Morphology of the Trypanosome Bilobe, a Novel Cytoskeletal Structure

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The trypanosome bilobe is a cytoskeletal structure of unclear function. To date, four proteins have been shown to localize stably to it: TbMORN1, TblLRP1, TbCentrin2, and TbCentrin4. In this study, a combination of immunofluorescence microscopy and electron microscopy was used to explore the morphology of the bilobe and its relationship to other nearby cytoskeletal structures in the African trypanosome procyclic trypomastigote. The use of detergent/salt-extracted flagellum preparations was found to be an effective way of discerning features of the cytoskeletal ultrastructure that are normally obscured. TbMORN1 and TbCentrin4 together define a hairpin structure comprising an arm of TbCentrin4 and a fishhook of TbMORN1. The two arms flank a specialized microtubule quartet and the flagellum attachment zone filament, with TbMORN1 running alongside the former and TbCentrin4 alongside the latter. The hooked part of TbMORN1 sits atop the flagellar pocket collar marked by TblBILBO1. The TbMORN1 bilobe occasionally exhibits tendrillar extensions that seem to be connected to the basal and probasal bodies. The TbMORN1 molecules present on these tendrils undergo higher rates of turnover than those for molecules on the main bilobe structure. These observations have been integrated with previous detailed descriptions of the cytoskeletal elements in trypanosome cells.

The Excavata are a eukaryotic “supergroup” comprised of diverse unicellular flagellates (2). One of the better-studied excavates is Trypanosoma brucei, a kinetoplastid euglenozoan, because of its importance as a human pathogen. This parasite is responsible for sleeping sickness in humans and nagana in cattle, and the closely related organisms Trypanosoma cruzi and Leishmania spp. are responsible for Chagas’ disease and leishmaniasis, respectively. Owing to this, the trypanosomes have been studied extensively in both cell biology and evolutionary contexts (16, 36).

T. brucei is an obligate parasite that cycles between an insect vector (the tsetse fly) and humans, cattle, or wild animals. In both its insect and mammalian hosts, it survives as an extracellular parasite, despite continuous exposure to the immune system. In vitro, it has been shown that there is a rapid endocytic uptake of bound immunoglobulins from an intracellular invagination of the plasma membrane termed the flagellar pocket; it is thought that this mechanism is key to the parasite’s survival in its mammalian host (12, 13). The basal body that acts as a microtubule organizing center for the single flagellum abuts this pocket, with a probasal body positioned orthogonally to it (22).

As is the case for many parasites compared to their free-living relatives, trypanosomes have a relatively streamlined morphology. This is particularly apparent in the cytoskeleton, which has been simplified considerably compared to those of other, free-living members of the Euglenozoa (9, 22, 24). The latter species have a complex basal body apparatus normally giving rise to two or more anteriorly inserted flagella and three microtubule roots, one of which extends to line a feeding apparatus. This feeding apparatus is separate from both the flagellar pocket and the cortical region of the cell. It has been retained in at least one parasite (T. cruzi) and is reduced in some free-living taxa, for example, the phototrophic euglenids (2, 24, 26). T. brucei, on the other hand, has a single flagellum emerging from a flagellar pocket near the posterior end of the cell and completely lacks an independent feeding apparatus. In T. brucei, the flagellum is attached to the cell’s surface via a flagellar attachment zone (FAZ). This consists of a group of four microtubules, termed the microtubule quartet (MTQ), and a FAZ filament. The MTQ, which is the only remnant of the microtubular roots observed in other excavates, extends from between the basal body and the probasal body. It then wraps around the flagellar pocket and travels beneath the flagellum for the length of the cell, to the anterior end (22, 23). The FAZ filament is nucleated above the flagellar pocket and runs parallel and immediately to the right of the MTQ, as far as the cell’s anterior pole. An electron-dense cytoskeletal barrier element, referred to as the flagellar pocket collar (FPC), encircles the flagellar pocket neck. Knockdown of its only known protein component, BILBO1, indicated that it is required for flagellar pocket morphogenesis (7). There is no known homologous structure in other euglenozoa or excavates, though an electron-dense region surrounding the flagellar pocket in at least one species of Bodo, the closest free-living relative to the trypanosomes, has been observed (4).

A particularly enigmatic cytoskeletal feature of T. brucei is the bilobe, a structure that localizes near the flagellar pocket and was originally proposed to mediate biogenesis of the Golgi complex (19). It was discovered serendipitously by an immunofluorescence assay using the pan-centrin monoclonal antibody 20H5 (14). The present work focused on a better understanding of this cytoskeletal structure. To date, there are four known protein components of the bilobe: TbCentrin2, TbCentrin4, TbMORN1, and TblLRP1 (19, 25, 33, 35, 41). Centrins are ubiquitous components of microtubule organizing centers, and TbCentrin2 and Tb-
Centrin4 are also correspondingly localized to the basal and pro-basal bodies (30, 35). TbMORN1 was the first protein to be identified that was localized exclusively to the bilobe (25). In spite of the considerable information obtained through immunofluorescence studies, no structure corresponding to the bilobe has ever been observed at an ultrastructural level. This is particularly intriguing given the extensive electron microscopy (EM) studies performed on *T. brucei* since the 1960s, culminating in recent, detailed tomographic reconstructions of the flagellar pocket region (15, 17, 22, 23, 34).

In this study, by focusing on TbMORN1 and taking advantage of the bilobe’s strong association with the flagellum, an ultrastructural analysis of the bilobe was carried out using a combination of immunofluorescence, transmission, and immunoelectron microscopy.

