

Insect Stage-Specific Adenylate Cyclases Regulate Social Motility in African Trypanosomes

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Sophisticated systems for cell-cell communication enable unicellular microbes to act as multicellular entities capable of group-level behaviors that are not evident in individuals. These group behaviors influence microbe physiology, and the underlying signaling pathways are considered potential drug targets in microbial pathogens. *Trypanosoma brucei* is a protozoan parasite that causes substantial human suffering and economic hardship in some of the most impoverished regions of the world. *T. brucei* lives on host tissue surfaces during transmission through its tsetse fly vector, and cultivation on surfaces causes the parasites to assemble into multicellular communities in which individual cells coordinate their movements in response to external signals. This behavior is termed “social motility,” based on its similarities with surface-induced social motility in bacteria, and it demonstrates that trypanosomes are capable of group-level behavior. Mechanisms governing *T. brucei* social motility are unknown. Here we report that a subset of receptor-type adenylate cyclases (ACs) in the trypanosome flagellum regulate social motility. RNA interference-mediated knockdown of adenylate cyclase 6 (AC6), or dual knockdown of AC1 and AC2, causes a hypersocial phenotype but has no discernible effect on individual cells in suspension culture. Mutation of the AC6 catalytic domain phenocopies AC6 knockdown, demonstrating that loss of adenylate cyclase activity is responsible for the phenotype. Notably, knockdown of other ACs did not affect social motility, indicating segregation of AC functions. These studies reveal interesting parallels in systems that control social behavior in trypanosomes and bacteria and provide insight into a feature of parasite biology that may be exploited for novel intervention strategies.

Trypanosoma brucei and other African trypanosomes are protozoan parasites that cause sleeping sickness in humans and related wasting diseases in wild and domestic animals. Sleeping sickness is recognized as one of the world’s most neglected diseases, with approximately 60 million people living at risk of infection, while livestock infections account for significant economic hardship in some of the most impoverished regions of the planet (1, 2). Dedicated efforts over the last decade have reduced the human health burden, but these parasites remain a continuing threat for reemergence owing to their capacity for explosive outbreaks and their historical ability to resist eradication (3, 4). Sleeping sickness is fatal if untreated, no vaccine exists, and current treatment options are toxic, antiquated, and increasingly ineffective (5, 6). Therefore, new perspectives on trypanosome biology, transmission, and pathogenesis are urgently needed to facilitate novel intervention strategies.

T. brucei is transmitted between mammalian hosts by blood-feeding tsetse flies. Transmission through the fly requires extensive interaction with host tissue surfaces, as parasites move across and through tissues en route from the fly midgut, through the alimentary tract and mouthparts, and then into the salivary glands (7). Once in the salivary gland, parasites colonize the gland epithelial surface and complete the final stages of development into forms infectious for mammals (8). Thus, as is the case for many microbes (9), *T. brucei* in its natural habitat lives in intimate and continuous contact with surfaces.

Despite the ubiquity of parasite-surface interactions during *T. brucei* transmission, studies of these organisms are almost exclusively conducted using suspension cultures. While such studies have yielded many important insights, they overlook an important and ubiquitous feature of trypanosome biology. To overcome this gap in knowledge, we utilized semisolid agarose matrices to

assess the influence of surface cultivation on parasite behavior (10). This led to the surprising discovery that rather than acting as individuals, surface-cultivated procyclic (insect-stage) trypanosomes assemble into multicellular groups that coordinate their movements across the surface (10). Initially, parasites collect into small groups, and these grow larger through recruitment of other cells. At the periphery of the inoculation site, parasites assemble in nodes of high cell density, and from there they advance outward. Movement is polarized such that cells move outward but not laterally, leading to the formation of thin projections radiating away from the center. When cells in radial projections encounter a separate group of parasites, they halt or divert their movement to avoid contact, implicating cell-cell signaling in the control of trypanosome group behavior. We termed this behavior “social motility” (SoMo), based on features shared with surface-induced social motility in bacteria (9, 11).

As is the case in bacteria (12–14), social motility in *T. brucei* requires cell motility and some ability of cells to sense and respond to external cues (10). Surface-induced group behaviors, such as

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social motility and biofilm formation, have been extensively studied in bacteria (11, 14), but molecular mechanisms that govern surface-induced group behavior in trypanosomes are completely unknown. In other microbes, collective activities of groups of cells are commonly controlled by cyclic nucleotides, which function as second messengers and as secreted signaling molecules. In *Dictyostelium discoideum*, for example, cyclic AMP (cAMP) acts as a chemoattractant and signaling molecule to direct surface motility of individual cells, enabling them to assemble into multicellular fruiting bodies (15). In several species of bacteria, cyclic-di-GMP (c-di-GMP) governs the transition between surface-induced swarming motility and biofilm formation (16–20). In this case, decreased intracellular c-di-GMP promotes swarming, while elevated c-di-GMP inhibits swarming and promotes biofilm formation (14, 18, 19, 21). In the yeast *Candida albicans*, cyclic nucleotides regulate dimorphic transitions connected to surface penetration and pathogenesis (22).

