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Trypanin is a cytoskeletal linker protein and is required for cell motility in African trypanosomes

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The cytoskeleton of eukaryotic cells is comprised of a complex network of distinct but interconnected filament systems that function in cell division, cell motility, and subcellular trafficking of proteins and organelles. A gap in our understanding of this dynamic network is the identification of proteins that connect subsets of cytoskeletal structures. We previously discovered a family of cytoskeleton-associated proteins that includes *GAS11*, a candidate human tumor suppressor upregulated in growth-arrested cells, and trypanin, a component of the flagellar cytoskeleton of African trypanosomes. Although these proteins are intimately associated with the cytoskeleton, their function has yet to be determined. Here we use double-stranded RNA interference to block trypanin expression in *Trypanosoma brucei*, and demonstrate that this protein is required for directional cell motility. Trypanin(−) mutants have an active flagellum, but are unable to coordinate flagellar beat. As a consequence, they spin and tumble uncontrollably, occasionally moving backward. Immunofluorescence experiments demonstrate that trypanin is located along the flagellum/flagellum attachment zone and electron microscopic analysis revealed that cytoskeletal connections between the flagellar apparatus and subpellicular cytoskeleton are destabilized in trypanin(−) mutants. These results indicate that trypanin functions as a cytoskeletal linker protein and offer insights into the mechanisms of flagellum-based cell motility.

Introduction

Although structural aspects of microtubules and other cytoskeletal filaments are well characterized (Vale and Milligan, 2000; Spudich, 2001), much less is known about proteins that mediate interactions between subsets of cytoskeletal structures (Cosson, 1996; Klymkowsky, 1999; Heald, 2000; Marte, 2000). The critical importance of these cytoskeletal linker proteins is evidenced by severe neurological and skin blistering diseases that result from defects in plakins, a family of coiled-coil proteins that physically link intermediate filaments with actin microfilaments and microtubules (Klymkowsky, 1999). These cross-linking proteins not only contribute to mechanical strength of the cytoskeleton, but also participate in dynamic rearrangements of the cytoskeleton (Andra et al., 1998). A better understanding of these cytoskele-

tal linker proteins therefore represents a key to understanding cytoskeleton organization and function (Klymkowsky, 1999).

African trypanosomes, e.g., *Trypanosoma brucei* and related subspecies, are protozoan parasites that cause fatal human sleeping sickness (Pepin and Donelson, 1999). These single-celled eukaryotes possess a canonical (9 + 2) microtubule-based flagellum and a unique subpellicular cytoskeleton that is comprised of a parallel array of interconnected microtubules and microtubule-associated proteins (Gull, 1999). The unusual cytoskeletal architecture of trypanosomes makes them an attractive model organism for dissecting cytoskeleton function (Schneider et al., 1988; Gull, 1999; Vaughan et al., 2000). Insights from studies on trypanosomes include the identification of novel microtubule-associated proteins (Rindisbacher et al., 1993; Schneider et al., 1988; Hill et al., 2000) and new tubulin isoforms (Vaughan et al., 2000).

Trypanin, formerly called T lymphocyte triggering factor (TLTF), is a 54-kD coiled-coil protein that is associated with the flagellar fraction of the *T. brucei* cytoskeleton (Hill et al., 2000). This fraction contains at least two different subsets of microtubules and associated proteins that func-
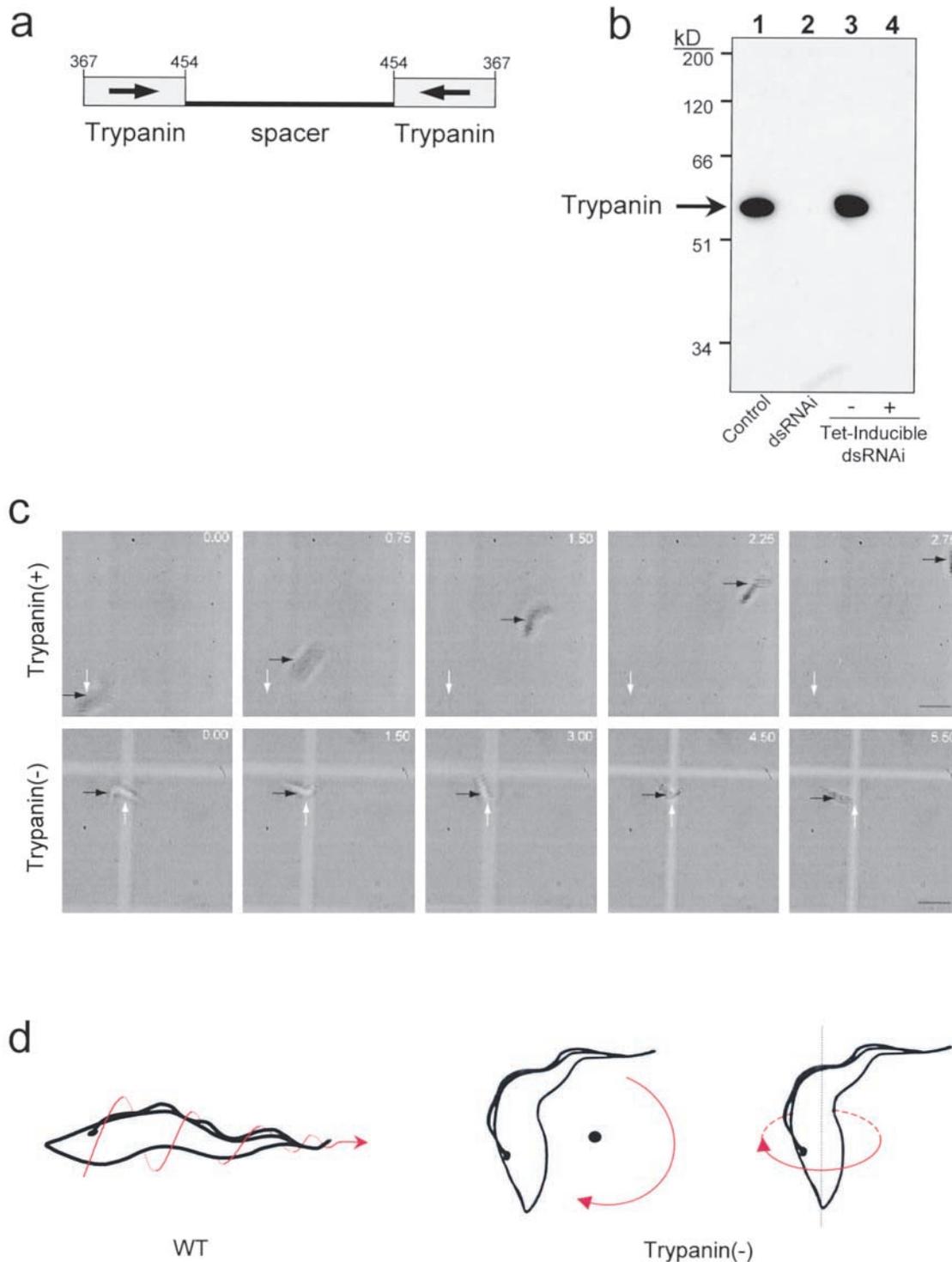
Figure 1. **Trypanin is required for directional cell motility.** (a) Schematic diagram of the trypanin-dsRNA construct. Inverted repeats correspond to the last 88 codons of the trypanin sequence. (b) Western blot probed with α-trypanin antibody α-Pep4. Samples were prepared from cells expressing a control DNA construct (lane 1) or KHTb5 cells (Materials and methods), which express trypanin-dsRNA constitutively (lane 2). Samples in lanes 3 and 4 were prepared from KHTb12 cells (Materials and methods), which express trypanin-dsRNA only when tetracycline is added to the growth medium. Cells were grown in the absence (−) or presence (+) of 1 μg tetracycline/ml as indicated. Staining with Ponceau S confirmed that equal protein amounts were loaded in each lane (unpublished data). (c) Time-lapsed video microscopy of KHTb12 cells grown in the absence (top) or presence (bottom) of 1 μg tetracycline/ml. The elapsed time in seconds is shown in each image. Arrows mark the midpoint of each cell at $t = 0$ (white arrows) and at each progressive time point (black arrows). Bar, 10 μm. Video 3, trypanin(+) (available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1); Video 4, trypanin(−) (available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1) (d) Wild-type trypanosomes, i.e., trypanin(+), are highly motile, moving with a distinctive auger-like motion (d, WT) at up to 20 μm/s (Fig. 2 a). In contrast, uncoordinated beating of the flagellum in trypanin(−) mutants drives them into one of two uncontrolled tumbling motions depicted schematically in d, Trypanin(−). Relative cell motion is indicated with an arrow and the rotational axis of trypanin(−) mutants is indicated with a black dot or dotted vertical line. For comparison, the spiral motion of wild-type (WT) trypanosomes is also depicted.

tion in cell motility, cytokinesis, establishment of cell polarity, and organelle inheritance (Robinson et al., 1991, 1995; Robinson and Gull, 1991; Bastin et al., 1998; Ngo et al., 1998; Gull, 1999). The broad significance of these findings was revealed by the discovery that trypanin represents a family of previously uncharacterized proteins that are present in organisms as diverse as protozoa, *Drosophila*, zebrafish, and humans, but that have not been identified in *Saccharomyces cerevisiae* (Whitmore et al., 1998; Hill et al., 2000).