**MATERIALS AND METHODS**

Antibodies and reagents. Anti-TbMORN1 and rabbit polyclonal anti-green fluorescent protein (anti-GFP) antibodies have been described previously (25, 31). A mouse monoclonal anti-TbCentrin4 antibody was raised against recombinant full-length TbCentrin4 and will be described in a forthcoming publication (20a). Rabbit polyclonal anti-TbBILBO1 antibodies were raised against a purified untagged recombinant fragment of TbBILBO1 (amino acids 1 to 110), and the antisera was affinity purified against the antigen. A mouse monoclonal anti-GFP antibody, a mouse anti-alpha-tubulin antibody, and the YL1/2 rat monoclonal antibody were purchased from Roche, Sigma-Aldrich, and Millipore, respectively. Anti-TbLRRP1 and anti-LdCentrin4 antibodies were kind gifts from Cynthia He (University of Singapore) and Hira Nakhasi (FDA), respectively.

**Cells line and generation.** The procyclic 427 Lister strain of *T. brucei brucei* was used for the generation of YFP::TbMORN1 and eGFP::TbMORN1 endogenous replacement cell lines and for experiments involving untagged TbMORN1. Endogenous replacement of one of the *TbMORN1* alleles with a YFP-TbMORN1- or mEGFP-TbMORN1-encoding allele was accomplished by double homologous recombination using previously published methods (3, 25, 29). In brief, the following targeting construct (5′ to 3′) was assembled in the pCR4Blunt-TOPO cloning vector—500 bp of the *TbMORN1* 5′-untranslated region (5′-UTR) plus the basicidin resistance gene plus the tubulin intergenic region plus the yellow fluorescent protein (YFP) or monomeric enhanced GFP (mEGFP) coding sequence plus 800 bp of *TbMORN1* coding sequence. The targeting construct was excised from the cloning vector by enzymatic digestion and introduced into *T. brucei* 427 cells by electroporation. Double homologous recombination in situ should produce a modified allele encoding the YFP (or mEGFP) tag at the 5′ end of *TbMORN1* and should confer basicidin resistance. Stable transformants were selected by growth in medium containing 10 μg/ml basicidin and cloned by limiting dilution. Putative clones were screened for recombination at the correct locus by PCR using genomic DNA, and expression of YFP or mEGFP-TbMORN1 was confirmed by immunoblotting and immunofluorescence analysis. Cells were cultured in SDM79 medium supplemented with 7.5 μg/ml hemin plus 20% heat-inactivated fetal calf serum (Sigma-Aldrich) at 27°C. The YFP/mEGFP::TbMORN1 cells were maintained with 10 μg/ml basicidin.

**Preparation of extracted cytoskeletons and isolated flagella.** Detergent-extracted cytoskeletons were prepared by incubating the cells in PHEME buffer [2 mM EGTA, 1 mM MgSO₄, 0.1 mM EDTA, 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid)–NaOH (PIPE-NaOH), pH 6.9] supplemented with 0.5% NP-40 (vol/vol) and Complete protease inhibitor cocktail (Roche) for 5 min at room temperature (RT). Isolated flagella were prepared by further incubating the cytoskeletons in PHEME buffer containing 1% NP-40, 1 M KCl, and Complete protease inhibitor cocktail (Roche) for 30 min on ice. These steps were incorporated into the general protocols for sample preparation for immunofluorescence or electron microscopy as described below.

**Immunofluorescence microscopy.** Cells were attached to coverslips by centrifugation (1,800 × g, 5 s). Intact cells were then fixed, washed once with phosphate-buffered saline (PBS), permeabilized with 0.25% (vol/vol) Triton X-100 in PBS (5 min, RT), washed three times with PBS, and blocked with 3% (wt/vol) bovine serum albumin (BSA) in PBS (30 min, RT). The coverslips were incubated with primary antibodies diluted in PBS in a humidified chamber (1 h, RT), washed three times with PBS, and then incubated with secondary antibodies diluted in PBS (1 h, RT). After three washes with PBS, the coverslips were rinsed with water and mounted on glass slides by use of DAPI (4′,6-diamidino-2-phenylindole) (Fluoromount G; Southern Biotech). When detergent-extracted cytoskeletons were required, the cells were incubated in PHEME buffer plus 0.5% NP-40 (5 min, RT) as described in the paragraph above, washed three times with PBS, fixed, blocked, and labeled as described for intact cells. When isolated flagella were required, the cells were incubated in PHEME buffer plus 0.5% NP-40 (5 min, RT) as described in the paragraph above and then washed three times with PHEME buffer plus 0.5% NP-40 to leave cytoskeletons. The cytoskeletons were then incubated in PHEME buffer with 1% NP-40 and 1 M KCl (30 min, ice) as described in the paragraph above, washed three times with the same buffer and once with PBS, fixed, blocked, and labeled by the standard method. Fixation was done with either 4% paraformaldehyde in PBS (20 min, RT) or ice-cold methanol (30 min, −20°C); the fixation method used is indicated in each figure legend. Epifluorescence images were obtained using an inverted microscope (Axio Observer Z1; Carl Zeiss MicroImaging Inc.) equipped with a PCO 1600 camera and using the manufacturer’s drivers in a custom C++ program. Image processing was carried out using ImageJ and Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA).

**Extraction and negative staining for electron microscopy.** Cells were harvested by slow centrifugation and resuspended in PBS. This solution was then adsorbed onto glow-discharged Formvar- and carbon-coated grids. To prepare whole extracted cytoskeletons, grids were incubated on droplets of PHEME buffer plus 0.5% NP-40 (3.5 min, RT) and subsequently rinsed four times in PHEME buffer. To prepare extracted flagella, cells on grids were first extracted in PHEME buffer plus 1% NP-40 (5 min, RT), rinsed with PHEME, and then extracted in PHEME buffer plus 1% NP-40 and 1 M KCl (15 to 20 min, 4°C). Grids were then rinsed four times in PHEME buffer. Grids were fixed in 2.5% glutaraldehyde in PHEME buffer (5 min, RT), rinsed four times in double-distilled water (ddH₂O), and negatively stained with 0.5% gold thioglycolate.

