Title
Investigation of Mechanisms Underlying African Trypanosome Social Behavior

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Publication Date
2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Investigation of Mechanisms Underlying African Trypanosome Social Behavior

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

By

Miguel Augusto Lopez

2013

ABSTRACT OF THE DISSERTATION

Investigation of Mechanisms Underlying African Trypanosome Social Behavior

by

Miguel Augusto Lopez

Doctor of Philosophy in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles 2013

Professor Kent L. Hill, Chair

African trypanosomes, including *Trypanosoma brucei* and related subspecies, are the causative agents of sleeping sickness in humans and nagana in cattle. These parasites are the source of significant human mortality and limit economic growth throughout sub-Saharan Africa. *T. brucei* has a complex life cycle with phases in an insect vector and a mammalian host. Although motility through fluids and on tissue surfaces is central to *T. brucei* development and disease pathogenesis, a complete description of how the parasite moves through fluids and behave on surfaces is not available. Through the use of novel techniques and classical molecular approaches applied towards the study of *T. brucei* motility in suspension media and on surfaces, we have discovered unappreciated aspects of protozoan parasite biology.

Though the use of high-speed imaging, we found that that *T. brucei* moves through suspension media via the propagation of kinks generated by the parasite's ability to alternate the chirality of its rotation. The observation that *T. brucei* moves by generating alternating chiralities

disproved the 150 year long notion that the parasite moves by spiraling in one direction and demonstrates that high-speed imaging can be a useful tool for uncovering important features of microorganism locomotion.

By studying *T. brucei* on agarose plates, we discovered that *T. brucei* engages in social behavior when exposed to surfaces: an unprecedented finding in protozoan parasite biology. This social behavior, termed Social Motility (SoMo) is characterized by the formation of multicellular communities that engage in polarized migrations across the agarose surface and cooperate to divert their movements in response to external signals. Investigation of the mechanisms underlying this social behavior revealed a novel role for a unique family of receptor-like proteins that were previously uncharacterized and implicates a role for cAMP signaling in the regulation of SoMo.

Lastly, our search for extra cellular factors that regulate this social behavior has led to the discovery of inter-kingdom cross-talk between *T. brucei* and bacteria. In short, the investigations presented here have uncovered novel aspects of *T. brucei* behavior that have transformed our understanding of protozoan biology and will serve as the basis for future investigations.

The dissertation of Miguel Augusto Lopez is approved:

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2013

DEDICATION

This dissertation is dedicated to my family. My parents have been a constant source of inspiration and support. It is on their shoulders that I stand. I also thank my brother. Having had the privilege of watching him grow and take on challenges while remaining levelheaded has inspired to face my own obstacles with resolve. I would also like to thank my fiancée Michelle. Since meeting her, my life has only improved and she has been a constant source of love, happiness and support. There are countless others that have helped me along this path that I am eternally indebted to. Thank you all.

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Honors and Awards:

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- 2007—University of California, Los Angeles, Department of Microbiology, Immunology and Molecular Genetics Departmental Retreat. Poster: *Role of receptor-type adenylyl cyclases in the cell-cell communication and social behavior of trypanosomes*
- 2007—University of California, Los Angeles, Molecular Biology Institute 2007 Lake Arrowhead Departmental Retreat; Poster: *Social motility and cell-cell communication in African trypanosomes*
- 2004—University of California, Los Angeles undergraduate Science Poster Day; Poster: *IL-6 is a positive regulator of hepcidin expression*
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Chapter 1

Introduction

Introduction to African Trypanosomes and Thesis

African trypanosomes, including *Trypanosoma brucei* and related subspecies, are the causative agents of sleeping sickness in humans and nagana in cattle. These parasites are the source of significant human mortality and limit economic growth throughout sub-Saharan Africa [1,2]. Sleeping sickness is recognized as one of the world's most neglected diseases and is a reemerging disease with ~60 million people at risk of infection. The disease is fatal if left untreated and current therapeutic options are insufficient as they are antiquated, toxic, difficult to administer and/or financially unattainable. The situation is further exacerbated by the development of drug resistance and there is a desperate need to identify new drug targets. Thus, *T. brucei* is a parasite of tremendous medical and economic importance and is recognized as a disease of poverty, as well as a cause of poverty.

T. brucei has a complex life cycle with phases in an insect vector and a mammalian host. Motility through fluids and on tissue surfaces in both instances is central to *T. brucei* development and disease pathogenesis. In the tsetse fly vector, the parasite must complete series of directional migrations to complete its development into a mammalian-infectious form [3]. In the mammalian host, trypanosome penetration of the blood brain barrier represents a critical and defining step of disease pathogenesis. African trypanosomes are extra cellular at all stages of infection and rely on their own flagellum for motility. In addition, since they move through at least two different hosts, they are faced with radically different environments and must accordingly adapt to these

environments. Thus, understanding mechanisms of flagellum-dependent cell motility and how these organisms interact with their environments is fundamental not only to building an understanding of parasite biology but also for the identification of targets for therapeutic intervention.

This thesis has focused on investigating how *T. brucei* moves through fluids and on surfaces. By dissecting the movement of the parasite in media of varying viscosities with and without the use high-speed imaging, we uncovered previously unappreciated complexities of *T. brucei* movement. These observations have changed the way we perceive the parasite's motility. In addition, our studies on the effects of surface-exposure on parasite motility revealed that these parasites engage in social behavior: an unprecedented finding in protozoan parasite biology. Investigation of the mechanisms underlying this social behavior have revealed a novel role for a unique family of proteins that were previously mostly uncharacterized. In addition, the search for extracellular factors that regulate this social behavior have led us to discover an unexpected cross-kingdom interaction between *T. brucei* and bacteria. In short, by using classic as well as novel laboratory techniques, the investigations presented here have uncovered novel aspects of *T. brucei* behavior that have transformed our understanding of protozoan biology.

History of African Trypanosomes

Although for centuries the people of regions of sub-Saharan Africa had been aware of nagana (a severe wasting disease in cattle) and sleeping sickness in humans, it was not until the efforts of David Bruce that the causative agent of these conditions became known. David Bruce was a bacteriologist hired by the governor of Zululand to investigate the cause of nagana. Through the application of Koch's postulate, in 1899 he became the first to identify a trypaonsome as the causative agent of nagana . This newly identified trypanosome became known as *Trypanosoma brucei*. Bruce went on to show that the tsetse fly acts as the insect vector for the parasite.

Trypanosomes, even at the time of David Bruce's discovery, were well known and are now considered to be one of the oldest lines of protozoa. Trypanosoma spp. (and Leishmania spp.) are placed in the order Kinetoplastida, family Trypanosomatidae, meaning that they have a single flagellum and a kinetoplast. The kineotplast is the hallmark feature of these organisms and is a self-replicating DNA-containing organelle adjacent to the mitochondrion. Because at some time in the life cycle trypanosomes invade the blood they are commonly referred to as hemoflagellates; all are parasitic. Hemoflagellates have the typical cell components of a eukaryote: nucleus, microtubules (cytoskeleton and flagellum), endoplasmic reticulum, Golgi apparatus and a single mitochondrion. In addition, they have another body, the kinetoplast, at the base of the flagellum. The African trypanosomes multiply by binary fission when in a non-arrested form.

Following David Bruce's work, several other discoveries were made including: the identification of *T. brucei gambiense* in a human patient, identification of trypanosomes in the blood and cerebral-spinal fluid of a patient with sleeping sickness, and the identification of *T. brucei rhodesiense*. These two subspecies of *T. brucei* cause two different versions of sleeping sickness in which the speed of disease progression is dictated by the particular subspecies of the parasite (see below).

Epidemiology of Sleeping Sickness

Sleeping sickness (also known as Human African Trypanosomiasis, or HAT) is caused by protozoan parasites of the genus trypanosoma and transmitted by the bite of the blood-sucking tsetse fly (of the genus Glossina). Sleeping sickness is now recognized as a neglected disease, and an estimated 60 million people are at risk of the disease throughout Sub-Saharan Africa (Figure 1-1) [4]. Sleeping sickness is almost always fatal if untreated and is a substantial cause of both mortality and morbidity in affected regions. Disease in humans progresses through two main stages, with mortality being associated with infection of the central nervous system during phase 2 of the infection (see detailed discussion below). Symptoms in patients manifest themselves in a variety of ways depending on the stage of the infection including: development of a large chancre, cyclical fever, and disruption of the sleep-wake cycle (see discussion below). In addition, because these parasites also infect livestock, thus they are not just a major source of

human morbidity and mortality, but also negatively impact sustained economic development in these regions [5].

Historically, in the past century sleeping sickness has occurred in the form of two large epidemics. Around the turn of the 20th century and when David Bruce's discoveries were made, sleeping sickness claimed the lives of hundreds of thousands of individuals within affected regions. Over the course of the next half-century, the disease was effectively controlled due to the execution of strong measures such as: mass animal culling, eradication of large forested regions of sub-Saharan Africa, advances in insect vector control and intensive human population surveillance for disease. In fact, by the 1960s, very few cases of sleeping sickness were reported [4].

After the 1960s, cases of sleeping sickness began to rise again as surveillance and vector-control became reduced due to war, famine, and the resultant socioeconomic instability. For the next three decades, the disease re-emerged to high levels and in 1998 the World Health Organization estimated that there were 300,000 cases of sleeping sickness [4]. In the past 10-15 years, efforts led by the WHO and other partner agencies, have led to advances in methods for detection of the disease and for the control of the tsetse fly. This has resulted in a sharp decrease in the amount of cases of sleeping sickness reported. By 2006, the WHO estimated that the number of cases of sleeping sickness had been reduced to 50,000 - 70,000. By 2009, fewer than 10,000 cases were reported. Although these improvements are impressive and significant, it should not go without mention that an unknown number of cases of sleeping sickness are probably not

reported or diagnosed for various infrastructural reasons and thus figures from the WHO or other surveillance agencies must be interpreted with caution. For example, in 2005 officials estimated that only 1 in 12 deaths due to sleeping sickness were actually reported [6]. In addition, due to the difficulty in accessing remote villages in rural regions, it is thought that many cases of sleeping sickness simply go undocumented. A study in 2007, for instance, estimated that just over half of all cases of sleeping sickness in the Democratic Republic of Congo went undiagnosed [7]. Lastly, there are increased cases of drug resistance to the few therapies used to treat the disease. Thus although the efforts from the WHO have led to promising results, we are still far from successfully controlling this lethal parasite.

Although the tsetse fly is limited to regions of sub-Saharan Africa, it should be noted that as westerners we are not immune from sleeping sickness. In fact, cases of sleeping sickness have been documented in Europe and the USA. Infection of Europeans and Americans usually affects travelers who have recently returned from sub-Saharan Africa. A recent detailed review noted that from 2000 - 2010 there were 94 cases reported in non-endemic countries [8]. Of these, 43% were diagnosed in Europe and 23% in the USA. In another study, sleeping sickness was reported in 83 travelers in non-endemic countries during 1998–2009 [9].

Life Cycle of *T. brucei*

As discussed previously, *T. brucei* has a complex life cycle with phases in an insect vector (the tsetse fly) and a mammalian host (Figure 1-2). A cursory summary of the life cycle is presented here with a more detailed examination of events within the insect vector and mammalian host to follow.

During the lifecycle of *T. brucei*, infection of a human by the parasite occurs following the bite of an infected, blood-sucking tsetse fly. After the bite, the parasite enters and spreads in the bloodstream, lymph nodes, and systemic organs. Parasites have been found to infect many organs during this early phase (or stage 1) of the disease including the spleen, heart, liver, eyes, and endocrine organs [6]. A painful, primary lesion called a trypanosomal chancre characterizes the site of the bite and appears between 5 and 15 days after the initial bite. Disease progression then depends on whether the patient is infected with *T. brucei rhodesiense* or *T. brucei gambiense*. In the case of sleeping sickness brought on by *T. brucei rhodesiense*, parasites cross the blood-brain barrier and enter the central nervous system within a few weeks. In the case of sleeping sickness cause by *T. brucei gambiense*, this event occurs a few months after the initial infection. Once the parasite enters the central nervous system (CNS), the disease has entered the late, or encephalitic, stage (also known as the CNS stage or stage 2). Pathologically, this stage of the disease is characterized by a meningoencephalitis, with extensive cerebral white matter infiltration with lymphocytes, macrophages, and plasma cells, marked perivascular cuffing with inflammatory cells, activation of both macrophages and astrocytes, and the appearance of morular (also known as Mott) cells, which are plasma cells containing IgM inclusions [10]. As mentioned above, the speed

with which the parasites cause disease and the duration of the two stages varies depending on wheter *T. brucei gambiense* or *T. brucei rhodesiense* is causing the disease. Whereas *T. brucei rhodesiense* infection causes an acute disease lasting several weeks if untreated, *T. brucei gambiense* infection typically lasts several months or even years [6].

Life in the mammal thus progresses through various stages and the parasite must interact with and adapt to varying environments. Life in the tsetse is no different, as the parasite must complete a series of specific migrations to different tissues within the tsetse in order for it to mature into a mammalian-infectious form. These events will be discussed in greater detail further below. For now, we will continue our discussion of sleeping sickness pathology in the mammal.

Pathology of Sleeping Sickness and Current Treatment

As discussed earlier, there are two forms of sleeping sickness, caused by two different subspecies of the African trypanosome, *Trypanosoma brucei*. A chronic and more common version of the disease found predominantly in West Africa is caused by *T. brucei gambiense*. A less common, acute version of the disease is caused by *T. brucei rhodesiense* in Eastern Africa. Although about 97% of cases of HAT are due to the *T. brucei gambiense* variant, the *T. brucei rhodesiense* variant, which causes a more acute and severe illness, is a hazard to travelers from Europe and the USA returning from visits to east African game parks [8,9]. Regardless of whether a patient's sleeping sickness is

cause by *T. brucei gambiense* or *T. brucei rhodesiense*, symptoms of HAT are described as occurring in early or late stages.

During the early-stage of the disease, symptoms tend to be non-specific and begin 1-3 weeks after the bite of an infected tsetse fly. Symptoms include headache, malaise, arthralgia, weight loss, fatigue, and intermittent fever with rigors. As the disease develops, patients might develop various features including lymphadenopathy; enlargement of the spleen, liver, or both; cardiac features such as myocarditis, pericarditis, and congestive cardiac failure; ophthalmological features such as iritis, keratitis, and conjunctivitis; endocrine dysfunction including menstrual abnormalities, impotence, alopecia, and gynaeco mastia; and fertility problems including sterility, prematurity, abortion, and stillbirths [6]. When posterior cervical lymphadenopathy occurs it is known as Winterbottom's sign, which is a typical feature of sleeping sickness caused by *T. brucei gambiense*.

During the late stage of disease a wide variety of symptoms can occur, with almost all regions of the nervous system potentially involved. Some of these symptoms include: mental disturbances, motor system disturbances, sensory system involvement, and abnormal reflexes [11]. In addition, ¾ of patients with sleeping sickness display deregulation of the sleep-wake cycle with nocturnal insomnia and daytime somnolence [12]. The deregulation of the sleep-wake cycle and the resulting symptoms give this disease its name.

Quick and reliable diagnosis of sleeping sickness is essential for therapeutic intervention as progression of the disease from the 1st stage to the 2nd stage severely limits the options for therapy. Accurate diagnosis of the disease is made difficult due to the lack of tools available to medical teams in the field as well as the difficulty in discerning the symptoms of sleeping sickness from other diseases prevalent in affected regions. Thin and thick smears of peripheral blood are reliable methods of detecting the parasite. Alternatively, lymph node aspirates can also be inspected for the presence of the parasite. Although more sophisticated methods exist for the detection of the parasites including the card agglutination test and polymerase chain reaction, they suffer from lack of accuracy or affordability (respectively) [13,14]. Once a patient is suspected to have progressed to stage 2, diagnosis is made by lumbar puncture and identification of parasites in the cerebral-spinal fluid.

Treatment of sleeping sickness depends on which stage the disease is at and whether the causing parasite is *T. brucei gambiense* or *T. brucei rhodesiense*. Once the parasite crosses the blood-brain barrier it becomes increasingly complicated to treat the infection and the drugs typically employed are much more toxic. Traditionally, the treatment of sleeping sickness has been unsatisfactory for many years. The four drugs traditionally used to treat the early or late stages of the diseases are unavailable orally, often toxic and sometimes ineffective [6].

Pentamidine is the first-line treatment for early-stage sleeping sickness caused by *T. brucei gambiense*. This drug was first used in 1940 and is usually administered by the

intramuscular route [14]. Although normally effective, pentamidine produces various side effects including hyperglycemia, hypoglycemia, hypotension, and gastrointestinal complications [14]. Late stage sleeping sickness caused by *T. brucei gambiense* is treated with eflornithine, an ornithine decarboxylase inhibitor. Interestingly, this drug was originally designed to combat cancer but was abandoned due to its inefficiency in treating cancer and high cost of manufacture. Due to efforts by the WHO and partners, intravenous doses of the drug were made widely available to patients in 2001 [15]. Recently, a combination of nifurtimox and eflornithine has been shown to be much more effective at treating *T. brucei gambiense*-induced sleeping sickness than eflornithine alone and has become the standard treatment for the 2nd stage of this disease. Although eflornithine is the first choice for treatment of stage 2 *T. brucei gambiense*-induced sleeping sickness, it has various drawbacks including that it is ineffective against *T. b rhodesiense* and can only be administered intravenously. Also, there are many side effects associated with the drug such as: bone marrow toxicity, alopecia, seizures, and gastrointestinal symptoms [16]. Lastly, there has been an increase in resistance to eflornithine.

Early-stage *T. b rhodesiense*-induced sleeping sickness is treated with intravenously administered suramin. This drug was first used in the 1920s and although usually effective it is associated with many side effects including: renal failure, skin lesions, anaphylactic shock, bone marrow toxicity, and neurological complications [14]. The only drug that is effective at present for treating late-stage *T. brucei rhodesiense*-induced sleeping sickness is arsenic-based drug melarsoprol. First used in 1949,

melarsoprol has to be given intravenously in propylene glycol because it has a very low solubility in water and cannot be delivered orally [17]. Although usually effective, melarsoprol use comes with serious drawbacks. To begin with, melarsoprol injections are extremely painful for the patient and the drug is very toxic, causing a post-treatment reactive encephalopathy in 10% of patients with death in half of these. The cause of the post-treatment reactive encephalopathy is not yet known, but it is accompanied by a rapidly developing coma, seizures or status epilepticus, and cerebral oedema. Aside from post-treatment reactive encephalopathy, melarsoprol treatment can also result in agranulocytosis, skin rashes, peripheral neuropathy, cardiac arrhythmias and a generalized inflammation [6]. In addition, resistance to the drug has been reported.

As seen from the discussion above, the long list of potentially fatal side effects caused by the current therapeutic options as well as the potential for the spread of drug resistant strains has resulted in a need for the development of new drugs and identification of new drug targets. The gross lack of development of alternative drugs is a result of a 50-year period of underinvestment by governments and the pharmaceutical industry into a disease for which treatment promised little or no prospect of financial returns. In the past decade, however, the situation has changed as a result of efforts by the WHO, non-governmental organizations, and partnerships between governments, universities, and the pharmaceutical industry. These efforts have resulted in the development of several more effective drug regimens and identification of new promising oral drug candidates [18]. A few promising drugs in the pipeline for the treatment of *T. brucei gambiense*-induced sleeping sickness include fexinidazole and oxaboroles [19,20].

In addition, investigators have been able to change the method of delivery of melarsoprol, resulting in an orally administrable version with reduced toxicity [6]. Lastly, advances in our understanding of *T. brucei* cell biology have led to the identification of several drug targets. Phosphodiesterases within the parasite flagellum, for example, have been found to be necessary for parasite viability and can be targeted by specific inhibitors [21,22]. Investigations in the next few years will prove critical in validating the efficacy of these newly identified compounds and in the identification of additional drug targets.

Although efforts to develop new therapeutics are increasing, there is still unfortunately no immediate promise for a vaccine against the parasite. The reason for this is the presence of variant surface glycoproteins (VSGs) on the surface of the parasite. VSGs are very interesting molecules that assist in the parasite's immune evasion of the mammal. Briefly, *T. brucei* has a large number of different genes for different VSGs in its genome [23], but expresses only one type of VSG at any given time. The parasite can then rapidly switch from the expression of one VSG to a different VSG. This process makes the generation of antibodies by the mammal's immune system to the parasite impossible as the pathogen is constantly switching the antigenic factor presented to immune cells. The immune system's response to *T. brucei's* VSGs and the periodic switching of these VSGs by the parasite result in the cyclical fevers characteristic of early stages of sleeping sickness and make vaccine design impossible at this time.

Life in the Tsetse

As previously mentioned, *T. brucei* spends part of its life in a mammalian host and another part in an insect vector, the tsetse (Figure 1-3). In these two different environments, the parasite is in constant contact with tissues and tissues surfaces. As discussed above, during a mammalian infection, parasites can be found in various host tissues and can penetrate tightly regulated areas such as the blood-brain barrier. Likewise, in the tsetse, parasites are in intimate contact with the insect's tissues. Interaction with these tissues and movement through and across their surfaces is well documented and essential for the parasite to mature into a mammalian-infective form. The study of the parasite within the tsetse has revealed many novel and surprising aspects of its biology.

As discussed earlier, David Bruce was the first to show that *T. brucei* was the causative agent of sleeping sickness and that the tsetse had a role in the transmission of the parasite. However, the first person to show that *T. brucei* must complete a phase of its lifecycle in the tsetse to infect a mammal was Kleine . Since the discovery that the tsetse acted as the insect vector for the parasite, investigators have noted that parasites derived from the insect vary greatly in their physical characteristics from those collected from human patients. In fact, the morphology of the parasite had been observed to vary not just between those present in a tsetse versus those in a mammal, but even between the different sections of the tsetse. It is now known that these changes in morphology by *T. brucei* are also accompanied by changes in the parasite's biochemistry [3,24]. These changes in morphology and biochemistry represent necessary changes that are made by the bug to adapt to the different environments within the tsetse. In addition, these

differentiation events are necessary for the parasite to adapt for life within the tsetse and then prepare for life with a mammal [3].

Upon entering the tsetse following a blood meal, *T. brucei* is channeled to the lumen midgut within the peritrophic matrix. To survive in this environment, the parasite must differentiate from its bloodstream form (present in the mammal) to a procyclic form (or insect-stage form). Along with visible changes in morphology, the parasite also undergoes metabolic changes resulting in adaptation from a glucose-rich environment in the mammal to a glucose-poor environment in the tsetse. In addition, differentiation into the procyclic form is accompanied with the shedding of the parasite's variant surface glycoproteins (VSGs). Following colonization of the midgut, invasion of the ectoperitrophic space occurs. Parasites from the midgut enter the ectoperitrophic space by passage through the membrane over the central two-thirds of the anterior midgut in the tsetse [3].

T. brucei then migrates up the ectoperitophic space to the tsetse fly's proventriculus where it colonizes the tissue. Parasite's *en route* to the proventriculus are longer and more slender than their midgut counterparts and are in G2 arrest until they reach the proventriculus [24]. Interestingly, once at the proventriculus and before migrating to the salivary gland, *T. brucei* undergoes an asymmetric division resulting in the formation of a long and a short cell referred to as the long or short daughter epimastigote (respectively) [24,25]. After migrating jointly to the salivary gland, the

longer daughter cell is thought to die off while the short epimastigote colonizes the salivary gland and differentiates to the mammalian-infective form of the parasite [26].

Once in the salivary gland, parasites are observed to fall in one of four categories: attached epimastigotes, premetacyclics, nascent metacyclics and mature free metacyclics [27]. Attachment of the parasite's flagellum to the tsetse salivary gland tissues is an integral part of *T. brucei's* maturation into an infective form [28]. The attachment occurs through an elaboration of the membrane of the flagellum [29] that forms junctional complexes with salivary gland epithelial cell microvilli. Attached epimastigotes are no longer cell arrested and actively divide. The differentiation of epimastigotes to premetacyclics occurs while the cells remain attached to the epithelium [29]. After this differentiation event, attached premetacyclics are characterized as nascent metacyclics until their release to form mature free metacyclics [29]. The free metacyclics are biochemically adapted for life within the mammalian host.

Although the migrations and differentiation events within the tsetse are well known and necessary for *T. brucei* maturation, not much is known about how the parasite interacts with its environment. In addition, although it is known that intimate contact between the parasite and tsetse tissues (such as those in the salivary gland) is necessary for specific events in the parasite's life cycle, it is not known how exposure to these surfaces impacts its behaviors(s) or what molecules mediate these interactions. Furthermore although the directed migrations within the tsetse (and within the mammal for that matter) are indications of tissue tropisms, it remains unclear what mechanisms are

employed by *T. brucei* to detect external signals. In other systems, the study of microbial movements in response to environmental signals has revealed much about how these organisms interact with their environments and themselves. In addition, the study of microbes on surfaces has revealed many complexities about their lives and has revolutionized the way we consider them (see below). Social behavior in response to surface exposure, for instance represents a vast and exciting field of research and the study of this behavior in pathogens has led to a new understanding of the diseases microbes cause how to treat them. Similar studies are needed in *T. brucei*. An understanding of how these parasites interact with their environments will undoubtedly be an important step in uncovering novel drug targets and further our understanding of how these parasites navigate through their hosts.

Microbial Social Behavior and *T. brucei*

As discussed above, *T. brucei* lives a complex life cycle through two hosts, the tsetse and a mammal. Although it is known that in both hosts the parasite interacts with host tissues and is constant intimate contact with these tissues, not much is known about the molecular mechanisms that mediate these interactions. Also, although the movements to specific tissues made by *T. brucei* within the tsetse imply tissue tropisms, we remain ignorant of what signal(s) might be mediating these tropisms. In other microbes, interaction with tissue surfaces and detection of extra cellular signals is successfully accomplished via the incorporation of social behaviors.

Social behaviors provide organisms in a community with a wide variety of advantages over solitary life. In metazoans, they are widely recognized to provide organisms with a means for communication and cooperation in a wide range of activities from navigation strategies and group hierarchies in insect communities to complex social networking in humans and other primates. At the cellular level, communication and cooperation among individuals in a group also occurs as can be noted in the collective motility of migrating cells during wound healing, tissue morphogenesis and tumor metastases. More recently, researchers have discovered that these social behaviors resulting in cell–cell communication and cooperation between individuals are not restricted to higher animals and are instead prevalent in and important to various microbial systems.

In microbes, social interactions give rise to multicellular groups having emergent behaviors that are not possible in single cells (Figure 1-4) [30]. For example, quorum sensing enables synchronization of gene expression and cellular activities to allow a population to act as a group [31]. Surface-associated behaviors such as biofilm formation and swarming motility allow microbes to establish communities with enhanced protection against external agonists and promote colonization and penetration of biotic and abiotic surfaces [32,33,34]. In addition, cell–cell signaling during sporulation in myxobacteria and slime molds directs group motility behaviors and developmental programs in which cellular differentiation gives rise to multicellular forms having distinct cell types with specialized functionalities, thereby enhancing survival through division of labor [35]. In

extreme cases, multispecies biofilms and microbial mats constitute complex microbial ecosystems where numerous microbes communicate, cooperate and battle with each other [36]. In any case, the goal is ultimately to enhance survival and proliferation of the organism and when the microbe is a pathogen, this has dire consequences for the host [31,33,34,37].

In the bacterial world, cell–cell communication is the rule and considering social behavior as a ubiquitous property of bacteria has transformed our view and understanding of microbiology. Social behaviors are also well documented in eukaryotic microbes [30,38]. However, despite the tremendous influence that the paradigm of 'sociomicrobiology' has had on our understanding of microbiology, one group of microbes, the parasitic protozoa, seem to have been left without an invitation to the party. Studies of these organisms generally consider them as individual cells in suspension cultures or animal models of infection, while social interactions are largely unstudied. Parasitic protozoa are etiologic agents of several major human maladies, including malaria, epidemic dysentery, Leishmaniasis and African sleeping sickness, that affect over half a billion people worldwide. Parasites also limit economic development in some of the poorest regions on the planet and are thus major contributors to the global human health and economic burden. Parasites have complex life cycles requiring transmission through multiple hosts, survival in diverse environments and a wide variety of cellular differentiation events. Hence, there are numerous facets of parasite biology that may benefit from, or may even depend upon, social interactions. A few examples follow.

