Extensive flagellar remodeling during the complex life cycle of Paratrypanosoma, an early-branching trypanosomatid

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Paratrypanosoma confusum is a monoxenous kinetoplastid flagellate that constitutes the most basal branch of the highly diverse parasitic trypanosomatids, which include human pathogens Trypanosoma and Leishmania. This makes Paratrypanosoma uniquely informative for the evolution of obligatory parasitism from free-living lifestyle and the evolution of human parasitism in some trypanosomatid lineages. It has typical promastigote morphology but also forms surface-attached haptomondads and amastigotes. Haptomondads form by attachment to a surface via a large bulge at the base of the flagellum, which is then remodeled into a thin attachment pad associated with flagellum shortening. Promastigotes and haptomondads multiply by binary division, and the progeny of a haptomondad can either remain attached or grow a flagellum and resume swimming. Whole-genome sequencing and transcriptome profiling, in combination with analysis of the cell ultrastructure, reveal how the cell surface and metabolism are adapted to parasitism and how characteristic cytoskeletal features are conserved. Our data demonstrate that surface attachment by the flagellum and the flagellar pocket, a Leishmania-like flagellum attachment zone, and a Trypanosoma cruzi-like cytostome are ancestral features, while evolution of extant trypanosomatids, including the human parasites, is associated with genome streamlining and diversification of membrane proteins.

Significance

Kinetoplastid flagellates are diverse and widespread protists, best known for serious human diseases caused by the trypanosomatid genera Trypanosoma and Leishmania. Most kinetoplastids are successful parasites, infecting a wide range of hosts and with unique and numerous adaptations to the host environment. It is proposed that disease-causing trypanosomatids with two-host (digenetic) life cycles (an insect vector and the mammalian or plant host) evolved from flagellates parasitizing solely insects (1). The earliest known branch of the trypanosomatid clade, predating its diversification, is Paratrypanosoma confusum, which infects mosquitoes (2). The free-living clade closest to trypanosomatids is the genus Bodo (3, 4).

Emergence of monoflagellated parasitic trypanosomatids from the biflagellated bacterivorous Bodo involved halving of the number of genes (3, 4). To identify further features associated with the evolution of parasitism, we analyzed the morphology of Paratrypanosoma and its adaptation to different in vitro environments. Combined with analysis of the genome and transcriptome, this allowed identification of genes potentially associated with these features. The single flagellum of trypanosomatids is a highly flexible structure used for locomotion, attachment, and sensing. Its structure is subject to substantial restructuring during the life cycle to adapt to different functions (5, 6) and is intimately associated with the vital flagellar pocket structure. Flagellar motility is also required for transmission, immune evasion, and cell division (7) of Trypanosoma brucei. Recently further flagellar functions, including production of extracellular vesicles that may mediate host interaction (8) and parasit–parasite interaction by membrane exchange or fusion (9), have been described. In the juxtaform morphological superclade (trypo- and epimastigotes), the flagellum is laterally attached to the cell by an extended flagellum attachment zone (FAZ). Alternatively, in the libiform morphological superclade, the flagellum may protrude from the flagellar pocket without an extended attachment (pro-, opistho-, and choanomastigotes) (10).

Here we report that Paratrypanosoma and stercorarian trypanosomases, including Trypanosoma cruzi and Trypanosoma grayi, retain more ancestral genes than other trypanosomatid clades. Despite having libiform morphology, Paratrypanosoma has flagellum–cell attachment via a small FAZ in the flagellar pocket similar to Leishmania (11). It also has a complex cytostome architecture similar to T. cruzi (12) but lost in T. brucei and Leishmania for transmission, immune evasion, and cell division (7) of Trypanosoma brucei. Recent further flagellar functions, including production of extracellular vesicles that may mediate host interaction (8) and parasit–parasite interaction by membrane exchange or fusion (9), have been described. In the juxtaform morphological superclade (trypo- and epimastigotes), the flagellum is laterally attached to the cell by an extended flagellum attachment zone (FAZ). Alternatively, in the libiform morphological superclade, the flagellum may protrude from the flagellar pocket without an extended attachment (pro-, opistho-, and choanomastigotes) (10).