**Immunogold labeling for electron microscopy.** After extraction and rinsing with PHEME buffer as described above, grids were moved through 5 subsequent drops of blocking solution comprised of 2% BSA in PBS. Grids were then incubated with primary antibodies diluted in blocking solution (60 to 80 min at RT or overnight at 4°C). After primary incubation, grids were blocked seven times as described above and then incubated with gold-conjugated secondary antibodies (British Biotech) as described for primary incubation. Grids were placed on a drop of blocking solution for 5 min and then fixed in 2.5% glutaraldehyde and negatively stained as described above. Grids were viewed on a JEOL 1210 transmission electron microscope operating at 80 kV. Images were taken using a Morada digital camera and iTEM imaging software (ResAlta Research Technologies). Cumulative ratios of gold particles to show bias in labeling between the two bilobe arms were calculated as follows. The TbCentrin4 arm was identified on the basis of being next to the FAZ filament and on the same side as the probasal body. The total number of gold particles on each arm was counted for each bilobe, and the cumulative ratio between the two arms was calculated in succession for all bilobes. Cumulative ratios were then plotted against the total numbers of bilobes. Samples were taken from at least three independent labeling experiments. When labeling densities are constant, the cumulative ratio quickly describes a damped oscillation around the cumulative mean. Total sample size was
determined as being at least twice the number of samples required to reach the cumulative mean.

**FRAP experiments.** All fluorescence recovery after photobleaching (FRAP) experiments were performed using a Zeiss LSM710 confocal microscope (Carl Zeiss, Germany) equipped with a 63×/1.4-numerical-aperture (NA) oil objective. Photobleaching and acquisition of time-lapse images were carried out using a 488-nm laser line (0.8% transmission for acquisition and 40% transmission in 2 iterations for photobleaching). The photobleaching area was selected using Zen software (Carl Zeiss, Germany). The mean fluorescence intensity of the bleached area was quantified using ImageJ (http://rsbweb.nih.gov/ij/), divided by the total mean fluorescence intensity of the cell, and then normalized to prebleach values. Cells used in all FRAP experiments were prepared for time-lapse microscopy as described previously (20).

**RESULTS**

It was previously shown that TbMORN1 localizes exclusively to the trypanosome bilobe and is a robust marker for it (25). In detergent-extracted cells, the endogenous protein is visible as a fishhook-shaped structure close to the point of flagellum entry into the cell (Fig. 1A). Unfortunately, anti-TbMORN1 antibodies are effective for immunofluorescence microscopy only when they are used to label detergent-extracted cells, which limits their usefulness. An endogenous replacement procyclic cell line which expressed YFP-TbMORN1 instead of one of its wild-type alleles was therefore generated by double homologous recombination. Integration of the targeting cassette at the TBMORN1 locus was confirmed by PCR using genomic DNA as the template, with primers annealing to the 5′ end of the YFP open reading frame (ORF) and outside the sequences used for homologous recombination (see Fig. S1A in the supplemental material). Immunoblots using anti-TbMORN1 antibodies showed the expression of wild-type TbMORN1 (40 kDa) and an extra protein species, of approximately 67 kDa, which was additionally recognized by anti-GFP antibodies (see Fig. S1B in the supplemental material). The YFP-tagged TbMORN1 expressed by these cells was found by immunofluorescence microscopy to recapitulate the localization and morphology of the wild-type protein (Fig. 1B). At the EM level, labeling of detergent-extracted cells with anti-GFP antibodies revealed a fishhook-shaped pattern in the expected region, consistent with the observations made by immunofluorescence microscopy (Fig. 1C, arrowheads). These data show that YFP::TbMORN1 cells are an appropriate tool for study of the trypanosome bilobe. Importantly, when these detergent-extracted cells were examined by transmission EM, a similarly shaped structure was not readily visible (Fig. 1D).

The bilobe is tightly associated with the flagellum skeleton, a fact that we decided to exploit for our ultrastructural studies (25). The flagellum skeleton can be purified readily from intact cells via a two-step procedure (28). First, the cytosol and membranes are
extracted using nonionic detergent and the cytoskeletons separated from the detergent-soluble fraction by centrifugation. Second, the cytoskeletal pellet is resuspended in a high-salt buffer to depolymerize the microtubule corset that lies beneath the plasma membrane and gives the cells their shape. Another centrifugation step separates the depolymerized subpellicular fraction from the flagellar fraction in the pellet. For brevity, we refer to the detergent- and salt-extracted flagellum skeleton preparations found in the pellet fraction as “isolated flagella.” This fraction also contains the bilobe, as shown by labeling with anti-TbMORN1 and the monoclonal antibody YL1/2 (which labels fibers emanating from the mature basal body) (37). The bilobe was readily visible as a structure distinct from the basal body and firmly associated with the flagellum (Fig. 1E, arrow). Preparations from the YFP::TbMORN1 cells gave identical results (Fig. 1F, arrows). The localization and morphology of TbMORN1 were therefore unaffected by the biochemical purification. Labeling with an antibody specific for the FAZ filament protein FAZ1 (21) showed that the posterior tip was present (data not shown). The various fractions obtained during the preparation of the isolated flagella (input, cytoplasm, cytoskeleton, high-salt supernatant, and purified flagella) were blotted with anti-tubulin and anti-GFP antibodies (see Fig. S1D in the supplemental material). As expected, two pools of tubulin—representing the soluble cytoplasmic pool and the corset microtubules—were successively solubilized by the two extraction steps and present in the S1 and S2 fractions, respectively. The remaining tubulin, corresponding to the axonemal microtubules, was present in the final pellet (P2). Almost all of the YFP:TbMORN1 was present in the P1 cytoskeletal fraction, consistent with its designation as a robust bilobe marker. Of that, around half remained associated with the purified flagellum pellet (P2) (see Fig. S1D). Collectively, these microscopy and biochemical data show that preparations of isolated flagella offer a viable avenue for investigation of bilobe ultrastructure.