The human representative of the trypanin family, GAS11, is upregulated by growth arrest (EMBL/GenBank/DDBJ under accession no. U19859; Whitmore et al., 1998), contains a novel microtubule-binding domain (Hill et al., 2000), and is encoded by a gene that is commonly deleted in breast and prostate cancer (Whitmore et al., 1998). The GAS11 microtubule-binding domain directs a green fluorescent protein (GFP)* fusion protein to the plus ends of trypanosome microtubules in vivo, at a position that corresponds to the last point of contact between two dividing cells (Sherwin and Gull, 1989; Hill et al., 1999). These findings, together with the fact that trypanin-like sequences have been highly conserved throughout evolution (Hill et al., 2000), suggest that this newly discovered protein family is required for fundamental cyotskeleton-dependent processes, e.g. cell growth and cell motility. To test this hypothesis, we used inducible double-stranded RNA interference (dsRNAi) to block trypanin expression in *T. brucei*. Our results demonstrate that trypanin is required for trypanosome cell motility and functions as a cytoskeletal linker protein, coupling the flagellum to the subpellicular cytoskeleton.

Results

Blocking trypanin expression by dsRNAi

Comparison of the trypanin amino acid sequence to partial protein sequences in the GenBank database led to the discovery of a new family of proteins implicated in cytoskeleton function/organization (Hill et al., 2000). This family of proteins is represented in protozoa, algae, *Drosophila melanogaster*, zebrafish, and humans (Hill et al., 2000). Trypanin from *T. brucei* and the human trypanin family member, GAS11 (Whitmore et al., 1998), are 35% identical throughout their length and exhibit several long stretches of nearly identical amino acids (Hill et al., 2000). Of particular interest is the observation that the last portion of the GAS11 microtubule-binding domain includes a region in which 54% of 59 contiguous amino acids are identical in all trypanin family members for which the sequence is known. This strict conservation of sequence in such diverse organisms suggests that these proteins participate in fundamentally important cellular processes.

To test the hypothesis that trypanin proteins mediate functions of the cytoskeleton, we employed dsRNAi to deplete *T. brucei* procyclic cells of trypanin protein. dsRNAi is

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*Abbreviations used in this paper: dsRNAi, double-stranded RNA interference; FAZ, flagellum attachment zone; GAS, growth arrest specific; GFP, green fluorescent protein; PFR, paraflagellar rod; SEM, scanning EM; TLTF, T lymphocyte triggering factor.

a potent and specific method for inhibiting gene expression in trypanosomes and other eukaryotic organisms (Ngo et al., 1998; Bosher and Labouesse, 2000; Wang et al., 2000). A trypanin-dsRNA construct (Fig. 1 a) was inserted into two integrative trypanosome expression vectors, one that drives constitutive expression (Biebinger et al., 1996), and one that allows for tetracycline-inducible expression (Wirtz et al., 1999). Constitutive- or tetracycline-induced expression of trypanin-dsRNA completely abolished trypanin expression, as determined by Western blot analysis (Fig. 1 b). The Western blot shown is intentionally overexposed to demonstrate that trypanin is completely absent in these mutants. Thus, expression of trypanin-dsRNA creates trypanin(−) mutants that are devoid of trypanin protein.

Trypanin is required for cell motility

Examination of trypanin(−) mutants by video microscopy revealed a surprising and profound defect in a critical microtubule-dependent function. Specifically, these mutants are incapable of directional cell motility (Figs. 1, c and d, and 2; Videos 1–5, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1). Trypanosomes containing trypanin "trypanin(+)" are highly motile (Figs. 1, c and d, and 2; Videos 1–3, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1), traveling long distances at velocities of up to 20 μm/s (Fig. 2 a; Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1). In marked contrast, trypanin(−) mutants spin and tumble uncontrollably, remaining primarily in one location (Figs. 1 and 2; Videos 2, 4, and 5, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1) or occasionally moving backward (Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1). The most striking aspect of the phenotype of trypanin(−) mutants is that these cells are not paralyzed. Instead, they have lost the ability to coordinate flagellar beat and can no longer harness this activity to drive productive cell motility. Motility traces of individual cells demonstrate the severe impact this has on the capacity for directional motility (Fig. 2, a and b).

The attached flagellum of African trypanosomes (see below) drives cell movement toward the flagellum tip in a distinctive corkscrew motion (Fig. 1 d; Videos 1 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1) (Walker, 1961). This auger-like motion is such a distinctive feature of trypanosomes that the Greek word for auger, trypanon, was adopted as the genus name (Trypanosoma = auger cell). The uncoordinated tumbling of trypanin(−) mutants, depicted schematically in Fig. 1 d, is profoundly different from the auger-like motility of trypanin(+) cells. Hence, we propose the name trypanin for the missing protein, based on the absence of this hallmark auger-like motility.

