Scale bars, 5 μm.

**FIGURE 6. The detached flagellum in overexpressing cells has an associated basal body.** Expression of the various Ty1-tagged TbBILBO1 constructs was induced for 2 days using 20 ng/ml tetracycline. Detergent-extracted cells were labeled with anti-Ty1 and anti-TbCentrin4 antibodies. DAPI was used to stain DNA. Boxed areas are enlarged in insets. A, in replicating cells overexpressing Ty1-TbBILBO1-FL, both Ty1-TbBILBO1-FL and TbCentrin4 had normal localizations and were associated with both old and new flagella. B–D, the detached new flagella in replicating cells overexpressing the other TbBILBO1 constructs are associated with TbCentrin4-positive basal bodies (arrowheads). Scale bars, 5 μm.

Loss or Mutagenesis of the TbBILBO1-NTD Is Lethal—Deletion or mutagenesis of the TbBILBO1-NTD was sufficient to cause inhibition of cell growth (Fig. 4H), but it was not clear whether this was due to cell cycle arrest or cell death. To test this, a propidium iodide/Annexin V viability assay was performed. Propidium iodide is a membrane-impermeant DNA dye, and a high signal indicates compromised cell viability; Annexin V binds to cells undergoing apoptosis. Overexpression of all four constructs was induced with 20 ng/ml tetracycline for 4 days, and the cells were then treated with fluorescein isothiocyanate-labeled Annexin V antibodies, stained with propidium iodide, and analyzed by FACS (Fig. 7A). In the absence of tetracycline, most of the cells were viable with the majority of the population giving a low signal in each channel (Fig. 7B). As a
positive control for cell death, wild-type *T. brucei* cells were cultured with the addition of 4 mM DTT for 24 h. DTT causes endoplasmic reticulum stress and induces a spliced leader RNA silencing pathway leading to apoptosis. This stress response cannot be recovered even after DTT removal (23). Under these conditions, few cells were viable with most of the population undergoing apoptosis/necrosis (Fig. 7C). Overexpression of Ty1-TbBILBO1-FL did not significantly affect the viability of the cells, similar to the negative control (Fig. 7D). Conversely, overexpression of Ty1-TbBILBO1-ΔNTD, -Mut1, and -Mut2 showed that many of the cells in the population were undergoing apoptosis or necrosis (Fig. 7, E–G). Quantification of the results confirmed that deletion of the NTD of TbBILBO1 had the most severe effect on cell viability, with only 34.7% viable cells compared with Ty1-TbBILBO1-Mut1 (42.9%) and -Mut2 (55.1%). In contrast, 96.3% cells overexpressing Ty1-TbBILBO1-FL were viable (Fig. 7H). Therefore, the dominant negative effects seen upon overexpression of constructs lacking or with mutagenized versions of the TbBILBO1-NTD led to cell death.

**DISCUSSION**

TbBILBO1 is the only known FPC protein (12). The results provided here show an essential requirement for a conserved surface patch in the NTD of this protein, which provides a basis for rational drug design and is expected to guide future studies on both TbBILBO1 and the FPC.

Transient expression of TbBILBO1 truncation constructs *in vivo* demonstrated that deletion of the NTD had no effect on targeting (Fig. 1F). This nonrequirement for correct localization implied that the NTD could have a functional role. Consistent with this hypothesis, the inducible overexpression of a TbBILBO1 construct lacking the NTD produced a dominant negative effect and completely inhibited cell growth in a dose-dependent manner (Fig. 2F). The structure of the NTD was solved by NMR and revealed to have a ubiquitin-like fold (Fig. 3). Importantly, this structural model revealed the presence of a conserved surface patch consisting of four aromatic and three basic residues that together form a crater-like structure (Fig. 4). Site-directed mutagenesis on the residues comprising either the rim or the crater bottom was sufficient to recapitulate the growth inhibition caused by deletion of the entire NTD when these constructs were overexpressed *in vivo* (Fig. 4H). Cells that were overexpressing constructs either lacking the NTD or carrying the mutant versions produced morphological phenotypes that were consistent with published TbBILBO1 RNAi data (12). FACS analysis on cells overexpressing the TbBILBO1-NTD deletion/mutant constructs confirmed that the effect was lethal, with an abundant production of necrotic cells (Fig. 7). Therefore, the NTD of TbBILBO1 appears to be essential for its function *in vivo*.