Cyclic nucleotide synthesis in *T. brucei* depends on a large family of receptor-type adenylate cyclases (ACs) that are localized to the flagellum membrane (23–26). Trypanosomal ACs are distinguished from mammalian ACs in that the intracellular catalytic domain is directly connected to an extracellular, putative ligand binding domain on a single polypeptide (24). As such, trypanosome ACs may be regulated by extracellular ligands directly rather than via upstream G protein-coupled receptors, as observed for mammalian ACs (24). The canonical *T. brucei* AC is expression site-associated gene 4 (ESAG4), a bloodstream stage-specific AC that modulates the host immune response to promote parasite survival during infection (27). Aside from ESAG4, functions for *T. brucei* ACs are unknown. Notably, several flagellar ACs were recently identified that are specifically upregulated in procyclic *T. brucei* (25), the parasite life cycle stage that undergoes social motility. Given the widespread use of cyclic nucleotides in control of microbial social behavior and the emerging role of the eukaryotic flagellum in coordinating cellular responses to external signals (28, 29), we asked whether *T. brucei* flagellar ACs function in social motility. Here we report that AC6, a procyclic stage-specific AC (25), is localized to the tip of the flagellum and regulates social motility. RNA interference (RNAi)-mediated knockdown of AC6 causes a hypersocial phenotype, while knockdown of several other flagellar ACs does not affect social motility. Importantly, point mutations in the AC6 catalytic domain phenocopy the knockdown, demonstrating that loss of activity, rather than loss of the protein, is responsible for the phenotype. These studies are, to our knowledge, the first to demonstrate function for trypanosomal ACs in the insect life cycle stage and extend the paradigm of cyclic nucleotide control of microbial social behavior to parasitic protozoa.

MATERIALS AND METHODS

Cell culture and motility assays. Procyclic cells derived from the 2913 cell line (30) were used for all experiments. Suspension cultures were maintained using Cunningham's semidefined medium (SM) as described previously (31). For RNAi lines, 2.5 mg/ml phleomycin, was included in the medium for selection of stable transfectants, and RNAi was induced by adding 1 mg/ml tetracycline. Lines carrying pMOT vector-based sequences for rescue and hemagglutinin (HA) tagging were cultured with 1 μ g/ml of puromycin. Motility in suspension cultures was assayed as previously described (31). Social motility assays were done as previously described (10). For quantitation, several independent assays were done, and the numbers of projections formed per plate were quantitated.

RNAi and quantitative reverse transcription-PCR (qRT-PCR). RNAi target regions for the ACs were chosen by using the Trypanofan RNAi algorithm (32) and were PCR amplified using the following primers: AC1&2KD_F, TTGATGATGATGGTAGCGGA; AC1&2KD_R, ACATACACCGCCTTACTGCC; AC3KD_F, GACGGTCTGTCCCTGTGT; AC3KD_R, TGGCTCTGAACAGTGAATGC; AC4KD_F, AGCTTACGAGGGCTGTGAAA; AC4KD_R, AAATACACTGCCCTTGTGCG; AC5KD_F, TCTGCTTATGCAGGACGATG; AC5_R, CCTCAAAGTCTCGAGGTGC; AC6KD_F, TGGAGCAGCAAATCTACGTG; AC6_R, TTTTCTCGGCTCTCCACTGT; AC6uKD_F, ATAAGCTTACGGGGTTCCCTCATTTAAC (restriction sites are italicized); AC6uKD_R, ATTCTAGAACAACAACAACCCCAAAAA.