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Significance

Kinetoplastids are a group of protists with unique morphology and molecular features. Many have developed a parasitic lifestyle and are economically and medically important causative agents of serious crop, animal, and human diseases. Evolutionarily, Paratrypanosoma confusum sits between parasitic trypanosomatids and free-living bodonids and therefore is uniquely informative for study of the emergence of parasitism. It is morphologically very flexible, as it forms three distinct life stages that can be studied separately. Particularly interesting is the haptomond stage in which it rebuilds its flagellum into an extensive adhesive plaque. As an adaptation to parasitism, Paratrypanosoma lost a plethora of enzymes involved in breakdown of macromolecules and the capacity of receptor-mediated endocytosis but has gained surface proteins and membrane transporters to obtain nutrients from the host.

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Promastigote attachment and replication as a haptomonad. Shown are frames from time-lapse videos of promastigote and haptomonad interconversion. Attachment of a promastigote to form a haptomonad and two successive divisions, first forming two haptomonads and then two promastigotes and two haptomonads. Time (hours:min) for each frame is shown in the top right, and a cartoon of cell arrangement is shown in the bottom left. (Magnification: 100×)

**Fig. 2.**

**Fig. 1.** Morphologies of *Paratrypanosoma.* (A) Different morphologies by light microscopy of Giemsa-stained cells (left) and SEM (right). Promastigotes have a long flagellum with a prominent bulge at its base (inset). The kinetoplast (K) and nucleus (N) are indicated. Neither haptomonads nor amastigotes have a long flagellum. (B–E) SEM of haptomonads. (B) Haptomonads attach perpendicular to the surface in dense clusters. (C) Haptomonad cluster detached from the surface by propylene oxide showing the underside of the attachment pad. (D) Haptomonads retain a bulge at the tip of their short flagellum. (E) Occasional long flagella are visible extending from the haptomonad attachment pad. (Scale bars: 5 μm (A, B, and C, except in A, Middle image, 10 μm, and inset, 1 μm), 1 μm (D), and 2 μm (E).)

**Results**

The discovery of *Paratrypanosoma* as an outgroup to all other trypanosomatids calls for a study focused on the distribution of morphological and molecular traits in the trypanosomatid tree. Light microscopy of live and Giemsa-stained cells and scanning electron microscopy (SEM) showed that in liquid medium axenic *Paratrypanosoma* attains both a motile promastigote with a long free flagellum (Fig. 1A) and a sedentary, surface-attached stage similar to haptomonads of *Leishmania* (16). The haptomonad stage formed extensive thin attachment plaques on plastic substrates (Fig. 1B–E). We have also observed these morphologies in the gut of the *Culex quinquefasciatus* host. On semisolid agar, *Paratrypanosoma* attained a third morphology: an amastigote without a noticeable free flagellum (Fig. 1A). These morphologies are typical of monoxenous trypanosomatids: haptomonads during surface attachment in the gut of the vertebrate host, and amastigotes when excreted. All *Paratrypanosoma* morphologies lack an extended FAZ, typical of the liberiform morphological superclass (11).

We have been unable to ascertain the relation of axenic culture cell morphologies to the *Paratrypanosoma* life cycle, which is not well understood. In *Culex quinquefasciatus* mosquitoes infected either by feeding on sugar or blood meal, *Paratrypanosoma* was detected in the gut and/or crop on the first (100% specimens, n = 8), second (100%, n = 9), and third day postinfection (90%, n = 10) but not after defecation. Gaining and retention of the infection from third or fourth larval instars through puptation to adult mosquitoes were tested by addition of promastigotes into water containing the larvae, where the promastigotes survive for at least 2 d. The adult mosquitoes of both sexes were never infected (n = 80). This may suggest defective infectivity of the culture-adapted parasites. *Paratrypanosoma* fail to survive at 37 °C for more than a few hours, suggesting a monoxenous lifestyle. We also attempted to infect four BALB/c mice by a combination of s.c. and i.p. injection of 10⁶ promastigotes, however 1–4 wk postinfection parasites were detected neither by cultivation nor PCR. Nonetheless, life cycles with the promastigote, surface-attached haptomonad, and amastigote stages are common among insect-parasitizing trypanosomatids (11), and promastigotes and haptomonads occur in *Leishmania* vectors (16), making analysis of *Paratrypanosoma* morphology and ultrastructure in vitro valuable.