Two types of processes of protozoan parasite life that would benefit from social behavior are interactions with environmental agents (including other cells) and coordination of population dynamics. In the mammal, parasites communicate with one another and their environment so as to successfully manage an infection and ensure their survival and transmission to a new host before exhausting the resources available in hand (Figure 1-5). In the mammal, the parasitemia of *T. brucei* is controlled partly via host immune defenses and from the differentiation of proliferating 'slender' form parasites into growth-arrested 'stumpy' forms [39]. Differentiation into non-dividing stumpy forms is irreversible in the bloodstream and premature commitment to this pathway would jeopardize maintenance of the infection [30]. Control is provided via a postulated quorum sensing-type system in which a soluble, parasite-derived 'stumpy induction factor' (SIF) accumulates as parasite cell density increases and triggers parasite differentiation only after a sufficient parasitemia has been achieved [40,41]. Stumpy-form parasites are pre-adapted for survival in the tsetse midgut, while slender forms are not. Thus, SIF-dependent slender-to-stumpy differentiation limits maximum parasite density in the mammalian host and simultaneously modulates parasite preparation for survival in the next host, optimizing probability of transmission. Although the specific nature of SIF remains unknown, recently investigators have developed a mathematical model using data from the study of *T. bucei* population dynamics during a mouse infection to suggest that SIF works via a quorum sensing mechanism [42]. Thus although components of traditional quorum sensing machinery have not been evaluated in *T. brucei* it is clear that the parasite is using some sort of social behavior to monitor and control its population density within a host.

T. brucei is not the only parasite that potentially uses social behavior to monitor and control population dynamics. *Plasmodium chabaudi* has been shown to adjust its sex ratio during infection [43]. During malaria, male and female gametocytes of the malaria parasites are produced in the mammalian bloodstream and taken up during a mosquito blood meal. Within the mosquito, gametocytes mature, then fuse and complete their life cycle in a series of steps that culminate in formation of infectious parasites in the mosquito salivary gland. The ratio of female to male gametocytes varies and is biased toward females. Although it is known that this sex ratio distribution contributes to parasite fitness and influences parasite evolution, the factors controlling it are unknown. In multicellular animals, gamete sex ratio distribution is governed by rules of social evolution theory, which predict that sex ratios are dictated by population diversity [44]. In essence, at low population diversity, female gametes outnumber males and as population diversity increases, the ratio of females to males decreases. Recently, investigators found that *Plasmodium* can adjust its sex ratio in response to the presence of unrelated genotypes in the parasite population. Thus, these studies indicate malaria parasites sense population diversity during an infection and adjust their behavior in response. In addition to resolving a longstanding question about *Plasmodium* biology, the studies offered a test of one of the basic tenets of social evolutionary theory, thus emphasizing another aspect of the value in applying social biology concepts to parasite biology.

Other aspects of parasite life that could benefit from social behavior are conflict, competition and cross-kingdom interactions. Wherever there is interaction among individuals, there is potential for conflict and competition. Bacteria engage in all manner of intercellular warfare and competition, ranging from growth inhibition and cytolysis of competing species, to bacterial cannibalism [30]. In an interesting case of sibling rivalry, neighboring colonies of *Paenibacillus dendritiformis* mutually inhibit each other's growth through secreted signaling molecules while growth inhibition does not occur in a single colony [45]. Interestingly, during mixed *T. brucei* infections in mice, mutual competitive suppression was observed between co-infecting *T. brucei* strains of varying virulence [46]. The authors report that mutual suppression of parasite growth in the host is correlated with extended host survival, suggesting that the less virulent strain reduces the pathogenic impact of the more virulent strain. The extent of mixed infections for *T. brucei* in the field is not known, but for some parasites, such as *Plasmodium*, the majority of natural infections are expected to involve multiple strains [47].

Social behaviors also facilitate cross-kingdom signaling in a variety of organisms. In microbial mats and multispecies biofilms, for example, microbes of different species compete for space and resources. In some cases, communication between these microbes results in symbiotic relationships while in other cases microbial warfare ensues [36]. Parasites are also in contact with cells from different kingdoms and these interactions influence their behavior. In the tsetse, for example, *T. brucei* is exposed to up to three different types of bacteria that can all reside within the insect [48]. Furthermore, studies

have shown that the success of a *T. brucei* infection in the insect correlates with the presence of one particular type of bacteria (see Chapter 6) [49].

Lastly, as previously discussed, exposure to surfaces results in the onset of several types of social behaviors in microbes including biofilm formation, swarming motility and hyphae formation. These behaviors assist in a wide variety of processes including increased resistance to environmental stresses and increased tissue penetration. As discussed earlier, *T. brucei* is in constant contact with host tissue surfaces. Although these interactions are well known and documented, little is known about how exposure to these surfaces affects *T. brucei* biology. One could very easily imagine that social behaviors could facilitate the parasite's ability to colonize tissues and resist external antagonists as occurs in other microbes.

Although social behaviors occur in most domains of life and are extremely variable in their manifestation, the underlying mechanisms employed by organisms to mediate them are universal and in general require organisms to: 1) sense external signals, 2) convert the signal(s) into a response and 3) cooperate with neighboring organisms to coordinate the community's efforts. The study of social behaviors in other organisms has uncovered novel pathways microbes use to sense their environments. As discussed above, although it is clear that *T. brucei* and other parasites could benefit from social behavior, it is unclear how they would accomplish it. When searching for potential molecules that could play a role in the regulation of a social behavior in *T. brucei*, a good place to start would be the parasite's flagella. As will be discussed below, the flagella is not only

required for motility of the parasite, but has also been implicated as a sensory organelle [50].

In summary, social behaviors provide microbes with numerous advantages. Although these behaviors are well studied in other systems, protozoan parasites remain unevaluated for social behaviors. Protozoan parasites have complex life cycles often in multiple hosts and are in constant contact with tissue surfaces and other microbes and could most certainly benefit from the advantages offered by social behavior. The study of social behaviors in protozoan parasites, especially those as amendable to laboratory techniques as *T. brucei*, could shed light on how these and other organisms accomplish biologic processes and cause disease.

The dual roles of the *T. brucei* flagella

As discussed earlier, *T. brucei* can reside in at least two different hosts. In both hosts the parasite is intracellular and must be able to move independently and sense external signals in order to migrate to certain tissues and escape immune cells. The eukaryotic flagellum/cilium is the perfect organelle to accomplish sensation and motility. As a mechanical component, the *T. brucei* flagellum propels the trypanosome. As a sensory organelle, it can potentially serve as a signaling center for the detection of external signals.

The *T. brucei* cell body has a vermiform shape with tapered ends. A single flagellum emerges from the basal body near the posterior end of the cell (Figure 1-6). The flagellum is surrounded by its own membrane and is attached along its length to the cell body, tracing a left-handed helical path from posterior to anterior, with the distal end extending a short distance beyond the cell body [51]. Within the flagellum are a canonical 9 + 2 microtubule axoneme and a lattice-like paraflagellar rod (PFR).

As a mechanical organelle, the *T. brucei* flagellum is the source of motility for the parasite. In fact, the flagellum plays such a prominent role in the motility of the parasite that the genus name *Trypanosoma* is derived from the Greek words *trypanon* and *soma* that were used to describe the auger-like movement noted by the parasite. This auger-like motion is due to the tip-to-base spiral waveform of the flagellum, which wraps around the cell body. Until recently, little was known about the molecular mechanisms of flagellar beat in *T. brucei* and this knowledge was based almost exclusively on analogy to other organisms. African trypanosomes are highly motile and have been described to move at speeds of up to 20 μm/s in media and even greater in viscous media [52]. The details of *T. brucei* motility are discussed in the subsequent chapter. For now, it is important to note that the flagellum acts as the sole source of motility for the parasite. This becomes increasingly significant when one considers that the parasite moves through various locations in the hosts. In the tsetse, these migrations are particularly important, as migrations to specific tissues are needed for maturation of the parasite into a mammalian-infective form. Thus as a mechanical component the flagellum is indispensable.

As discussed above, to be successful, trypanosomes must integrate extra cellular signals to direct parasite movements and developmental transformations within specific host compartments. While most evident in the tsetse, signaling also operates in the mammalian host, where autocrine signaling stimulates transformation of proliferating long-slender to nonproliferating short-stumpy forms [41]. In addition, penetration of the blood brain barrier involves paracrine signaling between parasite and host. For many years now, the eukaryotic flagellum has been a well-known sensory organelle [53]. Molecules on the cilia of microbes such as *Chlamydamonas reinhardtii* and *Caenorhabditis elegans*, for example, assist in the detection of photo and chemo signals (respectively). Also, ciliar-specific receptors on the photoreceptors and olfactory systems of mammals are essential for sight and smell (respectively). Thus the *T. brucei* flagellum is thought to be a major player in sensation by the parasite. The recent publication of the blood stream flagellar-membrane proteome [54] and our current efforts to isolate proteins from the procyclic form flagellum will aid greatly in identification of potential receptor molecules.

T. brucei **as a model system**

As can be gathered from the discussion above, *T. brucei* is a dynamic organism with a complex life. Due to the molecular architecture of this parasite, clinical importance and relation to other microbes, the study of this organism can profoundly impact our understanding of other organisms. As a model system, trypanosome biologists benefit

from having a great molecular tool kit to work with. To begin with, both major versions of the life cycle (the bloodstream and procyclic forms) can be cultured *in vitro* [55]. In addition, the publication of the genome has greatly facilitated the search for and study of genes of interest. Also, a great deal of reverse genetic approaches and cell culture assays exist and we can often generate and evaluate the localization and function of a protein within a few weeks [56]. Lastly, improvements to current techniques have also led the facilitation of forward genetics approaches using genetic or proteomics approaches [55]. Researchers in the field are constantly pushing the envelope on what can be done with these little critters.

As such, *T. brucei* has emerged as a model organism for many biological processes and for the study of different organelles. Of particular interest to our lab has been the use of the *T. brucei* flagellum not just as a platform from which to study parasite behaviors and functions but also as a model for the study of ciliary proteins found in other less accessible systems. Genetic screens, biochemical, genomic, and proteomic analyses in several organisms have led to the identification of hundreds of flagellar and putative flagellar proteins. A key challenge now is to determine where and how these proteins function individually and collectively to drive flagellar motility and other flagellum functions. In addition to its relevance to public health and economic development in some of the poorest places in the world, the molecular tools available in *T. brucei* make it an excellent experimental system in which to study flagellum protein function.

I have spent the past few years exploiting the molecular tools available to parasitologists in my study of *T. brucei*. Through the use of these tools in addition to the generation of novel assays, I have combined old and new techniques to uncover previously unappreciated aspects of *T. brucei* motility in liquid and on semi-solid surfaces. To begin with, along with a team of researcher led by Rodriguez et al., we have for the first time, described a detail description of trypanosome motility that is unique to this parasite [57]. In addition, with the assistance of Hill lab members, we have generated an assay to study *T. brucei* on semi-solid agarose plates. These studies were the first to show that protozoan parasites engage in social behaviors [58]. Through the incorporation of proteomics, we have also identified and characterized a family of potential receptors found on the *T. brucei* procyclic flagellum. Investigation of the function(s) of these proteins revealed a mechanism for the regulation of social behavior in *T. brucei* and unveiled previously unknown qualities of these proteins. Lastly, the study of *T. brucei* social behavior has revealed a previously unstudied and unanticipated cross-kingdom interaction between the parasite and bacteria. Work presented here has not only revolutionized the way we perceive these parasites but will also serve as a launch pad from which other studies will take off.

Figures

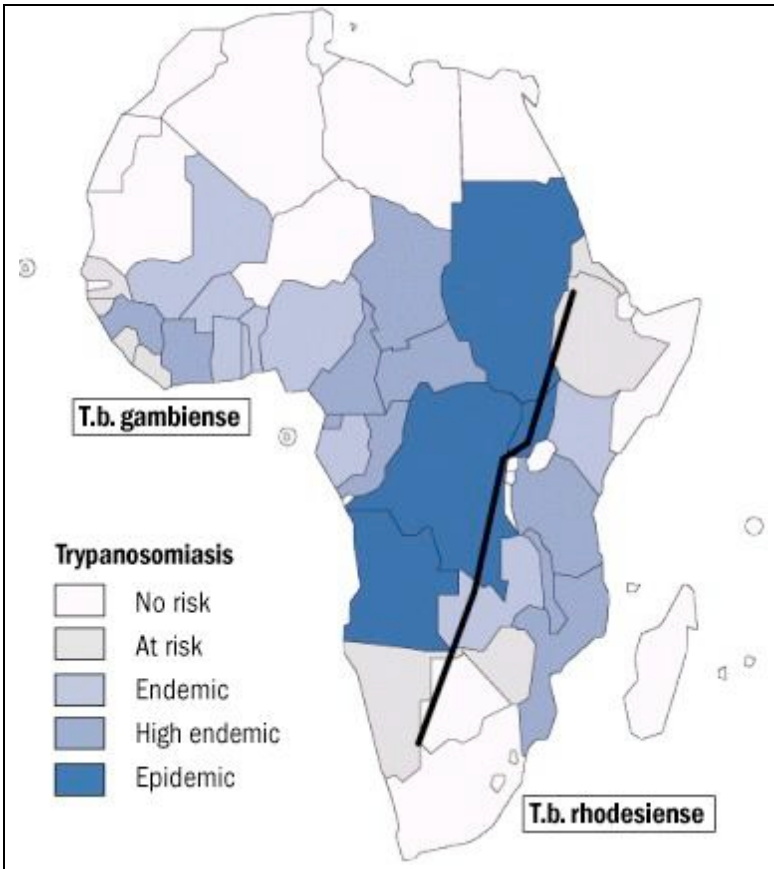

Figure 1-1. Sleeping sickness if found throughout regions of sub-Saharan Africa. This map shows the geographic distribution of the disease as well as the level of endemicity of infection. The black line serves to roughly divide regions where disease is caused by *T. brucei* gambiense vs *T.* brucei *rhodesiense*. From (http://www.infectionlandscapes.org/2011/04/trypanosomiasis-part-1-sleeping.html)

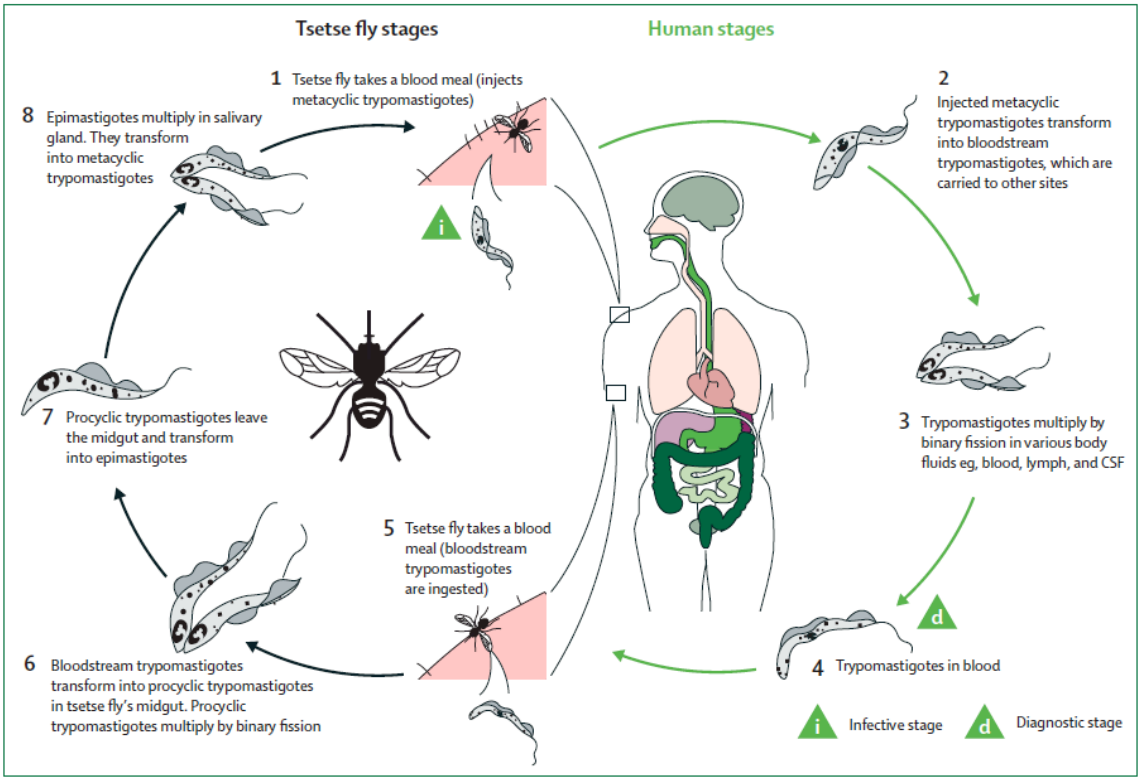

Figure 1-2. The lifceycle of African tyrpanosomes proceeds through two hosts, the insect and the mammal. Reproduced with permission from [6].

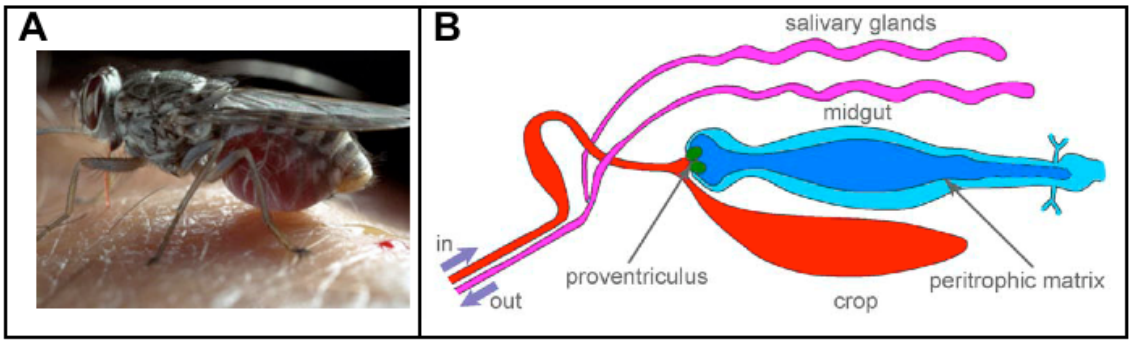

Figure 1-3. A) Tsetse fly taking a bloodmeal. B) Cartoon of the internal organs of the tsetse fly. *T. brucei* initially inhabits the endoperitrophic space of the midgut before moving to the ectoperitrophic space. From there the parasite moves to the proventriculus. *T brucei* then makes its way to the salivary glands where it adheres to epithelial cells to mature into a mammalian-infectious form. Reproduced with permission from [3]

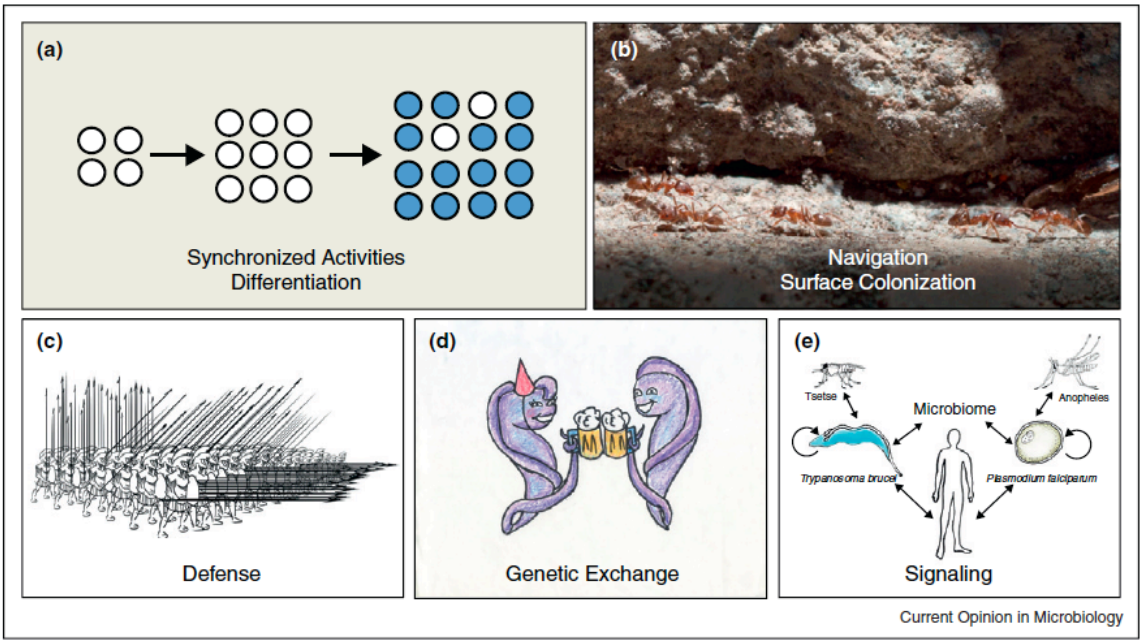

Figure 1-4. Social behaviors offer organisms a variety of advantages including: A) synchronization of group activities, B) colonization of surface tissues and navigation through host compartments, C) group defensive strategies for protection aainst external antagonists, D) opportunity for genetic exchange and E) facilitation of inter-kingdom signaling. Reproduced with permission from [30]

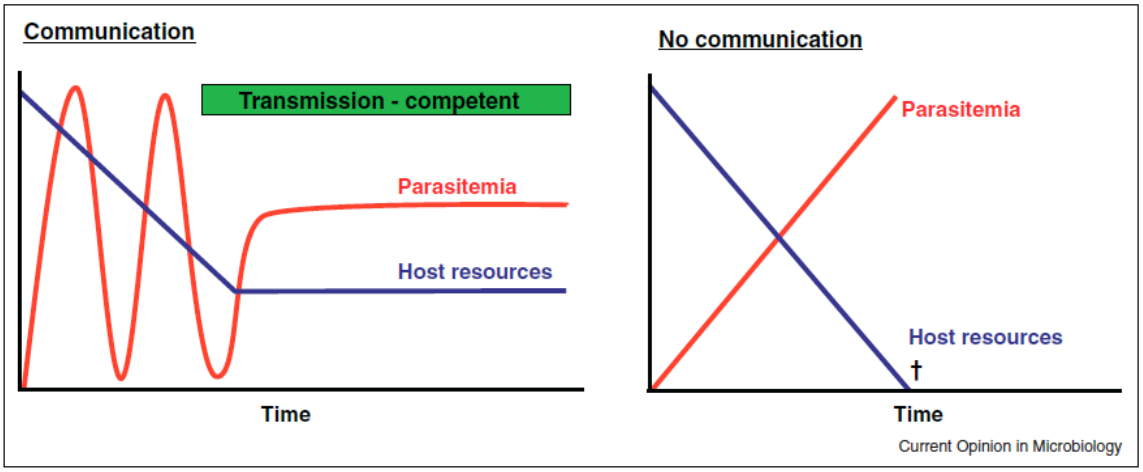

Figure 1-5. Cell–cell communication benefits *T. brucei*. Parasite–parasite communication (chart on left) via cell density-dependent signaling controls *T. brucei* differentiation from proliferating forms that are adapted for survival in the bloodstream to growth-arrested, transmission competent forms that are adapted for survival in the tsetse vector. By linking differentiation to population density, the parasite avoids depletion of host nutrients and prevents premature commitment to a developmental form that is not optimized for survival in the mammalian host. Without density-dependent cell–cell communication (chart on right), continued parasite proliferation would deplete host resources and thus reduce chances for transmission. Reproduced with permission from [30]

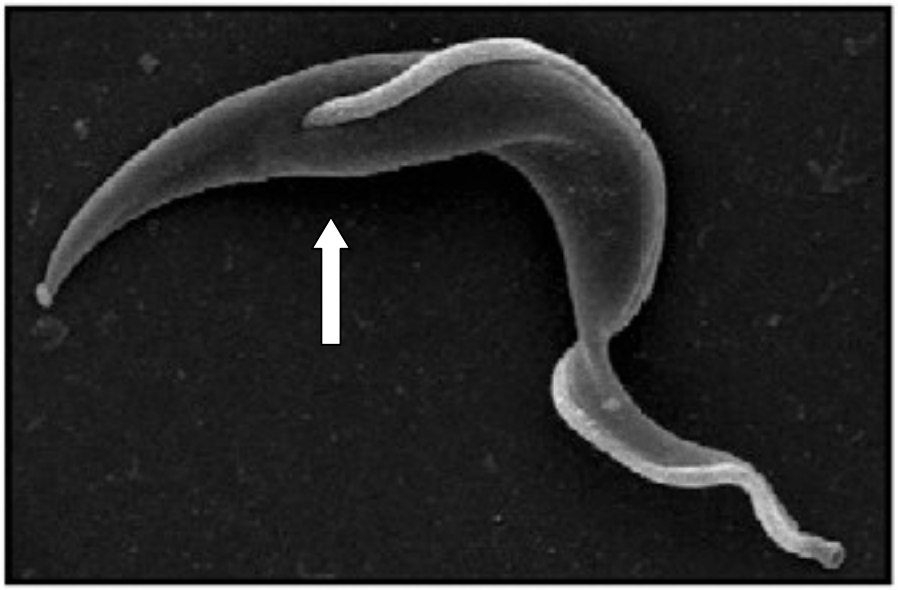

Figure 1-6. Electron micrograph of a procyclic *T. brucei* cell from suspension culture. The *T. brucei* cell body has a vermiform shape with tapered ends. A single flagellum emerges from the basal body near the posterior end of the cell (white arrow). The flagellum is surrounded by its own membrane and is attached along its length to the cell body, tracing a left-handed helical path from posterior to anterior, with the distal end extending a short distance beyond the cell body. Reproduced with permission from [59]

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Chapter 2

Characterization of *T. brucei* Motility Using High-Speed Video Microscopy and The Effects of Viscosity on Parasite Motility

The work presented in this chapter was a joint effort by many members from the labs of Dr. John Miao (UCLA Department of Physics), Dr. Manuel Penichet (UCLA Department of Surgery), Dr. Robijn Bruinsma (UCLA Department of Physics) and Dr. Kent Hill (UCLA Departments of Microbiology and the Molecular Biology Institute). This work was published in PNAS [1]. My specific contributions as the second author in the publication of these findings were the *in vitro* culture of PCF and BSF *T. brucei*, live cell imaging using the high-speed camera, generation of fluorescent cell lines for confocal microscopy and writing of specific sections of the manuscript. In addition, I observed that increased viscosity results in increased velocity by trypanosomes. This observation is in-line with that observed for other microbes such as the spirochetes that use a rotation-based motility to more effectively navigate through host tissues of high viscosity. The text and figures from [1] are provided below and the original paper is provided as Appendix B.

Introduction

The protozoan parasite *Trypanosoma brucei* is the causative pathogen of African sleeping sickness, a fatal disease indigenous to sub Saharan Africa where 60 million people are at risk of infection [2,3]. *T. brucei* is transmitted between human hosts by a tsetse fly vector, and parasite motility is important in both hosts. In the tsetse fly,

procyclic-form (PCF) parasites colonize the midgut and then migrate through the alimentary canal to the salivary glands, where maturation into human infectious forms occurs [4,5].

From the salivary gland, mature parasites can be injected into the blood of a mammalian host that has been bitten by the fly. In the mammalian host, migration of bloodstream-form (BSF) parasites through the blood-brain barrier initiates onset of the fatal course of the disease [6,7]. *T. brucei* is extra cellular at all stages of infection and depends on its own flagellum-mediated motility for dissemination. Flagellar motility of *T. brucei* in various environments is believed to be central not only to host-parasite interaction, but also to cell division, morphogenesis, and development [5,6,8,9,10,11,12,13,14,15,16].

The *T. brucei* cell body is roughly 20 μm long, with a relatively large posterior section tapering off into a long, narrow anterior section. It has a single flagellum, with the classic ‘‘9+2’’ microtubule axoneme architecture that is attached to the cell body along its length. Based on microscopy studies, it is believed that propulsion of *T. brucei* proceeds by left-hand (LH) helical waves propagating along the flagellum, from tip to base, driving the cell forward in a drill-like motion (Figure 2-1) [8,17,18]. The genus name of the parasite actually derives from this distinctive motility (from the Greek trypanon or auger, and soma or body), first described in 1843 [19]. Here I describe experiments done in collaboration with the lab Dr. Jianwei Miao from the Department of Physics at UCLA in which we used millisecond resolution differential interference contrast (DIC) microscopy, combined with other microscopy methods, to provide a quantitative analysis of *T. brucei* cell propulsion. Our results revealed that *T. brucei*

forward motility is characterized by the propagation of kinks separating helical waves of alternate chirality.

Results

Helical Waves with Alternating Chirality.