Fragments were amplified using genomic DNA from 2913 cells and ligated into the p2T7Ti-B RNAi plasmid (33). All sequences were verified by direct DNA sequencing. RNAi vectors were linearized, transfected, and selected with antibiotic as previously described (31). Clonal lines were generated by limiting dilution. Efficacy of knockdown was assessed via qRT-PCR as described previously (34) at 72 h postinduction. qRT-PCR results are reported as arithmetic means \pm standard deviations from at least two independent RNA preparations, each analyzed in duplicate and normalized against the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Tb927.6.4280/Tb927.6.4300) and RPS23 (Tb10.70.7020/Tb10.70.7030). Relative gene expression was determined using the $2^{-\Delta\Delta C}$ method as previously described (35, 36). Primers used for qRT-PCR were the following: AC1_F, CGTTGACTTCACGGCTTACA; AC1_R, ACATTCGTTCTCCACTGC; AC2_F, GCCATGTCGTTGATTTTACA; AC2_R, CCAACCAGACCACAGACCTT; AC3_F, ACTGATGGCGTCTTACACAA; AC3_R, GGATGCACTTTTCTTGGGCAAC; AC4_F, CTGCGAGTGCAGATTGGTGT; AC4_R, ACGTTCTCGGGTCTGAGTGC; AC5_F, CACATCTCAGCGCCAAAACTG; AC5_R, TAGACCGCATAATCGCCTCACA; AC6_F, TGCAGTTAAGTGGGTCACA; AC6_R, GATCCACCGCAGGATTAGAA; GAPDH_F, GGCTGATGTCTCTGTGGTGGAA; GAPDH_R, GGCTGTCGCTGATGAAGTCG; RPS_F, AGATTGGCGTTGGAGCGAAA; RPS_R, GACCGAAACCAGAGACGACGA.

Western blot assays and immunofluorescence. Protein extracts were prepared and analyzed by Western blotting as described previously (37). A total of 1×10^6 cell equivalents per lane were used. Monoclonal mouse anti-HA antibody from Covance (used at 1:5,000) and monoclonal anti- β -tubulin E7 hybridoma supernatant (used at a 1:7,000 dilution) were used as primary antibodies and detected by horseradish peroxidase-coupled goat anti-mouse antibodies (Bio-Rad) at a 1:5,000 dilution. Immunofluorescence was carried out on whole cells as described previously (25), with the monoclonal anti-HA antibody HA.11 (Covance) used at a 1:250 dilution and donkey anti-mouse secondary antibody coupled to AlexaFluor 488 (Molecular Probes) used at 1:2,500. Visualization of nucleic acid was done by staining with DAPI (4',6-diamidino-2-phenylindole).

Generation of AC6-Ri and AC6-Ri mutant cell lines.** AC6-Ri and AC6**-Ri cell lines were generated by expressing wild-type or mutant AC6 in the AC6 UTR knockdown line (AC6uKD) (34). To generate the AC6-Ri line, a 426-bp fragment corresponding to the 3' terminus of the AC6 open reading frame (ORF) was cloned immediately upstream of the α/β -tubulin intergenic region in the pMOTag plasmid (39). A fragment of the AC6 3'-untranslated region (UTR) was then cloned downstream of the puromycin resistance gene in this plasmid to generate the plasmid pMOT-AC6WT426. Primers used were the following: AC6_ORF_F, ATGGTACCGAGGCATATGTGGCGGATG; AC6_ORF_R, ATGTCCGACCTACTGCTTCCCCTTTTCTT; AC6_UTR_F, ATGGATCTAACAGCAGTAGTGAATTGAAG; AC6_UTR_R, ATTCTAGAACGTGGACTTCACCTTCATC (restriction sites are italicized).

Plasmid DNA sequences were confirmed by plasmid sequencing (Genewiz, Inc.) Tagging cassettes were excised by restriction digestion, purified, and transfected into AC6uKD cells. Stable transfectants were selected, and clonal lines were generated by limiting dilution.