Trypanin was previously called TLTF, as it was originally identified on the basis of its immunomodulatory activity in vitro (Vaidya et al., 1997). However, subsequent independent lines of evidence from biochemical (Hill et al., 2000), cell biological (Hill et al., 1999), and RNAi knockout studies (this work) demonstrate that this protein is an integral component of the trypanosome flagellar cytoskeleton and is required for directional cell motility. Previous references to
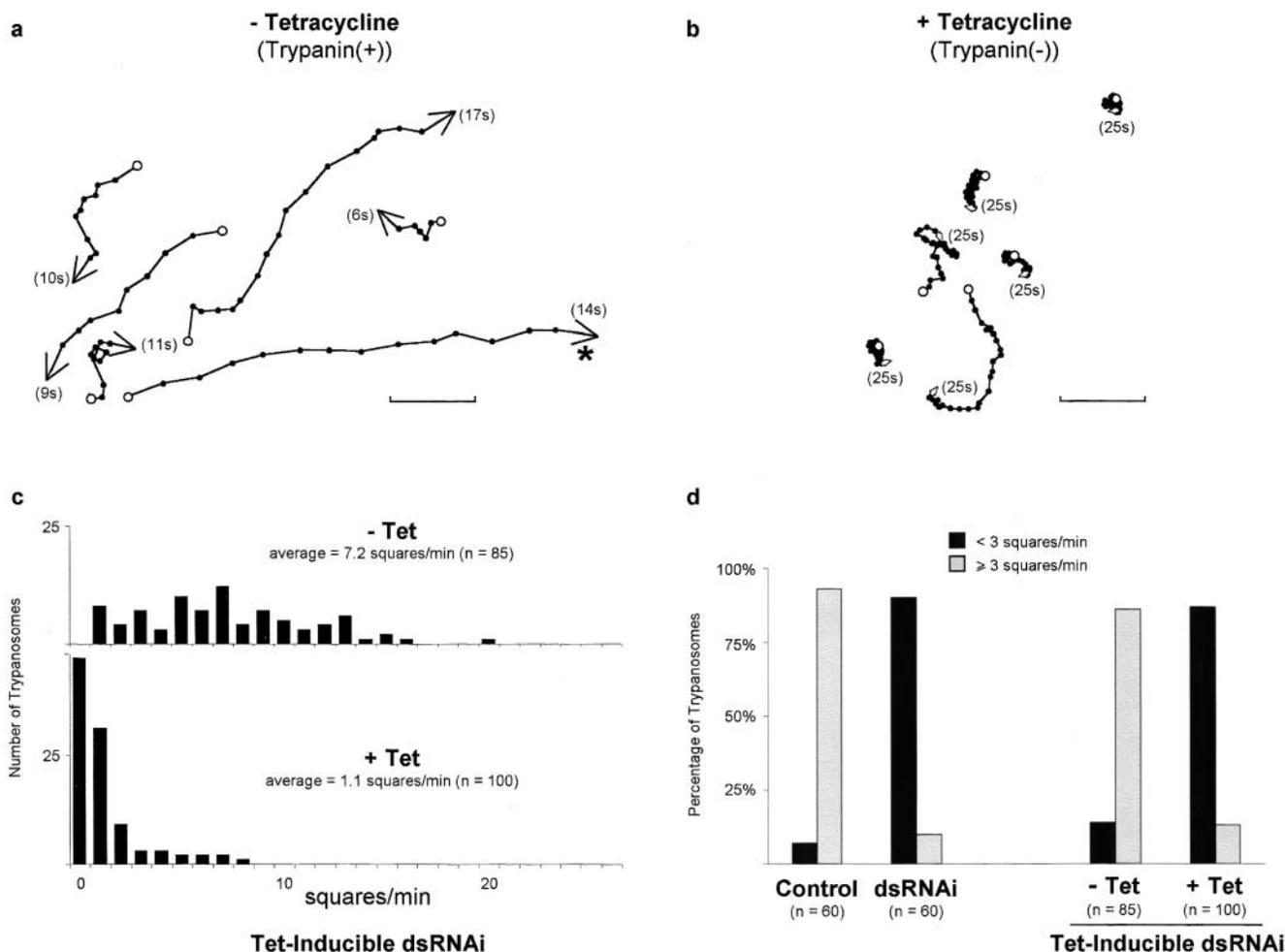
Figure 2. **Quantitative analysis of cell motility.** (a and b) Motility traces of trypanin(+) (a) and trypanin(−) (b) cells. The positions of individual cells are plotted at one second intervals (●). The starting position of each cell is marked with an open circle and the ending position is marked with an arrowhead. The number in parentheses indicates the time in seconds that a given cell was within the field of view. Bar, 50 µm. The average velocity of a wild-type/trypanin(+) cell moving primarily in one direction (a, *) is 20 µm/s, consistent with previous reports (Bastin et al., 1999). Video clips used to generate these motility traces (Videos 1, trypanin[+] and 2, trypanin[−]) are available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1. (c) Histograms show the range of motility of 85 trypanin(+) cells (− Tet) and 100 trypanin (−) mutants (+ Tet) in a quantitative motility assay that measures the number of squares (50 µm × 50 µm) each cell traverses per unit time. (d) Histogram shows the percentage of cells that migrate <3 squares/minute (black bars) or ≥ 3 squares/min (gray bars) in this assay. Assays were performed with cells constitutively expressing a control DNA construct (d, Control), or trypanin-dsRNA (d, dsRNAi), or with KHTb12 cells (a–c and d, Tet-Inducible dsRNAi) grown with (+) or without (−) 1 µg tetracycline/ml as indicated.

TLTF (Hill et al., 1999, 2000; Vaidya et al., 1997) refer to the same protein that we have now named TRYPANIN. For consistency, we have used the phenotype-based name of trypanin throughout this manuscript.

To quantitate cell motility, we developed an assay in which cells are monitored in a counting chamber and the number of squares (50 µm × 50 µm) traversed per minute is determined (Fig. 2, c–d). KHTb12 cells (Materials and methods) grown in the absence of tetracycline, i.e. trypanin(+), exhibit an average motility of 7.2 squares/min in this assay (Fig. 2 c). In contrast, trypanin(−) mutants remain primarily within one square (Fig. 2 c). Thus, the penetrance of this phenotype is virtually 100% and is qualitatively and quantitatively identical whether trypanin is depleted by constitutive (i.e., without tetracycline) or tetracycline-induced dsRNAi (Fig. 2 d). Time-course studies with tetracycline-inducible dsRNAi demonstrate that loss of

motility coincides with the loss of trypanin protein (see Fig. 5). Moreover, in one case, prolonged growth of KHTb5 cells (Materials and methods) under selection for constitutive expression of trypanin-dsRNA linked to the hygromycin-resistance gene (Biebinger et al., 1996) led to a coincident reversion to wild-type motility and renewed expression of trypanin protein (unpublished data). These results confirm that loss of trypanin is directly responsible for loss of motility.

Trypanin is localized to the flagellum/flagellum attachment zone

Two independent anti-trypanin antibody preparations were used in immunofluorescence assays with cytoskeletons prepared from trypanin(+) and trypanin(−) trypanosomes. Both of these antibodies stain trypanin(+) cytoskeletons along a line that corresponds to the site of flagellum attachment to the sub-
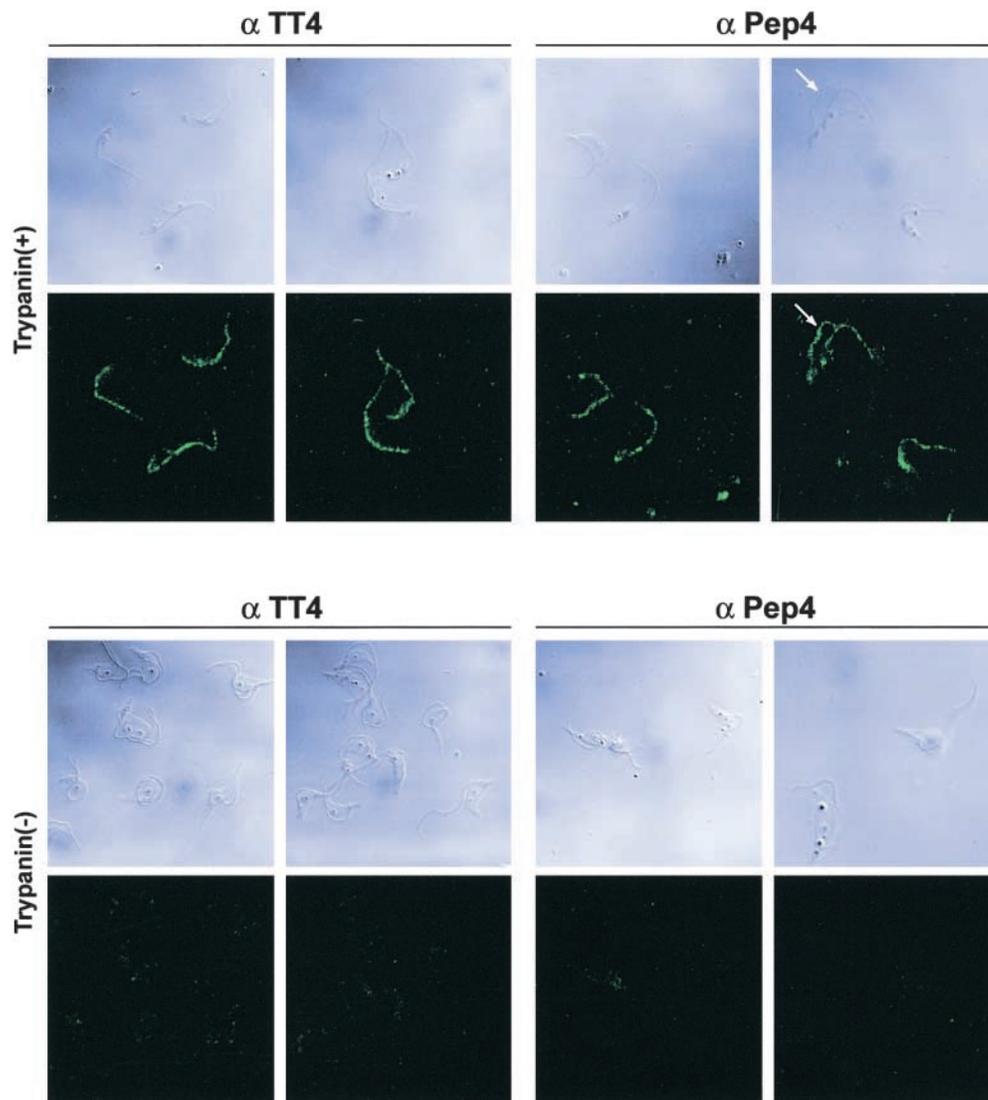
Figure 3. **Trypanin is localized to the flagellum/flagellum attachment zone.** Cytoskeletons from trypanin(−) cells were used to deplete nonspecific antibodies from α-TT4 and α-Pep4 antisera (Materials and methods). These preadsorbed antibodies were then used to stain cytoskeletons prepared from trypanin(+) or trypanin(−) cells as indicated. Staining is only observed in trypanin(+) cytoskeletons and corresponds to the region of the flagellum/flagellum attachment zone. As a negative control, no staining is observed in trypanin(−) cytoskeletons. Top panels for each sample show the corresponding DIC (differential interference contrast) images of the field used for immunofluorescence in the bottom panels. The white arrow points to an example where the flagellum has pulled away from the subpellicular cytoskeleton of a trypanin(+) cell (see text). Antisera were raised against the NH2-terminal 145 amino acids of trypanin (TT4) or to a synthetic peptide (Pep4) corresponding to the COOH-terminal 14 amino acids of trypanin (Hill et al., 2000) and were preadsorbed with trypanin(−) cytoskeletons to remove nonspecific antibodies as described in Materials and methods. The α-Pep4 staining is completely eliminated by addition of excess Pep4 peptide antigen, but not by a nonspecific peptide (unpublished data). Trypanin(+) and trypanin(−) cells are derived from a single culture of KHTb12 cells (Materials and methods) that was divided into two flasks and grown with (trypanin [−]) or without (trypanin [+]) 1 μg tetracycline/ml to induce trypanin-dsRNA expression.