Immuno-EM on the isolated flagellum preparations, using anti-GFP antibodies, revealed a fishhook-shaped pattern (Fig. 2A and B) consistent with that seen in the detergent-extracted cells (Fig. 1C). The labeled arm of YFP-TbMORN1 ran parallel to and at some distance from the posterior end of the FAZ filament, which appeared as a linear series of globular structures (Fig. 2A and B).

Transmission EM of the same isolated flagella gave much clearer views of the various cytoskeletal structures grouped near the base of the flagellum than those seen in detergent-extracted cells (cf. Fig. 1D to Fig. 2C to F). The basal body that acts as the microtubule organizing center for the flagellum axoneme is readily visible, with the probasal body sitting orthogonally to it. The MTQ that forms part of the FAZ originates between the basal body and probasal body and traces a helical path around the volume normally occupied by the flagellar pocket. It then passes through the horseshoe-shaped FPC, where it is joined by the FAZ filament. The FAZ filament is thus present on the same side of the flagellum as the probasal body; this makes it an important fiduciary marker. All of the cytoskeletal elements were consistently found to be in exactly the same relative positions as those previously reported in tomographic studies on whole trypanosome cells, further validating the use of the flagellum preparations (22, 23).

There was, however, an additional structure lying alongside the FAZ filament (Fig. 2C to F, asterisks) but distal to the MTQ (Fig. 2E and F, white arrows). Interestingly, this structure was not the arm heavily labeled for YFP-TbMORN1 but one that appeared to have much lower levels of labeling (Fig. 2A and B).

To determine whether this arm next to the FAZ filament contained other bilobe components, we considered two other known structural protein components of the bilobe—TbLRRP1 and TbCentrin4 (35, 41). Endogenous TbLRRP1 was found to colocalize with endogenous TbMORN1 in detergent-extracted cells (Fig. 3A). Similarly, TbLRRP1 copurified and colocalized with YFP-TbMORN1 in isolated flagellum preparations (Fig. 3B). Analysis of the isolated flagella by immuno-EM using anti-TbLRRP1 showed that TbLRRP1 also exhibited a bias in labeling between the two arms. As with TbMORN1, the arm next to the MTQ was more strongly labeled (Fig. 3C). In this case, though,
there was a smaller degree of bias and the gold label was present on the arm next to the FAZ filament. The bias in labeling was quantified by calculating the cumulative ratio of gold particles between the two arms over a random sequence of images (n).

Samples taken from four independent experiments showed a 2.1-fold enrichment of anti-TbLRRP1 antibodies on the arm next to the MTQ (n/H1100548; total ratio of gold particles in the two arms, 2,519:1,223; the cumulative ratio was stable for n values of n/H110228).

Colabeling of the isolated flagella with anti-GFP and anti-TbLRRP1 antibodies broadly recapitulated the colocalization seen at the level of immunofluorescence microscopy (Fig. 3D).

Centrins were integral to the discovery of the bilobe, as it was first observed using the pan-centrin monoclonal antibody 20H5. The colocalization of TbMORN1 with 20H5 labeling was taken as proof of its presence on the structure (19, 25). Separate studies recently showed that the TbCentrin4 isoform (also referred to as TbCentrin1) is a robust bilobe protein, in addition to localizing to the basal and probasal bodies (33, 35). Remarkably, it was found that TbCentrin4 did not overlap either endogenous (data not shown) or YFP-tagged TbMORN1 in detergent-extracted cells (Fig. 4A). Endogenous TbCentrin4 was labeled using polyclonal antibodies raised against the Leishmania ortholog (32). The situation became even clearer when the isolated flagellar preparations were examined. The TbCentrin4 arm intersected precisely with the tip of the posterior hooked part of TbMORN1 (Fig. 4B, panels i to iv). The two arms could sometimes become twisted around one another, but when they were laid flat, there appeared to be little, if any, overlap between the two proteins. To confirm these results, a monoclonal antibody against TbCentrin4 was used (20a). This antibody gave the same labeling pattern by immunofluorescence microscopy (Fig. 4B, panels v to viii).

Immunogold EM on the isolated flagellum preparations using the monoclonal anti-TbCentrin4 antibodies again showed differential labeling between the two bilobe arms. This time, however, it was the bilobe arm next to the FAZ filament that was labeled more strongly (Fig. 4C). Quantification of the labeling bias by calculating the cumulative ratio of the gold particles on the two bilobe arms over a random sequence of images showed a 2.1-fold enrichment on the arm next to the FAZ filament (n = 53; total ratio of gold particles in the two arms, 4,264:2,079; the cumulative ratio was stable for n values of >18). Double-label immuno-EM experiments using anti-GFP and anti-TbCentrin4 beautifully mirrored the results obtained by immunofluorescence microscopy. YFP-TbMORN1 presented a fishhook morphology, with the anterior arm lying next to the MTQ; TbCentrin4 was present in the other arm, lying alongside the FAZ filament (Fig. 4D and E). These data show that TbMORN1 and TbCentrin4 comprise distinct elements and that the structure observed in the transmission EMs discussed above (Fig. 2C to F, asterisks) represents only the TbCentrin4 arm.