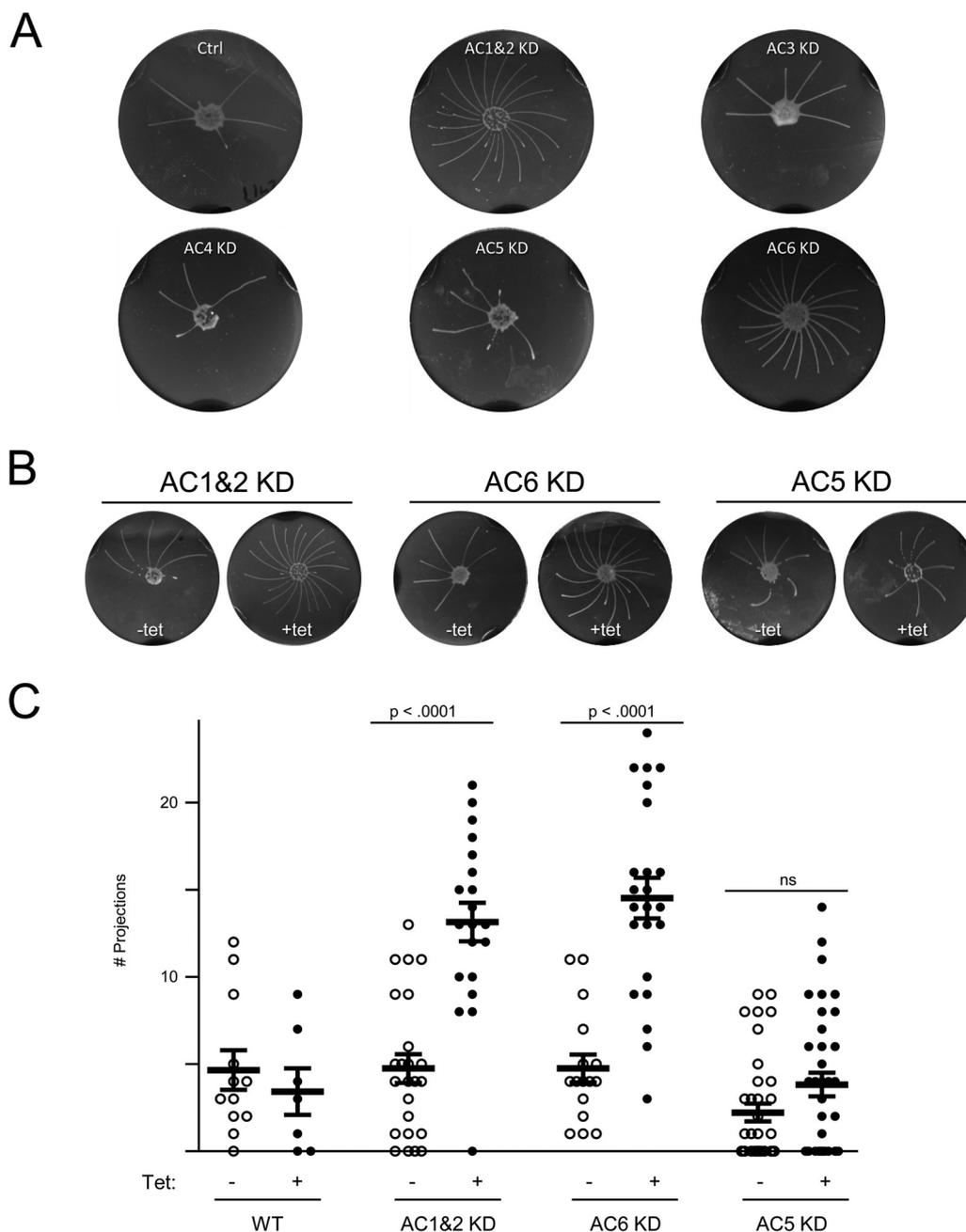


FIG 1 Social motility is regulated by a subset of procyclic stage-specific adenylate cyclases. (A) Gene-specific knockdowns targeting the indicated ACs were assayed for social motility in comparison with 2913 control cells (ctrl) on plates containing tetracycline to induce RNAi. (B) Social motility assay results are shown for the indicated knockdowns, maintained in the absence or presence of tetracycline (Tet) to induce RNAi. (C) Quantitation of the number of projections formed for wild-type cells (WT) or the indicated knockdown lines, maintained in the absence or presence of tetracycline. Mean values and standard errors are shown. *P* values (unpaired *t* test) are shown. Sample sizes: WT -Tet, *n* = 12; WT +Tet, *n* = 7; AC1 and -2KD -Tet, *n* = 24; AC1 and -2KD +Tet, *n* = 20; AC6KD -Tet, *n* = 16; AC6KD +Tet, *n* = 23; AC5KD -Tet, *n* = 36; AC5KD +Tet, *n* = 36.

Ri). Northern blotting demonstrated that AC6 mRNA was upregulated in procyclic cells (Fig. 2), consistent with qRT-PCR results (25). A single AC6 mRNA was observed in control and AC6-uKD cells, while AC6-Ri cells expressed two mRNAs, corresponding to endogenous and RNAi-immune transcripts (Fig. 2). Abundance of the endogenous transcript was substantially reduced upon RNAi induction, while the RNAi-immune transcript was unaffected (Fig. 2). Thus, AC6-Ri cells maintain AC6 expres-

sion following knockdown of the endogenous gene. Growth and individual cell motility in suspension culture were unaffected in AC6-Ri cells with or without RNAi induction (see Fig. S6 in the supplemental material). Maintenance of AC6 expression under RNAi-induced conditions rescued the hypersocial phenotype (Fig. 2), demonstrating that the hypersocial phenotype is a consequence of AC6-specific knockdown.