pellicular cytoskeleton (Figs. 3 and 4). As expected, no staining is observed in trypanin(−) mutants (Fig. 3).

To determine the location of trypanin relative to other flagellar structures, coimmunofluorescence experiments were performed using antibodies against the *T. brucei* paraflagellar rod (PFR) protein PFR-A (Kohl et al., 1999). The PFR is a lattice-like filament that runs alongside the axoneme in the flagellum of trypanosomes and other kinetoplastid parasites (Gull, 1999). In samples costained with antibodies against trypanin and PFR-A, trypanin exhibits an well-defined punctate distribution along one side of the PFR. This punctate trypanin staining pattern is decidedly different than the smooth, continuous line of the paraflagellar rod and is generally present only on the side of the PFR that is connected to the cell body (Fig. 4). In some cases, the flagellum becomes detached from the cell body and trypanin remains with the flagellum in these locations (e.g., arrows in Figs. 3 and 4). Trypanin extends further along the proximal end of the flagellum than the PFR does and is observed in the basal body/flagellar pocket region, where the flagellum emerges from the cell (Webster and Russel, 1993).
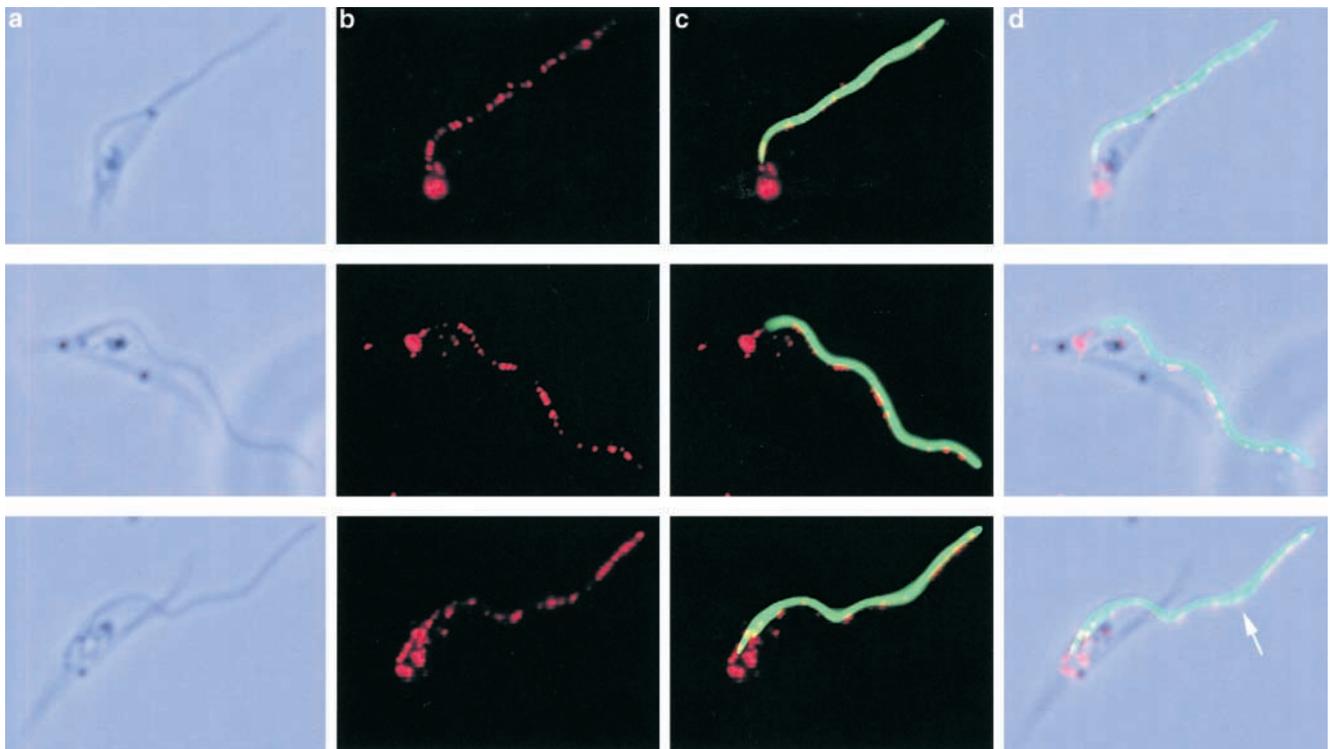
Figure 4. **Trypanin is found in punctate structures along a line between the PFR and the subpellicular cytoskeleton.** Cytoskeletons from trypanin(+) cells were costained with preadsorbed α-Trypanin-Pep4 antibodies (red) and α-PFR-A (green) antibodies (Kohl et al., 1999) as described in Materials and methods. Three representative examples are shown. For each sample, phase contrast (a), trypanin fluorescence (b), trypanin + PFR-A fluorescence (c) and merged (d) images of the same cell are shown. The white arrow points to an example where the flagellum has pulled away from the subpellicular cytoskeleton (see text).

The trypanin immunofluorescence staining pattern (Figs. 3 and 4), is consistent with previous biochemical fractionation studies (Hill et al., 2000) and is consistent with a cell motility function. This staining pattern also overlaps with the flagellar pocket localization of a trypanin–GFP fusion protein (Hill et al., 1999), but unlike the trypanin–GFP fusion protein, extends along the length of the flagellum/flagellum attachment zone. The simplest explanation for this difference is that trypanin is tightly associated with other components of the flagellum/flagellum attachment zone (FAZ) (Hill et al., 2000), and that GFP interferes with these interactions as the attachment zone extends away from the flagellar pocket region.

Trypanin is a cytoskeletal linker protein

The trypanosome flagellum consists of an axoneme and a lattice-like filament called the paraflagellar rod (Gull, 1999). One side of this flagellar apparatus is connected to the subpellicular cytoskeleton by regularly-spaced, transmembrane cross-links, forming an FAZ that runs parallel to the long axis of the cell (Sherwin and Gull, 1989; Hemphill et al., 1991). The cytoplasmic side of the FAZ consists of an electron-dense filament of unknown composition and four specialized microtubules that are connected to the FAZ filament and are biochemically distinct from other microtubules of the subpellicular cytoskeleton (Gull, 1999; Kohl et al., 1999). Disruption of any of these structures might affect cell motility, and research by others has shown

that loss of PFR-A, which leads to nearly complete ablation of the paraflagellar rod, results in cell paralysis (Bastin et al., 1998). The uncoordinated tumbling phenotype of trypanin(−) mutants differs from the paralyzed phenotype of PFR-A(−) mutants, but might similarly be due to a gross defect in flagellum and/or cellular ultrastructure. Alternatively, trypanin might play a regulatory role in coordinating flagellar beat and/or mediate dynamic interactions between the flagellum and the subpellicular cytoskeleton. To distinguish between these possibilities, we examined trypanin(−) mutants by EM.