To investigate *T. brucei* propulsion in standard culture conditions, we used high-speed DIC microscopy with a millisecond timescale (Appendix B). The millisecond frame-by-frame analysis revealed that cell propulsion of *T. brucei* is characterized by repeated reversals in the rotation direction of the flagellum tip, which produced helical waves of alternating chirality propagating tip to base (Figures 2-1B and Appendix B). The image sequence in Figure 2-2A (Top) shows such a bihelical wave in a PCF cell having RH chirality at the tip and LH chirality near the base of the flagellum. At a later time, this same cell initiated a beat with opposite chirality (see Figure 2-2A Bottom): that is, exhibiting LH chirality at the tip and RH chirality near the base of the flagellum. We quantified the frequency of LH and RH helical waves by tabulating how many times the flagellum tip flipped to initiate a wave in either direction. The average frequency, calculated from five cells, was 19$\pm$3 flips per second, with each flip representing a rotation of 180°. This was split approximately equally between LH (9.7$\pm$1.3 flips/s) and RH waves (9.0$\pm$2.0 flips/s), as illustrated by the representative example shown in Figure 2-3A. Typically, no more than three successive waves with the same chirality are

generated at the flagellar tip. Thus, there appears to be no systematic bias for LH or RH chirality in the motility dynamics.

Next, we examined BSF *T. brucei* cells to determine whether helical waves with alternating chirality are a shared feature of both life-cycle stages. The millisecond DIC images clearly demonstrate bihelical waves in the flagellum of BSF cells (Figure 2-2B and Appendix B). The image sequence in Figure 2-2B shows a BSF cell monitored over a 70-ms time period. The top panels show a bihelical wave with LH chirality at the tip and RH chirality near the base of the flagellum. As this wave propagates toward the flagellum base, a new RH helical turn is initiated at the flagellum tip, producing a wave with RH, LH, RH chirality from tip to base (Bottom).

Kinks

A segment of a filament connecting two helical segments of opposite chirality is known as a ‘‘kink.’’ We define a ‘‘plus’’ kink as one separating an anterior RH helical wave from a posterior LH helical wave, while a ‘‘minus’’ kink separates an anterior LH helical wave from a posterior RH helical wave. A kink separating two traveling helical waves, as in Figure 2-2A, will itself travel along the filament. Traveling kinks have been encountered earlier in motility studies of prokaryotes, such as *Escherichia coli* [20,21,22], where they appear to be associated with changes in course. In *Spiroplasma*, which do not have flagella [20], pairs of kinks traveling along the helical cell body cause the cell to swim in a zig-zag path. A theoretical study of *Spiroplasma* motility [23]

proposed that recoil against the motion of fluid carried backwards by traveling kinks actually is the propulsive mechanism of *Spiroplasma*.

Well-defined kinks could be observed in the millisecond DIC images of both PCF and BSF *T. brucei* cells (see the gray arrow in Figure 2-1B, Figure 2-2 and Appendix B). The kinks propagated down the flagellum along the cell body from tip to base (see Figure 2-2, and Appendix B), opposite to the direction of cell propulsion. Typical kink propagation velocities in *T. brucei* were 85±18 μm/s in PCF cells and 136±7 μm/s in BSF cells, more than an order of magnitude higher than the center-of-mass velocity of the cells (Figure 2-3B and Appendix B). The observation that a 1.6-fold increase of kink velocity in BSF cells versus PCF cells correlates with a 1.6-fold increase in the center-of-mass velocity of BSF versus PCF cells (see Figure 2-3B) suggests that kink motion is intrinsic to the propagation mechanism.

To further confirm the existence of helical waves and kinks in *T.brucei*, we used scanning electron microscopy (SEM) combined with a rapid-fixation technique that was optimized to preserve flagellar waveforms [24]. SEM images of rapid-fixed cells indeed revealed bihelical waves and kinks in both PCF and BSF cells (Figure 2-4A and B). Interestingly, SEM images indicate that the cell body is subject to torsional strain, possibly generated by the flagellum. At the flagellar pocket where the flagellum emerges from the cytoplasm, there is a preferred LH chirality for the flagellum, consistent with earlier studies [25]. As an independent test, we labeled the flagellum of PCF cells with a PFR2-GFP fusion protein and imaged these cells by three-dimensional (3D) confocal

microscopy. In a representative 3D image (Figure 2-4C and Appendix B), the flagellum (green) wraps around the surface of the cell body (red) and exhibits RH chirality near the base and LH chirality near the tip, forming a minus kink (white arrows).

Cell Body: Configurational Changes, Viscous Drag, and Torsional Stress.

Arrival of kinks at the posterior end of the cell appears to correlate with transitions of the main body of the cell between two dominant configurations (see Figures 2-1B and 2-2, and Appendix B). The configurational changes take place through alternating clockwise and counterclockwise rotations of the posterior end. In other words, the posterior end rocks back and forth about its own axis rather than completing full 360° rotations. The average frequency of the rocking motion of the posterior end was 5$\pm$3 flips per second (calculated from five cells and 34 individual flips), compared to 19$\pm$3 flips per second at the anterior end. The fact that the rotation frequency decreases significantly along the body of *T. brucei* is interesting. Different sections of a filament that supported a helical wave with a frequency gradient would, over time, be rotated with respect to each other over arbitrarily large angles, which for *T. brucei* would not be consistent with the mechanical integrity of the cell body. The reversals in rotation observed in *T. brucei* are thus necessary to maintain the frequency gradient.

The observation that the smaller anterior end of the body performs high-frequency complete rotations, while the larger posterior end only performs a low-frequency rocking motion, suggests a rationale why this may be an efficient mode of propagation for a

microorganism with the asymmetric cell structure of *T. brucei*, given that at low Reynolds number viscous forces dominate [26]. Note that a purely reciprocal motion (i.e., one that is symmetric under time reversal) cannot provide a net propulsive force to a microorganism, so the reciprocal rocking motion of the posterior end of *T. brucei* could not contribute a net propulsive force. On the other hand, the sequence of kinks traveling from the anterior to the posterior end separating helical sections with opposite chirality and opposite rotation direction obviously is nonreciprocal, and could therefore contribute a net propulsive force. Next, it follows from elementary hydrodynamics that a tapering cylindrical body rotating around its axis in a fluid is subject to a retarding viscous torqueper-unit length (τ_R) exerted by the surrounding medium that resists the rotation. This torque-per-unit length at a given point along the body is proportional to the local cross-section:

$$\tau_R = - 4\pi\eta R^2_B\omega_B$$

where η is the viscosity of the surrounding medium, R_B the radius of the cross section of the cell body at that point, and ω_B the rotation rate of the body. If, for example, we model the main body of *T.brucei* as a cylinder with a length L of 20 μm and a (constant) radius of 1 μm, then the power dissipated by viscous loss in water at the 20-Hz rotation rate of the tip would be substantial: about 104 times the ATP hydrolysis energy per second. Reducing this rotation frequency by a factor of four—the typical frequency reduction factor between posterior and anterior ends of *T. brucei*—reduces the power dissipation by more than an order of magnitude because power is proportional to the square of the

rotation frequency. For a case in which the radius of the cell body near the anterior end is about five times less than that near the posterior end, a reasonable estimate for *T. brucei*, the viscous torque-per-unit length near the anterior end is about 25 times less than that near the posterior end, thus roughly compensating for the frequency increase of the anterior end. If the flagellum produced helical waves of uniform chirality, then the posterior and anterior ends of the cell would necessarily rotate at the same ‘‘global’’ rate, which would be slowed greatly by the viscous torque on the cell body near its larger posterior end. The frequency gradient thus allows for high rotation rates at the smaller anterior end, which provides the traction force that pulls the cell body along, combined with reduced rotation rates toward the larger posterior end, which reduces viscous drag. Additionally, because the flagellum is attached laterally along the length of the cell body, a helical wave of the flagellum necessarily applies a torsional stress to the cell body, as also suggested by SEM images (Figure 2-4). Depending on the degree of viscoelasticity of the cell body, the cell body may well be able to store a significant amount of torsional elastic energy in reversible deformation of the microtubule cytoskeleton. This elastic energy could then be released when the flagellum started to rotate in the opposite direction, much like the rubber motor of a toy plane.

Rapid Swings of the Flagellar Tip of BSF Cells

Despite overall similarity, there is an interesting difference between the motilities of BSF and PCF cells. The anterior end of BSF cells exhibits more frequent and more pronounced movements than that of PCF cells under similar conditions (Figure 2-5A and

B). Rapid swings of BSF flagellar tips were observed following large angle bends of this portion of the flagellum (Figure 2-5C), a feature absent from PCF cells. To quantitatively analyze the rapid swings of the flagellar tip, we extracted 12 image sequences from two BSF cells undergoing fast forward locomotion. Each of the 12 trajectories (Figure 2-5D) represents the distance traveled by the flagellar tip in each image sequence plotted as a function of the time elapsed (a linear fit of the average trajectories is shown as a dashed line). The slope of each trajectory corresponds to the velocity. The highest recorded velocity of the flagellar tip is 673 μm/s, while the average velocity (i.e., the slope of the dashed line) is 510 μm/s (see Appendix B for details). Note that these observations could not have been made with conventional video frame rates, suggesting that millisecond DIC microscopy could be a useful tool for exploring flagellar and ciliary motility in other organisms [27,28,29,30].

BSF Motility in Infected Mouse Blood

Our studies of *T. brucei* motility so far were carried out in standard culture medium used for most studies of trypanosome motility, which has a measured viscosity close to that of water (0.95cSt). In contrast, the viscosity of human blood is about 20% higher than that of water. What is perhaps most relevant is that in blood, the dense distribution of erythrocytes presents to *T. brucei* a highly inhomogeneous, although deformable, labyrinth that it must negotiate. Typical sizes of the free spaces of this labyrinth can be small compared to the length of a single BSF cell. To examine *T. brucei* motility in a more native medium, we investigated BSF cells in whole blood obtained

from infected mice, 3 to 5 days after infection. Millisecond DIC images of actively swimming BSF *T. brucei* in infected mouse blood revealed the same motility motifs observed with cultured parasites (Figure 2-6A and B, Appendix B). Rapid swings of the flagellar tip were particularly striking, as they can be observed initiating contact with host blood cells and deforming them significantly (Figure 2-6C). Comparison with the measured force-deformation curves of erythrocytes [31] indicates that the flagellum is capable of exerting forces in the 300 pN range (see Appendix B).

Impact of Viscosity on Parasite Motility

As explained above, although most studies consider *T. brucei* motilty in suspension media that has a viscosity close to that of water, the parasite resides in the blood and fluids of a mammal that can have viscosities much greater than that. In addition, the parasite can penetrate and move within tissues of different densities. Other organisms that employ a rotation-based motility do so because it offers an advantage in viscous environments over other methods of motility. Spirochetes, for example have noted to move faster as a function of viscosity. To examine how viscosity impacts *T. brucei* motility, I looked at the average swimming velocity of PCF and BSF stage parasites in media with 0.25% or 0.5% methyl cellulose. Media plus 0.5% methyl cellulose was observed to have a viscosity of 12.47 cSt, which is more than 12 times that of water. Interestingly, the average velocities exhibited by PCF and BSF in these conditions increased as a function of viscosity (Figure 2-7). These results demonstrate that indeed the rotation-based motility of the parasite provides an advantage in

environments with increased viscosity and helps explain how these parasites remain such prolific swimmers in conditions that normally slow microbes down.

Discussion

Using millisecond DIC microscopy, supplemented by SEM and 3D confocal microscopy, we have found that forward *T. brucei* motility is characterized by (i) tip-to-base propagation of kinks separating LH and RH helical waves, (ii) very high motility of the flagellum tip in BSF cells, and (iii) a pronounced rotation-frequency gradient along the cell body, with the anterior end performing rapid full rotations while the posterior end rocks back and forth more slowly. This specialized form of motility appears to reduce viscous dissipation by minimizing rotary motion of the large posterior end of the cell, while allowing *T. brucei* to negotiate complex viscous environments, such as mammalian blood. Furthermore, this rotation-based movement appears to offer the parasite an advantage for increased motility in these environments.

As noted earlier, the closest resemblance between the motility of *T. brucei* and that of other microorganisms is the prokaryote *Spiroplasma* [20,32,33]. A comparison between these two widely divergent organisms is instructive. Both move in a zig-zag pattern, as kinks between waves of opposite chirality travel from the anterior to the posterior end. One can apply the theoretical analysis of kink motion in *Spiroplasma* [23] to *T. brucei* to argue that, also for *T. brucei*, the propulsive mechanism should be recoil

against the motion of fluid carried backwards by the traveling kinks. However, whereas in *Spiroplasma* there is a preferred timing difference between kinks, which is consistent with the theoretical analysis, we encountered a broad distribution of timing differences, ranging between 150 and 300 ms. In addition, typical kink velocities of *T. brucei* (Figure 2-3B) are about an order of magnitude larger than that of *Spiroplasma* (about 10 μm/s), which may be because of the fact that *T. brucei* can recruit the efforts of large numbers of dynein molecular motors distributed along the axoneme (see below), whereas *Spiroplasma* presumably can rely on only a few motors. Interestingly, despite the higher kink velocity in *T. brucei*, the center of mass velocities of the two organisms is similar (in the range of 10μm/s). This presumably reflects the larger viscous dissipative losses and also the larger mass of *T. brucei*, which by momentum conservation reduces the forward recoil velocity against the backward movement of fluid carried by the kinks. A key difference between *T. brucei* and *Spiroplasma* morphology is that *T. brucei* has a pronounced gradient in its body plan, while the body plan of *Spiroplasma* is so well described by a uniform helix (in the absence of kinks) that it is difficult to distinguish the anterior and posterior ends. In terms of motility, this translates into a uniform rotation frequency for *Spiroplasma* but a pronounced frequency gradient for *T. brucei*.

The eukaryotic axoneme is one of the most conserved structures in biology and was likely present in the last common ancestor of all extant eukaryotes [34]. Axoneme motility is mediated by thousands of dynein motors that drive sliding and, ultimately, bending of microtubule doublets in the axoneme [35,36]. The switch point hypothesis is a generally accepted paradigm for wave propagation along the axoneme [37,38]. At its

most basic, this hypothesis posits that axonemal dyneins are divided into two opposing groups, on either side of the axoneme, and that these groups are alternately activated or inactivated to cause axoneme bending in one direction or the other, thereby producing a plane wave. It has been demonstrated theoretically that arrays of coupled motor proteins subject to an external load can indeed switch collectively between two alternate directions of motion [39]. Similarly, helical waves could be generated by assuming that the dynein motors also apply a rotary twist on each pair of microtubules of either chirality, thereby imposing a net twist on the cylindrical array of all nine outer doublet microtubules that would turn the plane wave into a helical wave, as has been proposed for waves in cilia [40]. In such a model, the frequency of rotation would not be fixed but determined by the local, external load on the flagellum, determined in turn by the local radius of the flagellum. Because either helicity would be possible, there would now be dynamic instead of structural stability. Collective switching between these two helicity states, similar to the switch-point hypothesis, could then produce an array of moving kinks.

In summary, through quantitative and theoretical analysis of *T. brucei* motility, our results offer insights for considering propulsive mechanisms of microorganisms and provide new detail on an important, yet poorly understood, feature of trypanosome biology.

Materials and Methods

Please see Appendix B

Figures

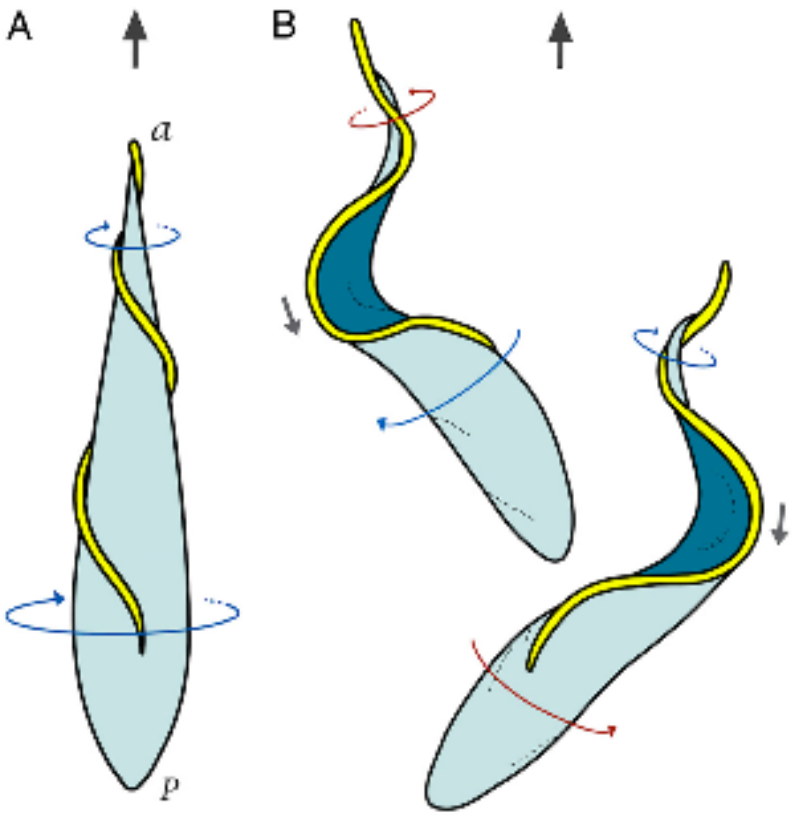


Figure 2-1. Models of forward motility for *T. brucei* in aqueous media. (*A*) The traditional model: propulsion is caused by helical waves propagating from the tip to the base of the flagellum with LH chirality, resulting in a drill-like motion of the cell body (‘‘*a*’’ and ‘‘*p*’’ represent the anterior and posterior end of the cell). (*B*) The bihelical model in which alternating LH and right hand (RH) helical waves propagate down the flagellum separated by a kink. The flagellum of the bottom cell exhibits a LH helical wave (*blue arrow*) at the tip and a RH helical wave (*red arrow*) near the base, separated

by a ‘‘minus’’ kink (*gray arrow*). The flagellum of the top cell shows a RH helical wave at the tip and a LH helical wave near the base, separated by a ‘‘plus’’ kink. Kinks propagate in a direction opposite to that of cell propulsion. Two dominant cell-body configurations are associated with the propagation of kinks, with the cell body rocking back and forth between the two alternating configurations.

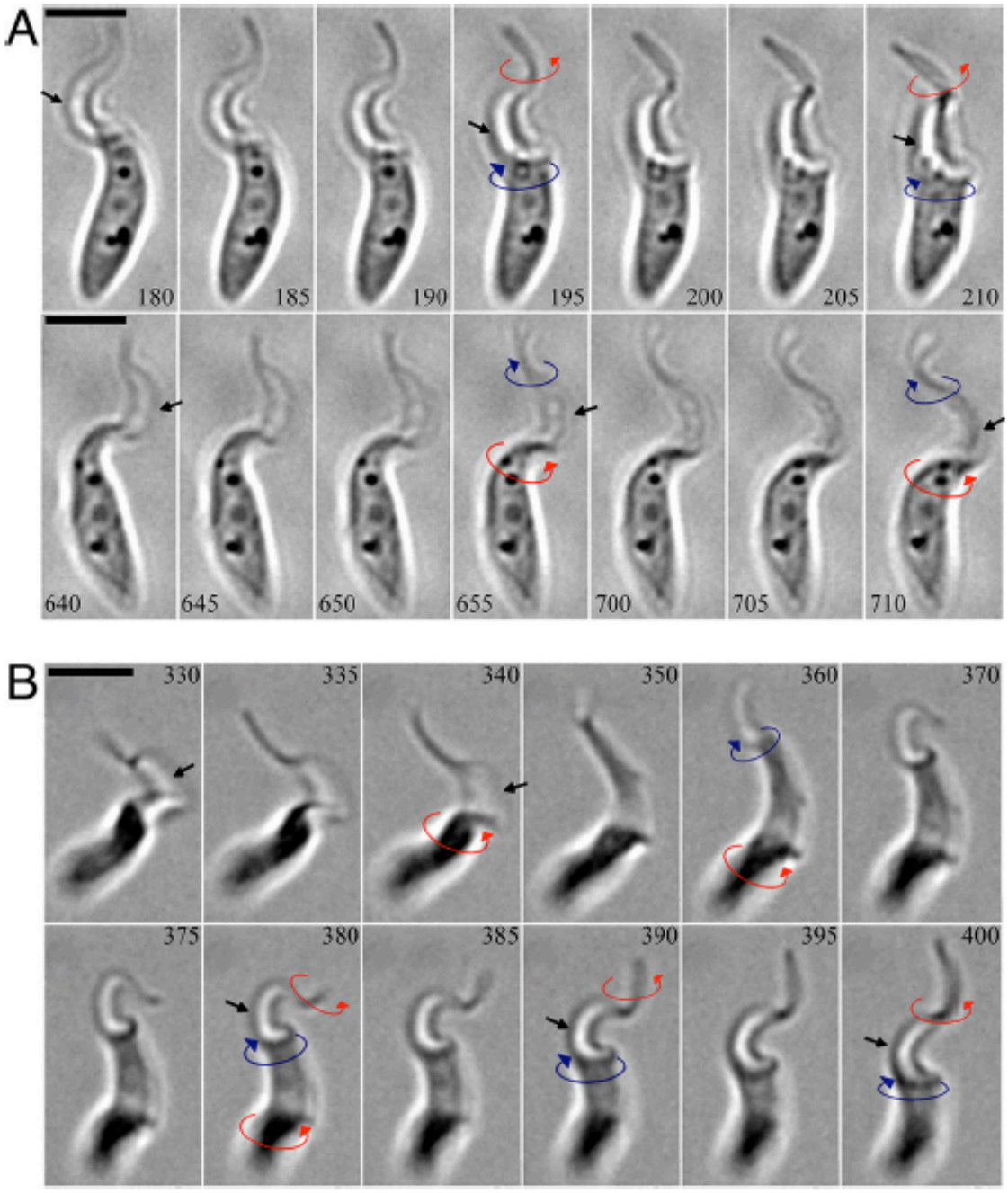


Figure 2-2. Millisecond DIC microscopy imaging of PCF and BSF cell motility. (*A*) (*Top*) Image sequence of a PCF cell showing a RH (*red arrow*) helical wave at the tip and a LH (*blue arrow*) helical wave near the base of the flagellum, separated by a plus kink (*black arrow*). (*Bottom*) Image sequence of the same cell showing a LH helical

wave at the tip and a RH helical wave near the base separated by a minus kink. The numerical values are in milliseconds. (Scale bars, 5 μm.) (*B*) Image sequence of a BSF cell showing bihelical waves that are separated by a minus kink (*Top*) immediately followed by a plus kink (*Bottom*).

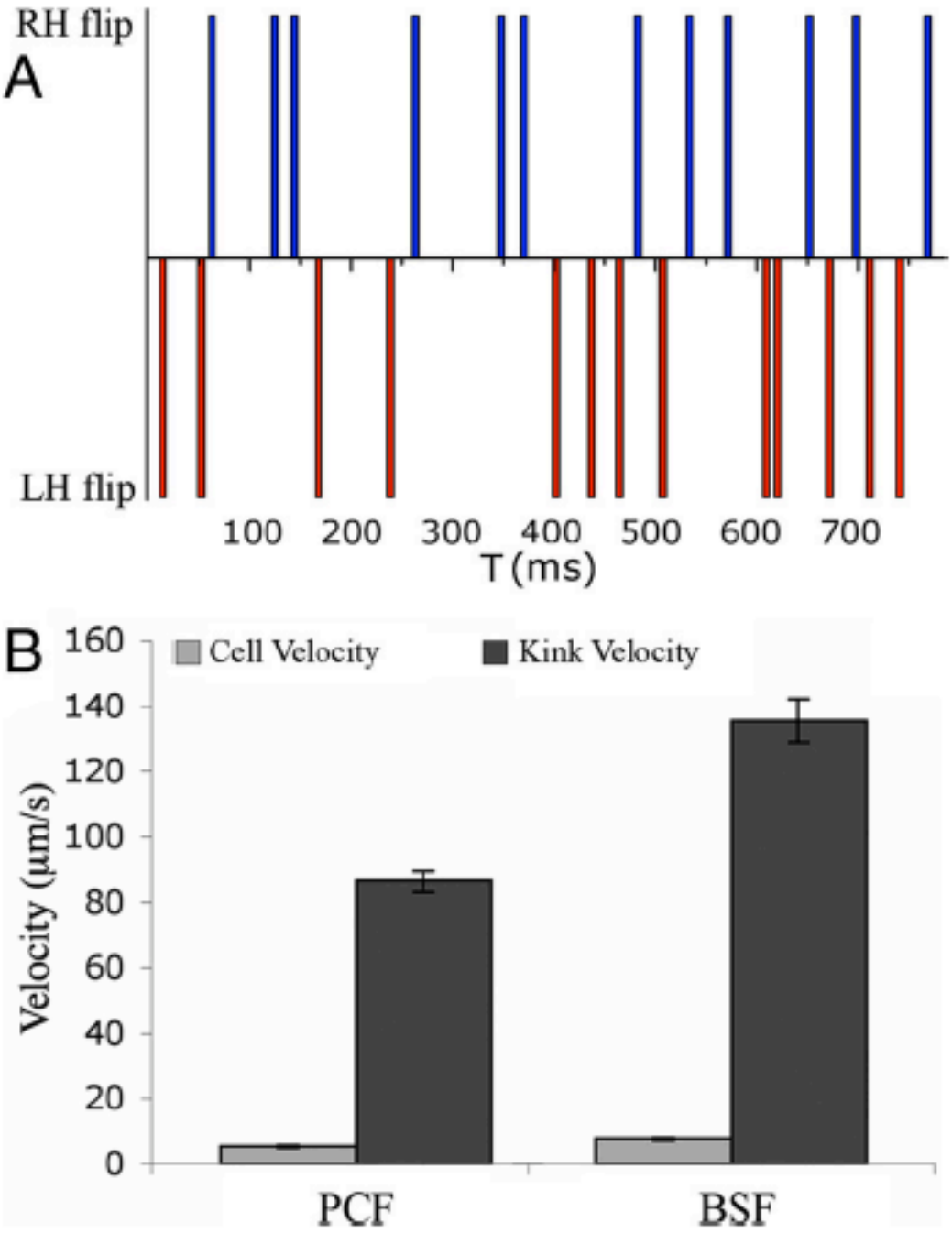


Figure 6-3. Analysis of cell propulsion in PCF and BSF *T. brucei*. (*A*) Representative plot showing the distribution of the LH and RH helical waves at the anterior end of a PCF cell within a time interval of about 800 ms. The number of waves was determined by monitoring how many times the flagellum flipped to initiate a RH or LH wave, as

indicated with blue and red vertical bars, respectively. Each flip represents a rotation of 180°. No more than three successive flips in the same direction are generated at the anterior end. Note that the rotation frequency in this example is somewhat higher than the average frequency of 19±3 flips per second. (*B*) Cell velocities were calculated from 50 PCF (5 ± 2 μm/s) and 50 BSF (8 ± 2 μm/s) cells undergoing directional motion. The kink velocities were obtained from 27 kinks in five PCF cells (86 ± 3 μm/s) and 24 kinks in eight BSF cells (136±7 μm/s), respectively. The ratio of the kink velocity to the cell velocity is about 16 for PCF and 18 for BSF cells.