RNAi alone does not distinguish between phenotypes caused

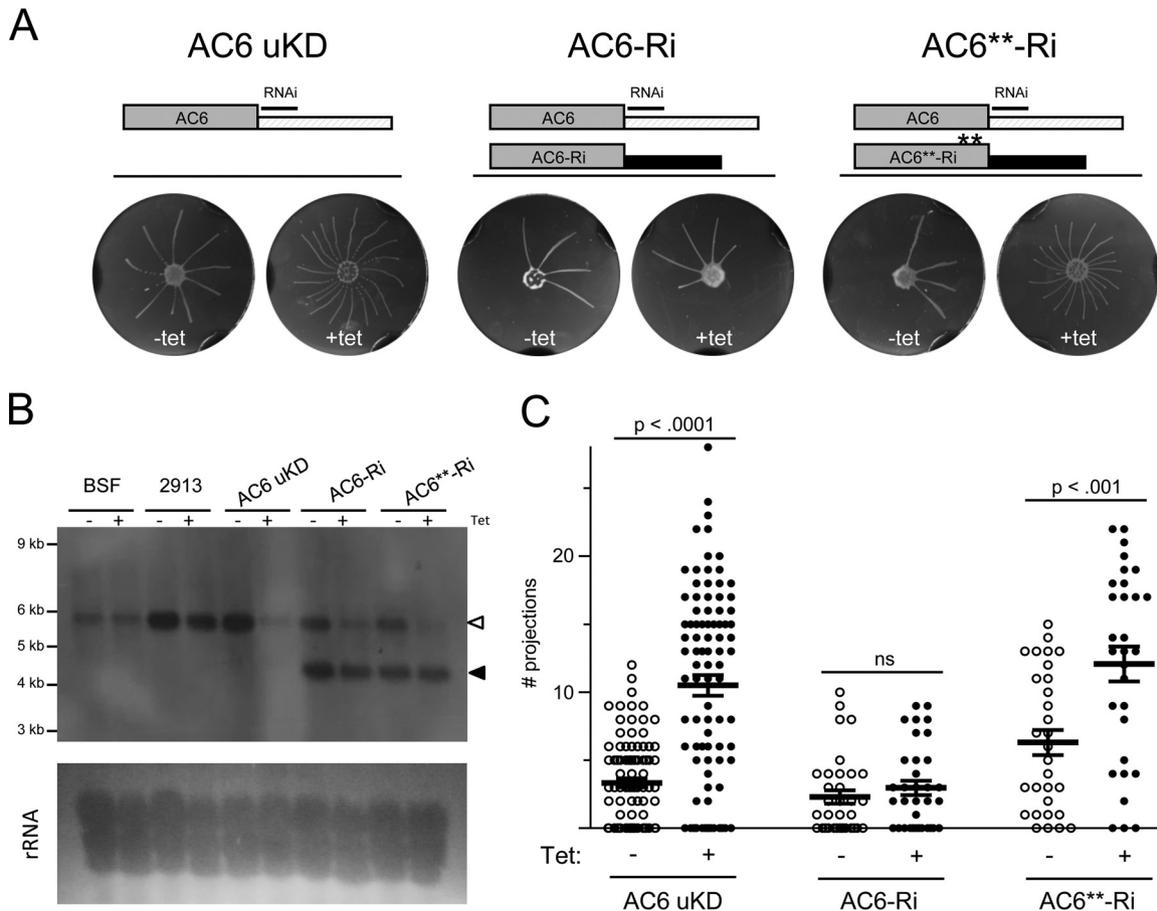


FIG 2 Social motility is modulated by AC6 catalytic activity. (A) Social motility assays on AC6-UTR-knockdown cells (AC6 uKD), AC6-RNAi-immune cells (AC6-Ri), or the RNAi-immune catalytic domain mutant cells (AC6** -Ri). Cells were maintained in the absence or presence of tetracycline (Tet) to induce RNAi. Schematics at the top of the panel illustrate the region targeted for RNAi. RNAi targets the 3' UTR of the endogenous AC6 gene, while transgenes are immune to RNAi, owing to the presence of an alternate UTR. (B) Northern blot probed with an AC6-specific probe (top). RNA was prepared from bloodstream cells (BSF), procyclic 2913 control cells (PCF), AC6-uKD cells, AC6-Ri cells, or AC6** -Ri cells maintained with or without Tet as indicated. The position of the endogenous AC6 mRNA (open arrowhead) and transgene mRNA (closed arrowhead) are indicated. Total RNA was visualized by UV illumination of the blot (bottom). (C) Quantitation of the number of projections formed for the indicated cell lines maintained in the absence or presence of Tet. Mean values and standard errors are shown. *P* values (unpaired *t* test) are indicated. Sample sizes: AC6uKD -Tet, *n* = 90; AC6uKD +Tet, *n* = 90; AC6-Ri -Tet, *n* = 34; AC6-Ri +Tet, *n* = 34; AC6** -Ri -Tet, *n* = 30; AC6** -Ri +Tet, *n* = 30.