Surface attachment is widespread among trypanosomatids, yet the development of the plaque used for attachment (Fig. 1B–E) has not been studied in detail. Therefore, we analyzed transition of *Paratrypanosoma* between the promastigote and haptomonad morphologies by time-lapse light microscopy in culture (Fig. 2) with reference to the morphology of haptomonads by SEM (Fig. 1B–E). Surface attachment of promastigotes occurred by the base of the external flagellum: the “bulge” (Fig. 1A and D). Following attachment, the flagellum shortened until it barely extended from the flagellar pocket, taking around 1 h (Fig. 2 and Fig. S14). This was associated with the cell repositioning to an upright orientation and the bulge spreading into a thin extended pad attached to the surface. A colony of haptomonads could

*Leishmania mexicana.* Furthermore, it is able to restructure the flagellum to attach to surfaces, generating an extensive flagellum-derived adhesive pad, and does so readily in culture. This allowed an analysis of this process, which occurs in most trypanosomatid lineages (13–15). Judging by the distribution of morphological traits in trypanosomatids, we propose that *Paratrypanosoma* morphology is close to that of the last common ancestor of trypanosomatids.
cover a large area by these extended pads. Time-lapse light microscopy indicated haptomonads can divide while attached, generating daughter cells that may remain attached or grow an extended flagellum and detach (Fig. 2 and Fig. S1B and Movies S1 and S2). The cell cycle took ∼6 h at 20 °C, while elongation of the flagellum and detachment took 1–2 h (Fig. 2).

Transformation from promastigotes into haptomonads was promoted by nutrient availability and alkaline pH. The digestive tract of a mosquito is nutrient-rich and typically alkaline (17) and is thus suitable for haptomonads. The attachment pad was highly resistant to our attempts to degrade it enzymatically (Dataset S1), indicating it could confer strong attachment even under harsh digestive conditions in the mosquito gut. Formation of haptomonads, like life cycle transitions in other species, includes significant remodeling of the flagellum. We therefore sequenced the Paratrypanosoma genome for analysis of evolution of the flagellum and flagellum-related cytoskeleton and as a reference for transcriptome analysis of different morphological stages for insight into metabolism.

Using paired-end and mate-pair Illumina reads, we assembled a 31.4 Mbp draft genome with 31x average coverage and 2,114 scaffolds (maximum length, 2.99 Mbp; N50 of 438 Kbp). Using Augustus trained on a set of unambiguous gene models for conserved genes and RNA-seq (RNA-seq) read mapping, we annotated 8,666 protein-coding genes, 66 rRNAs, and 122 tRNA genes. This gene complement is comparable to other trypanosomatids (18). Most core eukaryotic genes (72.3%) were present (a similar proportion to high-quality T. brucei (74.9%), Leishmania major (73.6%), and Leptomonas pyrrocoris (72.6%) genomes), indicating a complete genome assembly (19). RNA-seq comparison of gene expression between haptomonads and promastigotes revealed 327 and 264 genes significantly up-regulated, respectively (false discovery rate corrected P value < 0.05, fold-change > 2) (Dataset S2). The most up-regulated genes in haptomonads were associated with the ribosome or translation (Dataset S3A), while those up-regulated in promastigotes were mostly part of intermediary metabolism or redox processes (Dataset S3B).