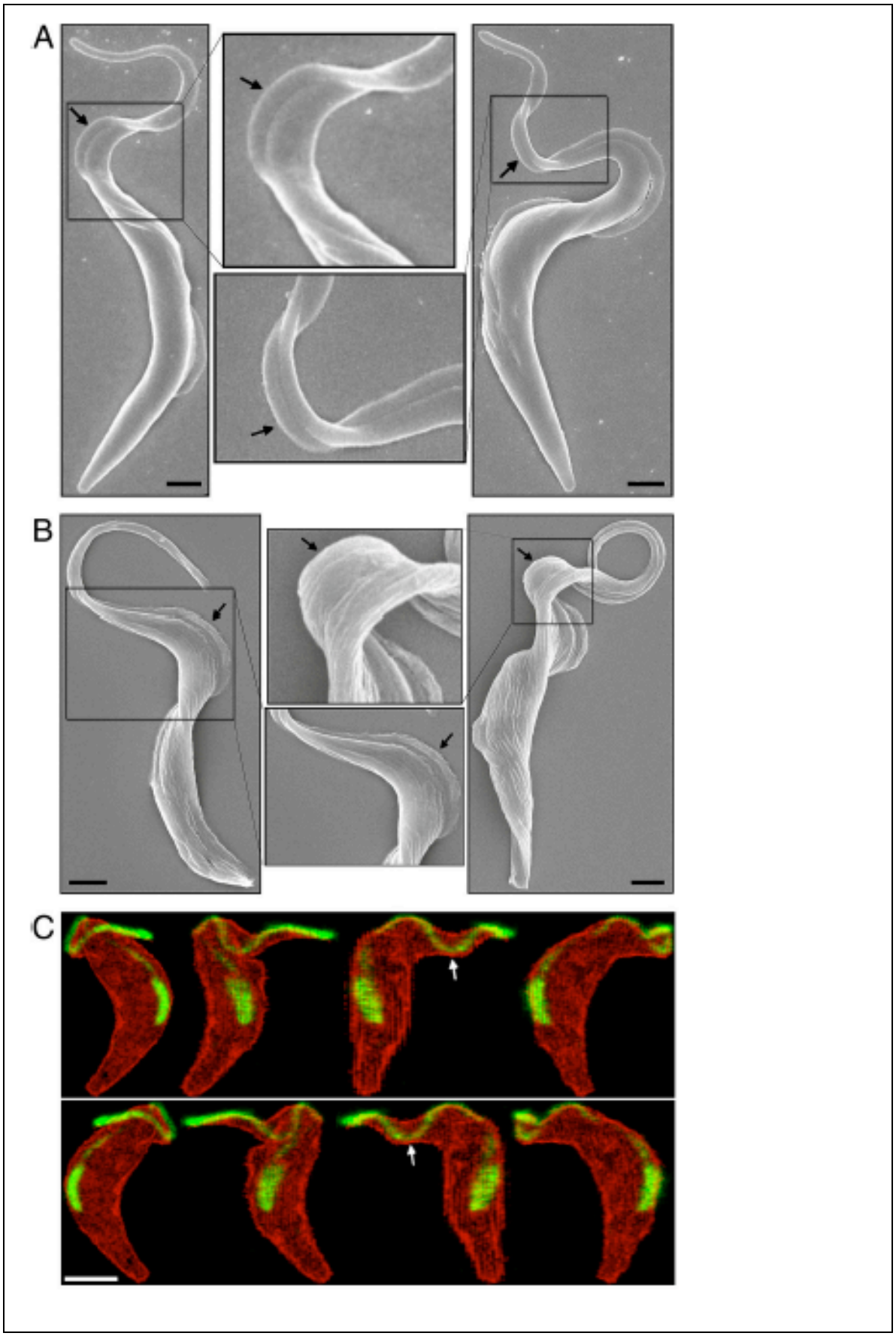
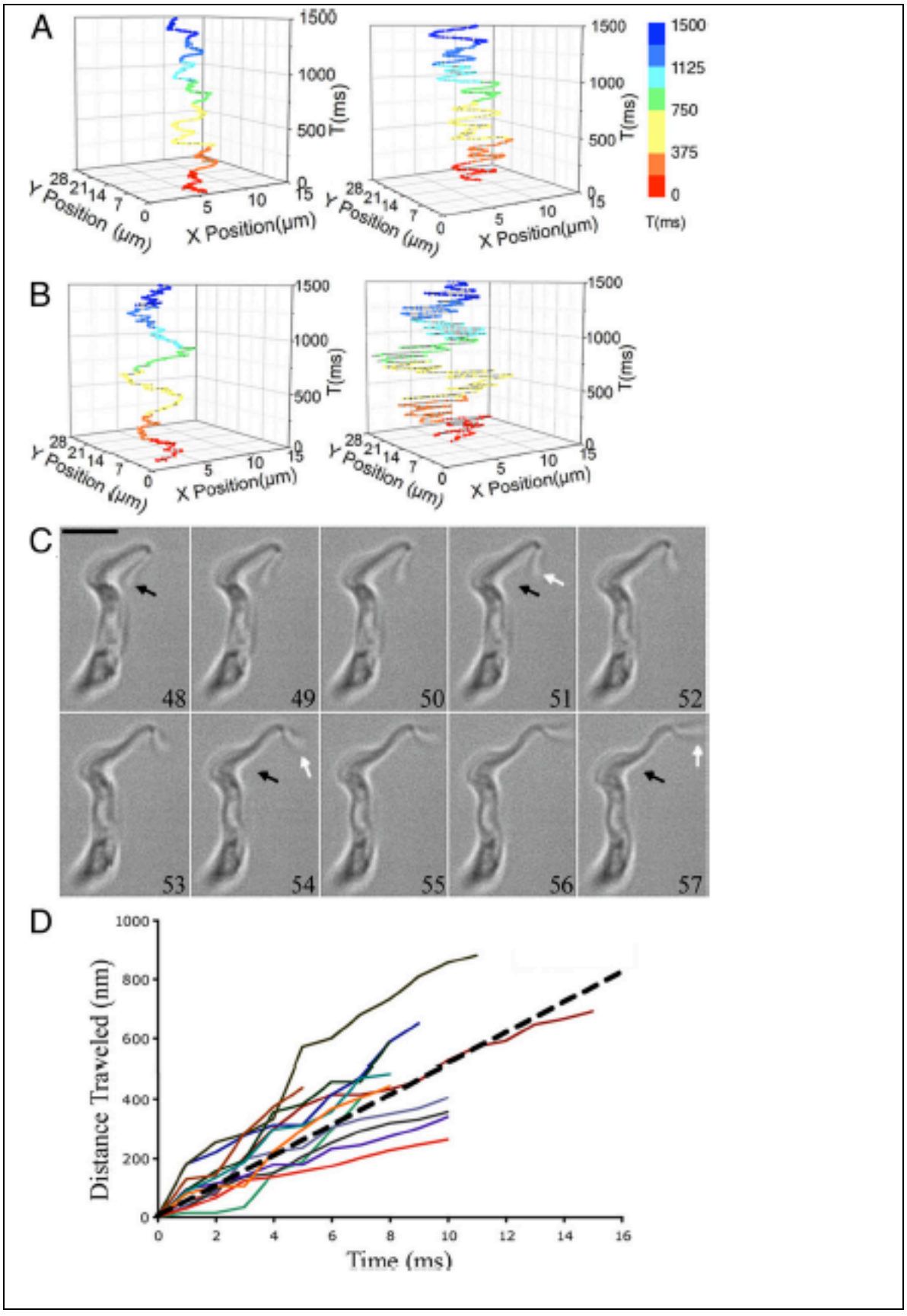

A
B
C

Figure 2-4. SEM and confocal microscopy imaging of rapid-fixed PCF and BSF cells. (*A*) SEM images of PCF cells with bihelical waves separated by a plus (*Left*) or a minus (*Right*) kink. (*B*) SEM images of BSF cells with bihelical waves separated by a plus (*Left*) or a minus (*Right*) kink. The zoom-in views illustrate torsion of the cell body induced by the bihelical waves and kinks. (Scale bars, 1 μm.) (*C*) Confocal microscopy images of a GFP-labeled flagellum in a single PCF cell. The cell is rotated along the vertical axis with 45° per image. The flagellum (*green*) wrapping around the surface of the cell body (*red*) exhibits RH chirality near the base and LH chirality near the tip, forming a minus kink (*white arrows*). (Scale bar, 3 μm.)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Y Position (μm)
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Distance Traveled (nm)
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Figure 2-5. Quantification of the motility of *T. brucei* cells. (A) Trajectory of the anterior (Right) and posterior (Left) end of a PCF cell as a function of time, where each color represents a different time period. (B) Trajectory of the motion of the anterior (Right) and posterior (Left) end of a BSF cell as a function of time. (C) Rapid motion of the BSF flagellar tip with a speed of 673 μm/s. White arrows show motion of the flagellar tip and the black arrows point to the initial tip position. The numerical numbers are in millisecond. (Scale bar, 5 μm.) (D) Twelve trajectories of rapid swings of the flagellar tip from two BSF cells of which the slopes represent the velocities of the flagellar tip swings. The average velocity (i.e., the slope of the dashed line) is 510 μm/s.

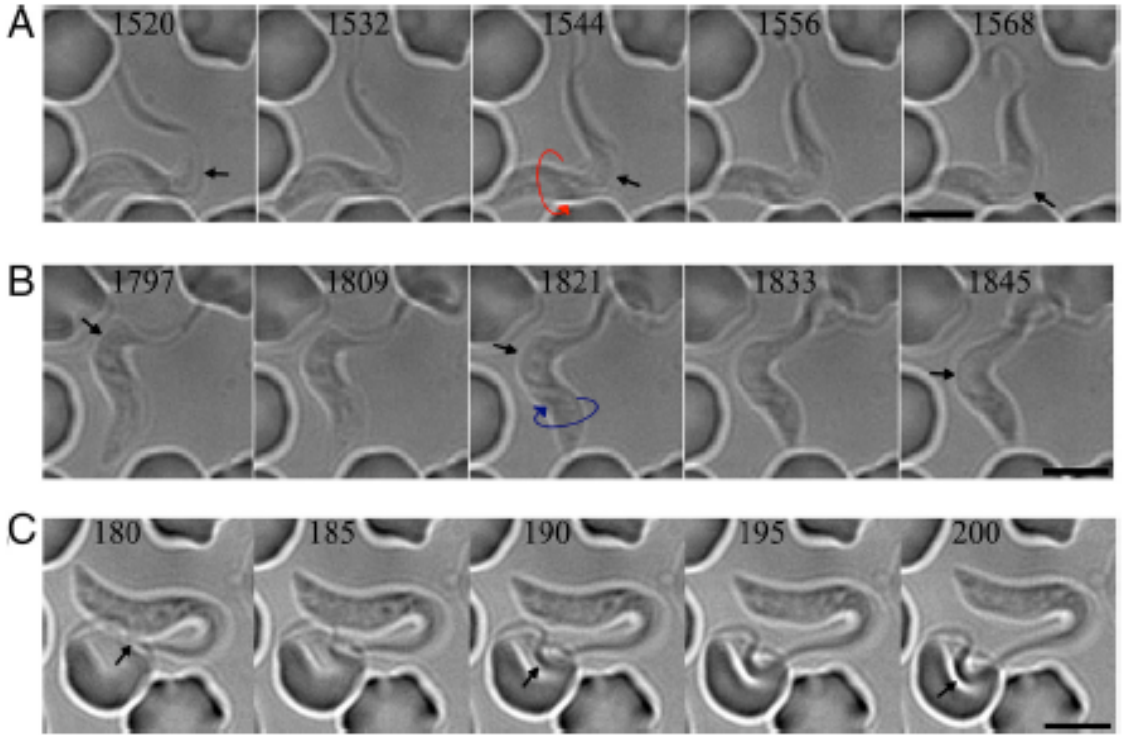

Figure 2-6. Millisecond DIC microscopy imaging and analysis of BSF cell motility in infected mouse blood. (*A*) Image sequence of a BSF cell exhibiting bihelical waves

separated by a plus kink, where the posterior end rotates clockwise, as indicated by the visible flagellum. The arrows pointing to the center of the kink show the kink propagation from the flagellar tip to base. The numerical values are in milliseconds. (Scale bars, 5 µm.) (*B*) Image sequence of the same BSF cell showing a minus kink where the posterior end rotates countclockwise. (*C*) Rapid swing of the flagellum tip of the BSF cell was observed initiating contact with a host red blood cell over a time period of 20 ms.

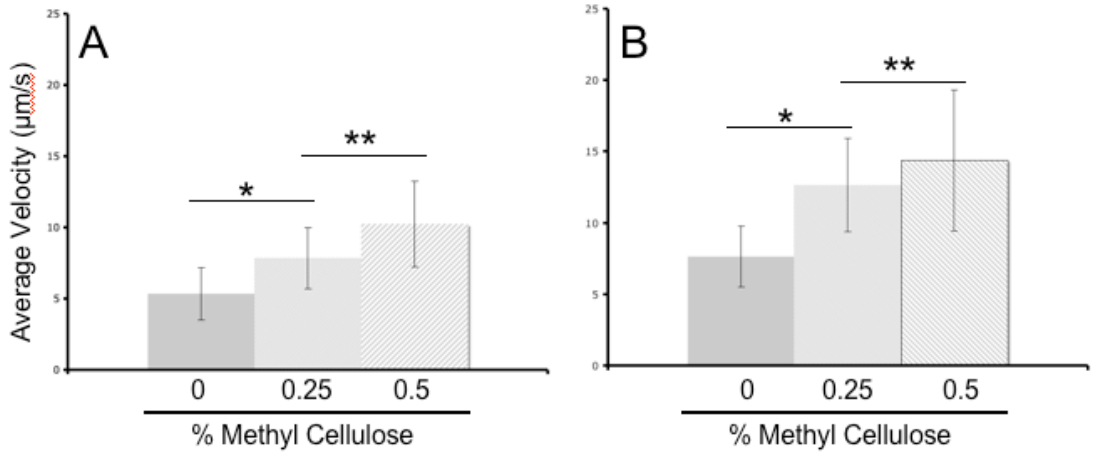

Figure 2-7. Average velocity increases as a function of viscosity. PCF (A) or BSF (B) cells were incubated in media with varying concentrations of methyl cellulose and assessed for their average velocities via motility assay [41]. A) Average PCF velocity increases from 5.4 µm/s to 7.9 µm/s to 10.3 µm/s. B) Average BSF velocity increases from 7.7 µm/s to 12.7 µm/s to 14.4 µm/s. The increases in velocity were significant as confirmed by t-test. (n=50 for all conditions)

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Chapter 3

Social Motility in African Trypanosomes

Social Motility in African Trypanosomes

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Abstract

African trypanosomes are devastating human and animal pathogens that cause significant human mortality and limit economic development in sub-Saharan Africa. Studies of trypanosome biology generally consider these protozoan parasites as individual cells in suspension cultures or in animal models of infection. Here we report that the procyclic form of the African trypanosome Trypanosoma brucei engages in social behavior when cultivated on semisolid agarose surfaces. This behavior is characterized by trypanosomes assembling into multicellular communities that engage in polarized migrations across the agarose surface and cooperate to divert their movements in response to external signals. These cooperative movements are flagellum-mediated, since they do not occur in trypanin knockdown parasites that lack normal flagellum motility. We term this behavior social motility based on features shared with social motility and other types of surface-induced social behavior in bacteria. Social motility represents a novel and unexpected aspect of trypanosome biology and offers new paradigms for considering host-parasite interactions.

Citation: Oberholzer M, Lopez MA, McLelland BT, Hill KL (2010) Social Motility in African Trypanosomes. PLoS Pathog 6(1): e1000739. doi:10.1371/journal.ppat.1000739

Editor: Marilyn Parsons, Seattle Biomedical Research Institute, United States of America

Received October 5, 2009; Accepted December 22, 2009; Published January 29, 2010

Funding: Funding for the work was provided by grants from the NIH-NIAID (AI052348), Burroughs Wellcome Fund, the Beckman Young Investigator program and the Ellison Medical Foundation to KLH. MAL is the recipient of an NIH-NRSA (GM07185) fellowship. MO is the recipient of a Swiss National Science Foundation Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Studying microbial life under conditions that promote a single-cell lifestyle has proven very effective for uncovering important aspects of microbial physiology. However, microbes are social organisms, capable of communicating with one another and engaging in cooperative behavior [1–3]. Well-characterized social activities include biofilm formation, social motility, fruiting body development and quorum sensing [1,3–10]. Social interactions among cells in a population effectively give rise to multicellular communities having specialized functionalities and offering advantages over a unicellular lifestyle. Some of these advantages include increased protection from external antagonists, such as desiccation or host defenses, access to nutrients, exchange of genetic information and enhanced ability to colonize, penetrate and migrate on surfaces [1,2,11]. In bacterial and fungal pathogens, social interactions have major influences on microbial physiology and disease pathogenesis and considering multicellularity as a general property of bacteria has profoundly changed our understanding of microbiology [1–3,6].

Most microorganisms, particularly pathogens, are intimately associated with surfaces in their natural environments and preferentially engage in social behavior when exposed to semisolid surfaces [2,7,10,12–14]. Commonly, this is manifested as various forms of social motility, including swarming, gliding and twitching [12,15]. Each of these surface-induced motilities is influenced by environmental and genetic factors and driven by overlapping yet distinct mechanisms that are not completely understood. The defining feature is cooperative movement of groups of bacteria across a surface, requiring active motility and cell-cell communication among members of the group in response to external stimuli. Once studied only in a few bacteria, such as Proteus mirabilis and Serratia marcescens, surface-induced cooperative motilities are now known to be widespread among both Gram-negative and Gram-positive bacteria, including several important pathogens, such as Salmonella and Pseudomonas spp. [13,16–18]. Surface-induced social interactions have also been observed in yeasts and fungi, including the opportunistic pathogen Candida albicans [5,6]. Thus, various types of surface-induced social behavior are widespread among microorganisms and applying this conceptual framework to studies of bacterial biology has yielded many novel insights. Surprisingly, the paradigm of social behavior has not previously been applied to parasitic protozoa.

African trypanosomes, i.e. Trypanosoma brucei and related species, are protozoan parasites that cause significant human mortality and limit economic development in sub-Saharan Africa [19]. T. brucei is transmitted to the bloodstream of a mammalian host through the bite of an infected tsetse fly vector. Parasite motility is important in both hosts and this is especially apparent in the tsetse, where parasites undergo an ordered series of directional migrations that are critical for parasite survival and completion of the life cycle [20–23]. Trypanosomes first colonize the midgut, then migrate into the ectoperitrophic space and advance back up the alimentary canal to the mouthparts and from there, to the salivary glands [21,23]. Throughout this process, parasites are in intimate contact with tissue surfaces of the tsetse fly. Once in the salivary glands, epimastigotes colonize the epithelial surface, stimulating the final stage of differentiation into mammalian-

Author Summary

African trypanosomes, e.g. *Trypanosoma brucei*, and related kinetoplastid parasites cause morbidity and mortality in several million people worldwide. Trypanosomes are protists and are thus generally considered to behave as single-celled microorganisms. In other microorganisms, social interactions among individuals lead to development of multicellular communities with specialized and advantageous capabilities versus single cells. The concept of bacteria acting as groups of cells communicating and cooperating with one another has had a major impact on our understanding of bacterial physiology and pathogenesis, but this paradigm has not been applied to parasitic protozoa. Here we report that *T. brucei* is capable of social behavior when exposed to semisolid surfaces. This behavior, termed social motility, is characterized by the assembly of parasites into multicellular communities with emergent properties that are not evident in single cells. Parasites within communities exhibit polarized movements and cooperate to coordinate their movements in response to an external stimulus. Social motility offers many potential advantages, such as facilitating colonization and navigation through host tissues. The identification of social behavior in *T. brucei* reveals a novel and unexpected aspect of parasite biology and provides new concepts for considering host-parasite interactions.

infective trypomastigotes [22–24]. Thus, throughout the tsetse stage of its life cycle *T. brucei* is in intimate contact with host tissue surfaces and exhibits an implicit requirement for sensing and signaling to guide parasite migration and differentiation. Currently, little is known about how surface contact modulates trypanosome behavior or motility [25].

Here we report that *T. brucei* engages in social motility when cultivated on semisolid agarose surfaces. This behavior is characterized by the formation of multicellular communities that sense external stimuli and communicate with one another to coordinate movement of the population. *T. brucei* social motility shares features with surface-induced social behavior in other microorganisms and represents a novel form of motility and intercellular communication not previously observed in these pathogens. As such, our findings present a novel and unprecedented feature of trypanosome biology and provide new concepts for considering development and pathogenesis of parasitic protozoa.

Results

Surface-induced changes to microbial motility and behavior are common among diverse bacteria and protists [2,5,7,26,27]. *T. brucei* spends much of its life cycle in contact with host tissue surfaces and interaction between parasite and tsetse epithelia is well documented [22,23,28], yet studies of *T. brucei* motility to date mainly utilize suspension cultures and do not provide information about how parasite behavior, e.g. motility, is affected by contact with surfaces. As part of our ongoing investigations into trypanosome motility, we thus cultivated procyclic form *T. brucei* on semisolid agarose plates [29]. We focused on procyclic forms because we know more about the motility apparatus and have more mutants available in this life cycle stage than in bloodstream forms and because the potential impact of parasite motility is most pronounced in this stage [30]. We found that procyclic trypanosomes formed groups of densely-packed cells within 24h post-plating (Fig. 1). The approximate doubling time on plates was 24 hours (Fig. S3), indicating that these groups did not arise simply through clonal expansion of single cells. Individuals within each group remained highly motile and actively moved out and back from the group. Interestingly, parasites were often arranged in distinct patterns on the agarose surface, with large, tightly packed groups surrounded by a zone of clearance and then a perimeter of smaller groups (Fig. 1). To investigate how these patterns arose, we established a system to monitor parasite movements over several hours using time-lapse and video microscopy (Materials and Methods). Time-lapse imaging revealed a striking behavior in which groups of hundreds to thousands of parasites moved *en masse* across the agarose surface, recruiting neighboring cells and enabling mergers of large groups (Fig. 1B–F, Video S1). This confirmed that the assembly of large communities was an active process and not simply the result of clonal expansion.

The *en masse* movement of large groups of trypanosomes across the agarose surface suggested some form of cooperation among individuals in the population. We therefore investigated this behavior more closely using increased magnification and greater time resolution (Fig. 2, Video S2). These analyses showed that recruitment of individual parasites into a community followed a specific sequence of events as described here. Cells at the periphery of the group were highly motile and moved out and back from the community. We refer to these cells as "scouts". When scouts came into contact with cells located adjacent to the community, they returned and induced polarized movement of the community outward at this position, forming a multicellular "pseudopod" that extended to recruit the external parasites (Fig. 2, Video S2). Mergers of large groups of cells followed essentially the same sequence of events (Fig. S1, Video S3). First, single trypanosomes advanced and returned randomly from the group periphery. Second, contact of scouts with an adjacent group biased their movement and initiated a period of reciprocal exchange. This led to formation of a multicellular "pseudopod" between the groups that intermittently broke down and reformed. Ultimately, stable contact was made and the groups merged along a path defined by the "pseudopod". Thus, the arrangement of cells on the agarose surface resulted from the cooperative movement of parasites into groups, which then expand through recruitment of neighboring cells.

Long-term cultivation of social bacteria on semisolid agarose gives rise to large macro-communities that form complex patterns on the agarose surface [2,15,17]. *T. brucei* formed macrocommunities within three to six days following inoculation (Fig. 3A). A characteristic feature of this process is that parasites initially collected into small clusters that were distributed around the perimeter of the inoculation site. Parasites in these clusters then advanced outward from the site of inoculation, forming symetrical arrays of radial projections, with a median of 13 projections per inoculum. This pattern is similar to that produced during social motility in *Pseudomonas aeruginosa*, *Myxococcus xanthus and Paenibacillus dendritiformis* [7,13,18,31–33], as shown by others (Fig. 3C). Movement of trypanosome projections was polarized, with a single leading edge that advanced at a steady rate on the order of a few microns per minute (Fig. S2, Video S4). The leading edge was characterized by a bulbous accumulation of densely packed cells, while the proximal region maintained a constant width (Fig. 3B). Cells along the lateral edge readily moved out and back (Fig. S2, Video S5), demonstrating they are not physically restrained. Therefore, polarized migration of projections is governed by parasite actions, rather than physical restrictions on parasite movement.

To determine whether social motility requires directional motility, we employed a trypanin RNAi knockdown line that is

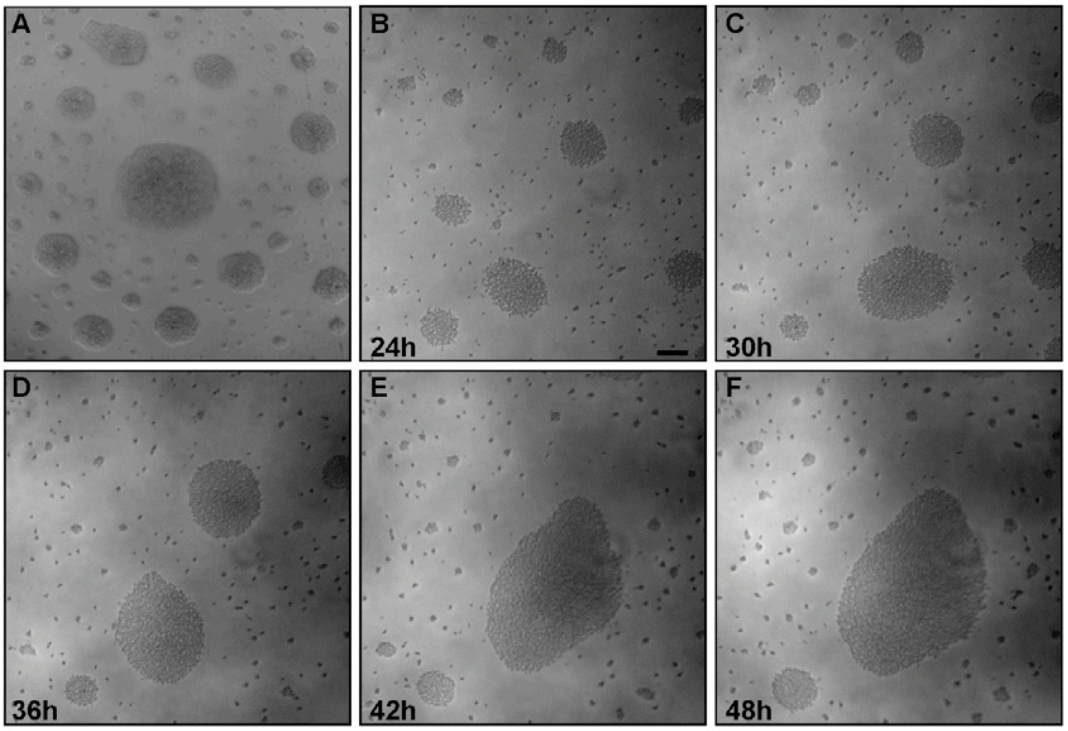

Figure 1. Trypanosome communities assemble through recruitment of neighboring cells. Trypanosome suspension cultures were transferred to semisolid agarose plates and monitored over time. (A) 48h post plating. (B–F) Time-lapse images show communities of parasites recruiting and merging with nearby individual cells and communities. The accompanying video (Video S1) shows groups of cells migrating en masse over the agarose surface. Scale bar is 100μm. Images are taken from Video S1 at the indicated time points post plating.
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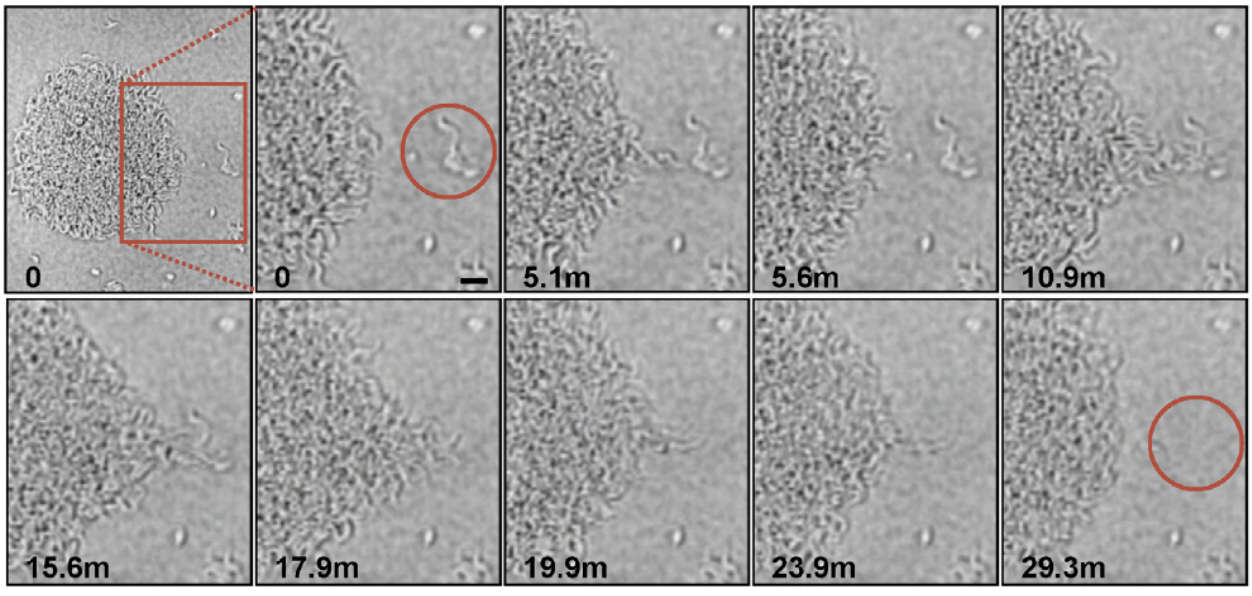

Figure 2. Parasite recruitment. Time-lapse images show active recruitment of individual cells into larger communities. Cells at the periphery of the community (termed "scouts") move out and back. When these scouts come into contact with a group of cells outside the community and return (time points 5.1–5.6 min), they stimulate coordinated movement of cells at the community edge outward, toward the neighboring cells, leading to their recruitment into the community (time points 10.9–29.3min). The image series was initiated approximately 24 hours after plating and time points, in minutes, of individual images are indicated. Scale bar is 20μm. Images are taken from Video S2.
doi:10.1371/journal.ppat.1000739.g002

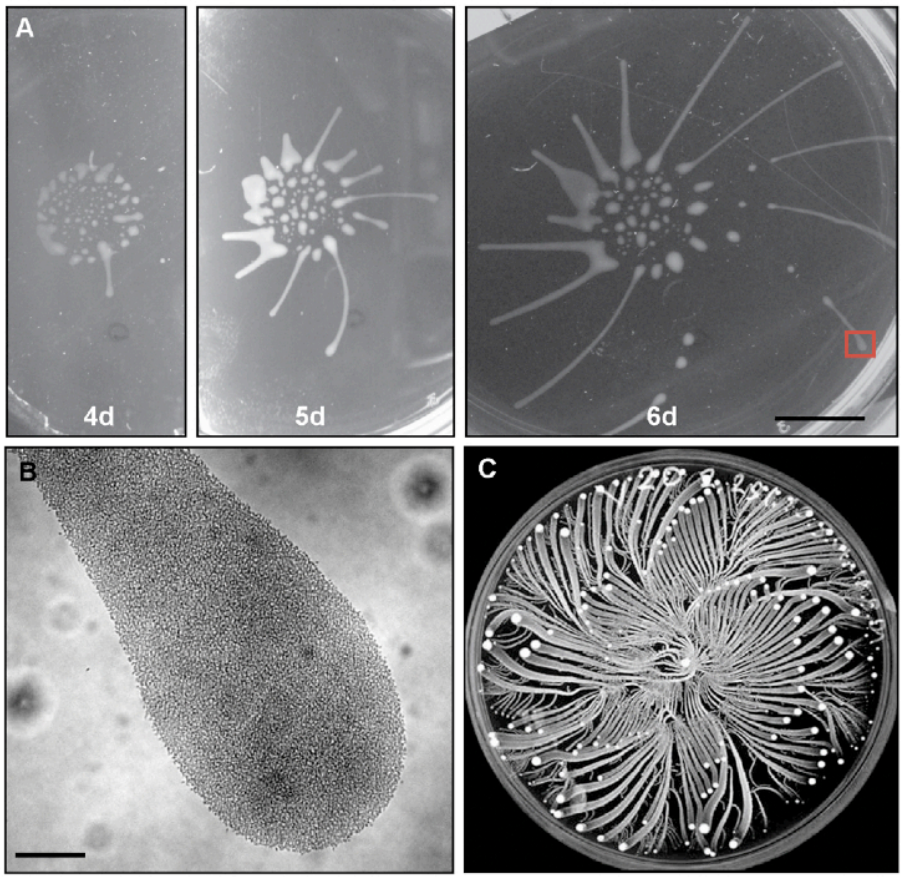

Figure 3. *T. brucei* social motility results in polarized migration outward from the site of inoculation. (A) Trypanosome communities 4 days (4d), 5 days (5d) and 6 days (6d) post plating. Parasites accumulate at the periphery of the inoculation site (4d, also compare to mot+ samples in Fig. 4A). Groups at the periphery then move outward, forming characteristic radial projections (5d and 6d). (B) Close up of the radial projection leading edge, (boxed region in panel A6d) shows characteristic bulbous accumulation of densely-packed cells. (C) Comparison to social motility patterns formed by *Paenibacillus vortex*, as shown by Ingham and coworkers [33]. Scale bars are 1cm (panel A), 200μm (panel B). Panel C adapted from [33], with permission.
doi:10.1371/journal.ppat.1000739.g003

incapable of directional motility [34]. Trypanin knockdown cells were evenly distributed at the site of inoculation and did not accumulate at the perimeter, as seen for cells having wild type motility (Fig. 4A). Moreover trypanin knockdown cells did not form radial projections (Fig. 4A–D). Cell doubling continued normally, as indicated by the roughly equivalent increase in cell density over time versus control cells (Fig. S3). Trypanin knockdown and control cells from the same plate were collected and assayed for (i) cell number, (ii) RNAi induction and (iii) motility. Both groups demonstrated an approximately equal cell doubling (Fig. S3) and trypanin protein was undetectable in the knockdown cells (Fig. 4F). Absence of directional motility in trypanin knockdowns was confirmed by direct microscopic examination (data not shown). Therefore, social motility in trypanosomes requires directional motility and is an active process.