Transmission EM of transverse and longitudinal sections prepared from whole cells revealed no obvious defects in the ultrastructure of trypanin(−) mutants (see Fig. 6, e–f; unpublished data). Specifically, the flagellar axoneme, paraflagellar rod, flagellum attachment zone and subpellicular microtubules (Gull, 1999; Kohl et al., 1999) all appear normal. Likewise, scanning EM (SEM) revealed that the left-handed helical twist (Gull, 1999) of the flagellum and subpellicular cytoskeleton are normal (Fig. 5, a and b), as are organelle inheritance and cell division (unpublished data). In spite of this apparently normal ultrastructure, examination of whole cells by SEM revealed a partially detached flagellum (Fig. 5 b, arrow) in 15 of 53 (28%) trypanin(−) cells examined. Although similar regions of flagellum detachment can be observed in trypanin(+) cells (unpublished data), the frequency is significantly less, 4 of 52 (8%) cells examined. The extent of flagellum detachment is relatively minor in
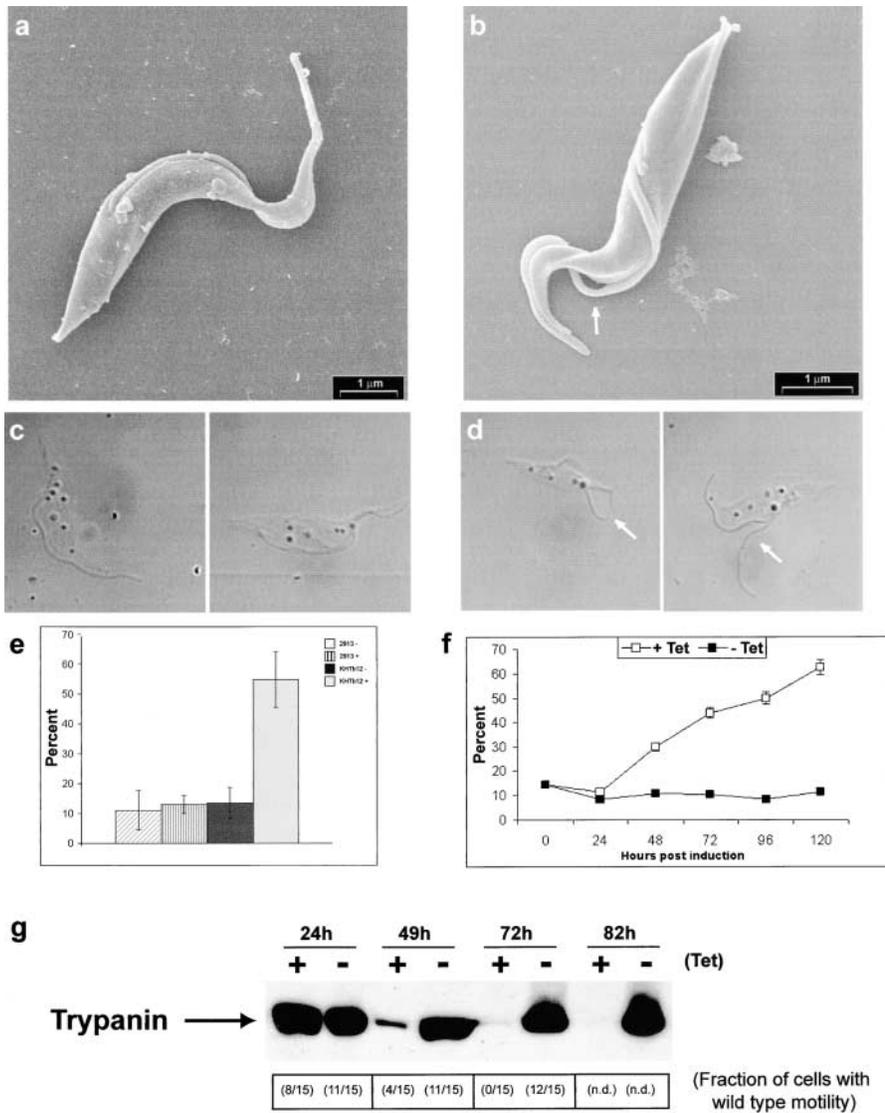
Figure 5. **Cells that lack trypanin are deficient in flagellum attachment.** Panels (a) and (b) show SEM images of trypanin(+) and trypanin(−) cells, respectively. The arrow in (b) points to a region of flagellum detachment that was observed in 15 out of 53 (28%) trypanin(−) cells examined by SEM. Similar regions of flagellum detachment were occasionally observed in trypanin(+) cells examined by SEM (unpublished data), although the frequency, 4 out of 52 (8%), was significantly less. Panels (c) and (d) show DIC images of detergent-extracted cytoskeletons prepared from trypanin(+) (c) and trypanin(−) (d) cells. Regions of flagellum detachment are indicated by white arrows. Removal of cellular membranes by detergent extraction leads to pronounced flagellum detachment (d), which can be quantitated by light microscopy (e and f). Quantitation was conducted on cytoskeletons prepared 96 h (e) or at the indicated time points (f) after supplementation with (+) or without (−) tetracycline. *T. brucei* 29–13 is the parental cell line used to generate the KHTb12 cell line (Materials and methods). In KHTb12 cultures grown without tetracycline (e, black bar), as well as 29–13 cells grown without (e, diagonal hatches) or with (e, vertical hatches) tetracycline, flagellum detachment is observed in ~10% of detergent-extracted cytoskeletons. KHTb12 cells grown in the presence of tetracycline for 96 h (e) exhibit a fivefold increase in the observable frequency of flagellum detachment (gray bar). Averages from three independent experiments are shown, with error bars representing standard error. (f and g) Time-course experiments demonstrate that flagellum detachment (f) coincides with loss of trypanin protein (g) and loss of motility (g). Western blots were performed on total protein extracts prepared at the indicated time points after addition of 1 μg tetracycline/ml to KHTb12 cultures. At the indicated time points a parallel sample from each culture was monitored for motility as described in the legend to Fig. 2, and the number of cells showing wild-type motility (≥3 squares/min) is shown.

trypanin(−) whole cells, but becomes more pronounced when cellular membranes are removed by detergent extraction (Fig. 5, c–e). In these detergent-extracted cytoskeletons, flagellum detachment is readily visible using light microscopy, making it possible to assay for this defect as a function of time after the addition of tetracycline to block trypanin expression (Fig. 5 f). These time-course experiments demonstrate that flagellum detachment parallels the loss of trypanin protein and loss of cell motility (Fig. 5 g).

Close examination of detergent-extracted cytoskeletons prepared from trypanin(−) mutants revealed that the flagellum attachment zone is disrupted and lacks the highly structured organization seen in cytoskeletons from trypanin(+) cells (Fig. 6, a–d). Interestingly, the cytoplasmic FAZ filament, specialized microtubules and transmembrane cross-bridges that comprise the flagellum attachment zone (Sherwin and Gull, 1989; Hemphill et al., 1991; Gull, 1999; Kohl et al., 1999) appear relatively unperturbed before de-

tergent extraction (Fig. 6, e and f). Therefore, these data suggest that trypanin facilitates direct coupling of the flagellar cytoskeleton to the subpellicular cytoskeleton, and that previously described desmosome-like junctions between the flagellar membrane and plasma membrane (Sherwin and Gull, 1989; Balber, 1990; Hemphill et al., 1991; Gull, 1999) contribute significantly to the stability of this attachment complex. In the absence of trypanin, the cytoskeleton connection is destabilized, though not completely destroyed, and subsequent removal of the flagellar membrane and plasma membrane leads to complete disruption of the attachment complex.