Having defined the identities of the two arms of the bilobe at an
ultrastructural level, we next considered its posterior hooked part in more detail. Given that the anterior arms of the bilobe coincide with the posterior end of the FAZ filament (Fig. 2 to 4), it seemed likely that the posterior hook was positioned above the flagellar pocket.

The flagellar pocket is formed from an invagination of the plasma membrane. This invagination has two subdomains—a cylindrical neck region that tightly encircles the membrane of the flagellum and the balloon-shaped bulge of the flagellar pocket itself (22). A horseshoe-shaped cytoskeletal barrier element, the FPC, demarcates the top of the flagellar pocket. In cross-sectional EMs through the cell posterior, the FPC is readily visible as a region of electron-dense material (Fig. 5A, arrows). The only known component of the FPC is TbBILBO1 (7). To better characterize the relationship between the bilobe and the FPC, polyclonal antibodies against a recombinant fragment of TbBILBO1 were used to label isolated flagella from YFP::TbMORN1 cells. Where visible, TbCentrin4 labeling of the basal body and probasal body is indicated with an arrowhead. TbCentrin4 was labeled using anti-LdCentrin4 rabbit polyclonal antibodies (i to iv) or monoclonal antibodies to TbCentrin4 (v to viii). Images are maximum-intensity z projections. Each z stack was analyzed in single sections, and overlap was observed only if the two bilobe arms became twisted around one another. (C) TbCentrin4 is present largely on the bilobe arm adjacent to the FAZ. Anti-TbCentrin4 antibodies were used to label isolated flagella from YFP::TbMORN1 cells, followed by gold-conjugated secondary antibodies (10 nm). The MTQ, basal body (black arrowhead), and probasal body (white arrowhead) are indicated for orientation. (D and E) YFP-TbMORN1 and TbCentrin4 define separate arms of the bilobe. Isolated flagella from YFP::TbMORN1 cells were labeled with anti-GFP (20-nm gold particles) and anti-TbCentrin4 (10-nm gold particles) antibodies. There appears to be minimal overlap between the hook-shaped YFP-TbMORN1 labeling pattern and the TbCentrin4 distribution. Bars, 1 μm (A and B) and 500 nm (C to E).

**FIG 4**  TbMORN1 and TbCentrin4 are on separate arms of the bilobe. (A) YFP-TbMORN1 and TbCentrin4 do not overlap in detergent-extracted cells (the third panel shows merged images with a differential interference contrast [DIC] overlay). (B) Image gallery showing minimal to no overlap between YFP-TbMORN1 (green) and TbCentrin4 (red) in purified flagella isolated from YFP::TbMORN1 cells. Where visible, TbCentrin4 labeling of the basal body and probasal body is indicated with an arrowhead. TbCentrin4 was labeled using anti-LdCentrin4 rabbit polyclonal antibodies (i to iv) or monoclonal antibodies to TbCentrin4 (v to viii). Images are maximum-intensity z projections. Each z stack was analyzed in single sections, and overlap was observed only if the two bilobe arms became twisted around one another. (C) TbCentrin4 is present largely on the bilobe arm adjacent to the FAZ. Anti-TbCentrin4 antibodies were used to label isolated flagella from YFP::TbMORN1 cells, followed by gold-conjugated secondary antibodies (10 nm). The MTQ, basal body (black arrowhead), and probasal body (white arrowhead) are indicated for orientation. (D and E) YFP-TbMORN1 and TbCentrin4 define separate arms of the bilobe. Isolated flagella from YFP::TbMORN1 cells were labeled with anti-GFP (20-nm gold particles) and anti-TbCentrin4 (10-nm gold particles) antibodies. There appears to be minimal overlap between the hook-shaped YFP-TbMORN1 labeling pattern and the TbCentrin4 distribution. Bars, 1 μm (A and B) and 500 nm (C to E).
corresponding to its first 111 amino acids were generated. The affinity-purified antibodies recognized a protein with the expected molecular mass of approximately 67 kDa in immunoblots of trypanosome whole-cell lysates (Fig. 5B). In immunofluorescence analysis, the antibodies bound to a horseshoe- or ring-shaped structure close to the flagellum base (Fig. 5C, arrow). Interestingly, in immunofluorescence colabeling experiments with anti-GFP and anti-TbBILBO1 in YFP::TbMORN1 cells, the antibodies were present on discrete structures. Viewed from the flagellar side of the cell, which we refer to as the top of the cell based on figure conventions in the literature (see Fig. 8), the posterior hook part of TbMORN1 and the TbBILBO1 ring were superimposed, as previously documented (25). At more oblique angles, however, the degree of overlap was revealed to be comparatively slight (Fig. 5D). By immuno-EM, this slight disparity in relative localization was confirmed—the anti-TbBILBO1 antibodies were consistently found to label a more distal region than the anti-GFP ones, although both sets described a ring/hook in the FPC region (Fig. 5E and F, arrows). We tentatively concluded that the bilobe is a separate structure that sits atop the FPC, in the neck region of the flagellar pocket.