Radial projections advanced in parallel and did not cross paths (Fig. 4A–D). Moreover, when projections from a control group approached a non-motile group, their movement was either halted or was diverted so as to avoid contact (Fig. 4A–D). When diverted, projections did not cross, rather they continued in parallel, implying that cells in each projection are capable of sensing each other and coordinating their movements. Avoidance of non-motile communities occurred within a radius of approximately 0.5–1 cm (Fig. 4E). To determine if this avoidance was uniquely a response to non-motile cells, we inoculated two groups of control cells on opposing sides of a culture plate and followed their development and migration over the course of several days (Fig. 5). Opposing radial projections either halted advancement, or diverted paths so as to avoid contact with one another. As a negative control, radial projections from a single community of motile cells did not divert their path of migration over time (Fig. 5F). The combined data thus indicate that *T. brucei* can sense and respond to external signals and that parasites in a community can sense other parasites and may choose to include them in the group or to avoid them.

Discussion

The impact of cell-cell communication and a multicellular lifestyle on the physiology and pathogenesis of bacteria is now well-established and related phenomena operate in yeast and fungi [1,3,5,6,35]. To date however, the paradigm of microbial social interactions has not been applied to parasitic protozoa. We report here that *T. brucei* is capable of social behavior in which parasites communicate with one another and assemble into multicellular

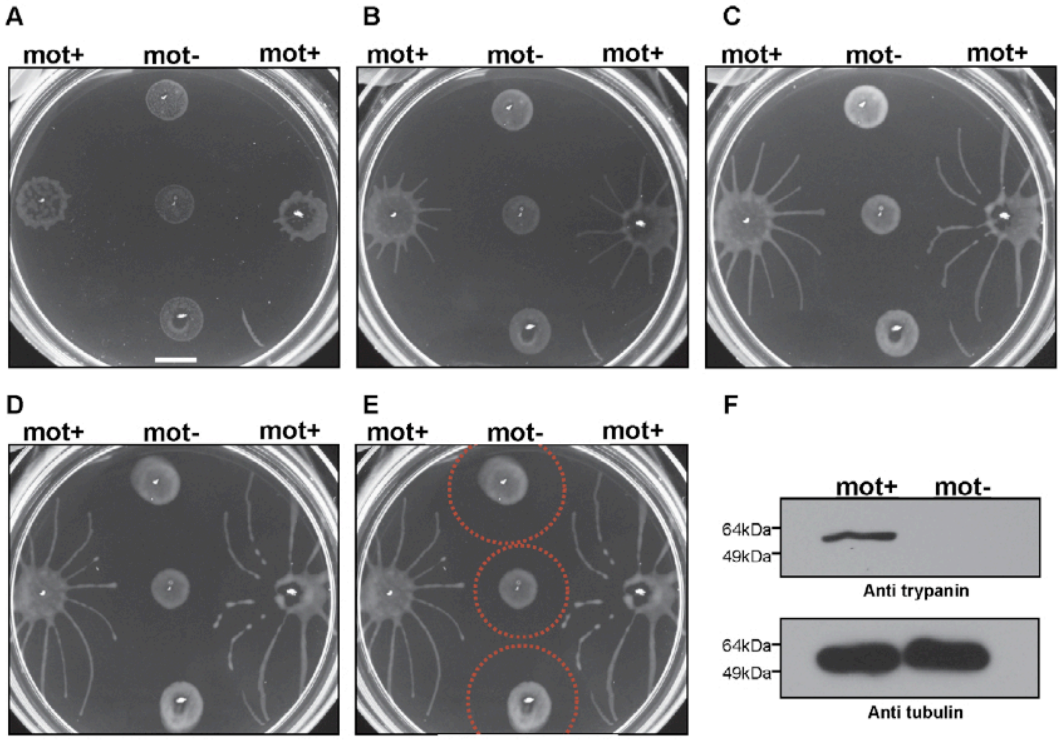

Figure 4. Social motility requires directional cell motility. Suspension cultures of control cells (mot +) or trypanin knockdown cells (mot 2) were inoculated on semisolid agarose plates and monitored over time. (A–E) Images taken 3 (A), 4 (B), 5 (C), or 6 (D, E) days post plating. (E) Indicated by red circles is the approximate zone of inhibition. (F) Western blot analysis on proteins extracted from control (mot +) and trypanin knockdown (mot 2) cells grown on the same agarose plate. Tubulin is used as a loading control. Scale bar in panel a is 1cm.
doi:10.1371/journal.ppat.1000739.g004

communities with emergent properties that are not evident in single cells. This behavior manifests as groups of parasites engaging in cooperative movement across the surface of semisolid agarose and altering course in response to an external stimulus. We term this behavior social motility, based on analogy to social motility in bacteria. These results demonstrate a novel feature of trypanosome biology and reveal a level of complexity and cooperativity to trypanosome behavior that was not previously recognized. Given the widespread distribution of social interactions among other microbes, we expect our findings to have broad relevance among parasitic protozoa.

Social interactions among microbes are manifested in a variety of forms and represent complex behavioral responses for which the underlying molecular mechanisms are not well-understood. As is the case for bacteria [2], social motility in *T. brucei* requires directional motility, involves some form of cellular differentiation upon exposure to a semisolid surface and culminates in cooperative cell migration in response to external signals. At early stages parasites merge into groups, while at later stages the behavior has the added feature of groups avoiding one another. This suggests some form of differentiation and is consistent with different stages of social motility observed in some bacteria, such as *Paenibacillus* spp. [33]. In most cases where it has been investigated, social motility requires a combination of external and internal, i.e. genetic, factors and it is likely that this is also the case in trypanosomes. Based on our observations and what is known in other organisms, a minimum requirement for social motility in *T. brucei* would be directional motility, the ability to sense an external signal and to transduce this signal into a cellular response and communication between parasites in a group. Trypanosomes are certainly capable of directional motility [36,37] and must integrate host-derived and parasite-derived signals to complete their life cycle [24,38–40], although their signaling and sensory capacities are poorly understood. The trypanosome genome encodes several components of classical signal transduction pathways, as well as numerous predicted cell surface proteins of unknown function that might serve sensory and/or signaling roles [40–45]. The contribution of these proteins to cell-cell signaling or other sensory functions is not known and efforts to address this question have been limited by the lack of a defined in vitro assay for cell-cell signaling. Social motility assays therefore provide an opportunity to test the requirement of trypanosome signaling systems in social motility and overcome a major barrier to dissecting signaling and sensory mechanisms in trypanosomes.

Within the tsetse, close contact between parasites, as well as intimate interactions with host tissue surfaces are readily observed [20,22,23], indicating that surface-induced social behavior might operate in vivo. However, until appropriate mutants are available for direct investigation, we can only speculate on potential physiological roles for social motility. In this context it is informative to consider whether there are features of the parasite life cycle that might benefit from social motility or related behavior. *T. brucei* development within the tsetse fly requires parasite migration across and through a variety of host tissues. These migrations lead ultimately to colonization of the tsetse salivary gland epithelia, which the parasites must reach in order to complete development into mammalian-infective trypomastigotes. Trypanosomes progress through specific tsetse tissues in a well-defined order, but the mechanisms responsible for tissue tropisms are

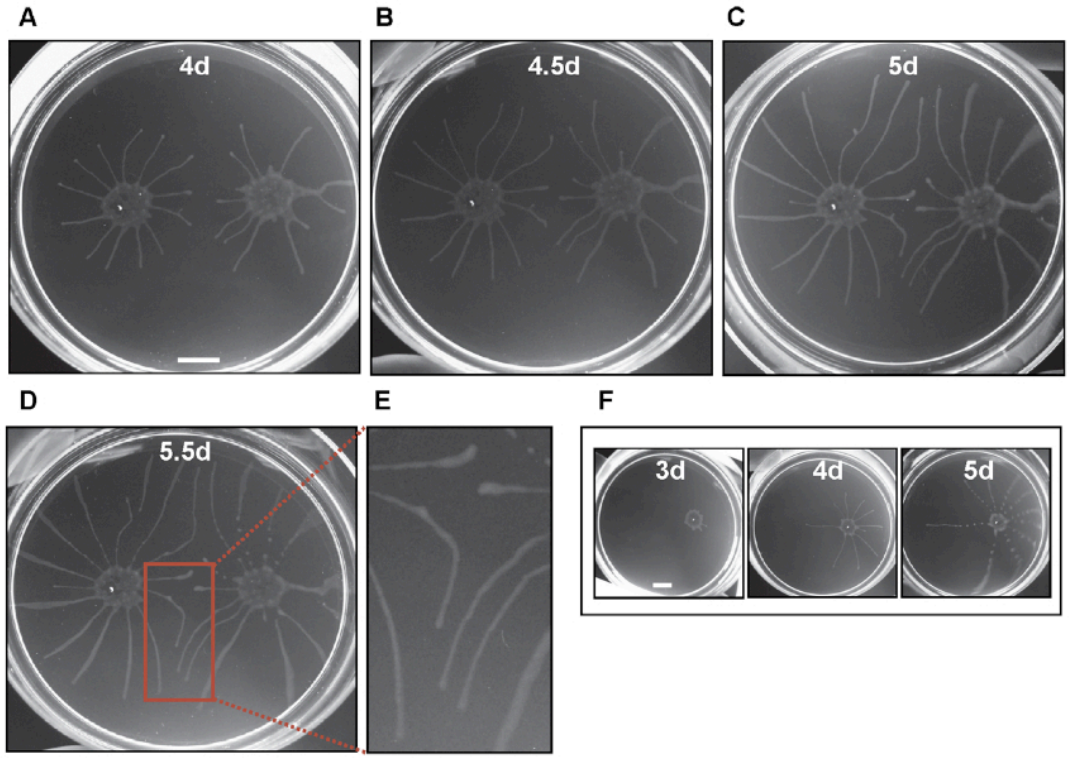

Figure 5. Trypanosomes sense nearby communities and change their course of migration. (A–D) Cells were inoculated on opposing sides of the agarose plate and monitored at the number of days post plating indicated in each panel. Projections radiating from each inoculation site advance outward in a generally straight path unless they come in proximity with opposing projections, at which point they halt progression, or redirect their movement. (E) Close up of outlined area in panel D. (F) As a control, cells from a single inoculation advance outward in a generally linear path to the edge of the culture dish. Scale bars are 1cm.
doi:10.1371/journal.ppat.1000739.g005

unknown. Social motility offers a system in which groups of cells coordinate their movements in response to an external stimulus and thus could provide a mechanism for parasite navigation through host tissues. In bacterial pathogens, cell-cell signaling, assembly into multicellular communities, social motility and other types of surface-induced behavior provide several advantages. Groups of bacteria feed cooperatively, resist hostile environments, prey on other microbes, exchange genetic information and develop functional specializations [1,2]. Quorum sensing and biofilm formation induce programs of virulence gene expression, facilitate colonization of host tissues and provide resistance to immune and physical defenses [3]. We speculate that trypanosome cell-cell communication and social behavior may have similar impacts on development and pathogenesis of T. brucei. For example, assembly into groups might facilitate resistance against host defenses in the tsetse [24], as well as promote tissue colonization and invasion. Social motility might also provide a means to bring parasites together for genetic exchange [46,47]. Finally, signaling pathways required for social motility are expected to overlap with host-parasite signaling pathways, about which very little is known. In summary, the identification of social motility in T. brucei reveals a novel and unexpected aspect of parasite biology and provides entirely new conceptual approaches for considering host-parasite interactions.

Materials and Methods

Trypanosome cell lines and suspension culture

Three procyclic T. brucei brucei cell lines, Antar 1 R5 Pro/G ITM [21], 29-13 double marker [48], and trypanin RNAi (KHTb12) [34], were used for these studies. While each experiment was not duplicated for each cell line, social motility was observed for both Antar and 29-13 lines. Suspension cultures were maintained using Cunningham's semi-defined medium (SM), supplemented with 10% heat-inactivated fetal calf serum as described previously [49]. For 29-13 cells, the medium was further supplemented with 15μg/ml G418 (Gibco) and 50μg/ml Hygromycin (Gibco). For the trypanin RNAi line, 2.5μg/ml Phleomycin, 15μg/ml G418 (Gibco) and 50μg/ml Hygromycin (Gibco) were included in the medium and RNAi was induced by adding 1μg/ml tetracycline. Cell doubling was monitored using a Z1 Coulter Particle Counter (Beckman Coulter, USA).

Plating on semisolid agarose plates

Cultivation on semi-solid agarose plates was adapted from [29]. Four percent (w/v) agarose (SeaPlaque GTG Agarose, Cambrex-LONZA, ME, USA) solution was made in MiliQ water, autoclaved and cooled to 65°C. A 1:10 dilution of this 4% stock solution was prepared in pre-warmed (42°C for 20min) SM culture medium supplemented with the appropriate antibiotics for selection. The resulting 0.4% agarose solution was cooled to 37°C for 1h. In most cases ethanol (final concentration 1%) was added to the medium. A 13ml aliquot was poured into Petri Dishes (100×15mm), which were then dried without lids for 1.5h in a laminar flow hood at room temperature. For inoculation onto the plate, 5μl of cells from a suspension culture at a density of 1.5×10^7cells/ml were added on the agarose surface. For the experiments in Fig. 2 and S1, 50ul of cells were spread on the

surface by gently rotating and rocking the plate. Trypanin RNAi lines were induced for 72h with 1μg/ml tetracycline in suspension culture prior to plating. Inoculated plates were dried for 3 min without lids, closed and sealed with parafilm and incubated as for suspension cultures at 27uC.

Imaging of plates

For Fig. 1A, the plate was imaged using a Zeiss Axioskop II microscope with a 2.5×LD Plan NeoFluor objective and Zeiss Axiocam camera. For Fig. 1B–F (Video S1), the plate was imaged using a Zeiss Axiovert 200M microscope with a 2.5×LD Plan NeoFluor objective and a COHU RS-170 high performance CCD camera (COHU, Inc.). Images were captured at 1 frame per 10min at room temperature using Adobe premiere Elements 1.0 (Adobe Systems). Time stamps are indicated in the panels. For Video S1, images were compiled into a movie using NIH-ImageJ (http://rsbweb.nih.gov/ij). The playback speed is 5 frames per second (3000× original speed) and elapsed time is 24h.

For Fig. 2 (Video S2) and Fig. S1 (Video S3), plates were maintained at 28uC, 5% CO_2 in a CTI humidified live cell cultivation chamber equipped with heating insert and CTI 3700 controller from Zeiss, Inc. This chamber allows independent control of humidity, temperature and CO_2 on the microscope stage. Plates were monitored on a Zeiss Axiovert 200M microscope, using a 10×LD Plan NeoFluor objective and a COHU RS-170 High performance CCD camera (COHU, Inc.). For Fig. 2 (Video S2), images were captured once every 5 sec and played back at 10 frames per second (fps), giving a final playback speed of 50×. For Fig. S1 (Video S3), the video was recorded in real-time using a VCR, then digitized in AVI format at 30 frames per second (fps) using an in-line Sony Handycam digital camera as an analog/digital converter and Adobe Premier Elements (Adobe Systems). Individual images were extracted at 1 fps, exported into QuickTime video format using the Sorenson TM CODEC within Adobe Premier Elements, and played back at 30 fps. The final playback speed is thus 30× real speed and elapsed time is 10 minutes 57 seconds.

For Fig. 3A, 4A–E and 5, plates were imaged at the indicated times post plating using an Olympus Stylus 770 SW digital camera and processed using Adobe Photoshop 8.0. For Fig. 3B, the plate was imaged as described above for Fig. 1B–F.

For Fig. S2 (Videos S4 and S5), time-lapse images were captured and compiled into video as described above for Fig. 1B–F. The playback speed is 6429× and elapsed time is 21.43 hours for Video S4 and 8.9h for Video S5.

Western blotting

Cells were collected in PBS from the agarose plate, counted and washed two times in PBS. The equivalent increase in opaqueness of control and trypanin RNAi communities on plates, Fig. 4, indicated that they continued doubling at equivalent rates and direct cell counting confirmed this. Protein samples were prepared and subjected to Western blot analysis as described [49], using 1×10^6 cell equivalents per lane. Monoclonal anti-trypanin antibody [50] was used at 1:5000, and monoclonal anti-b-tubulin E7 hybridoma supernatant was used at 1:5000. The anti-b-tubulin antibody was developed by Michael Klymkowsky, University of Colorado and was obtained from the Developmental Studies hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibody was horseradish peroxidase-coupled goat anti-mouse (BioRad) used at 1:2500.

Supporting Information

Figure S1 Merger of large communities occurs via the same defined sequence of events that drive recruitment of individual cells. High frame-rate (30 frames/sec) video analysis demonstrates that groups of parasites detect the presence of adjacent groups and then merge. Mergers occur in discrete stages defined as follows. Cells migrate out and back from each group giving an undulating appearance to the group periphery (A). Contact with an adjacent group initiates a period of reciprocal exchange (B–F), followed by stable contact, then rapid and massive cell movement as the two groups merge (G–H). Cell movement between groups generates multicellular pseudopod-like projections (black arrow). These projections only form between adjacent groups and only after contact. Therefore, crosstalk between groups by just a few cells initiates directed and coordinated movement of the entire group. Essentially the same sequence of events drives recruitment of individual cells into a group (Fig. 2). Scale bar is 20μm. Time-lapse image series taken from Video S3.
Found at: doi:10.1371/journal.ppat.1000739.s001 (1.94 MB TIF)

Figure S2 Radial projections advance exclusively at the leading edge, even though cells at the lateral edge are free to move out and back. (A, B) Snapshots of the leading edge (*) of a migrating community at time point 0 (A), and 21.3 hours later (B). Projections advanced at a steady rate of 2.3μm/min, as determined from this movie (Video S4). Scale bar 1cm. (C) Close-up of the region boxed in B. Cells at the lateral edge freely move in and out (black arrows). Scale bar is 1 cm. Image taken from Video S5.
Found at: doi:10.1371/journal.ppat.1000739.s002 (1.88 MB TIF)

Figure S3 Cell doubling of control and trypanin RNAi strains on semi solid agarose. Plates were inoculated with the same number (6.5×10^4 cells) of 29-13 control (mot+) or trypanin RNAi (mot −) cells. Cells from each community were collected by rinsing with PBS at the indicated number of days (3d, 4d and 5d) post inoculation and counted using a hemacytometer (A). At each time-point, the plates were imaged (B) prior to harvesting cells. The data show averages and standard deviations calculated from four communities for each time-point for each cell line. Scale bar in panel B is 1cm.
Found at: doi:10.1371/journal.ppat.1000739.s003 (1.48 MB TIF)

Video S1 Trypanosome communities assemble through recruitment of neighboring cells. This movie corresponds to the time-lapse images in Fig. 1B–F and shows merger of cells and communities of cells. The elapsed time is 24 hr.
Found at: doi:10.1371/journal.ppat.1000739.s004 (1.85 MB MOV)

Video S2 Parasite recruitment. Video S2 corresponds to the time-lapse images in Fig. 2 and shows high magnification of a trypanosome community recruiting neighboring cells. The elapsed time is 29.3 minutes.
Found at: doi:10.1371/journal.ppat.1000739.s005 (3.83 MB MOV)

Video S3 Merger of large communities occurs via the same defined sequence of events that drive recruitment of individual cells. This movie corresponds to the time-lapse images in Fig. S1 and shows specific stages of merger between trypanosome communities. The elapsed time is 10 minutes, 57 seconds.
Found at: doi:10.1371/journal.ppat.1000739.s006 (3.56 MB AVI)

Video S4 Merger of large communities occurs via the same defined sequence of events that drive recruitment of individual cells. This movie corresponds to the time-lapse images in Fig. S1

and shows specific stages of merger between trypanosome communities. The elapsed time is 10 minutes, 57 seconds.
Found at: doi:10.1371/journal.ppat.1000739.s007 (10.39 MB MOV)

Video S5 Cells at the lateral edge of radial projections are free to move out and back. This movie corresponds to the image in Fig. S2C and shows that cells along the lateral edge of advancing radial projections freely move out and back. The elapsed time is 8.9 hours.
Found at: doi:10.1371/journal.ppat.1000739.s008 (0.84 MB MOV)

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Acknowledgments

We would like to thank Jan van den Abbeele (Institute of Tropical Medicine Antwerp) for generously providing the Antar 1 R5 Pro/G ITM cell line and George Cross (Rockefeller University) for the 29-13 cell line. We would also like to thank Wenyuan Shi (UCLA) for many helpful scientific discussions.

Author Contributions

Conceived and designed the experiments: MO MAL BTM KLH. Performed the experiments: MO MAL BTM. Analyzed the data: MO MAL KLH. Wrote the paper: MO MAL KLH.

Chapter 4

Identification and Characterization of *T brucei* Insect-Stage Specific Adenylate Cyclases

The work presented in this chapter was an effort by multiple members of the lab of Dr. Kent Hill (UCLA Department of Microbiology and Molecular Biology Institute) and was spearheaded by Dr. Pius Kabututu and Edwin Saada. The results have been prepared into a manuscript authored by many members of Dr. Kent Hill's lab including myself. My specific contributions as a coauthor to the work included: 1) QRTPCR analysis of the expression of the 6 adenylyl cyclases (ACs) identified in bloodstream versus procyclic cell cultures, 2) epitope tagging and immunofluorescence assay of ACP1, P3 and P6, 3) generation of multiple AC knockdown cell lines (including the ACP1 KD cell line used in this study) and 4) evaluation of growth and motility phenotypes in these knockdown cell lines. These results are presented and discussed here and in Chapter 5.

Introduction

African trypanosomes, including *Trypanosoma brucei* and related subspecies, are the causative agents of sleeping sickness in humans and nagana in cattle. These parasites are the source of significant human mortality and limit economic growth throughout sub-Saharan Africa [1,2]. Sleeping sickness is recognized as one of the world's most neglected diseases [1] and is a reemerging disease with ~60 million people at risk of infection. The disease is fatal if left untreated and current therapeutic options are insufficient as they are antiquated, toxic, difficult to administer and/or financially unattainable. The situation is further exacerbated by the development of drug resistance and there is a desperate need to identify new drug targets. Thus, *T. brucei* is a parasite of

tremendous medical and economic importance and is recognized as a disease of poverty, as well as a cause of poverty.

T. brucei has a digenetic lifecycle, being transmitted to a mammalian host through the bite of an infected tsetse fly. In both hosts, infection is characterized by migration to and colonization of different tissues by the parasite. In addition, throughout infection trypanosomes remain extra cellular and in the constant presence of extra cellular host and parasite-derived factors. Successful infection by the parasite depends on its ability to navigate through the hosts and respond to these extra cellular factors. In the mammal, for instance, trypanosomes replicate within the blood during the first phase of infection before penetrating the blood brain barrier to invade the central nervous system and cause the second stage of the infection [1]. The presence of the parasite within the cerebrospinal fluid is associated with the mortality of the disease. Within the tsetse, migrations to different tissues are even more apparent as *T. brucei* undergoes a series of directional migrations necessary for development and differentiation into a human-infectious form [3]. Parasites entering the fly with the bloodmeal first colonize the tsetse midgut as procyclic forms. They then penetrate the peritrophic membrane and migrate anteriorly along the GI tract their to he salivary glands where they attach to and colonize the epithelial surface, stimulating the final stage of differentiation into human-infectious forms [3]. Though the specific mechanisms underlying these migrations remain unknown, it is thought that tissue tropisms play a key role in the migration of the parasite to specific tissues. Thus far, neither factors nor receptors have been identified that could play a role in these tissue tropisms.

As *T. brucei* remains extra cellular throughout its life cycle, it is exposed to a variety of host and parasite-derived factors. Sensation of these factors influences parasite biology and behavior. In the mammalian host, for instance, autocrine signaling stimulates transformation of proliferating long-slender to nonproliferating short-stumpy forms [4,5,6,7]. Transformation of the parasite from the rapidly dividing long-slender form to the nonproliferating short-stumpy form is believed to be an important step in preventing premature depletion of resources within the host which might result in death of the host prior to successful transfer of the parasite to the insect vector. In addition, penetration of the blood brain barrier involves paracrine signaling between parasite and host. Autocrine signaling has also been observed to play a role in the biology of the insect-stage form of the parasite. We recently reported that procyclic form (or insect-stage) *T. brucei* engages in social behavior when inoculated on semi-solid agarose plates. Throughout this process, the parasites sense and communicate with one another to form macroscopic communities capable of coordinated actions [8]. Specifically, these macroscopic communities can move *en masse* and can detect and avoid one another through unknown mechanisms [8].