Discussion

Although a great deal is known about molecular motors that drive movement of the eukaryotic flagellum, little is known about mechanisms that regulate/coordinate flagellar beat

Figure 6. **Loss of trypanin causes disruption of flagellum attachment complexes.** Electron micrographs of the FAZ (see text) are shown for intact, detergent-extracted cytoskeletons (a–d) or transverse sections of whole cells (e and f) prepared from trypanin(+) (a, c, and e) or trypanin(−) (b, d, and f) cells. Images are oriented with the flagellum on top. In cytoskeletons from trypanin(+) cells, the flagellum is tightly coupled to the subpellicular cytoskeleton by regularly spaced, electron dense cross-links (a and c, black arrows) (Gull, 1999). These crossbridges connect the flagellar apparatus directly to the FAZ filament (see text), which can be seen in transverse sections of intact cells (e and f, white arrowheads). The FAZ filament is in turn connected to a group of specialized, reticulum-associated microtubules (e and f, brackets) that lie immediately adjacent to the FAZ filament on the cytoplasmic side of the flagellum attachment zone (Gull, 1999). Cytoskeletons from trypanin(−) cells exhibit abnormalities in this ordered array of connections between the flagellum and the subpellicular cytoskeleton (b and d, black arrows). Disruption of flagellum attachment complexes in the absence of trypanin is not evident in sections of intact cells (e, f, and unpublished data), but becomes very pronounced upon removal of cellular membranes (a–d). Cytoskeletons were prepared from KHTb12 cells grown with (Trypanin [−]) or without (Trypanin [+]) 1 μg tetracycline/ml. Ax, axoneme; FAZ, flagellum attachment zone; PFR, paraflagellar rod; PMT, subpellicular microtubules. Bars: (a, c, and d) 250 nm; (b) 500 nm; (e and f) 125 nm.
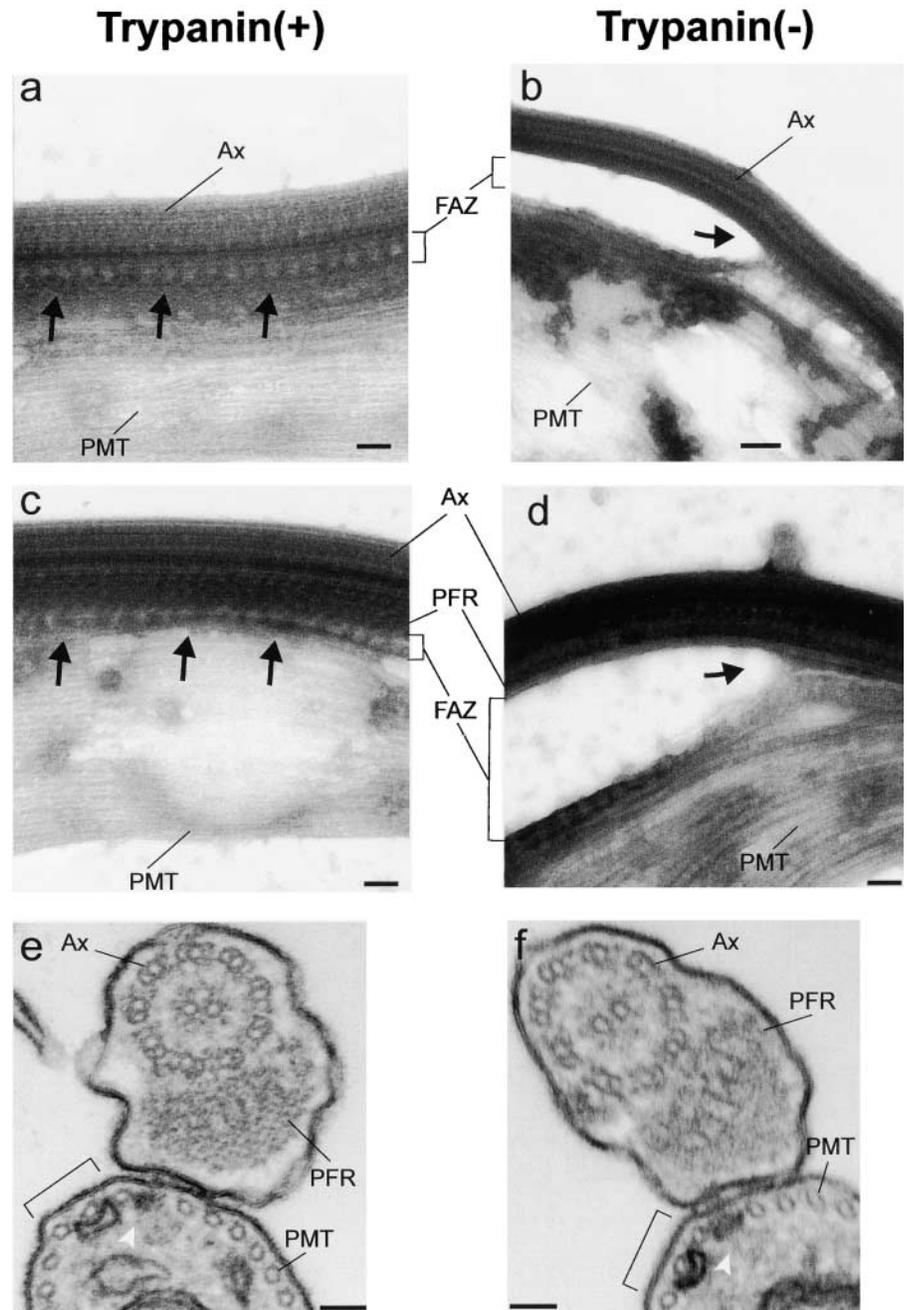
(Cosson, 1996). Our results demonstrate that trypanin is required for directional cell motility in *T. brucei*. EM studies revealed that the unusual cell motility defect of trypanin(−) mutants results from uncoupling of the flagellar apparatus from the subpellicular cytoskeleton. The punctate distribution of trypanin along the cell body side of the paraflagellar rod (Fig. 5) supports the interpretation that trypanin is part of the attachment complex that connects the flagellum to the subpellicular cytoskeleton. Our data further indicate that this flagellum attachment complex has two components, a cytoskeletal component, of which trypanin is a part, and a membrane component that operates even in the absence of trypanin and stabilizes the direct cytoskeleton connection (Balber, 1990; Hemphill et al., 1991). The only other protein shown to be required for flagellum attachment in *T.*

brucei is the membrane-associated glycoprotein, FLA1, a homologue of GP72 from *T. cruzi* (Cooper et al., 1993; Nozaki et al., 1996; LaCount et al., 2000). Loss of FLA1/GP72 leads to flagellum detachment even in intact cells (Cooper et al., 1993; LaCount et al., 2000), suggesting that FLA1 is required for establishment of both the membrane and cytoskeletal components of the flagellum attachment zone.

Immunofluorescence experiments show that trypanin remains associated with the PFR in samples where the flagellum has detached from the cell. This is different than what is observed for a large (>200 kD) component of the FAZ described previously (Kohl et al., 1999). Because we cannot rule out the possibility that some cytoplasmic components of the FAZ have been pulled away with the flagellum in these sam-

ples, these experiments do not address whether trypanin is associated directly with the flagellum, or with structures that are outside the flagellum but within the flagellum attachment complex (Gull, 1999; Kohl et al., 1999). We are currently working to distinguish between these possibilities using immuno-gold EM. These studies will also be used to determine whether the punctate trypanin staining corresponds to a recognizable flagellum or FAZ structure and to investigate the intense trypanin staining at the proximal end of the flagellum.

It should be emphasized that the motility defect of trypanin(−) mutants is fundamentally different than that reported for PFR-A and FLA1 mutants (Bastin et al., 1998; LaCount et al., 2000; unpublished data). Therefore, our data illuminate a previously unrecognized mechanistic advantage for flagellum attachment in *T. brucei*, i.e., that minor pertubations of this attachment can profoundly affect the capacity for directional cell motility without causing cell paralysis. Because African trypanosomes are extracellular at all stages of infection, cell motility is likely to play an important role in their ability to infiltrate the mammalian host's central nervous system and for migration from the midgut to the salivary gland within the tsetse fly vector (Van Den Abbeele et al., 1999). Trypanin(−) mutants provide an opportunity to test this hypothesis directly.

The membrane/cytoskeleton interface of the *T. brucei* FAZ resembles similar structures in higher eukaryotes (Vickerman, 1969; Vickerman and Peterson, 1979; Balber, 1990) and trypanin-related proteins are present in a wide variety of organisms, including humans (Hill et al., 2000). Our results provide the first in vivo demonstration of function for this new family of cytoskeleton-associated proteins. Additional proteins in this family might contribute to flagellum/cilium function in other cells, e.g., sperm or ciliated epithelial cells. Alternatively, they might participate in other activities of the cytoskeleton. The finding that trypanin-related proteins are expressed in muscle and cells/tissues that lack flagella (Whitmore et al., 1998) (unpublished data), supports this possibility and previous studies on the human trypanin family member (Whitmore et al., 1998; Hill et al., 2000) are consistent with a cytoskeletal milieu for this protein. Therefore, in addition to providing information relevant to pathogenesis of parasitic disease and trypanosome development in the tsetse fly, our results have direct relevance to understanding cytoskeleton function in other eukaryotic organisms.