We used comparative genomics to investigate whether Paratrypanosoma has retained more ancestral features than other trypanosomatids. Using OrthoFinder (20), we defined orthologous groups (OGs) of proteins for a large set of trypanosomatids, three bodonids (free-living Bodo saltans and Neobodo designis, and parasitic Trypanoplasma borelli), endosymbiotic Perkinsiella sp., and Naegleria gruberi, a heterolobosean (Fig. 3 and Dataset S4). Next, we counted the number of ancestral OGs (OGs shared with any bodonid or Naegleria) (Fig. 3 and Dataset S4). For this group was significantly different to universally conserved genes (Dataset S7). Ancestral genes inherited only by sterecorarian trypanosomatids and Paratrypanosoma tended to be up-regulated in haptomonads: 84 genes in this group had significant changes in their expression, of which 55 were up-regulated in haptomonads: 84 genes in this group had significant changes in their expression, of which 55 were up-regulated in haptomonads (P value = 1.22 × 10−5; Dataset S7). Stercorarian trypanosomes were lost in the other trypanosomatids and (ii) proteins of the dispersed gene family 1 (DGF1) (21) are unique to Paratrypanosoma and sterecorarian trypanosomes. This is remarkable, as DGFs are the fifth largest protein family in T. cruzi. They are long membrane proteins of unknown function stored in intracellular vesicles, with their extracellular domains secreted during transformation to the amastigote (21, 22). The DGF1 proteins are up-regulated in the T. cruzi amastigote relative to the trypomastigote and epimastigote (21), while in Paratrypanosoma they tended to be up-regulated in the haptomonad (Dataset S2).

Finally, we looked at the relationship of phyletic patterns and functional categories for OGs. Using a one-way ANOVA with Tukey's honest significance test, we saw that differential gene expression in the haptomonad and promastigote stages depended on phyletic patterns of corresponding OGs (P value = 1.22 × 10−5; Dataset S7). Ancestral genes inherited only by sterecorarian trypanosomatids and Paratrypanosoma tended to be up-regulated in haptomonads: 84 genes in this group had significant changes in their expression, of which 55 were up-regulated in haptomonads and 29 in promastigotes (Dataset S2). Stage-specific expression of this group was significantly different to universally conserved genes, genes shared by Paratrypanosoma and both Trypanosoma clades only (but not bodonids), and Paratrypanosoma-specific genes (P values adjusted for multiple testing = 0.012, 0.006, and 8 × 10−5, respectively). Genes within the two latter groups tended to be up-regulated in promastigotes. Overall, ancestral genes were typically constitutively expressed in Paratrypanosoma or up-regulated in haptomonads, while trypanosomatid-specific genes tended to be up-regulated in the promastigote (Dataset S7). This...
suggests that the haptomonad stage might be an ancestral character-
istic of trypanosomatids.

The dramatic morphological change between promastigotes and haptomonads is formation of the adhesive pad from the bulge at the base of the promastigote flagellum. As this may involve restructuring of the paraflagellar rod (PFR), FAZ, and flagellar axoneme, we analyzed conservation across kinetoplasmas of proteins known to form these structures (Fig. S2). The FAZ proteins are of particular interest as they have adaptable functions in cell morphogenesis. They were first identified in the extended FAZ of Trypanosoma but are also components of the flagellar pocket neck in Leishmania promastigotes (11).

Using TEM and electron tomography, we analyzed the structure of the pocket and base of the flagellum to determine the ultrastructural features responsible for the haptomonad morphological adaptation. TEM revealed extensive attachment of the bulge to the cell body by desmosome-like structures in promastigotes and haptomonads, comparable to the Leishmania flagellar pocket neck, albeit covering a larger area (Fig. S3 A and B). This suggests the proteins involved in bulge-cell attachment are the FAZ proteins, and RNA-seq showed FAZ mRNAs were present in both promastigotes and haptomonads. Attachment is particularly complex in the distal pocket region, likely mediated by FAZ10, and this attachment was elaborated in haptomonads (Fig. S3B). Immunofluorescence using the anti-T. brucei FAZ antibody Dot1 identified a structure in promastigotes near the expected localization of FAZ10, which also showed elaboration in haptomonads (Fig. S3C). The Paratrypanosoma genome encodes orthologs of almost all FAZ proteins (Fig. S2A), while Bodo and Neobodo have orthologs of around half. The trypanosomatids therefore appear to have diversified FAZ proteins. Given the ancestral trypanosomatid was likely liberform, we propose that the FAZ proteins originally evolved to generate the haptomonad morphotype. The extended FAZ of the juxtaform Trypanosoma later arose in that lineage. Some FAZ proteins are often lost among liberforms (FAZ4, FAZ13), and some OGs (FLA, FLABP, FAZ11) show duplication among juxtaforms. Further candidates for forming the extended FAZ may be identified among OGs gained at the Trypanosoma node (Datasets S7 and S8).