It should be noted that the precise role of TbBILBO1 remains unclear. Based on published RNAi data (12) and the dominant negative overexpression data provided here, it is clearly involved in FP and FPC biogenesis, but its purpose in interphase cells is undetermined. Whatever that function is, the data provided here suggest that the NTD could possibly provide it. Interdomain pulldowns revealed that the NTD does not interact directly with other parts of TbBILBO1. It is therefore pos-
sible that the conserved surface patch on the NTD may be the binding site for either an unknown essential component of the FPC or a key regulatory protein. Mutations disrupting their interaction might therefore have a catastrophic impact on the integrity and/or function of the FPC, leading to the same phenotype as the NTD deletion (Fig. 4).

It was reported previously that overexpression of untagged full-length TbBILBO1 is nontoxic (12). The small size of the Ty1 epitope tag used in the in vivo studies here is presumably sufficient to prevent any deleterious effects on full-length TbBILBO1 function. The previous study also stated that overexpression of either N- or C-terminally EGFP-tagged TbBILBO1 caused a growth arrest and a dominant negative phenotype similar to that seen following RNAi depletion of the protein, namely a detached flagellum in an abnormally elongated cell posterior (12). As here, no FPC was reported to be present at the detached posterior flagella. The authors postulated that this growth arrest was due to a dominant negative effect. Given the large size of the EGFP tag (37 kDa), it seems likely that this growth arrest was due to the EGFP module interfering with the normal function of the NTD. The fact that this arrest was seen also in C-terminal EGFP-tagged TbBILBO1 constructs supports the notion that the NTD (or EF-hand motifs) might communicate with the C termini of other TbBILBO1 molecules assembled in the FPC.

The data provided here not only corroborate these statements but also extend the conclusions to single-residue resolution. The fact that mutations of the aromatic and basic residues arranged in a surface patch on the NTD were sufficient to recapitulate the effect of deleting the entire domain strongly suggests that this is a key functional region of the molecule (Fig. 4).

As such, this specific region represents a good candidate for pharmacological blockade. Given that orthologs of TbBILBO1 with high sequence conservation are present in other pathogenic trypanosomatids and that no orthologs exist in humans, the protein could present an attractive pan-trypanocidal drug target.

One further hypothesis is prompted by the structure of the TbBILBO1-NTD (Fig. 8A). As noted, the Par6 protein also exhibits a ubiquitin-like domain and binds atypical protein kinase C (aPKC; Fig. 8B). The structure of this complex has been solved (24), allowing superimposition of the TbBILBO1-NTD onto the Par6-PB1 in the Par6-aPKC complex. The C-terminal loop of the TbBILBO1-NTD obscures the surface corresponding to the aPKC binding site on Par6. The conserved surface patch of the TbBILBO1-NTD is also distant from the corresponding aPKC binding site on Par6. D, two views of the superimposed structures with the TbBILBO1-NTD shown in white surface plot.

In summary, this work has provided a structural insight into TbBILBO1, the first characterized and only known cytoskeletal protein in the T. brucei FPC. The conserved surface patch identified in the TbBILBO1-NTD appears to be essential for the proper function of TbBILBO1 in the parasite and therefore rep-
represents an attractive candidate site for therapeutic drug design. Future studies will try to identify the interacting partner(s) of the TbBILBO1-NTD, its functional mechanism in vivo, and its possible regulatory interaction with the EF-hand motifs in the assembly and operation of the FPC.

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