by loss of protein activity, versus indirect consequences arising from loss of the target protein (38). For example, while the AC6 knockdown phenotype might be a direct result of impaired cAMP output, it might alternatively be due to changes in potential interaction partners of AC6 caused by loss of the protein. To distinguish between these possibilities, we asked if blocking AC catalytic function without altering protein levels altered social motility. The *T. brucei* AC active site contains a highly conserved NMAART domain that is required for catalytic activity (40). We therefore mutated the NMAART domain to all alanines to generate a catalytically inactive version, termed AC6**, and introduced an RNAi-immune copy of AC6** into the AC6 uKD line. This cell line is referred to as AC6** -Ri. Following tetracycline induction, endogenous AC6 mRNA levels were substantially reduced, while AC6** -Ri mRNA levels were unaffected. Growth and motility in suspension culture were unaffected in AC6** -Ri cells (see Fig. S6 in the supplemental material), but expression of the AC6** catalytic mutant failed to compensate for loss of endogenous AC6 (Fig. 2).

It is possible that the phenotype of AC6** -Ri mutants might be due to altered expression levels and/or localization of the mutant protein. To test this, we compared expression and localization of wild-type AC6 to that of AC6**. For this, we used *in situ* tagging (39) to place a hemagglutinin (HA) epitope at the C terminus of the wild-type and mutant proteins. Western blot analysis showed a single band of approximately equal abundance in AC6-HA and AC6** -HA cells (Fig. 3). Immunofluorescence revealed that wild-type AC6-HA localized to the flagellum, but interestingly, was restricted to the flagellar tip (Fig. 3). Interestingly, tip localization for AC6 was predicted previously based on sequences within a C-terminal domain that is required for flagellum targeting (25). Importantly, the AC6** catalytic mutant gave a flagellum tip distribution that was indistinguishable from that of the wild type (Fig. 3), indicating that the hypersocial phenotype of AC6** -Ri cells is a consequence of lost activity and is not caused by altered expression or mislocalization.

We next asked whether knockdown of AC6, or expression of AC6**, affected total cellular cAMP levels (see Fig. S7 in the sup-

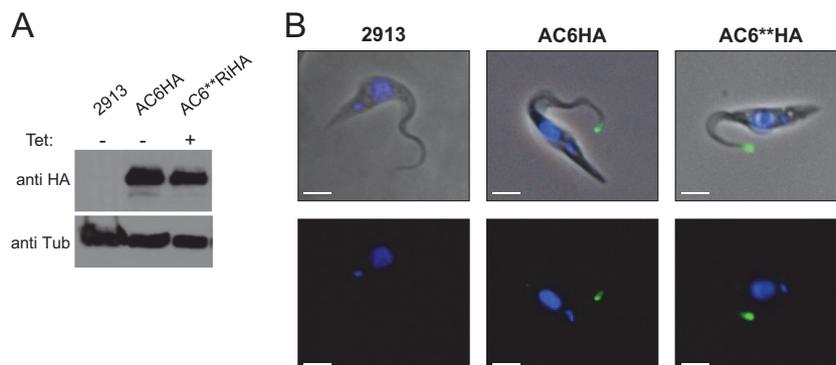


FIG 3 AC6 is localized to the tip of the trypanosome flagellum. (A) Western blot of protein extracts from 2913 cells and from cells expressing wild-type HA-tagged AC6 (AC6HA) or catalytically inactive AC6 (AC6**HA). Blots were probed with anti-HA antibody, or anti-tubulin antibody as a loading control. (B) Control cells (2913) or cells expressing HA-tagged wild-type AC6 (AC6HA) or the catalytic domain mutant (AC6**HA) were subjected to immunofluorescence with anti-HA antibody (green) and DAPI (blue). Bar, 2 μ m.

plemental material). Although there was a trend toward reduced total cellular cAMP following RNAi induction in AC6uKD and AC6**-Ri cells, the changes were not statistically significant. Given the discrete localization of AC6 to a tiny subdomain of the parasite surface and the fact that this is just one of several ACs expressed in this life cycle stage, it is not surprising that RNAi did not cause gross perturbations of total cellular cAMP.

DISCUSSION

Recognition of social behavior and cell-cell communication as ubiquitous properties of bacteria (9, 13) transformed our view of microbiology and microbial pathogenesis, but protozoan parasites have not generally been considered in this context. Recently, we showed that the paradigm of microbial social behavior applies to the protozoan parasite *T. brucei* when cultivated on surfaces to mimic their natural environment (10). Here we provide the first molecular dissection of mechanisms underlying social motility in trypanosomes, and our results suggest that cAMP signaling systems in the flagellum control *T. brucei* social behavior. As discussed below, our studies reveal intriguing parallels in systems used by trypanosomes and bacteria for controlling surface-induced social behavior. Moreover, by discovering an accessible readout for receptor-type adenylate cyclase function in *T. brucei*, our findings provide a foundation for dissecting structure-function relationships among these enigmatic proteins and, by extension, cAMP signaling in trypanosomes.