SEM of promastigotes revealed a cystosome-like indentation near the cell anterior (Fig. 1 A and Fig. S4A). TEM of the pocket structures (Fig. S4B) and 3D reconstruction of the pocket organization by electron tomography showed that overall the pocket was typical of promastigotes, including those of Leishmania, with a simple invagination surrounded by complex electron-dense areas and sets of microtubules (Fig. 4). There were two sets of specialized microtubules: a quartet similar to the FAZ quartet of T. brucei and Leishmania (23), and a highly decorated set of microtubules associated with the cystosome. These ran from the pocket neck around the preoral ridge, back to a dip in the cell surface from which microtubules extend into the cytoplasm (Fig. 4 and Fig. S4A). This structure is comparable to the T. cruzi cystosome/cytopharynx (11). This suggests the Leishmania-like flagellar pocket/FAZ structure and microtubule quartet were ancestral and later extended into the long Trypanosoma FAZ. It also indicates the cystosome was present in the ancestral trypanosomatid; has been lost in Leishmania, many monoxenous trypanosomatids, and salivarian trypanosoma lineages; but retained in stercorean trypanosomes, some monoxenous parasites (including Crithidia fasciculata), and B. salts (24). No gains or losses of FAZ OGs suggested a function in formation of the cystosome.

The Paratrypanosoma promastigote flagellum has the canonical 9+2 axoneme and, based on the genome sequence, a canonical molecular composition (Fig. S2). Both haptomonads and amastigotes have greatly shortened flagella. In Leishmania, flagellar shortening during transition to the amastigote is associated with loss of the central pair, distal motor proteins, and radial spokes, giving a transition from a 9+0 to a collapsed 9v (variable) axoneme (6). We used TEM to check whether similar axoneme restructuring occurred in Paratrypanosoma (Fig. 5). Longitudinal sections through the promastigote, haptomonad, and amastigote flagellum base showed a basal plate and central pair, while longitudinal sections and Markham rotational averaging showed a presence of the central pair, radial spokes, and inner and outer dynein arms in all three stages (Fig. S5). RNA-seq data confirmed this result, with central pair, radial spoke, and dynein arm light and intermediate chains not significantly regulated between promastigotes and haptomonads (Dataset S9). Essentially all axonemal components were conserved in all species analyzed (except Perkinsella, which has lost its flagellum), providing no putative markers for the 9v axoneme formation.

The PFR is normally present in trypanosomatid flagella but is usually lost in amastigotes. We therefore asked if the PFR is present in different developmental stages and whether it restructures to form the haptomonad adhesive pad. TEM showed the PFR is present in promastigotes but was shortened or absent in haptomonads and amastigotes (Fig. 5A–C). Immunofluorescence using an antibody recognizing PFR2, a major PFR component, showed a similar PFR in promastigotes to Trypanosoma and Leishmania and uneven loss of the PFR in haptomonads (Fig. 5D). The characteristic quasi-crystalline structure of the PFR was only visible immediately next to the axoneme in the promastigote bulge and haptomonad attachment plaque (Fig. 4), suggesting that elaboration of the flagellum does not involve expansion of the PFR. RNA-seq also showed similar PFR mRNA levels between promastigotes and haptomonads (Dataset S9). Comparative genomics revealed that Bodo and Paratrypanosoma possess almost all known PFR components, indicating greater conservation of the PFR than the FAZ.