Materials and methods

DNA constructs
Standard cloning methods were used to place oppositely oriented copies of the last 263 bp of the trypanin open reading frame (Vaidya et al., 1997) at either end of the GFPmut3 gene (Cormack et al., 1996) (Fig. 1 a). This construct was inserted into the trypanosome expression vector pHD496 (Biebinger et al., 1996) for constitutive expression, and into pLEW100 (Wirtz et al., 1999) for tetracycline-inducible expression. As a control, the GFP open reading frame was inserted into pHD496, which employs a constitutive promoter to drive expression of a dicistronic transcript containing the reporter gene (either trypanin-dsRNA or GFP) and a downstream hygromycin-resistance gene (Biebinger et al., 1996). The plasmid pLEW100 employs a tetracycline-inducible promoter to drive expression of a monocistronic transcript containing trypanin-dsRNA, whereas a phleomycin-resistance gene is transcribed constitutively in the opposite direction from a separate promoter (Wirtz et al., 1999).

Cell culture and transfection
Procyclic trypanosomes (YTAT for pHD496 vectors; and 29–13 [Wirtz et al., 1999] for pLEW100:trypanin-dsRNA) were transfected (Hill et al., 1999) with NotI-linearized plasmids, and stably transfected cells were obtained by selection with 60 µg hygromycin/ml (pHD496) or 2.5 µg phleomycin/ml (pLEW100). Constitutive trypanin-dsRNA expressers (designated KHTb5) or tetracycline-inducible trypanin-dsRNA expressers (designated KHTb12) were maintained under constant drug selection. For all tetracycline induction experiments, a culture of KHTb12 was split into two flasks, which were maintained in the absence or presence of 1 µg tetracycline/ml to induce trypanin-dsRNA expression. For all samples, Western blot analysis confirmed trypanin was present in trypanin(+)/(− tetracycline) cells and absent from trypanin(−)/(+ tetracycline) cells as expected (unpublished data). Tetracycline does not affect the motility of wild-type trypanosomes (unpublished data), nor has any such effect of tetracycline been reported in other studies using the tetracycline-inducible system employed here (Wirtz and Clayton, 1995; Wirtz et al., 1998, 1999). Total protein extracts were analyzed by Western blotting (Hill et al., 1999) using affinity-purified α-Pep4 antibodies (Hill et al., 2000). 3×10^6 cell equivalents were loaded in each lane. Detergent-extracted cytoskeletons were prepared as described previously (Robinson et al., 1991; Hill et al., 2000).

Video microscopy and quantitative motility assays
Trypanosomes were monitored by video microscopy using a Zeiss Axioskop microscope equipped with a 100× oil immersion objective (Fig. 1 c; Videos 3–5, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1) or a 40× objective (Fig. 2, a and b; Videos 1–2, available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1). Images were captured on a Panasonic GP-KR222 digital camera running live S-video at 60 frames/s. All videos are shown in real time. For motility assays, cells were placed in a hemacytometer with counting grid squares that measure 50 µm on each side. Individual cells were monitored for 1.5 min or until they moved off the counting grid. The depth of the counting chamber is 100 µm (∼10 cell lengths) and trypanin(+)/wild-type cells move readily in and out of the focal plane. Because this assay monitors movement in two dimensions, the motility traces and histograms in Fig. 2 underestimate the motility of trypanin(+) cells. Trypanin(−) parasites remain primarily in a single focal plane throughout the experiment.

Indirect immunofluorescence assays
Previous efforts at immunofluorescence localization of trypanin were hampered by nonspecific cross-reactivity of anti-trypanin antisera (Hill et al., 2000). To overcome this problem, trypanin(−) mutants were used to deplete cross-reactive antibodies from two independent anti-trypanin antisera. The polyclonal α-trypanin antisera, α-TT4 and α-Pep4, have been described previously (Hill et al., 2000). Cytoskeletons were isolated (Hill et al., 2000) from trypanin(−) mutants and used to deplete cross-reactive antibodies from α-TT4 and α-Pep4 antisera. For these experiments, trypanin(−) cytoskeletons were incubated with α-trypanin antisera for 1 h, and then removed by centrifugation (Hill et al., 2000). The resulting supernatant was subjected to three more rounds of depletion. After the fourth round of depletion, the supernatant was centrifuged twice at 16,000 g for 15 min to remove debris. This final supernatant contains preadsorbed α-trypanin antibodies that were used for immunofluorescence.

For immunofluorescence, a single KHTb-12 culture was split into two flasks, which were maintained in the absence or presence of 1 µg tetracycline/ml to induce trypanin-dsRNA expression. Cytoskeletons prepared from these trypanin(+) and trypanin(−) cells were settled onto poly-L-lysine– coated coverslips. Unattached cytoskeletons were aspirated and remaining samples were fixed with 2% paraformaldehyde for 10–20 min, and then washed three times with PBS containing 100 mM glycine. Primary antibodies were added at a 1:5 dilution (preadsorbed α-TT4 or α-Pep4) or 1:500 (α-PFR-A, Kohl et al., 1999) for 1–4 h. After three washes with PBS containing 0.1% Triton X-100 (PBS-T), secondary antibodies conjugated to FITC, Alexa-Flor488, or Cy3 (Molecular Probes) were added at a dilution of 1:300 in PBS-T and incubated for 1 h. For coimmunofluorescence experiments (Fig. 4), polyclonal α-trypanin antibodies and monoclonal α-PFR-A antibodies were detected simultaneously using appropriate α-rabbit and α-mouse secondary antibodies, coupled to Cy3 and FITC, respectively. Samples were washed three times with PBS-T, mounted in vectashield™ (Vector Labs), and visualized on a Zeiss LSM 510 inverted laser-scanning confocal microscope (Fig. 3) or Zeiss Axioskop 2 (Fig. 4). Red, green, phase contrast, and DIC Images of the same field were captured independently, and then merged using Adobe Photoshop® (v. 5.5). The α-Pep4 staining is completely eliminated by addition of excess Pep4 peptide antigen,

against which this α-Pep4 antiserum was raised, but not by a nonspecific peptide. Secondary antibodies alone give no signal.

EM

EM procedures were based on the work of Sherwin and Gull (1989). For transmission EM of whole cells (Fig. 5, e and f), cells were washed twice with PBS, fixed in half-strength Karnovsky's solution (1% paraformaldehyde, 1% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.2) containing 1% tannic acid, rinsed, and then postfixed with 1% osmium tetroxide plus 1.5% potassium ferrocyanide. Dehydration through an acetone gradient was performed, followed by infiltration and embedment in Eponate 12 (Ted Pella, Co). Sections were cut on a Reichert Ultracut E, poststained with uranyl acetate and lead citrate, and imaged using a Hitachi H-7000 transmission electron microscope. For SEM (Fig. 4, a and b), cells were fixed as above, allowed to adhere to poly-L-lysine–coated coverslips, and then rinsed, postfixed with 1% osmium tetroxide in 0.1 M Na cacodylate buffer, and dehydrated with ethanol. Drying was achieved using hexamethyldisilazane. Samples were sputter coated with a mixture of gold/palladium (60/40), and imaged with a Hitachi S-4000 FESEM. Images were captured using a Kevex Sigma and 4855 digital beam control unit (Thermo Noran) digital image acquisition system.

For transmission EM of detergent-extracted cytoskeletons (Fig. 5, a–d), cytoskeletons were NP-40 extracted but not centrifuged, and a single drop was applied to negatively charged carbon-coated colloidian grids for 30 s. Liquid was removed with blotting paper and cells were immediately fixed with PEME (Robinson et al., 1991; Hill et al., 2000) containing 2.5% glutaraldehyde for 30 s. Fixative was aspirated and the cytoskeletons were stained with 1% ammonium molybdate for 15 s before being aspirated and air dried. Grids were examined with a Mitsubishi T600 transmission electron microscope at the University of Iowa Central Microscopy Research Facility (Iowa City, IA).