Although the typical morphology of TbMORN1 is a fishhook shape, more exotic conformations were frequently observed. Common to these morphological variants were one or two fingerlike projections radiating from the posterior hook part and apparently extending toward the kinetoplast (Fig. 6A and B, arrows). These projections were observed only in cells with a single nucleus and kinetoplast (mitochondrial genome), although the kinetoplast DNA was often in the process of replicating. They are thus present only at an early stage of the cell cycle. The projections could also be observed in isolated flagellum preparations. Colabeling with YL1/2, which labels fibers radiating from the mature basal body (37), showed that the projections (arrows) appear to contact the basal and probasal bodies (arrowheads) (Fig. 6C and D). Since the probasal body in Fig. 6D is also labeled with YL1/2, it must have recently matured, and the flagellum therefore derived from a cell that was at an early stage of the cell cycle. The types of commonly observed YFP-TbMORN1 projections were analyzed.

FIG 5 The posterior hooked part of TbMORN1 sits above the flagellar pocket collar. (A) The flagellar pocket collar is visible as an electron-dense region at the neck of the flagellar pocket in an EM (arrows). (B) Anti-TbBILBO1 antibodies recognize an approximately 67-kDa polypeptide in immunoblots of trypanosome whole-cell lysates. (C) Anti-TbBILBO1 antibodies label a ring-shaped structure close to the point of flagellum entry into the cell (arrow). Detergent-extracted cells were fixed with methanol and labeled with anti-TbBILBO1. (D) YFP-TbMORN1 and TbBILBO1 overlap only partially at oblique angles. Isolated flagella from YFP::TbMORN1 cells were labeled with anti-GFP and anti-TbBILBO1. (E and F) The posterior hooked part of YFP-TbMORN1 overlaps with TbBILBO1 in immunogold experiments. TbBILBO1 labeling is always more posterior to YFP-TbMORN1 labeling (arrows), Bars, 500 nm (A, E, and F) and 1 μm (C and D).
and grouped into various categories. These consisted of single thin tendrils (Fig. 6E, panel i), single thick tendrils (Fig. 6E, panel ii), coat-hanger shapes (Fig. 6E, panels iii and iv), and figure-eight shapes (Fig. 6E, panel v). Upon reexamination of the unlabeled images of isolated flagellum preparations, it was found that a component that appeared to be a tendril of material linking the basal body and maturing probasal body. Flagella extracted from YFP::TbMORN1 cells were labeled with anti-GFP antibodies and the YL1/2 antibody. YL1/2 labels fibers emanating from the mature basal body. The probasal body and the basal body are both labeled (arrowheads), indicating that the probasal body has matured. (E) Gallery showing various morphological forms of the YFP-TbMORN1 tendrils. Variously, these are single thin tendrils (i), single thick tendrils (ii), coat-hanger shapes (iii and iv), and figure-eight structures (v). All images show isolated flagella from YFP::TbMORN1 cells, fixed with paraformaldehyde. (F) A tendril emanating from the probasal body is occasionally seen in EM images of isolated flagella. The MTQ, originating between the basal body (black arrowhead) and the probasal body (white arrowhead), is indicated. The FAZ is marked for orientation. (G) Both YFP-TbMORN1 and TbLRRP1 can be found on the tendril and as part of the MTQ. Isolated flagella from YFP::TbMORN1 cells were labeled with anti-GFP (20-nm gold particles) and anti-TbLRRP1 (10-nm gold particles). Only half of the MTQ (bar) appears to be labeled. (H) The YFP-TbMORN1 tendril (long arrow) does not appear to contain alpha-tubulin (short arrow). The probasal body (arrowhead) is indicated in the DIC overlay. Isolated flagella were fixed with paraformaldehyde and labeled with anti-GFP and anti-alpha-tubulin antibodies. All immunofluorescence images are maximum-intensity z projections. Bars, 1 μm (A to E and H) and 500 nm (F and G).

FIG 6 Tendrillar extensions of TbMORN1. (A and B) YFP-TbMORN1 (arrowheads) is sometimes seen to exhibit tendrillar extensions (arrows) to its normal hook-shaped pattern. YFP::TbMORN1 cells were fixed with paraformaldehyde, permeabilized with detergent, and labeled with anti-GFP antibodies. (C and D) The YFP-TbMORN1 tendrils (arrows) contact the basal body and maturing probasal body. Flagella extracted from YFP::TbMORN1 cells were labeled with anti-GFP antibodies and the YL1/2 antibody. YL1/2 labels fibers emanating from the mature basal body. The probasal body and the basal body are both labeled (arrowheads), indicating that the probasal body has matured. (E) Gallery showing various morphological forms of the YFP-TbMORN1 tendrils. Variously, these are single thin tendrils (i), single thick tendrils (ii), coat-hanger shapes (iii and iv), and figure-eight structures (v). All images show isolated flagella from YFP::TbMORN1 cells, fixed with paraformaldehyde. (F) A tendril emanating from the probasal body is occasionally seen in EM images of isolated flagella. The MTQ, originating between the basal body (black arrowhead) and the probasal body (white arrowhead), is indicated. The FAZ is marked for orientation. (G) Both YFP-TbMORN1 and TbLRRP1 can be found on the tendril and as part of the MTQ. Isolated flagella from YFP::TbMORN1 cells were labeled with anti-GFP (20-nm gold particles) and anti-TbLRRP1 (10-nm gold particles). Only half of the MTQ (bar) appears to be labeled. (H) The YFP-TbMORN1 tendril (long arrow) does not appear to contain alpha-tubulin (short arrow). The probasal body (arrowhead) is indicated in the DIC overlay. Isolated flagella were fixed with paraformaldehyde and labeled with anti-GFP and anti-alpha-tubulin antibodies. All immunofluorescence images are maximum-intensity z projections. Bars, 1 μm (A to E and H) and 500 nm (F and G).
TbMORN1 contains tubulin, isolated flagellum preparations were labeled with anti-alpha-tubulin antibodies. The YFP-TbMORN1 tendril signal and the alpha-tubulin signal did not overlap (Fig. 6H, arrows).