Several independent analyses point to a specific role for cAMP produced by AC6 in social motility. First, a hypersocial phenotype was observed in two independent AC6 knockdown lines targeting distinct regions of the AC6 mRNA, while expression of other ACs was unaffected. Second, the phenotype was rescued by expression of wild-type AC6, but not by a catalytic domain mutant. Thus, normal social motility depends on AC6 activity, and the mutant phenotype is not due to off-target effects of knockdown.

cAMP is implicated in the *T. brucei* transmission cycle as well as pathogenesis, and elevated cAMP is lethal for bloodstream-form cells, making cAMP pathways a target of drug discovery efforts (46). Pathways of cAMP signaling in these organisms remain poorly characterized, however, and lack familiar features found in mammalian cells (44). One outstanding question is why trypanosomes have so many AC genes, ~ 75 compared to ~ 10 in mammals, for example (47). Some *T. brucei* ACs may substitute for

others (48), suggesting some redundancy or overlap in function. However, the presence of ACs that are specific to different life cycle stages and that exhibit distinct subcellular localizations (24, 25) argues against strictly redundant functions for these proteins. The idea of distinct functions for individual ACs is also supported by the fact that expression of AC1 to AC5 is unaltered in AC6 knockdowns but does not accommodate for loss of AC6. Likewise, normal expression of AC6 does not accommodate for loss of AC1 and -2. At least two possibilities may explain the inability of AC6 to compensate for loss of AC1 and -2 or vice versa. One possibility is that normal social motility requires interaction between different AC isoforms, such that each isoform must be present to form an active complex. It is also possible that rather than direct interaction, independent signaling through each is required, and different isoforms function as coincidence detection modules.

Simultaneous expression of several ACs in a single cell presents a conundrum if each AC controls a different pathway, because cAMP output must be segregated to avoid interference. In mammalian cells, this problem is mitigated via a microdomain model, in which spatial separation of cAMP signaling output enables different ACs to control distinct cellular responses (49). Such a model has been suggested for *T. brucei* (25, 50) and is supported by our results. In this model (Fig. 4), social motility is regulated by local levels of intraflagellar cAMP specifically derived from an AC6 microdomain at the flagellum tip. When levels are high, social motility is attenuated, and when levels are low social motility ensues. In wild-type cells (Fig. 4A), AC6 activity is downregulated in response to a specific signal, e.g., the presence of an inhibitor or loss of an activator, resulting in reduced local cAMP levels and initiation of social motility. Upon constitutive inactivation of AC6, e.g., through knockdown or expression of a catalytic mutant (Fig. 4B), a signal-independent decrease in local cAMP within the AC6 microdomain results in a precocious, hypersocial phenotype. A key tenet of this model is that a cAMP-specific phosphodiesterase localized throughout the flagellum provides a diffusion barrier to shield the AC6 tip domain from cAMP generated by other flagellar ACs (50). As such, the model predicts that if local cAMP levels were constitutively elevated through loss of the diffusion barrier, social motility would be blocked. In support of this model, in separate work we found that genetic or pharmacological inhibition of flagellum-localized cAMP-specific phosphodiesterase