Online supplemental material

Video clips 1–5 (available at http://www.jcb.org/cgi/content/full/jcb.200201036/DC1) are supplied in QuickTime format in real time. In contrast to the highly directed motility of trypanin(+) trypanosomes (Videos 1 and 3), trypanin(−) mutants (Videos 2, 4, and 5) spin and tumble uncontrollably, occasionally moving backward, i.e., away from the flagellum tip.

References

Andra, K., B. Nikolic, M. Stocher, D. Drenckhahn, and G. Wiche. 1998. Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev.* 12: 3442–3451.

Balber, A.E. 1990. The pellicle and the membrane of the flagellum, flagellar adhesion zone, and flagellar pocket: functionally discrete surface domains of the bloodstream form of African trypanosomes. *Crit. Rev. Immunol.* 10:177–201.

Bastin, P., T. Sherwin, and K. Gull. 1998. Paraflagellar rod is vital for trypanosome motility. *Nature.* 391:548.

Bastin, P., T.J. Pullen, T. Sherwin, and K. Gull. 1999. Protein transport and flagellum assembly dynamics revealed by analysis of the paralyzed trypanosome mutant snl-1. *J. Cell Sci.* 112:3769–3777.

Biebinger, S., S. Rettenmaier, J. Flaspohler, C. Hartmann, J. Pena-Diaz, L.E. Wirtz, H.R. Hotz, J.D. Barry, and C. Clayton. 1996. The PARP promoter of *Trypanosoma brucei* is developmentally regulated in a chromosomal context. *Nucleic Acids Res.* 24:1202–1211.

Bosher, J.M., and M. Labouesse. 2000. RNA interference: genetic wand and genetic watchdog. *Nat. Cell Biol.* 2:E31–E36.

Cooper, R., A.R. de Jesus, and G.A. Cross. 1993. Deletion of an immunodominant *Trypanosoma cruzi* surface glycoprotein disrupts flagellum-cell adhesion. *J. Cell Biol.* 122:149–156.

Cormack, B.P., R.H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene.* 173:33–38.

Cosson, J. 1996. A moving image of flagella: news and views on the mechanisms involved in axonemal beating. *Cell Biol. Int.* 20:83–94.

Gull, K. 1999. The cytoskeleton of trypanosomatid parasites. *Annu. Rev. Microbiol.* 53:629–655.

Heald, R. 2000. A dynamic duo of microtubule modulators. *Nat. Cell Biol.* 2:E11–E12.

Hemphill, A., D. Lawson, and T. Seebeck. 1991. The cytoskeletal architecture of *Trypanosoma brucei. J. Parasitol.* 77:603–612.

Hill, K.L., N.R. Hutchings, D.G. Russell, and J.E. Donelson. 1999. A novel protein targeting domain directs proteins to the anterior cytoplasmic face of the flagellar pocket in African trypanosomes. *J. Cell Sci.* 112:3091–3101.

Hill, K.L., N.R. Hutchings, P.M. Grandgenett, and J.E. Donelson. 2000. T lymphocyte triggering factor of African trypanosomes is associated with the flagellar fraction of the cytoskeleton and represents a new family of proteins that are present in several divergent eukaryotes. *J. Biol. Chem.* 275:39369–39378.

Klymkowsky, M.W. 1999. Weaving a tangled web: the interconnected cytoskeleton. *Nat. Cell Biol.* 1:E121–E123.

Kohl, L., T. Sherwin, and K. Gull. 1999. Assembly of the paraflagellar rod and the flagellum attachment zone complex during the *Trypanosoma brucei* cell cycle. *J. Eukaryot. Microbiol.* 46:105–109.

LaCount, D.J., S. Bruse, K.L. Hill, and J.E. Donelson. 2000. Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. *Mol. Biochem. Parasitol.* 111:67–76.

Marte, B. 2000. From rigid structures to dynamic networks. *Nat. Cell Biol.* 2:E1.

Ngo, H., C. Tschudi, K. Gull, and E. Ullu. 1998. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei. Proc. Natl. Acad. Sci. USA.* 95: 14687–14692.

Nozaki, T., P.A. Haynes, and G.A. Cross. 1996. Characterization of the *Trypanosoma brucei* homologue of a *Trypanosoma cruzi* flagellum-adhesion glycoprotein. *Mol. Biochem. Parasitol.* 82:245–255.

Pepin, J., and J.E. Donelson. 1999. African Trypanosomiasis (Sleeping Sickness). *In* Tropical Infectious Diseases: Principles, Pathogens and Practice. Vol. 1. R. Guerrant, D.H. Walker, and P.F. Weller, editors. Churchill Livingstone, Philadelphia, PA. 774–784.

Rindisbacher, L., A. Hemphill, and T. Seebeck. 1993. A repetitive protein from *Trypanosoma brucei* which caps the microtubules at the posterior end of the cytoskeleton. *Mol. Biochem. Parasitol.* 58:83–96.

Robinson, D.R., and K. Gull. 1991. Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. *Nature.* 352:731–733.

Robinson, D., P. Beattie, T. Sherwin, and K. Gull. 1991. Microtubules, tubulin, and microtubule-associated proteins of trypanosomes. *Methods Enzymol.* 196:285–299.

Robinson, D.R., T. Sherwin, A. Ploubidou, E.H. Byard, and K. Gull. 1995. Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle. *J. Cell Biol.* 128: 1163–1172.

Schneider, A., A. Hemphill, T. Wyler, and T. Seebeck. 1988. Large microtubule-associated protein of *T. brucei* has tandemly repeated, near-identical sequences. *Science.* 241:459–462.

Sherwin, T., and K. Gull. 1989. The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. *Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci.* 323:573–588.

Spudich, J.A. 2001. The myosin swinging cross-bridge model. *Nat. Rev. Mol. Cell Biol.* 2:387–392.

Vaidya, T., M. Bakhiet, K.L. Hill, T. Olsson, K. Kristensson, and J.E. Donelson. 1997. The gene for a T lymphocyte triggering factor from African trypanosomes. *J. Exp. Med.* 186:433–438.

Vale, R.D., and R.A. Milligan. 2000. The way things move: looking under the hood of molecular motor proteins. *Science.* 288:88–95.

Van Den Abbeele, J., Y. Claes, D. van Bockstaele, D. Le Ray, and M. Coosemans. 1999. *Trypanosoma brucei* spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. *Parasitology.*

118:469–478.

Vaughan, S., T. Attwood, M. Navarro, V. Scott, P. McKean, and K. Gull. 2000. New tubulins in protozoal parasites. *Curr. Biol.* 10:R258–R259.

Vickerman, K. 1969. On the surface coat and flagellar adhesion in trypanosomes. *J. Cell Sci.* 5:163–193.

Vickerman, K., and T.M. Peterson. 1979. Comparative Cell Biology of the Kinetoplastid Flagellates. *In* Biology of the Kinetoplastida. Vol. 1. W.H.R. Lumsden, and D. Evans, editors. Academic Press, London. 35–130.

Walker, P.J. 1961. Organization of function in trypanosome flagella. *Nature.* 189: 1017–1018.

Wang, Z., J.C. Morris, M.E. Drew, and P.T. Englund. 2000. Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J. Biol. Chem.* 275:40174–40179.

Webster, P., and D.G. Russel. 1993. The flagellar pocket of trypanosomatids. *Parasit. Today.* 9:201–206.

Whitmore, S.A., C. Settasatian, J. Crawford, K.M. Lower, B. McCallum, R. Seshadri, C.J. Cornelisse, E.W. Moerland, A.M. Cleton-Jansen, A.J. Tipping, et al. 1998. Characterization and screening for mutations of the growth arrest-specific 11 (GAS11) and C16orf3 genes at 16q24.3 in breast cancer. *Genomics.* 52:325–331.

Wirtz, E., and C. Clayton. 1995. Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. *Science.* 268:1179–1183.

Wirtz, E., M. Hoek, and G.A. Cross. 1998. Regulated processive transcription of chromatin by T7 RNA polymerase in *Trypanosoma brucei. Nucleic Acids Res.* 26:4626–4634.

Wirtz, E., S. Leal, C. Ochatt, and G.A. Cross. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei. Mol. Biochem. Parasitol.* 99:89–101.