To test if the tendrillar projections represent a transient population of TbMORN1 molecules, FRAP experiments were carried out. Due to the photostability and reversibility drawbacks of YFP, a GFP tag was preferred for these experiments (5). A monomeric EGFP::TbMORN1 endogenous replacement cell line was generated in the same manner as the YFP::TbMORN1 cell line (see Fig. S1A to C in the supplemental material). A specific area containing either the main bilobe structure (Fig. 7A, upper panels) or tendrillar projection(s) (Fig. 7A, middle panels) was then photobleached, and the recovery of the fluorescence signal in the bleached area was monitored. FRAP analysis revealed that mEGFP-TbMORN1 was present more transiently on the tendrillar projections than at the main bilobe structure (Fig. 7B, left panel) and no significant recovery was observed at the main bilobe structure (Fig. 7B, right panel) (n = 5 independent experiments in all cases). It was observed that the mEGFP-TbMORN1 signal at the tendrillar projections recovered to ~80% of prebleach levels approximately 20 min after photobleaching (Fig. 7B, middle panel) (n = 5 independent experiments). In contrast, no significant recovery was observed at the main bilobe structure (Fig. 7B, left panel) (n = 5 independent experiments). Next, we tested whether the mobility of TbMORN1 was altered by the presence of tendrillar projections. The main bilobe structure adjacent to a tendrillar projection was photobleached, and recovery of the mEGFP-TbMORN1 signal was monitored. TbMORN1 recovery occurred in a manner similar to that observed upon photobleaching of the main bilobe alone (Fig. 7A, lower panels, and B, right panel) (n = 5 independent experiments). These experiments suggest that the main bilobe and tendrillar projections contain two dynamically distinct populations of TbMORN1.

**DISCUSSION**

In this project, the trypanosome bilobe was studied at high resolution. In so doing, a better appreciation of its morphology, composition, and relationship to other cytoskeletal elements in the vicinity of the flagellar pocket was obtained. A key innovation was
the use of isolated flagellum preparations for EM, which permits a less restricted view of the cytoskeletal structures clustered near the flagellum root, away from any obscuring factors in the cytoplasm. This is a relevant consideration because despite several decades of examination of trypanosomes by use of EM, the bilobe eluded notice until its serendipitous discovery in 2005 by immunofluorescence microscopy (19). Even in unlabeled cytoskeletons in which the cytoplasm and membranes have been removed by detergent extraction, the bilobe is not readily visible without close scrutiny (Fig. 1C). With foreknowledge of the bilobe’s likely location, however, it is occasionally possible to discern a structure near the FPC with the FAZ filament snaking away from it. In isolated flagellum preparations, however, the bilobe becomes much more amenable to observation (Fig. 2).

Following this line of enquiry, we clarified the spatial relationship of TbMORN1 to the other previously characterized bilobe components, TbLRRP1 and TbCentrin4, and also to the FPC. We found that TbMORN1 and TbLRRP1 colocalize in a fishhook-shaped structure whose anterior stem lies alongside the MTQ and whose posterior hook sits atop the FPC (Fig. 3). In contrast, TbMORN1/TbLRRP1 and TbCentrin4 do not, as previously thought, reside on the same structure. Instead, TbCentrin4 is present mostly on a single rod that runs parallel to the TbMORN1/TbLRRP1 stem and alongside the FAZ filament (Fig. 4). The two structures clearly inhabit the same cellular volume, but it is not clear if they are separate entities or subdomains of a single heterogeneous assemblage. The cellular volume in question is that of the flagellar pocket neck, a cylindrical plasma membrane subdomain which is contiguous with the ballooned membrane of the flagellar pocket proper, as noted in an earlier electron tomography study (22).

The same study identified a filament, annotated the “neck microtubule,” in the flagellar pocket neck region. Interestingly, this filament is similar in both length and location to the TbCentrin4 arm. If these are synonymous structures, then the microtubule must be coated heavily with TbCentrin4 (and perhaps other proteins), as it is clearly texturally distinct from nearby microtubules such as those in the MTQ. Further work will be needed to resolve this issue.

It is curious that while the TbCentrin4 arm is comparatively visible, the TbMORN1/TbLRRP1 fishhook is almost invisible. This situation seems to be exacerbated by the use of gold thiogluco during staining, a condition which provides the highest levels of ultrastructural detail by negative staining. During earlier experiments in which we employed uranyl acetate as a staining agent, we achieved equal staining of both the TbMORN1 and TbCentrin4 components of the bilobe, although this came at a high cost in terms of maintaining ultrastructural detail. In these pictures, the bilobe appeared as a single hairpin-shaped structure (see Fig. S2 in the supplemental material). The relative lack of visibility of the TbMORN1/TbLRRP1 fishhook after gold thioglucose staining offers a plausible reason for why this structure eluded description for so long, despite the presence of many excellent electron microscopy studies published in the literature.

In addition to considering the relationships between the known components of the bilobe, the relative localizations of TbMORN1 and the FPC were also explored (Fig. 5). In the initial characterization of TbMORN1, an overlap was documented between it and the FPC protein TbBILBO1 (25). On closer examination here, however, it was found that such an overlap is seen only when the two structures are viewed from above or below and that when they are viewed from an angle, the degree of overlap is marginal. This supports the localization of TbMORN1 above the FPC and in the flagellar neck region of the cell.