Promastigote to haptomonad interconversion was modulated by the growth medium, suggesting links between morphological adapta-
tion and metabolism. We analyzed which metabolic pathways are likely active in Paratrypanosoma and which were lost early in

![Fig. 4. Paratrypanosoma has a cytostome similar to T. cruzi and a pocket architecture similar to Leishmania. 3D model of the pocket/cytostome of Paratrypanosoma assembled from an electron tomogram, with the approximate position of the tomographic volume within a promastigote cell indicated. Virtual sections through the preoral ridge (1), the cytostome (2), the cytostome microtubules exiting the pocket (3) and the FAZ-associated microtubule quartet (4), the PFR (5), and axoneme next to the PFR (6) are shown. The quasi-crystalline structure of the PFR (5) was only present immediately next to the axoneme (6) in the promastigote bulge. (Scale bars, 100 nm.)](Image)
the evolution of trypanosomatids (Dataset S10). In comparison with \textit{B. saltans}, \textit{Paratrypanosoma} has lost many proteases, peptidases, cathepsins, and enzymes for hydrolysis of complex sugars. This suggests a loss of enzymes for digestion of complex energy sources early in the evolution of parasitism. With the exception of xanthine–guanine phosphoribosyltransferase, \textit{Paratrypanosoma} encodes all components of the purine salvage pathway, which is thus likely operational (Dataset S10). However, it lacks arginases needed for the urea cycle and has lost ornithine aminotransferases and xanthine dehydrogenases and therefore may indicate the urea cycle and oxidative metabolism of purines are not possible (Dataset S10). Concerning lipid metabolism, it possesses the methylmalonyl pathway, which converts propionyl-CoA, a product of odd chain fatty acid oxidation, into succinyl-CoA. This pathway has been lost in salivarian trypanosomes and \textit{Phytomonas} (Dataset S10). \textit{Paratrypanosoma} has all of the enzymes needed for ether–lipid biosynthesis except 1-acyl-sn-glycerol-3-phosphate acyltransferase, which may indicate an inability to perform the second acyltransferase reaction of phosphatidic acid formation. It also encodes a pathway needed for phospholipid formation carried out by phosphoenolpyruvate mutase, previously identified only in \textit{B. saltans} and \textit{T. cruzi}. However, ATP citrate lyase and synthase are absent from all trypanosomatids including \textit{Paratrypanosoma}, implying that they are unable to convert mitochondrial acetyl-CoA to citrate. Only \textit{Paratrypanosoma} and Leishmaniinae are able to convert and subsequently oxidize methionine into succinyl-CoA. Finally, the tryptophan degradation pathway is present in \textit{N. gruberi}, \textit{T. boreli}, \textit{B. saltans}, as well as \textit{Paratrypanosoma}, but lost from all other trypanosomatids (Dataset S10).

**Discussion**

We have demonstrated that \textit{Paratrypanosoma}, the most basal-branching trypanosomatid derived from free-living bodonids (2), assumes three different morphotypes characteristic of trypanosomatids. In a liquid cultivation medium, it alternates between motile promastigote and surface-attached haptomonad morphologies, both capable of division. Transfer to an agar plate triggers transformation into another distinct morphotype: an amastigote. As these morphotypes are common among mono- and dixenous trypanosomatids, this indicates that the ancestral trypanosomatid likely had morphological flexibility, advantageous when faced with dramatically different conditions during the evolution of parasitism of invertebrate and vertebrate hosts. Therefore, the trait of extensive interstage morphological transformation of \textit{Leishmania} and \textit{Trypanosoma} in their mammalian and insect hosts likely existed in their monoxenous predecessor; the wide array of trypanosomatid morphotypes (10) did not originate within the context of dixenous parasitism but predated the two-host lifestyle (25).