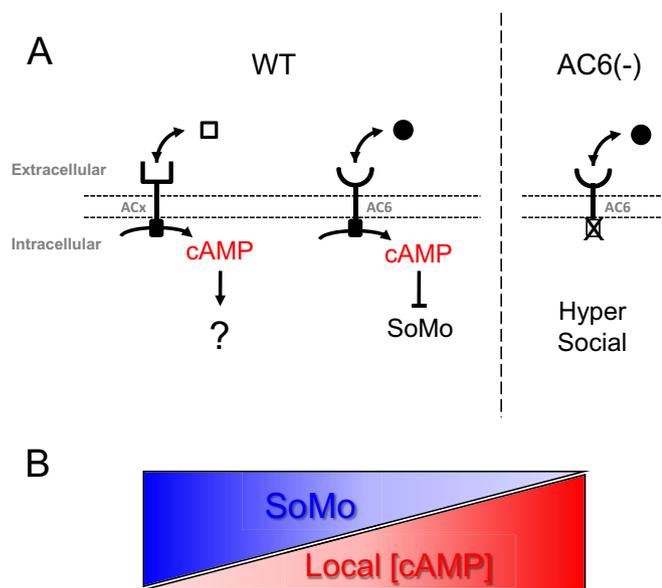


FIG 4 Model for cAMP microdomain regulation of social motility. (A) Schematic model for AC6-dependent control of social motility in wild-type cells (WT) and AC6 knockdown or catalytic mutant cells [AC6(-)]. AC6 is one of several ACs in the trypanosome flagellum. The model posits that ACs recognize different ligands, depending on their divergent extracellular domains, and that ligand binding regulates AC activity. cAMP produced specifically by AC6 acts to inhibit social motility, and when AC6 activity is reduced through ligand-mediated regulation (WT), this results in reduced cAMP and activation of social motility. Constitutive inactivation of AC6, e.g., through knockdown or expression of a catalytic mutant [AC6(-)], causes a signal-independent decrease in local cAMP, resulting in a precocious, hypersocial phenotype. (B) Reciprocal relationship between social motility (SoMo) and cAMP concentration.

leads to elevated cAMP and a complete block of social motility (M. Oberholzer and K. M. Hill, submitted for publication).

Our findings extend the concept of cyclic nucleotides as regulators of microbial social behaviors to parasitic protozoa and reveal parallels with c-di-GMP regulation of bacterial swarming motility. Two specific examples are the SadC/BifA system in *Pseudomonas aeruginosa* and the ScrABC system in *Vibrio parahaemolyticus* (14, 19). *Pseudomonas* and *Vibrio* species undergo surface-dependent swarming motility. In *P. aeruginosa*, swarming is regulated by the combined activities of SadC, a diguanylate cyclase (DC) that synthesizes c-di-GMP, and BifA, a phosphodiesterase (PDE) that specifically degrades c-di-GMP (19). Knockout of the cyclase reduces intracellular c-di-GMP levels and causes hyper-swarming, while knockout of the phosphodiesterases results in elevated c-di-GMP levels and blocks swarming (19). In *V. parahaemolyticus*, swarming motility is regulated by the ScrABC system. Here, the ScrC protein is a c-di-GMP cyclase and phosphodiesterase that is regulated by the periplasmic binding protein ScrB (14). The ligand for ScrB is an extracellular “S-signal” that accumulates in a cell density-dependent fashion and binds ScrB. Once bound to its ligand, ScrB neutralizes the c-di-GMP cyclase activity of ScrC and simultaneously activates its phosphodiesterase activity. This causes intracellular c-di-GMP levels to drop and stimulates swarming. At low density, in the absence of S-signal, the cyclase activity of ScrC predominates, resulting in elevated c-di-GMP and inhibition of swarming. Thus, in both bacteria and trypanosomes, the extent of social motility on surfaces is inversely

proportional to levels of intracellular cyclic nucleotide, as controlled by reciprocal regulation of cyclase and phosphodiesterase activities. In bacteria, an expanded repertoire of DC genes is common and makes it possible to use c-di-GMP signaling for responding to a wide variety of signals (16, 17).

In addition to pathway architecture, the domain structure of ScrC in *Vibrio* spp. is similar to that of trypanosome ACs, having a surface exposed ligand binding domain coupled via a transmembrane segment to a cytoplasmic nucleotide cyclase domain (14, 44). Ligand binding to trypanosome ACs has not been demonstrated, but the AC extracellular domain bears homology to bacterial periplasmic binding proteins (52), such as ScrB. Bacterial periplasmic binding proteins bind a variety of small-molecule ligands and control chemotaxis and swarming motility (14, 53), raising the intriguing possibility that small-molecule ligands might control social motility through AC6 in *T. brucei*. In bacteria, where social motility has been well-studied, this behavior facilitates rapid colonization of surfaces, antibiotic resistance, and efficient use of nutrients (9, 54, 55). Although the *in vivo* correlates of social motility in *T. brucei* are not yet known, similar activities would be beneficial to parasites in the tsetse fly (10). More broadly, studies of bacterial surface-associated group behaviors have provided tremendous insights into signaling mechanisms used by these organisms (13, 14). Likewise, our findings present an important advance by providing the first demonstrated requirement for ACs in procyclic-form parasites and establishing a foundation for dissecting cAMP signaling systems in these organisms in a readily accessible *in vitro* assay.

ADDENDUM IN PROOF

Recent work has shown that *T. brucei* social motility is a property of a distinct life cycle stage within the fly transmission stage (S. Imhof, S. Knüsel, K. Gunasekera, X. L. Vu, and I. Roditi, *PLoS Pathog* 10:e1004493, 2014, <http://dx.doi.org/10.1371/journal.ppat.1004493>).

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