In summary, on the basis of the results presented here, we can redefine the bilobe as a bipartite entity that approximates a hairpin shape (Fig. 8A). One arm of this hairpin contains TbCentrin4 and might be synonymous with the previously described “neck microtubule” (22). The remainder of the hairpin, forming a fishhook shape, contains TbMORN1 and TbLRRP1. Whether this hairpin is indeed a single heterogeneous entity or two distinct structures sharing a similar cellular volume remains an open question. A model in which the TbMORN1/TbLRRP1 fishhook and TbCentrin4 bar simultaneously interact with each other at their tips and, where possible, with the FPC might represent the most parsimonious interpretation of the available data (Fig. 8B). Regardless, our original conception of the “bilobe” as a bowling pin- or skittle-shaped object probably merits an update, at the very least in terms of nomenclature.

Finally, the existence of more exotic TbMORN1 morphologies, in which the protein extends its localization to include the microtubule quartet or a tendril contacting the probasal body, was documented. The tendril is not labeled with anti-alpha-tubulin antibodies and, indeed, appears to be texturally different from the microtubules of the quartet by immuno-EM. By FRAP, it was shown that TbMORN1 in these tendril projections has a higher mobility than that of the TbMORN1 molecules present in the bilobe. The timing of replication (duplication plus segregation) of the TbMORN1 bilobe relative to that of the kinetoplast has been quantified using images of fixed cells (data not shown). In this analysis, it was found that TbMORN1 consistently forms two resolvable structures before the duplicated kinetoplasts segregate. Cells with two kinetoplasts and only one TbMORN1 bilobe are never observed. Since the exotic morphologies are observed only in cells with a single (or replicating) kinetoplast and a single nucleus, this supports the idea that they are present during an early phase of the cell cycle. The earliest known event in the cycle is the maturation of the probasal body and outgrowth of the new MTQ (23). It is tempting to speculate that the tendril observed here precedes the outgrowth of the new MTQ. The flagellar pocket itself is duplicated following outgrowth of the new microtubule quartet, during which the new basal (formerly probasal) body undergoes a rotation around the flagellar pocket, folding the membrane around the old microtubule quartet in the process (23). One tantalizing possibility is that the tendrils observed here form a protein scaffold that facilitates the duplication of the flagellar pocket and is disassembled following this process. It should be stressed that when TbMORN1 levels in procyclic cells were depleted to 20% of wild-type levels by RNA interference, there were no observed defects in flagellar pocket or organelle biogenesis, so even if it is involved in this process, TbMORN1 may not act alone (25). An alternative, equally speculative interpretation is that the tendril projections represent newly synthesized molecules of TbMORN1 trafficking to the bilobe along the old and newly forming MTQs. Such behavior would be analogous to intraflagellar transport, the bidirectional movement of protein complexes along the microtubules of the flagellum axoneme. This process has been documented for trypanosomes and has been shown to be essential for biogenesis of new flagella (1). Such an interpretation implies that the microtubules of the quartet are fully com-
petent as transport highways, in addition to performing structural roles. In Fig. 6H, it appears that only half of the old microtubule quartet contains TbMORN1 molecules; if the trafficking hypothesis is true, this could imply that the individual microtubules of the quartet are licensed for either anterograde or retrograde transport.

The work presented here also has a broader context in terms of eukaryotic diversity and evolution. The trypanosomes and other parasitic kinetoplastids are part of the more inclusive phylum Euglenozoa. This phylum also includes free-living kinetoplastids, as well as the diplomonads, euglenid microalgae and their heterotrophic relatives, and a novel clade known as the Symbiontida (8, 40). Although the trypanosomes and phototrophic euglenids have been studied extensively ultrastructurally (18, 24), the cytoskeletons of free-living kinetoplastids, heterotrophic euglenids, and diplomonads are not as well understood.

The cryptic nature of the bilobe and the fact that it evaded detection for decades raise the question of whether there are homologous structures in other taxa. Centrin-containing fibers have been found in other excavate groups, such as trichomonads (10), retortamonads (39), and diplomonads (6), and other flagellates, including the chrysomonads (11), the dinoflagellates (27), and Chlamydomonas (38), but this in itself is not strong evidence for the existence of bilobe homologs. At this point, taxonomic sampling is too widespread and infrequent to make any reasonable inferences regarding the evolution of a morphologically and biochemically complex structure. Given the relative ease of preparing the isolated flagellar complexes used in this study, on the other hand, it might be possible to examine whole mounts of the flagella and associated structures in euglenozoan taxa such as Leishmania, Bodo, Diplonema, and Euglena. Indeed, a centrin-containing structure is present in Leishmania donovani promastigotes, and its location near the top of the flagellar pocket is consistent with what would be expected of a bilobe homolog (32). Potential bilobe homologs could then be investigated biochemically or genomically and, eventually, phylogenetically. From an evolutionary standpoint, the inclusion of free-living taxa would help to clarify whether the bilobe is part of the cellular adaptations necessary for

FIG 8 Morphological model of the trypanosome bilobe. (A) Drawing showing the bilobe’s position relative to other key cytoskeletal components. The locations of the bilobe and nearby cytoskeletal elements relative to the anterior-posterior (A-P), top-bottom (T-B), and left-right (L-R) axes of the whole cell are indicated in the inset. (B) Schematics of the isolated bilobe. The positions of the MTQ, FAZ filament, and apparent central projection of the FPC (arrowheads) are shown in flagellar (“top”) (i), “bottom” (ii), left (iii), and right (iv) views. Comparison with previously published tomograms indicates that the arms of the bilobe and the hook/flagellar pocket collar are oriented at an obtuse angle to one another. Furthermore, the left end of the flagellar pocket collar may extend partway along the ventral face of the MORN1/LRRP1 hook.
a parasitic lifestyle and may provide much-needed direction in pinpointing a clear cellular function for the structure.

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