The capacity to firmly but transiently attach to a substrate by the flagellum, apparently preventing their discharge from the host, is common in trypanosomatids. Bodonids can also undergo surface attachment (24). This feature might have predisposed trypanosomatids for their initial radiation in insects (1), which required fixation to the host gut. Attachment associated with extensive remodeling of the flagellum, as in other trypanosomatids (22), seems to be a central feature in the life cycle of \textit{Paratrypanosoma}. Attachment and flagellum shortening could occur without a division event, while we only observed flagellum growth and detachment following division of a haptomonad. This has similarities to both \textit{Leishmania}, in which flagellum shortening can occur without division, and \textit{T. brucei}, in which division to generate disimilar daughters is used for life cycle stage transition. Remodeling of the flagellum in haptomonads involved expansion of flagellum/cell attachment in the distal pocket region, suggesting the FAZ proteins may contribute to surface attachment.

OG gains and losses indicate \textit{Paratrypanosoma} has diverged significantly from the common ancestor of trypanosomatids (Fig. S6), however it has retained more ancestral OGs than any trypanosomatid lineages except stercorarian trypanosomes (Fig. 3). Recent comparison of the \textit{B. saltans} and trypanosomatid genomes revealed that metabolic losses accompanied the emergence of obligatory parasitism (3) with little further gene loss or streamlining.

**Fig. 6.** Evolution of the flagellar pocket/cytostome complex of human infective trypanosomatids. Cartoon summarizes the likely loss of the cytostome and extension of the microtubule quartet and FAZ to generate \textit{Leishmania}, \textit{T. brucei}, and \textit{T. cruzi} pocket/cytostome morphology from an ancestral \textit{Paratrypanosoma}-like morphology.
of the genome occurring later (4, 25). Indeed, our analyses revealed a massive loss of proteases, peptidases, and cathepsins involved in the breakdown of polypeptides. Paratrypanosoma and other trypanosomatids have also lost receptor-mediated endocytosis of macromolecules, cobalamin biosynthesis, and lysosomal pro-X exopeptidase and ammonium transporter, compelling trypanosomatids into foraging nitrogen from other sources. Paratrypanosoma has gained or expanded several gene families that were not present in its free-living predecessor, including transmembrane transporters suitable for scavenging amino acids and other metabolites from the host (3, 4, 18). An interesting gene family expansion, which likely occurred early in the evolution of trypanosomatids, are the DGF1 genes, present in Paratrypanosoma and the stercorarian trypanosomes only. These abundant secreted proteins may play a role in host–parasite interactions (21, 22).

The ultrastructure of Paratrypanosoma includes a Leishmania-like FAZ including a microtubule quartet and a T. cruzi-like cytostome. This supports the hypothesis that the extended FAZ evolved once in the Trypanosoma lineage and indicates that the cytostome was an ancestral feature, retained in T. cruzi but lost in T. brucei, Leishmania, Phytomonas, and several monoxenous lineages (Fig. 6). This implies that there has been a streaming of ultrastructure analogous to the streamlining of the genome, with the notable exception of innovation to generate an extended FAZ in Trypanosoma.

The differences in transcriptome of Paratrypanosoma promastigotes and haptomonads coexisting in culture are comparable in magnitude to the differences between L. mexicana amastigotes (mammalian host) and promastigotes (sandfly vectors) (18, 23).

Our analysis of the morphologies Paratrypanosoma can attain in culture, its genome and transcription profile, and the ultrastructure of the flagellar pocket/cytostome complex has uncovered features likely present in the ancestors of the three human-infective trypanosomatid lineages (Fig. 6). Future studies of this very interesting protist will be particularly informative in regard to how trypanosomatid parasites have evolved from the free-living bodonids.

Materials and Methods

Promastigotes and haptomonads were cultured at 27 °C in a 1:1 mixture of RPMI 1640 and M199, pH 7.0, with 10% (v/v) FCS, 2% sterile human urine, 10 μg/mL hemin, and penicillin-streptomycin, and interconversion was analyzed by time-lapse microscopy in culture. Amastigotes were generated by culture on agarose plates. For SEM, haptomonads were grown and fixed on coverslips. For TEM, they were grown in culture flasks, treated with propylene-oxide treatment to dissolve the substrate, and then prepared by high-pressure freezing. Detailed culture and EM methods, genome assembly and annotation, transcriptome analysis, and gene family/ontology methods are available in SI Materials and Methods.

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