Thus, although it is clear that *T. brucei* infection depends on navigation to different tissues and that the parasite senses extra cellular factors, there remains no identification of factors or receptors that could mediate navigation or sensation. When evaluating how *T. brucei* might sense external factors, it is important to consider the parasite's flagellum. The eukaryotic flagellum is an important organelle with many roles including motility and sensation. As a mechanical organelle, the eukaryotic flagellum is

well studied and has long been regarded as important for the motility of many organisms and cells as well as the regulation of several processes in metazoans [9]. More recently, exciting work has uncovered that this organelle plays an integral role in the sensation of external signals as well [10]. For example, molecules on the cilia of microbes such as *Chlamydamonas reinhardtii* and *Caenorhabditis elegans*, for example, assist in the detection of photo and chemo signals (respectively). Also, ciliary receptors on the photoreceptors and olfactory systems of mammals are essential for sight and smell (respectively). Given the role of sensation by eukarytoic flagellum in other organisms, it is therefore not unlikely that *T. brucei* would employ its flagellum for similar processes. That being said, there is little knowledge in the field about what kinds of receptors are present on the surface of the *T. brucei* flagellum.

The *T. brucei* flagellum is a fascinating organelle. It emerges from the flagellar pocket at one end of the parasite and is attached along its length to the parasite's cell body. The flagellum is surrounded by its own membrane and is attached to the cell by a network of cytoskeletal and membranous connections that collectively make up the flagellum attachment zone (FAZ) [11]. Within the flagellum are a canonical 9 + 2 microtubule axoneme and a lattice-like paraflagellar rod (PFR). The flagellum has been implicated as integral to the parasite and defects in its function and structure result in severe defects to the parasite. As such, the organelle and the proteins that comprise it have emerged as viable drug targets. Much work has been done to characterize the proteins to that make up the *T. brucei* flagellum. However, until recently much remained unknown about the proteins that are found on the flagellum's surface thus hindering

efforts to identify molecules that could play a role in the organelle's role in the mediation of sensation by *T. brucei*. In an effort by Oberholzer et al., a proteome of the *T. brucei* blood stream form flagellar membrane was published [12]. Through the incorporation of many techniques (some of which described here) this work became the first to identify transmembrane, surface-exposed proteins that were specific to the flagellum [12]. The efforts of this investigation will undoubtedly assist in the identification of receptors used by the parasite to sense its environment.

In a similar effort, here we decided to look for proteins that are specific to the procyclic form (insect-stage) *T. brucei* flagellum and could thus mediate sensation by this form of the parasite. Our investigation uncovered the presence of six adenylate cyclases (ACs) on the flagellum of the procyclic form of the parasite. Trypanosomal ACs are unique structures with a cytoplasmic catalytic domain near the C-terminus connected by a single transmembrane domain to a large, extracellular N-terminal domain. The N-terminal domain varies among family members and exhibits homology to a superfamily of proteins involved in nutrient sensing in bacteria and ligand binding in mammalian receptors. Architecturally, these molecules resemble membrane-bound type I guanylyl cyclases which act as receptor molecules to generate cGMP in response to extra cellular signals [13,14]. Additionally, unlike related kinetoplastids, African trypanosomes show a remarkable expansion of receptor-like ACs [15]. The unique structure of trypanosomal ACs as well as their expansion within the genomes of African trypanosomes has lead to the tantalizing hypothesis that these molecules function as receptors to extracellular signals [7,13,14].

Of the approximately 60 family members, very few *T. brucei* ACs have been previously studied or characterized. The canonical AC, ESAG4, is expressed specifically in BSF parasites and has recently been shown to function in modulation of the host immune response and parasite viability [16,17]. Other AC genes remain largely unstudied and have been considered to be constitutively expressed and redundant in function [13]. Here, we sought to characterize the 6 ACs identified through different molecular and biochemical approaches. Our results have uncovered novel information about this family of proteins and challenges earlier conceptions of the roles ACs might have within the parasite. Our results also suggest that ACs represent a highly regulated set of proteins that might function as receptors to stage-specific ligands.

Results

Proteomic analysis of flagella from procyclic-form T. brucei identifies stage-specific adenylate cyclases

We previously reported isolation of intact flagella and proteomic analysis of flagellar membrane and matrix proteins from bloodstream form (BSF) *T. brucei* [12]. We attempted a similar analysis using procyclic culture form (PCF) *T. brucei*. MudPIT analysis of the PCF flagellum preparation indicated a high level of contamination by cellular proteins, likely owing to the use of sonication in removal of flagella from cell bodies (methods). Among the proteins identified were a group of receptor-type adenylate cyclases that were not detected in our prior BSF flagellum preparations (Table 4-1).

These adenylate cyclases are members of a previously described family of "genes related to ESAG 4" (GRESAG4), based on their sequence similarity to the expression site associated gene 4 (ESAG4) [18]. ESAG4 is expressed only in BSF parasites, while GRESAG4 genes examined previously were expressed in both BSF and PCF parasites [19,20,21]. We were therefore surprised to uncover a group of GRESAG4s that were detected only in PCF samples and we investigated these further. We termed these proteins ACP1 through ACP6, to reflect the fact that they were found in PCF, but not BSF, proteomic analyses.

T. brucei encodes approximately 64 adenylate cyclases , each having a large extracellular domain at the N-terminus, followed by a single transmembrane region and a cytoplasmic catalytic domain [14] (Figure 4-1). The catalytic domain is followed by a short C-terminal region of approximately 150-175 amino acids. Amino acid sequence diversity among ACP1 through P6 is highest within the N-terminal and C-terminal regions (Figure 4-1). Pairwise alignments revealed that ACP1 and P2 are 90% identical in amino acid sequence, with differences lying primarily within the C-terminal 154 amino acids. The other ACs identified here exhibit considerable sequence differences between one another.

Our BSF [12] and PCF proteomic data suggested ACP1 – ACP6 are expressed only in the procyclic life cycle stage. However, sequence similarities among this large protein family make it difficult to unambiguously identify any individual member using proteomics alone. For example, proteomic analyses of BSF flagella identify two peptides that mapped to ACP5, but these two peptides also mapped to 17 other ACs, meaning ACP5 could not be unambiguously identified in BSF parasites. As another example is

ACP1 and ACP2 are 89% identical at the amino acid level and were identified with five peptides that mapped to both proteins, as well as a single peptide that mapped uniquely to ACP1. ACP1, P3-P6 were each identified by one or more peptides that are unique to that protein, with the exception of ACP2. Attempts to epitope tag it in BSF were unsuccessful (data not shown). We therefore used quantitative reverse-transcriptase PCR (qRT-PCR) with gene-specific primers to directly interrogate the developmental expression profile for ACP1 through P6. ACP1 and P3-P6 were each expressed primarily in procyclic-form parasites (Figure 4-2), while ACP2 expression was nearly equivalent in both life cycle stages. Thus ACP1, and P3-P6 show developmental regulation of expression distinct from that reported for other GRESAG4 genes [19,21,22].

In situ epitope tagged Adenylate cyclases exhibit localization to different domains of the flagellar membrane

The large size of the AC gene family, together with extensive sequence homology among individual genes has complicated efforts to analyze any single AC gene or protein. Our proteomic analyses identified only a small subset of ACs as being expressed in procyclic-form parasites, thereby allowing prioritization of individual genes for direct analysis. Furthermore, the subcellular localization of most adenylate cyclases in *T. brucei* have not been determined. Two individual ACs, ESAG4 [20] and FS24 [12], have been shown to localize along the length of the flagellum in bloodstream form parasites. Immunofluorescence using pan-specific antibodies showed localization along the length of the flagellum for a group of GRESAG4 proteins in procyclic form parasites, but individual proteins were not examined in this life cycle stage [20]. To investigate the localization of the 6 ACs we found, homologous recombination was used to deliver an *in*

situ HA epitope tag to the 3' ends of each of the genes using the previously described plasmid constructs [23]. Western blot analysis of cell lysates demonstrated a single HA tagged protein of the expected size was expressed in each HA-tagged cell line (not shown). Southern blotting demonstrated correct integration of the HA epitope tag at the expected AC locus in each case (not shown).

Using immunofluorescence with anti-HA antibody, we found all 6 ACs to be localized exclusively to the flagellum (Figure 4-3). Interestingly however, the specific distribution within the flagellum was different for each AC. ACP1, P3, P4 and P6 were localized almost exclusively to the distal tip of the flagellum, whereas ACP2 was evenly distributed along the entire length of the flagellum. ACP5 was concentrated at the distal tip with weaker, but significant, signal along the flagellum length. The tip-specific localization of ACP1, P3, P4 and P6 distinguishes these from trypanosomal ACs studied previously and is to our knowledge a novel finding for a transmembrane protein in *T. brucei*.

To assess whether the HA epitope tag influenced protein localization, we used ACP1-specific antibody to determine the location of the endogenous protein. Specificity of the anti-ACP1 antibody was tested using a gene-specific RNAi knockdown of ACP1. qRT-PCR demonstrated specific and efficient knockdown of ACP1, without affecting expression of ACP2, which is the most closely related protein to ACP1 (Figure 4-4 and not shown). Knockdown of ACP1 did not affect growth or motility (not shown). Western blots with affinity-purified anti-ACP1 antibodies detected a single band of the expected size that was lost following induction of RNAi. The anti-ACP1 antibody failed to detect any signal in lysates from bloodstream form parasites, corroborating the qRT-PCR results

demonstrating that ACP1 is a procyclic specific protein (Figure 4-4B). These results further indicate that the antibody distinguishes ACP1 from ACP2, as ACP2 is expressed in ACP1 knockdowns (not shown) and in bloodstream form cells (Figure 4-2). Immunofluorescence with ACP1-specific antibody showed that endogenous ACP1 is located at the distal tip of the flagellum (Figure 4-4C), demonstrating that the HA-tagged protein correctly reports localization of the endogenous protein.

Localization of individual trypanosomal ACs to discrete regions within the flagellar membrane supports the emerging model that cAMP signaling is compartmentalized within specific membrane microdomains [24].

Discussion

There are approximately 60 adenylate cyclase genes in the *T. brucei* genome [15], but only a few of these have been studied directly. Among the ACs identified in our study, ACP4 corresponds to GRESAG 4.2/4.3 [16]. ACP1 and P2 correspond to two ESAG4-like genes that were identified as being upregulated in BSF parasites following knockdown of ESAG4 [17], though they were not examined in PCF parasites. ACP3, P5 and P6 do not correspond to previously studied GRESAG4 genes [17](Table 4-1). Of the six ACs identified in our proteomic analysis, five are up-regulated in procyclic-stage parasites based on qRT-PCR analysis. Western blots using isoform-specific antibodies further demonstrate that ACP1 expression is restricted to procyclic-form parasites. Procyclic-specific expression is a novel finding, as all ACs examined previously have been found to be either BSF-specific, ESAG4 [17] and FS24 (Figure 4-2) [12], or constitutively expressed GRESAG4.1, 4.2/3 and 4.4 [19,20,21,22,25]. Prior analysis of

GRESAG 4.2/4.3, which corresponds to ACP4, reported equal expression in BSF and PCF parasites by Northern blot [21], while our qRT-PCR analysis shows six-fold upregulated expression in PCF versus BSF parasites. We suspect that the discrepancy may lie in greater capacity for gene-specific analysis in qRT-PCR owing to the availability of the annotated *T. brucei* genome. Earlier studies may have underestimated potential for cross-reactivity in Northern blots as the size and extent of sequence similarity among the AC/GRESAG gene family was not fully known. Thus, our studies reveal a new paradigm for trypanosomal ACs through identification of a group of AC genes upregulated in the procyclic life cycle stage, indicating a specific role within the tsetse fly.

Despite being discovered more than 20 years ago, the biological function of trypanosomal ACs remains unclear. Very recent work demonstrated a role for bloodstream-specific ESAG4 in manipulation of host immune responses to trypanosome infection [16]. Additional recent work has suggested ESAG4 and/or other ACs are required for cytokinesis in bloodstream form parasites [17]. However, the functions of GRESAG4 ACs and the reason for expansion of the AC gene family remain unknown. The large size of the AC gene family and the propensity of trypanosomes for gene duplication, has led to suggestions that these proteins may perform redundant functions [17]. Our data suggest otherwise, because we see developmentally regulated expression patterns that differ from expression profiles described for ESAG4, and other GRESAG4 genes. Distinct functions for individual AC proteins is also supported by the finding that individual proteins show distinct distributions within the flagellar membrane. Finally, there is significant sequence diversity among *T. brucei* ACs, suggesting diversified

functions [14,26]. AC sequence differences are concentrated in the N-terminal domain, which is predicted to be extracellular and shares sequence similarity to periplasmic binding proteins ("PBP") of bacteria. PBPs function in chemotaxis and signaling in bacteria through recognition of diverse small molecule ligands. Therefore, we favor the idea that individual ACs direct cellular responses to specific ligands [13,14,26] and hypothesize that procyclic-specific ACs P1 and P3-P6 identified here direct responses to extracellular signals that are specifically found in the tsetse fly. Tissue-specific signals within the tsetse are hypothesized to drive parasite movements and differentiation [27] and receptor-type ACs are ideally suited to function in perception of these signals. Importantly, identification of a small subset of procyclic-specific genes within *T. brucei's* cohort of ~60 AC genes allows one to now focus on this subset to test for requirement in transmission through the tsetse-fly vector.

Previous studies showed flagellum localization for ACs in procyclic parasites, but the antibodies used recognize multiple ACs and thus could not distinguish between isoforms [20]. By using gene-specific epitope tagging, we were able to determine the unique distribution of individual AC proteins. Surprisingly, we discovered three distinct patterns of localization. ACP2 was distributed along the flagellum, as seen previously for ESAG4 and FS24 in bloodstream trypanosomes [12,16,20] and for a group of GRESAGs in procyclic trypanosomes [20]. In contrast, ACP1, P3, P4 and P6 were localized to the flagellum tip, while ACP5 was enriched at the tip, but also found along the length of the flagellum. This distribution differs from that reported for all previously studied *T. brucei* ACs [20]. As discussed above, ACs are postulated to function as receptors for diverse ligands. Divergent ligand binding domains would confer ligand specificity, but each

receptor employs the same primary signaling output, cAMP, and this presents a problem because of potential interference between pathways. Differential localization of individual AC proteins would provide a mechanism to overcome this dilemma, because the signaling output for each protein is spatially restricted and thus would impinge on distinct effector proteins. Such a model is consistent with the micro-domain model for cAMP signaling in eukaryotic cells [24,28]. In this model, adenylate cyclases, phosphodiesterases and effectors are in close proximity to one another, resulting in confinement of cAMP production to distinct foci within the cell [7,24,28]. Importantly, the trypanosome flagellum also houses cAMP-specific phosphodiesterase, TbrPDEB1, which is distributed along the length of the flagellum [12] and thus is positioned such that it can act as a diffusion barrier to limit cAMP to the site of the adenylate cyclase that generates the signal [24]. Spatial restriction of cAMP signal initiation serves a variety of purposes including reduction of stochastic fluctuations of the signal and aberrant regulation of cellular processes due to non-specific signaling or cross-talk between different signaling units [28]. In this way, segregating individual ACs to distinct flagellum sub-compartments would allow two different receptors to initiate signal-specific responses despite using a common primary signaling output.

Differential localization of ACP1 versus P2 is of particular interest, because these two proteins contain almost identical N-terminal domains (Figure 4-1), suggesting they might bind the same ligand. Differential localization of two receptors with the same ligand specificity would allow the cell to detect spatial gradients of ligand and thus provide a potential mechanism for orienting itself with respect to the location of a ligand source. Such a mechanism would, in turn, provide a means for parasite chemotaxis and

navigation within diverse host environments, which is of central importance for *T. brucei* transmission through the tsetse fly [29].

Localization of a subset of ACs to the flagellum tip offers insight into potential functions of these proteins. For example, the flagellum tip is important for parasite development and host-parasite interaction within the tsetse fly vector. *T. brucei* development in the tsetse fly is a complex process and culminates with differentiation into human infectious, metacyclic trypomastigotes in the salivary gland. Differentiation into metacyclics is initiated by attachment of the trypanosome flagellum tip to the salivary gland epithelium, followed by extensive interdigitation of epithelial and flagellar membranes. Localization of procyclic-specific receptor ACs to the flagellum tip puts them in an ideal position to coordinate signaling events that drive these processes.

The tip is a specialized region of the flagellum, both structurally and functionally. The tip extends free of the cell body and is not constrained by the FAZ (flagellum attachment zone), which extends along the rest of the flagellum length. The tip is also the site for turnaround of the intraflagellar transport (IFT) machinery that is used for flagellum assembly [30,31]. IFT particles transition at the flagellar tip from the anterograde, kinesin-dependent transport, to retrograde, dynein-dependent transport and this requires signaling events to coordinate dynein and kinesin motor activity. In PCF parasites, the tip of the growing daughter flagellum is also distinguished by the presence of the flagellum connector, a mobile cytoskeletal structure that connects the growing daughter flagellum to the existing old flagellum [32,33,34]. The flagellum tip further plays an important role in cell division because it marks the site of cleavage furrow formation [34]and is the location of polo-like kinases suggested to be important in cell

division initiation [34,35,36]. Cell division and flagellum assembly must be coordinated, but the underlying signaling systems are unknown. One can imagine that these systems may be impacted by signaling through tip-localized ACs, though it is not clear in such a situation what roles would be played by the extracellular domain.

Several hundred proteins have been identified in the *T. brucei* flagellum [12,37,38]. However, only two proteins have been shown to be specifically localized to the flagellum tip. One of these is a kinesin that is associated with the axoneme and postulated to function in flagellum length regulation [39]. The other is calpain-x, which associates with membrane via dual acylation [40]. The function of calpain-x is not known. Proteins localized or enriched at the tip of the flagellum membrane have been characterized in other organisms and are known to play important functions in cell signaling. Examples include polycystins and Gli proteins in vertebrates, which function in Ca++ and hedgehog signaling, respectively [41]. Gli proteins are particularly interesting, because their dynamic delivery to and from the flagellum tip is a key event in hedgehog signaling responses that control vertebrate development. Examples of flagellum-tip proteins in protists include flagellar agglutinins in *Chlamydomonas* that mediate attachment of gametes to initiate cAMP signaling that drives mating [42]. Despite the importance of flagellum tip-localized proteins for flagellum function and cell physiology, targeting sequences and mechanisms that direct localization to specific flagellum sub-compartments are virtually unknown. In this regard, ACP1 and P2 in *T. brucei* offer potential insights. ACP1 and P2 are ~90% identical in amino acid sequence and differences are primarily restricted to a 42-amino acid region at the C-terminus of each protein (not shown). This region is expected to be intracellular and thus accessible

to targeting machinery. As such, sequence differences in the C-terminus may constitute targeting signals that direct differential localization ACP1 and P2 within the flagellum. The characterization of these unique regions and investigation of their protiential roles in localization of these ACs is the focus of current Hill lab efforts.

Trypanosomal ACs are postulated to coordinate intracellular cAMP signaling in response to engagement by extracellular ligands [7,14]. Although not demonstrated in this chapter, we have several lines of evidence supporting this idea. Other lab members have found the ACs to be detergent-soluble and surface-exposed, as would be required for receptors of extracellular ligands. These results are consistent with prior studies on ESAG4 in BSF cells [20] and a group of anti-ESAG4 cross-reactive GRESAG4 ACs in procyclic cells [20]. In addition, the lab has demonstrated that these ACs are glycosylated, also a common feature of many cell surface receptors.

Cyclic AMP signaling plays important roles in *T. brucei* development and pathogenesis [7,13,16]. Adenylate cyclases are the source of cAMP production within the cell and therefore play a central role in the initiation of cAMP signal transduction. In addition to providing for synthesis of cAMP, trypanosomal ACs have a unique receptor-like structure that provides an opportunity for integrating regulatory inputs from outside the cell. Together with earlier studies by others, our findings reveal that all of the adenylate cyclases so far studied in *T. brucei* are restricted to the flagellum, indicating that the flagellum is a key hub of cAMP signaling. Future studies aimed at understanding AC function as well as mechanisms of targeting to the flagellum and specific flagellum sub-compartments therefore offer opportunities for understanding key aspects of trypanosome biology and host-parasite interaction.

Materials and Methods Section

Cell Culturing

Procyclic-form trypanosome line "29-13" were used for all experiments [43]. Cells were cultured in Cunningham's SM medium complemented with 10% heat-inactivated fetal bovine serum [44]. Transfected cell lines were selected for and maintained as described below. Cell densities were monitored by using a Z1 Coulter Counter (Beckman).

Alignments/Domains

All protein sequences were obtained from TriTrypDB.org and GeneDB.org. Alignments were done using the AlignX module of VectorNTI Advance v10.5 (Invitrogen). Domains are presented as annotated by NCBI Blast and SMART [45].

Generation of RNAi Lines

The RNAi target region for ACP1 was chosen by the Trypanofan RNAit algorithm, and was PCR-amplified using the following primers:

ACP1-RNAi-F: at*aagctt*tccttctggcttcgtcactt

ACP1-RNAi-R: at*ctaga*ttcatcccggaacaaaactc

Restriction sites are italicized. Resulting DNA was ligated into the p2T7 RNAi vector, which uses opposing tetracycline-controlled T7 promoters. Insertion was verified by sequencing by Genewiz, Inc. The p2T7-ACP1-RNAi vector was linearized with Not1, and transfected into the 29-13 cell line, and selected for using phleomycin as previously described [44]. Clonal lines were generated by limiting dilution.

Proteomics

Procyclic *T. brucei* Fla1 cells were induced for 25 hours with 1g/ml tetracycline to a cell density of 4 x10^6 cells/ml. After addition of 0.2M sucrose for cushion, cells were sonicated for 6 min and spun at 2,000 g for 5 min to separate flagella in the supernatant from pelleted cell bodies. The flagella-containing supernatant was further spun down at 2,000 g for 5 min to remove debris in the pellet and then subjected to a high-speed centrifugation at 20,000 g for 35 min. The precipitated flagella were resuspended in PBS, layered on top of a 13 ml step 10/20/30/40/55/68% density gradient of sucrose, and then centrifuged at 245,000 g for 4 hours at 4°C in a Beckman Optima L-90K ultracentrifuge using a SW 41 rotor. 14 fractions of ~1 ml each were collected from the top of the gradient and spun further at 14,000 g for 1 hour at 4°C to concentrate the fraction samples for phase-contrast microscopic analyses. Purified flagella were visualized in fractions 8 and 9 corresponding to the region that includes the interface between 40 and 55% sucrose layers. To solubilize flagellar membranes, purified flagella were incubated for 10 min at RT with 0.1% NP40 in PBS and then centrifuged at 10,000 g for 10 min to separate the axoneme-containing pellet from the supernatant harboring flagellar membranes and the matrix. Since NP40 detergent can degrade the quality of the MS spectra, contaminating NP40 was removed after precipitation of proteins from the flagellar membranes/matrix fraction by TCA (trichloroacetic acid) treatment and 2 washes with acetone prior to the MS analysis by MudPIT approach [12]. Cell bodies were disrupted by hypotonic lysis (Kubata B. et al., 2000), sonicated for 2 min, and incubated for 10 min at RT with 0.1% NP40 in PBS. Proteins from cell bodies were

precipitated by TCA, washed by acetone to remove contaminating NP40, and subjected to MS analysis by MudPIT approach.

Quantitative Real-Time PCR

Cells were grown to midlog growth, and harvested at $5x10^6$ cells/ml. For ACP1KD, cells were grown with and without tetracycline for 72 hours prior to harvesting. Total RNA was extracted using Qiagen's RNAeasy kit, and treated with amplification grade DNAse (Invitrogen). cDNA was generated using 2ug total RNA using Superscript II Reverse-Transcriptase with oligo(dT) primers (invitrogen) per manufacturer's instructions. qRT-PCR was conducted on a DNA Engine Opticon 2 (MJ Research, Bio-Rad) according to the manufacturer's instructions. Gene-specific primer sets were designed using the Trypanofan RNAit algorithim and NCBI Primer-Blast.

Primers used were:

ACP1-F: CGTTGACTTCACGGCTTACA

ACP1-R: ACATTTCGTTCTCCCACTGC

ACP2-F: GCCATGTCGTTGATTTCACA

ACP2-R: CCAACCAGACCACAGACCTT

ACP4-F: AGCTTACGAGGGCTGTGAAA

ACP4-R: AAATACACTGCCCCTTGTCG

ACP5-F: TCTGCTTATGCAGGACGATG

ACP5-R: CCTCAAAAGTCTCGAGGTGC

FS24-F: GCGCTAGCATAAGACGTGGT

FS24-R: GAACCGTTCTCACACCAACA

ISG65-F: CATGACAGAGGAGTGGCAGA

ISG65-R: CATGCTCGGTTGAAGCACTA

qRT-PCR was performed in duplicate on at least two independent RNA preparations, and values were normalized against two genes in common to both PCF/BSF. Primers used were:

TERT-F: GAGCGTGTGACTTCCGAAGG

TERT-R: AGGAACTGTCACGGAGTTTGC

PFR2-F: GAAGTTGAAGGTGTTGTGAGTCC

PFT2-R: CCTCCAGCGTGATATCTGTTACC

Relative gene expression was determined using the 2 delta-delta CT method as previously described [44]

In situ epitope tagging

In situ tagging allows for introduction of an epitope-tagged gene into its endogenous chromosomal locus. In brief, 300 to 600bp fragments of DNA homologous to the target gene's open reading frame or 3'UTR were PCR-amplified and cloned upstream of the 3xHA tag, or downstream of the puromycin resistance marker of the pMOTag2H vector, as described previously [23]. Sequences were verified at the UCLA Sequencing and

Genotyping Core center. Tagging cassettes were excised by restriction digestion, purified, and transfected into 29-13 procyclic cells using standard methods. Transformants were selected using 1ug/ml puromycin, and clonal lines were created by limiting dilution [44].

ACP1 antibody generation

Antibody generation and purifications were performed by Pacific Immunology, Inc. (PacificImmunology.com). The peptide CAVGERNVSTPKEEN corresponds to the unique 3'end of the ACP1 ORF. This was synthesized and used for antibody production in two New Zealand rabbits. Affinity purification of production bleeds and ELISA analysis to determine titer of the immunopurified anti-ACP1 antibodies were performed by Pacific Immunology.

Immunofluorescence microscopy

Immunofluorescnece was carried out on whole cells as described previously [12], with the monoclonal anti-HA antibody HA.11 (CoVance) used at 1:250 dilution, using a donkey anti-mouse secondary coupled to AlexaFluor488 (Molecular Probes) at 1:2,500. Immunofluorescence Assays with the anti-ACP1 antibodies were done at 1:500, using a secondary donkey anti-rabbit coupled to AlexaFluor488 (Molecular Probes) at 1:2,500. Samples were mounted in VectaShield containing DAPI (Vector Laboratories), allowing visualization of DNA. Sample imaging was performed on a Zeiss Axioskop II compound microscope using a 100x oil-immersion objective.

Figures

Protein	Chromosome	GeneID	AA	Predicted kDa	Relatedness (REF)
AC-P1	11	Tb927.11.17040	1253	137.5	ESAG4-Like
AC-P2	10	Tb927.10.16190	1254	137.9	ESAG4-Like
AC-P3	7	Tb927.7.7470	1205	132.9	GRESAG 4.2/4.3
AC-P4	10	Tb927.10.13040	1208	133.3	GRESAG 4.2/4.3
AC-P5	11	Tb927.11.13740	1214	133.8	-
AC-P6	9	Tb927.9.15660	1242	137.5	-

Table 4-1. *T. brucei* adenylate cyclase genes

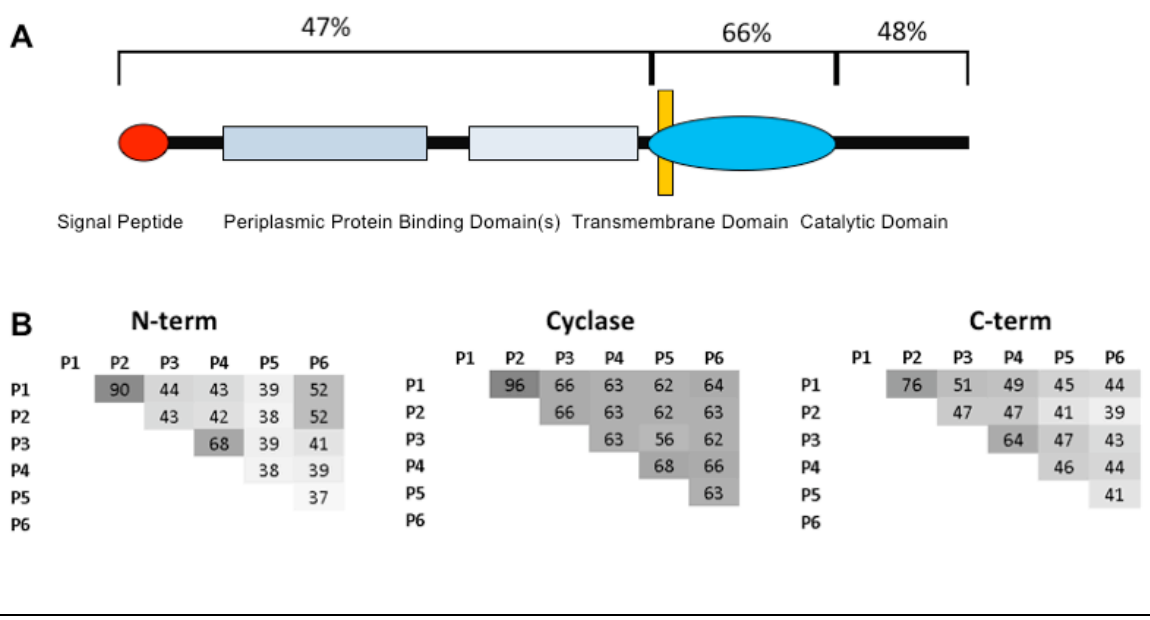

Figure 4-1: Domain architecture and identity between *T. brucei* AC P1 through P6. A) Architecture of African Trypanosome adenylate cyclases. Similarity in the different regions of the peptides between the 6 identified ACs in our study is indicated as a percent value. B) PAIRWISE comparison of domain similarity between individual ACs as indicated by percent values.

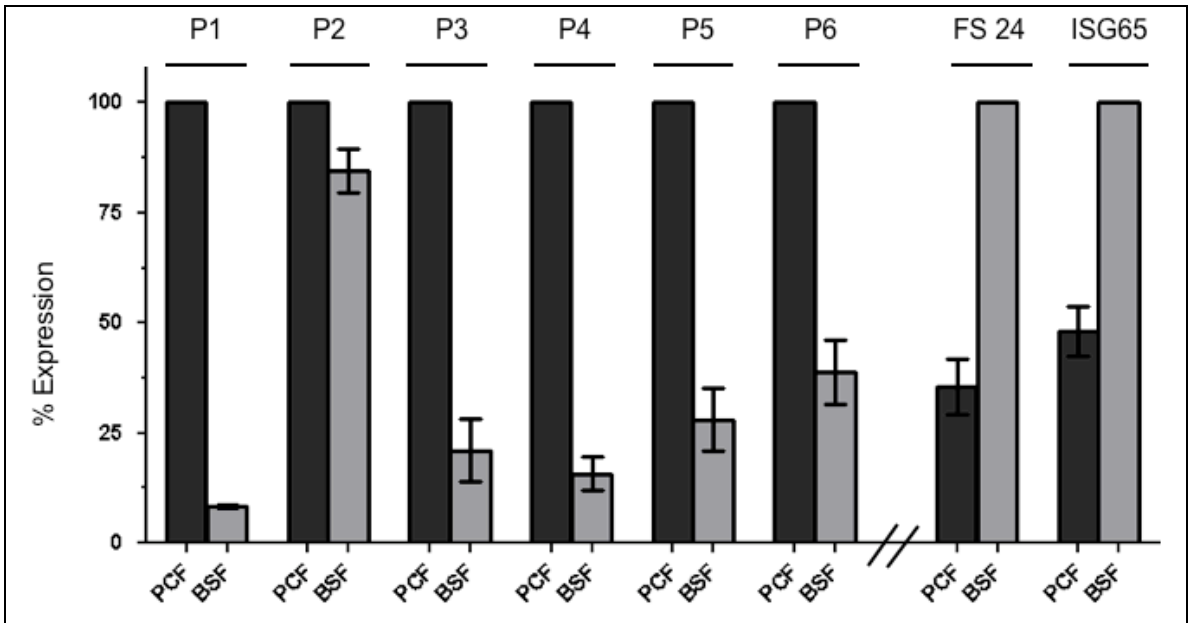

Figure 4-2: *T. brucei* adenylate cyclases display life-stage specific expression by qRT-PCR. ACP1, 3, 4, 5 and 6 are all specific to the insect-stage form of the parasite. As predicted, FS24 and ISG65 (two proteins known to be expressed in the bloodstream stage of the parasite) display increased expression in the BSF versus PCF stage.

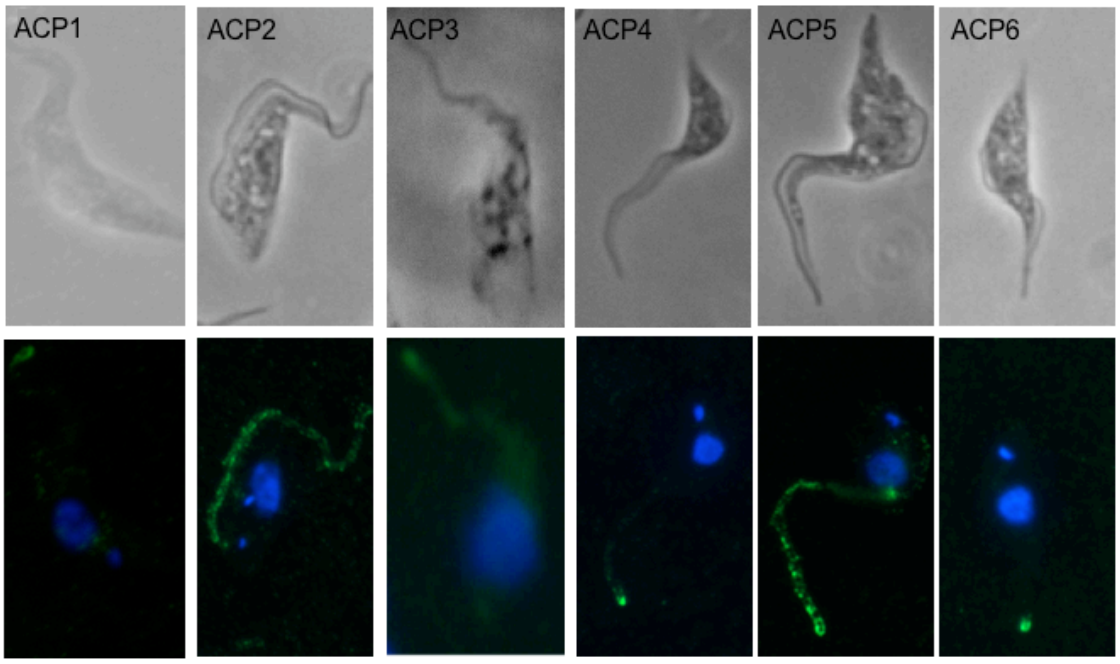

Figure 4-3. ACs P1-P6 localize to the *T. brucei* flagellum. Top panels are DIC images. Bottom panels are fluorescent images. HA-tagged protein in green, DAPI staining in blue.

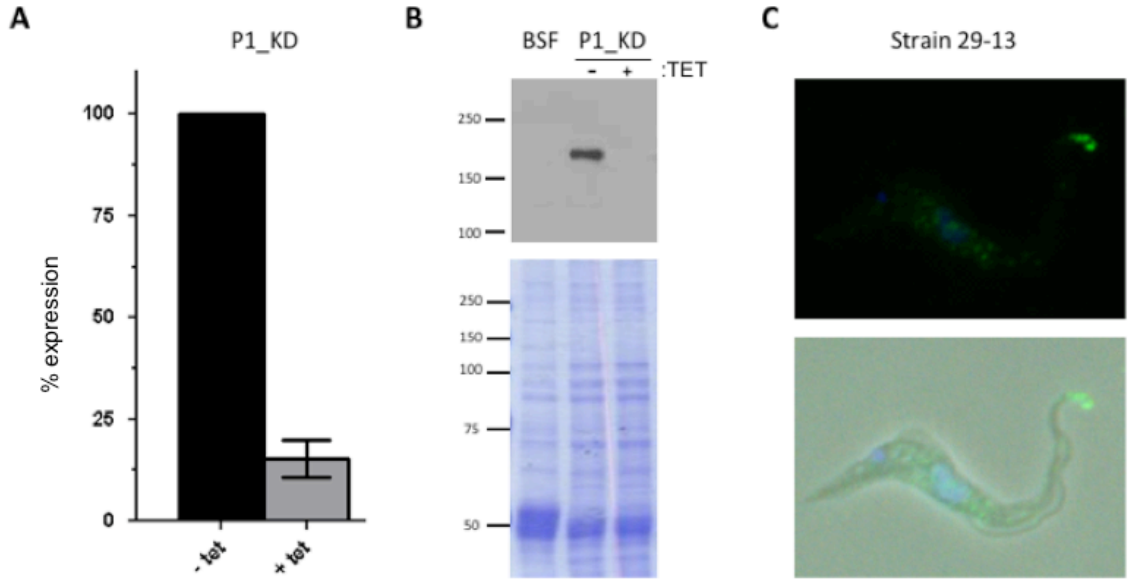

Figure 4-4. Endogenous AC P1 localizes to the flagellum tip. A) QRTPCR showing KD of ACP1 following tetracycline induction of RNAi KD. B) Western with ACP1-specific antibody confirming KD of ACP1 in KD cell line and specificity of ACP1 antibody. ACP1 is not expressed in Blood Stream Form (BSF) *T. brucei* (Figure 4-2) and this is confirmed by the lack of detection by the ACP1 antibody. C) IF of WT *T. brucei* cells with ACP1-specific antibody. Top panel is fluorescent image with antibody labeling in green and DAPI in blue. Bottom panel is DIC and fluorescent overlay.

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Chapter 5

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

Introduction

African trypanosomes, including *Trypanosoma brucei* and related subspecies, are the causative agents of sleeping sickness in humans and nagana in cattle. These parasites are the source of significant human mortality and limit economic growth throughout sub-Saharan Africa [1,2]. Sleeping sickness is recognized as one of the world's most neglected diseases and is a reemerging disease with ~60 million people at risk of infection. The disease is fatal if left untreated and current therapeutic options are insufficient as they are antiquated, toxic, difficult to administer and/or financially unattainable. The situation is further exacerbated by the development of drug resistance and there is a desperate need to identify new drug targets. Thus, *T. brucei* is a parasite of tremendous medical and economic importance and is recognized as a disease of poverty, as well as a cause of poverty.

T. brucei has a digenetic lifecycle, being transmitted to a mammalian host through the bite of an infected tsetse fly. In both hosts, trypanosomes are extracellular and in constant contact with host tissues. Within the tsetse, *T. brucei* undergoes a series of directional migrations necessary for development and differentiation into human-infectious forms [3]. Parasites entering the fly with the bloodmeal first colonize the tsetse midgut as procyclic forms. They then penetrate the peritrophic membrane and migrate anteriorly along the GI tract to the salivary glands where they attach to and colonize the epithelial surface, stimulating the final stage of differentiation into human-infectious forms [3]. Throughout this process, the parasites are in direct contact with host tissue

surfaces and undergo specific developmental changes in each host compartment. In the mammalian host, trypanosomes are also in contact with host tissues and must respond to cues from the host environment to stimulate parasite development and tissue colonization. Thus, interaction with host tissue surfaces and the ability to sense external cues are key aspects mediating *T. brucei* development, survival and pathogenesis.

Traditionally, studies of trypanosomes consider the parasites as individual cells grown in suspension cultures or in animal models. This has uncovered important aspects of trypanosome biology, but does not consider the effect of tissue surfaces on parasite biology. In other microbes, surface exposure induces dramatic changes that manifest themselves in a variety of ways and enable the organisms to perform distinct biological processes that are otherwise unavailable to single cells in the absence of these behaviors[4,5,6,7,8,9,10,11,12,13]. In pathogenic microbes, surface induced social behaviors such as biofilm formation and swarming motility lead to increased antibiotic resistance and tissue penetration [9,12,14]. The study of these social behaviors has revolutionized the way we consider microbes and treat the conditions they cause.

In an attempt to better understand how surface-exposure affects *T. brucei*, we cultivated procyclic (insect-stage) *T. brucei* on semi-solid agarose plates [15]. We found that surface exposure induced profound changes in trypanosome behavior, causing individual cells to assemble into multicellular communities with properties that are not evident in single cells. We termed this behavior “social motility”, based on analogies with similar surface-induced social interactions in bacteria. During initial stages of social

motility trypanosomes assemble into communities that grow through the active recruitment of nearby cells [15]. Continued recruitment, merger with other communities and replication results in the formation of macroscopic communities 3 to 4 days post inoculation. Parasites then undergo polarized migration outward from the site of inoculation, forming radial projections reminiscent of bacterial social motility. These projections are evenly spaced and consistent in number. They can also change their course of migration in response to extracellular factors, as projections avoid one another when they are in close proximity. Thus tyrpanosomes can sense and communicate with one another and coordinate their efforts to engage in a social behavior.

The discovery of social behavior in trypanosomes was a novel finding and we decided to investigate the mechanisms underlying its regulation. As discussed above, social behaviors in response to surface exposure are widespread and manifest in a variety of different ways. Furthermore, the study of these behaviors in pathogenic microbes has influenced the way we consider and treat diseases caused by them. Although the mechanisms that regulate these behaviors are diverse, they generally require organisms to be able to sense external signals, transduce the signal to generate a response and cooperate with surrounding organisms. One particular class of molecules that play a major role in the regulation of social behaviors is cyclic nucleotides. In the social amoeba *Dictyostelium discoidium,* for example, cAMP signaling plays a key role in fruiting body formation [5]. Also, in bacteria, c-di-GMP signaling regulates a variety of social behaviors including biofilm formation, swarming motility and adherence to tissue surfaces [16,17,18,19].

In trypanosomes, cyclic nucleotide signaling is important for a variety of processes including differentiation, cytokenesis and pathogenesis [20,21]. In *T. brucei,* the flagella is speculated to be a center for cAMP production and signaling [22,23]. Accordingly, adenylate cyclases (ACs) and phosphodiesterases (PDEs) have been localized to the parasite flagella [24,25,26,27]. *T. brucei* phosphodiesterases are fairly well studied and characterized and have been implicated in cell viability [24]. In addition, in recent years they have emerged as candidates for targeting by therapeutics [28].

In contrast to PDEs, not as much is known about trypanosomal ACs. Although localization and biochemical studies have been done on these molecules [20,22,27,29,30,31,32,33], much about their expression and function(s) remains unknown. Trypanosomal ACs have a cytoplasmic catalytic domain near the C-terminus, connected by a single transmembrane domain to a large, extracellular N-terminal domain (Figure 5-1). The N-terminal domain varies among family members and exhibits homology to a superfamily of proteins involved in nutrient sensing in bacteria and ligand binding in mammalian receptors. Architecturally, these molecules resemble membrane-bound type I guanylyl cyclases which act as receptor molecules to generate cGMP in response to extracellular signals [22]. Additionally, unlike related kinetoplastids, African trypanosomes show a remarkable expansion of receptor-like ACs [34]. The unique structure of trypanosomal ACs as well as their expansion within the genomes of African trypanosomes has lead to the tantalizing hypothesis that these molecules function as receptors to extracellular signals [20,22].

Of the approximately 60 family members, very few *T. brucei* ACs have been previously studied or characterized. The canonical AC, ESAG4, is expressed specifically in BSF parasites and has recently been shown to function in modulation of the host immune response and parasite viability [25,26]. Other AC genes remain largely unstudied and have been considered to be constitutively expressed and redundant in function [22]. Recently, our lab identified 6 previously uncharacterized ACs that localize to distinct subdomains of the *T. brucei* flagella. Five of these six ACs were preferentially expressed in the procyclic (insect-stage) form of the parasite. This data demonstrated for the first time that these genes are not constitutively expressed and their localization to distinct subdomains of the flagella implied that they are not redundant in function, as was previously thought. Given their increased expression in the procycylic form of the parasite, localization to the flagella (a known sensory organelle) and potential role as receptor molecules, we decided to investigate their possible role(s) in the regulation of Social Motility (SoMo). Here we report that SoMo is regulated by ACs and cAMP signalling. These findings dispel the notion that trypanosomal ACs are redundant in function and prove the importance of cyclic nucleotide signaling in the regulation of *T. brucei* social behavior.

Results

Knockdown (KD) of insect-stage specific ACs has no effect on parasite viability or motility

To assess the function of the six ACs uncovered in the *T. brucei* procyclic form flagellar proteome, we generated cell lines carrying constructs targeting the open reading frame (ORF) of ACs 1 and 2, 3, 4, 5 or 6 for RNAi-mediated KD using a tetracycline-inducible system [35]. Real-Time Quantitative Reverse Transcription PCR (qRTPCR) was used to assess KD of the targeted ACs in the five KD cell lines (Figure 5-2A). Growth and forward translocation in suspension culture was unaffected in the KD cells (Figure 5-2B and not shown). Thus KD of ACs 1 and 2, 3, 4, 5 or 6 does not impact parasite growth or motility.

KD of ACs 1,2 or 6 results in a "hyper SoMo" phenotype

To assess the function of ACs 1-6 in the regulation of Social Motility (SoMo), we decided to plate the KD cell lines using the SoMo assay previously described [36]. Briefly, *T. brucei* SoMo is characterized by the formation of multicellular communities on soft-agarose plates. After 3-4 days these communities move *en masse* across the surface of the agarose to form macroscopic patterns. These patterns are generated by the

movement of individual rafts of cells (or "projections") away from the site of inoculation. The number of these projections and the spacing between them is consistent across assays, with a median value of 4 projections formed per inoculation (Figure 5-3).

Interestingly, the KD of ACs 1 and 2 or 6 results in an increased formation of projections as compared to control cells and conditions (Figure 5-3). AC1,2 KD and AC6 KD cell lines generate a median value of 14 projections per inoculation when induced for KD (in the presence of tetracycline) versus 5 or 4 (respectively) when uninduced. We termed this phenotype "hyper SoMo" due to the increased or hyper formation of projections exhibited by the KD lines. KD of AC 3, 4 or 5 does not result in a hyper SoMo phenotype (Figure 5-4 and not shown). Thus different ACs have different effects on SoMo; KD of ACs 1, 2 or 6 causes a hyper SoMo phenotype while KD of AC 3, 4 or 5 does not affect SoMo.

KD of AC6 by targeting the 3'UTR results in hyper SoMo

Next we sought to further characterize the roles of ACs 1, 2 and 6 in the regulation of SoMo. ACs 1 and 2 are very similar proteins sharing 90% homology. To avoid potential downstream issues and complications in discerning the role of AC1 versus 2 due to the similarity of the two proteins, we focused our efforts on the study of AC6. To facilitate expression of RNAi-immune constructs in an AC6 KD background line, we generated a line (AC6 uKD) where the 3' untranslated region (UTR) of AC6 is

targeted for RNAi-mediated KD. This line is different from the previously used AC6KD line because rather than targeting the open reading frame of the gene (as was done in the AC6KD line), we targeted the 3' UTR. Expression of AC6 in AC6 uKD was assessed via qRTPCR (Figure 5-4)**.** Expression of ACs 1, 2, 3 and 5 in this cell line remained unaffected as demonstrated by qRTPCR thus indicating specific KD to AC6 (not shown). Growth and forward translocation in suspension culture was unaffected in AC6 uKD cells (Figure 5-4 and not shown).

The hyper SoMo phenotype of the AC6 KD line was also present in the AC6 uKD line (Figure 5-4). AC6 uKD cells generate a median value of 14 projections per inoculation when induced for KD (in the presence of tetracycline) versus 5 when uninduced. By comparison, AC5 KD cells produce a median of 3.5 or 6 projections in induced versus uninduced conditions, respectively (Figure 5-4). Thus KD of AC6 via two independent approaches results in hyper SoMo.

Restoration of AC6 expression in AC6 uKD rescues hyper SoMo while expression of a catalytically inactivated version of AC6 does not

To assess the specific contribution of AC6 in the regulation of SoMo, we decided to generate two RNAi-immune constructs for transfection into the AC6 uKD line. The first would serve to restore expression of a wild-type copy of AC6 (AC6 Ri) and the second would serve to test the importance of the catalytic domain of AC6 in the

regulation of SoMo (AC6** Ri). In both cases, immunity to RNAi was achieved by placing the intergenic region (IgR) of alpha/beta tubulin dowstream of the AC6 stop codon in either the AC6 Ri or AC6** Ri constructs. Following transfection into the AC6 uKD line, homologous recombination gives rise to cell lines carrying either a wild-type copy or catalytically inactive version of AC6 immune to RNAi. To generate a catalytically inactive version of AC6, highly conserved residues within the catalytic domain were mutated via site-directed mutagenesis (see Materials and Methods). Mutation of the residues we targeted have been previously reported to reduce the catayltic activity of *T. brucei* ACs by greater than 90% [32,33].

Expression of the RNAi-immune AC6 constructs and native AC6 was assessed via Northern Blot (Figure 5-5). Replacing the native 3'UTR of AC6 with the alpha/beta tubulin IgR results in a size difference between native AC6 and the AC6/AC6** Ri variants. AC6 is preferentially expressed in the procyclic form (PCF) of *T. brucei* versus the bloodstream form (BSF), in line with previous results from our lab. Native AC6 expression in the AC6 uKD line is reduced in induced conditions, as previously seen by qRTPCR (Figure 5-4) and in the AC6 Ri and AC6** Ri cell lines. Expression of the introduced versions of AC6 or AC6** in the AC6 Ri or AC6** Ri lines remains constant in induced and uninduced conditions. Growth and motility in suspension culture are unaffected in the AC6 Ri and AC6** Ri cell lines (not shown).

Restoration of AC6 expression in the AC6 uKD cell line (AC6 Ri) rescues the hyper SoMo phenotype (Figure 5-5). In uninduced or induced AC6 KD conditions, AC6

Ri cells generate a median value of 3.5 projections per innoculation. Conversely, expression of the AC6** in the AC6 uKD cell line (AC6** Ri) fails to rescue the hyper SoMo phenotype. AC6** Ri cells generate a median value of 14 projections when induced for AC6 KD versus 7 when uninduced. Thus AC6 is sufficient to regulate SoMo.

Effect of cAMP and catalytic activity of AC6 on regulation of SoMo

To test the role of intracellular cAMP levels in the regulation of SoMo an ELISA was done to assess cAMP levels in the AC6 uKD, AC6 Ri and AC6** Ri cells. Intracellular cAMP levels were slightly reduced in the AC6 uKD and AC6** Ri cells in induced conditions as compared to uninduced controls (Figure 5-6). There was no difference in intracellular cAMP levels in AC6 Ri cells in uninduced versus induced conditions. Although these differences are not significant, the data points to a clear trend. Furthermore, when one considers the current micro domain model of cAMP signaling (see discussion), it is unclear if large changes in intracellular cAMP levels would be observed if only one AC is inhibited. According to the micro domain model of cAMP signaling, small regional changes in cAMP levels would constitute significant changes in biology.

To ensure that the hyper SoMo phenotype in AC6 uKD cells and AC6** Ri cells was due to reduced AC6 function and not mislocalization, homologous recombination was used to deliver and HA-tagged version of version of AC6** into AC6 uKD cell lines. The localization of HA-tagged AC6** was compared to that of HA-tagged native AC6. HA-tagged AC6** localizes to the flagellar tip as does the wild-type HA-tagged AC6

(Figure 5-7). Thus the hyperSoMo phenotype observed in the AC6** line is not a result of mislocalization of the AC6** protein.

Discussion

The results presented in this study are the first to demonstrate regulation of SoMo at the molecular level. In addition, these are the first studies showing that trypanosomal ACs do not serve redundant roles as previously thought. The generation of AC 1 and 2, 3, 4, 5, and 6 KD cell lines was important in evaluating the different contribution(s) of these molecules in the regulation of SoMo. The observation that only 2 of the 5 generated KD cell lines (AC1,2 KD and AC6 KD) produced a hyperSoMo phenotype points to a distinct role for *T. brucei* ACs in the regulation of cellular processes.

Further characterization of the hyper SoMo phenotype observed in the AC6 KD cell line demonstrates a clear role in the regulation of SoMo by AC6. First of all, generation of two independent KD lines targeting distinct regions of the AC6 mRNA for RNAi KD indicates that the hyper SoMo phenotype observed is the result of AC6 KD and not off-target effects; QRTPCR results demonstrating that expression of other ACs in the AC6 KD and AC6 uKD lines is unaffected in addition to the ability to rescue the hyper SoMo phenotype via the introduction of an RNAi-Immune version of AC6 in the AC6 uKD line supports this hypothesis.

The substitution of key catalytic residues of AC6 was an important step in identifying the molecular mechanism by which AC6 regulates SoMo. Substitution of all amino acids 231 - 237 to alanine led to an inability to rescue the hyperSoMo phenotype. The mutation of these key residues was previously reported to substantially decrease the catalytic activity of another *T. brucei* AC [32,33]. To test if cAMP levels were affected in the AC6** Ri cell lines and AC6 uKD cell lines we perfomed an ELISA to measure cAMP levels in these cells. The reduction of cAMP levels in the AC6** Ri line indicates a reduced activity in the catalytic activity of AC6**. Although this data is not significant, it supports the micro domain model of cAMP signaling in which small regional changes to cAMP levels can influence cellular processes. In addition, the decrease of cAMP levels in AC6 uKD and AC6** Ri cells implicates a role for cAMP in the regulation of SoMo. This hypothesis is supported by recent work in the Hill lab in which SoMo is shown to be inhibited by the disruption of PDE activity (see discussion below).

The observation that the cyclic nucleotide cAMP is an important player in the regulation of SoMo is exciting and in line with other studies demonstrating the importance of this class of molecules in the regulation of other social behaviors. Since its discovery more than 50 years ago by Earl Sutherland, cAMP has emerged to be one of the most important messengers in biology and has been observed to regulate a wide variety of cellular processes. In fact, the list of biological processes that are influenced by cAMP signaling is ever expanding. Given the tremendous importance of this molecule and the vast amount of studies conducted in various organisms, we in the trypanosomal

community are still in the early stages of understanding the role(s) of cAMP in trypanosome biology.

As discussed in the introduction, cAMP has previously been implicated to play a role in the differentiation, cytokenesis and pathogenesis of *T. brucei* [20,25,26,37]. Here, we report that a single AC (AC6) can regulate SoMo. In addition, our lab has also observed that disruption of the function of a flagellar-specific phosphodiesterase (TbrPDEB1) results in a null SoMo phenotype. It is important to note that AC6 and TbrPDEB1 both localize exclusively to the *T. brucei* flagellum. This is an important finding for two reasons: 1) the eukaryotic flagellum is a known sensory organelle [38], and 2) the *T. brucei* flagellum is considered to be a center for cAMP signaling for the parasite [22,39,40]. These 2 points along with the observations from our lab discussed above and the thought that tryponsome ACs act as receptors have led us to generate a model to explain how cAMP signaling influences SoMo (Figure 5-8). From a very simplistic standpoint, decreased cAMP levels (as a result of AC6KD) result in a hyperSoMo phenotype whereas increased levels of cAMP (from decreased TbrPDEB1 function) result in a null SoMo phenotype.

Though the specifics of social behaviors vary greatly form organism to organism, in general, the regulation of these behaviors requires sensation of external signals, transduction of this signal to generate a response and cooperation with surrounding organisms. In our model, a flagellum-associated AC (in this case AC6) detects a signal, prompting it to produce the appropriate level of cAMP to promote SoMo. cAMP would

then work directly or through an effector to influence a cellular process that would influence SoMo. At the same time, a local PDE (in this case TbrPDEB1) would degrade the cAMP signal once an action has been conducted and prevent cAMP from stimulating nonspecific targets through diffusion. Positioning of the AC and the PDE within close proximity of each other is based on the micro domain model of cAMP signaling in which ACs, PDEs and effectors are all in close proximity to one another resulting in the confinement of cAMP production to distinct foci within the cell [21,39,41]. The spatial restriction of cAMP production accomplishes a variety of purposes including reduction of stochastic fluctuations of the signal and aberrant regulation of cellular processes due to non-specific or off-target signaling. When AC6 is not present or inhibited, the appropriate amount of cAMP is not produced. This results in a decreased amount of cAMP and a hyperSoMo phenotype. Conversely, if TbrPDEB1 function is inhibited, the cAMP produced by AC6 is not degraded. This results in a higher amount of cAMP and an inhibition of SoMo.

Interestingly, our observations with an inverse correlation between cAMP and extent of SoMo parallels work relating to c-di-GMP and the regulation of the swarming behavior of *Pseudomonas aeriginosa* [17,18]. In these studies, knock out of a c-di-GMP phosphodiesterase results in decreased swarming motility [17], whereas knock out of a diguanylate cyclase results in hyper swarming behavior [18]. Indeed, in a growing number of bacterial systems, it has been shown that intracellular levels of c-di-GMP influence a variety of bacterial behaviors. In general, the accumulation of c-di-GMP promotes sessile behaviors, such as biofilm formation, while the breakdown of c-di-GMP

and subsequent decrease in cellular levels of this signal favors motile behaviors, such as swarming motility [42,43,44,45,46,47]. Thus our observations are in line with current findings in the microbial field regarding the role of cyclic nucleotide signaling and the regulation of social behaviors.

The next step in our work will be to further evaluate our model by looking for the signal(s) that mediate AC6 activity as well as identification of effectors that translate the cAMP signal intracellularly. As discussed previously, due to their distinct architecture and similarity to membrane-bound receptor-type guanylyl cyclases, *T. brucei* ACs have been speculated to be receptors for extracellular ligands [20,22]. Indeed, this thought is supported by the fact that these molecules possess a periplasmic binding protein domain and localize to the parasite's flagellum. However, to date no ligands have been identified that bind to *T. brucei* ACs. Periplasmic binding protein domains bind a variety of different ligands and are present in a variety of different receptors including guanylyl cyclases. Thus, the search for ligands that might bind to *T. brucei* ACs is a complicated endeavor due to the possible number of potential ligands. These sorts of investigations have been further complicated by the lack of an assay to monitor the parasite's response to different stimuli. Our findings show that in the absence of AC6 activity, an increased amount of social behavior occurs. Thus under normal conditions, signal(s) detected by AC6 result in: activation of the enzyme, production of the appropriate amount of cAMP, and suppression of hyperSoMo. Given the reproducibility of the SoMo assay, the ease in interpreting the results due to the macroscopic nature of the assay, and the overall manipulability *T. brucei* as a model system, we are confident that this assay can be used

to identify a ligand for AC6. Investigations looking for inhibitors and activators of different *T. brucei* ACs will be the focus of future experiments in our lab.

Identification of molecules downstream of AC6 will also be important in furthering the understanding of SoMo. cAMP exerts its influence on cellular processes via a variety of different effectors. One of the most common effectors used by cAMP in mammals is Protein Kinase A (PKA). In mammals, PKA is found in an inactive state, with two catalytic subunits (PKA-C) bound in a heterotetramer to two regulatory subunits (PKAR). cAMP binds to the regulatory subunits, resulting in release of PKA-C subunit. Although a putative regulatory subunit of PKA has been identified in *T. brucei*, this molecule was paradoxically found to be stimulated by cGMP as opposed to cAMP [48]. That being said, it would be interesting to test the role this protein might have in SoMo. In addition, the *T. brucei* genome contains several other proteins with putative cNMP binding domains; these molecules are currently being evaluated in our lab for their role(s) in the regulation of SoMo.

In summary, the results presented in this paper are the first to show a mechanism for the regulation of SoMo and that *T. brucei* ACs are not redundant in function. This work, in addition to unpublished results from the Hill lab point to a distinct role for cyclic nucleotide signaling in the regulation of SoMo. Furthermore, these findings will serve as a starting point from which we can begin dissecting the mechanisms underlying *T. brucei* social behavior. Given the parallels with other systems, work done to investigate SoMo will clearly further our understanding of microbes and how they interact with their

environments.

Material and Methods

Nomenclature and GeneDB ID

From: http://tritrypdb.org/tritrypdb/

AC1= Tb11.01.8820, AC2= Tb927.10.16190, AC3= Tb11.01.5310, AC4= Tb927.7.7470, AC5= Tb11.01.5310, AC6= Tb09.244.2380

Cell culture, Viability and Motility assays

Suspension cultures were maintained using Cunningham's semi-defined medium (SM), supplemented with 10% heat-inactivated fetal calf serum as described previously [35]. For 29-13 (Wild-type cells), the medium was further supplemented with 15mg/ml G418 (Gibco) and 50mg/ml Hygromycin (Gibco). For the RNAi lines, 2.5mg/ml Phleomycin, 15mg/ml G418 (Gibco) and 50mg/ml Hygromycin (Gibco) were included in the medium and RNAi was induced by adding 1mg/ml tetracycline. Media for lines carrying pMOT vector for rescue and/or tagging was prepared as discussed above for the 29-13 cells plus the addition of 1 µl/ml of puromycin. Cell growth in the presence or absence of tetracycline was monitored using a Z1 Coulter Particle Counter (Beckman Coulter, USA) and plotted using Microsoft Excel. Motility on the various cell lines was assayed via the Motility Trace method as previously reported [35]. Knockdown cell lines were assayed following a 72 hour incubation with tetracycline.

RNAi cell-line generation and QRTPCR

The RNAi target regions for the different ACs were chosen by the Trypanofan RNAit algorithm, and were PCR-amplified using the following primers:

For the AC1,2 KD line:

F- TTGATGATGATGGTAGCGGA, R- ACATACACCGCCTTACTGCC

For the AC3KD line:

F- GACGGTTCTGTCCCTGTTGT, R- TGGCTCTGAACAGTGAATGC

For the AC4KD line:

F- AGCTTACGAGGGCTGTGAAA, R- AAATACACTGCCCCTTGTCG

For the AC5KD line:

F- TCTGCTTATGCAGGACGATG, R- CCTCAAAAGTCTCGAGGTGC

For the AC6KD line:

F- TGGAGCAGCAAATCTACGTG, R- TTTTCTCGGCTCTCCACTGT

For the AC6 uKD line:

F- AT*AAGCTT*ACGGGGTTCCCTCATTTAAC

R- AT*TCTAGA*ACAACAACAACCCCCAAAAA

Restriction sites are italicized. Resulting DNA was ligated into the p2T7 RNAi vector, which uses opposing tetracycline-controlled T7 promoters. Insertion was verified by sequencing by Genewiz, Inc. The p2T7-AC-RNAi vectors were linearized with Not1,

and transfected into 29-13 cells and selected for using phleomycin as previously described [35]. Clonal lines were generated by limiting dilution.

Efficiency of KD was confirmed via Quantitative Real-Time PCR. Cells were grown to midlog growth, and harvested at $5x10^6$ cells/ml. For the KD lines, cells were grown with and without tetracycline for 72 hours prior to harvesting. Total RNA was extracted using Qiagen's RNAeasy kit, and treated with amplification grade DNAse (Invitrogen). cDNA was generated using 2ug total RNA using Superscript II Reverse-Transcriptase with oligo(dT) primers (invitrogen) per manufacturer's instructions. qRT-PCR was conducted on a DNA Engine Opticon 2 (MJ Research, Bio-Rad) according to the manufacturer's instructions. Gene-specific primer sets were designed using the Trypanofan RNAit algorithim and NCBI Primer-Blast.

Primers used were:

For AC1:

F- CGTTGACTTCACGGCTTACA, R- ACATTTCGTTCTCCCACTGC

For AC2:

F- GCCATGTCGTTGATTTCACA, R- CCAACCAGACCACAGACCTT

For AC3:

F- ACTGATGGGCGTCTTCACACAA, R-GGATGCACTTTTCTTGGGCAAC

For AC4:

F- CTGCGAGTGCGAGTTGGTGT, R- ACGTTCTGCGGTGCTGAGTG

For AC5:

F- CACATCTCAGCGCCAAAAACTG, R- TAGACCGCATAATCGCCTCACA

For AC6:

F- TGCAGTTAAGGTGGGTCACA, R- GATCCACCGCAGGATTAGAA

qRT-PCR was performed in duplicate on at least two independent RNA preparations, and values were normalized against the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Tb927.6.4280/ Tb927.6.4300) and RPS23 (Tb10.70.7020/Tb10.70.7030).

GAPDH primers:

GGCTGATGTCTCTGTGGTGGA, GGCTGTCGCTGATGAAGTCG

RPS primers:

AGATTGGCGTTGGAGCGAAA, GACCGAAACCAGAGACCAGCA

Relative gene expression was determined using the 2 delta-delta CT method as previously described [35]

SoMo Assay

Cultivation on semi-solid agarose plates was peformed as previously described [36]. Visualization of the plates at the indicated time points was accomplished using an Olympus Stylus 770 SW digital camera as previously described [49].

Western Blots

Protein extracts were prepared and analyzed by western blotting. $1x10^6$ cell equivalents per lane were used for western blotting analysis. Monoclonal mouse anti-HA antibody from Convonace (used at 1:5000) and monoclonal anti-ß-tubulin E7 hybridoma supernatant (used at 1:7000 dilution) were used at as primary antibodies to detect HA-tagged cells or ß-tubulin, respectively. The secondary antibody used was horseradish peroxidase-coupled goat anti-mouse (BioRad). Visualization of proteins was done using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific).

Generation of AC6 Rescue and Mutant Cell lines

The AC6 Ri and AC6** Ri cell lines were generated in the AC6 uKD cell line using the pMOTag HA-tag plasmid [50]. To generate the AC6 Ri line, the HA-tag was removed from the pMOTag HA-tag plasmid. A portion of the AC6 open reading frame was then cloned into cloning site upstream of the plasmid's α/ß intergenic region sequence. Primers used to amplify the final 426 bp segment of the AC6 ORF were:

F- ATGGTACCGAGGCATATGTGGCGGATG,

R- ATGTCGACCTACTGCTTCCCCTTTTCCT

Following confirmation of proper integration of the appropriate fragment by sequencing via GeneWiz, the plasmid was prepared for transfection into the AC6 uKD line and cells were selected for the integration of the construct from homologous recombination by growth under puromycin [35]. Clones were then prepared by serial dilution and assayed for AC6 expression in the presence of tetracycline via QRTPCR and Northern Blot analysis.

AC6** Ri plasmid was prepared by cloning a fragment of AC6 ORF into a pMOTag HA-tag vector where the HA tag was removed as described above. Primers used for amplification of the final 1332 bp of the AC6 ORF were:

F- ATGGTACCACGCGCATTAGTGTGTGGTC

R- ATGTCGACCTACTGCTTCCCCTTTTCCT

Following confirmation of proper integration of the appropriate fragment by sequencing via GeneWiz, the plasmid was prepared for mutagensis by the Stratagene Quickchange Kit. The stratagene quickbase primer design software was used to design the following primers:

F- ggatatgactattacggtcaaacggcagccgcggctgccgccgcggagagcattgcgaa

R- ttcgcaatgctctccgcggcggcagccgcggctgccgtttgaccgtaatagtcatatcc

These primers were designed to introduce mutations to amino acids 232 - 237 on the AC6 peptide which represent the highly conserved NMAART domain. Previously, the

mutations N232A and R236A were observed to cause >90% decrease in the catalytic activity of a trypanosome AC [32]. We therefore mutated all amino acids from A231 and T237 to alanine. Sequencing via GeneWiz was performed on the mutated plasmid prior to transfection into the AC6 uKD line. Clonal lines were generated and selected for as described above. Expression of the AC6** was confirmed by Northern blot.

Epitope tagging and Immunofluorescence

Cell lines carrying HA-tagged versions of AC6 and AC6** were generated using the pMOTag HA plasmid [35]. The final 426 bp of the AC6 ORF were amplified by PCR using the following primers:

F- ATGGTACCGAGGCATATTGTGGCGGATG

R- ATCTCGAGCTGCTTCCCCTTTTCCTCC

The fragment was then cloned into the cloning site of the pMOTag HA plasmid upstream of the HA tag. Following confirmation of proper integration of the appropriate fragment by sequencing via GeneWiz, the plasmid was prepared for transfection into 29-13 cells were selected for the integration of the construct from homologous recombination by growth under puromycin. Clones were then prepared by serial dilution [35] and assayed for expression of an HA-tagged AC6 via Western blot and Immunofluorescence.

For generation of a cell line carrying an HA-tagged version of AC6**, the AC6** segment was amplified from the AC6** plasmid from above using the following primers:

F- ATGGTACCACGCGCATTAGTGTGTGGTC

R- ATCTCGAGCTGCTTCCCCTTTTCCTCC

The fragment was then cloned into the cloning site of the pMOTag HA plasmid upstream of the HA tag. Following confirmation of proper integration of the appropriate fragment by sequencing via GeneWiz, the plasmid was prepared for transfection into AC6 uKD cells were selected for the integration of the construct from homologous recombination by growth under puromycin. Clones were then prepared by serial dilution and assayed for expression of an HA-tagged AC6** in the presence or absence of tetracyline via Western blot and Immunofluorescence.

Immunofluorescence was carried out on whole cells as described previously [51], with the monoclonal anti-HA antibody HA.11 (CoVance) used at 1:250 dilution, using a donkey anti-mouse secondary coupled to AlexaFluor488 (Molecular Probes) at 1:2,500. Immunofluorescence Assay with the anti-ACP1 antibodies were done at 1:500, using a secondary donkey anti-rabbit coupled to AlexaFluor488 (Molecular Probes) at 1:2,500. Sample imaging was performed on a Zeiss Axioskop II compound microscope using a 100x oil-immersion objective.

cAMP ELISA

CyclicAMP levels were measured using the Cyclic-AMP Direct ELISA Kit (EnzoLife Sciences). Cell lines were treated with or without tetracycline for 24 hours as

described above. $4x10^7$ cells were harvested, washed in PBS, and re-suspended into a hypotonic lysis buffer (1 mM Hepes, 1 mM EDTA, 1x SigmaFAST Protease Inhibitors) and left on ice for 10 minutes. Samples were then passed through a 25±3/8G needle ten times, before the addition of 1M HCL to give a final concentration of 0.1M HCL. Cells were spun at 14,000xg at 4^0C for 10 minutes, and supernatant fractions were saved for analysis. 100ul of supernatant corresponding to $2.5x10^7$ cells was used per well. The ELISA was done following the manufacturer's suggested protocols, and output was read at 405nM and analyzed using MasterPlex2010 (MiraiBio Group of Hitachi America). A best-fit curve using 5PL logistics was utilized. All samples and controls were done and measured in duplicate.

Northern Blot

Cell lines were treated with or without tetracycline for 72 hours as described above, and total RNA was extracted using a RNeasy Miniprep Kit (Qiagen). Northern blots were performed on RNA samples (10ug/lane) from each line as previously described [52], with the exception of using digoxiginin-labelling and detection methods in place of 32-P probes. rRNA was visualized under UV light. A probe unique to the AC 6 ORF was created using the PCR-DIG Probe Synthesis Kit (Roche) with primers F-TGTGCTTTTGTTTGGTGCTC and R-AGTAGTTCGGGTCCGTGATG and suspended into DIG-EasyHyb Buffer (Roche). This corresponds to a 305 bp probe region that is AC 6 specific according to RNAit and NCBI-BLAST. Blots were visualized using the DIG Nucleic-Acid Detection Kit (Roche).

Figures:

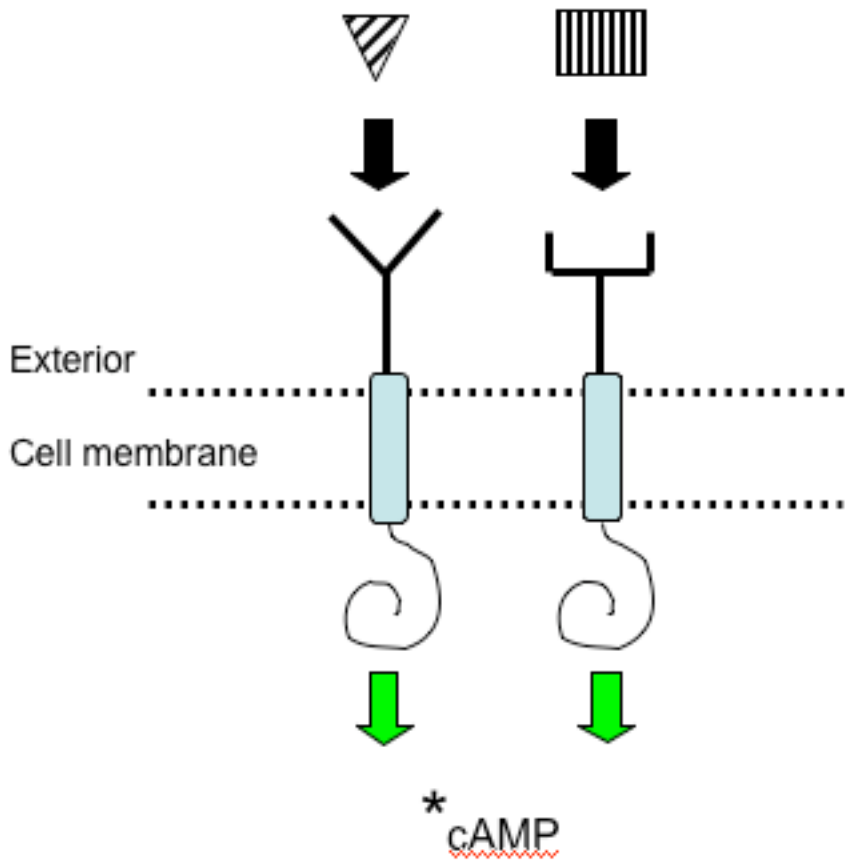

Figure 5-1. *T. brucei* adenlyl cyclases (ACs) are receptors to extracellular signals. *T. brucei spp* have an expanded array of ACs. These molecules are characterized by a large, extracellular, variable N-terminal domain, a transmembrane domain and a highly conserved intercellular catalytic C-terminal domain. Due to their expanded family size, variable extracellular domain and similarity to guanylyl cyclases, *T. brucei* ACs are

speculated to be ligand-binding proteins that can detect environmental cues. In the simplistic illustration, two theoretical ACs have the capacity to bind different ligands. Interaction with these ligands influences their production of cAMP.

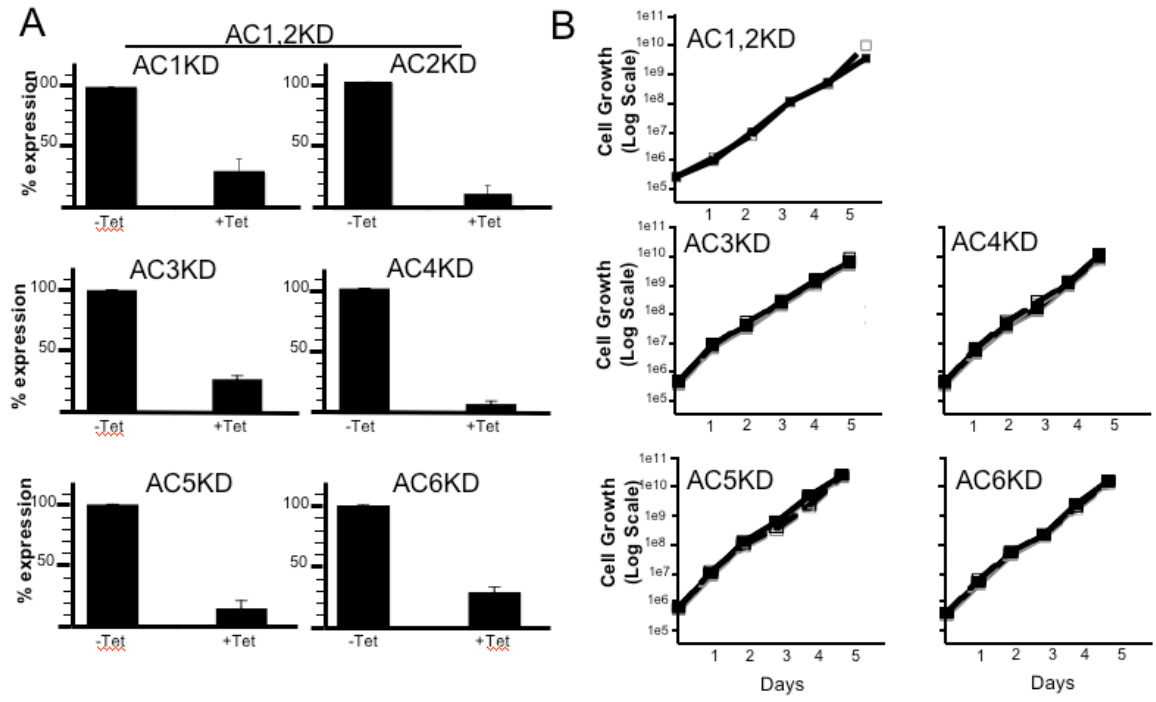

Figure 5-2. Knockdown (KD) of insect-stage specific ACs has no effect on parasite viability. A) QRTPCR results of AC 1, 2, 3, 4, 5 or 6 in various AC KD cell lines. Top 2 graphs represent AC1 and 2 expression in the AC1,2KD line. In each case, expression of the RNAi-targeted AC in induced (+Tet) conditions is normalized to expression of the AC in uninduced (-Tet) conditions. B) Graphs of the cumulative growth of various AC KD cell lines in induced (solid black line) vs uninduced (dashed black line) conditions.

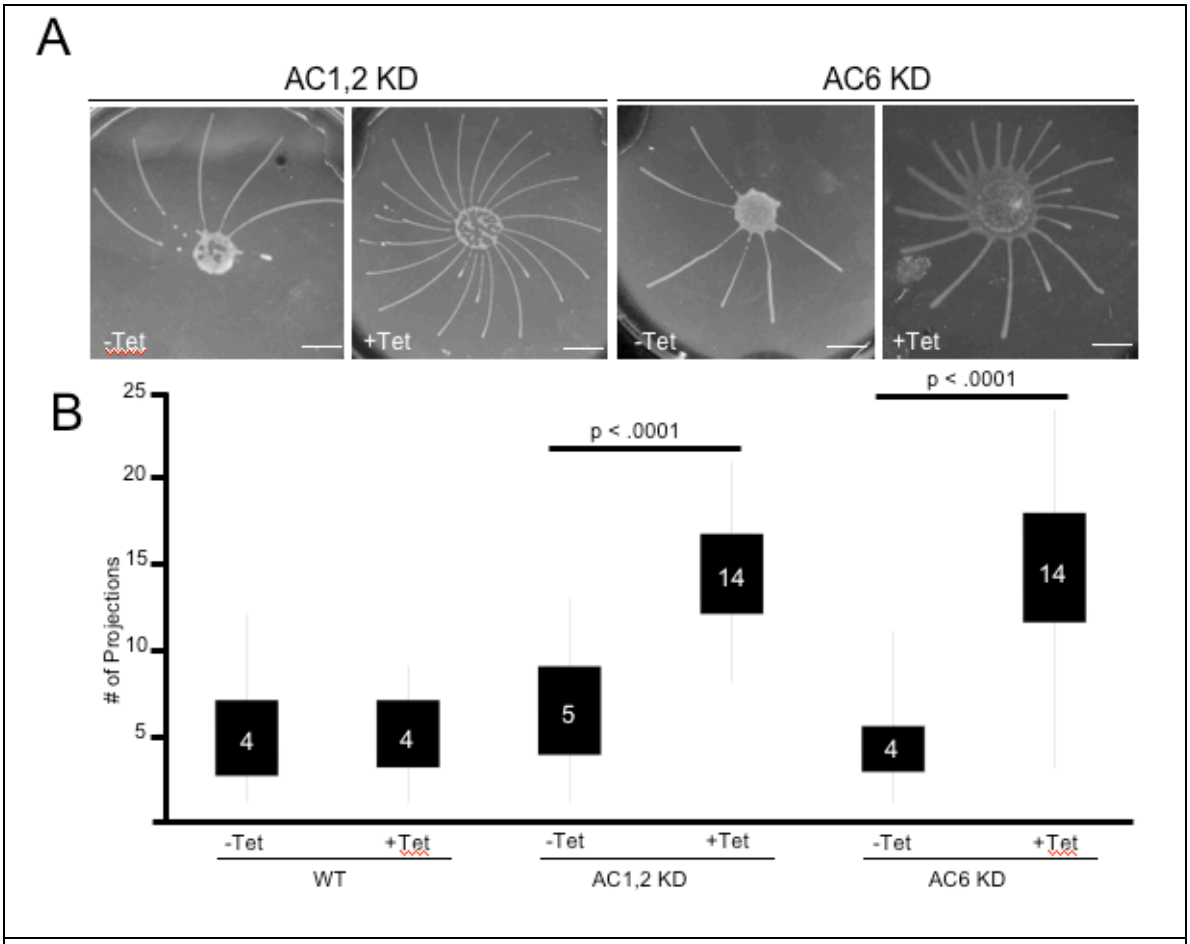

Figure 5-3. Knockdown of ACs 1,2 or 6 results in a hyper SoMo phenotype. A) Knockdown of ACs 1,2 or 6 results in a hyper SoMo phenotype characterized by the formation of an increased number of radial projections in induced conditions vs uninduced conditions (Scale bar = 1cm). B) Box plot of the number of radial projections formed by various AC KD cell lines in induced vs uninduced conditions. The median value of total projections formed by each cell line is indicated in white. AC 1,2 KD and AC 6KD cell lines generate about 3x as many radial projections in induced conditions as compared to control cells (WT n≥10, AC 1,2KD n≥ 18, AC 6KD n≥16).

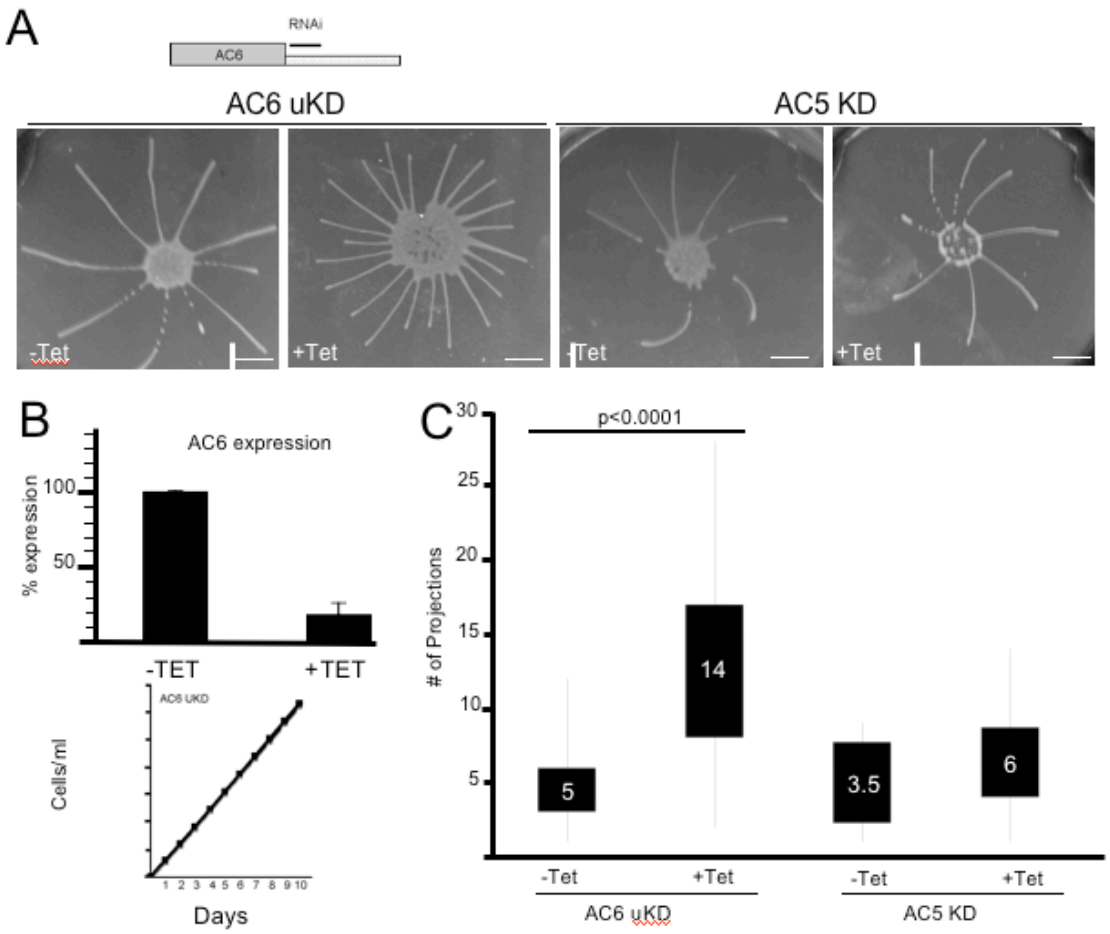

Figure 5-4. Knockdown AC 6 by targeting the 3' UTR results in a hyper SoMo phenotype. A) The 3' untranslated region (UTR) of AC6 was targeted for RNAi-mediated KD of AC6. KD of AC6 in this case results in a hyper SoMo phenotype as observed previously when the open reading frame (ORF) was targeted by RNAi (Figure 3). Knockdown of AC5 does not impact social behavior. (Scale bar = 1cm). B) QRTPCR showing AC6 levels in AC6 uKD (top panel) and growth rates (bottom). C) Box plot of the number of radial projections formed by AC6UKD and AC5KD cell lines in induced vs uninduced conditions. The median value of total projections formed by each cell line is indicated in white. AC6UKD cell lines generate 3x as many radial projections in

induced conditions as compared to uninduced conditions (AC6UKD n≥58, AC 5KD n≥18).

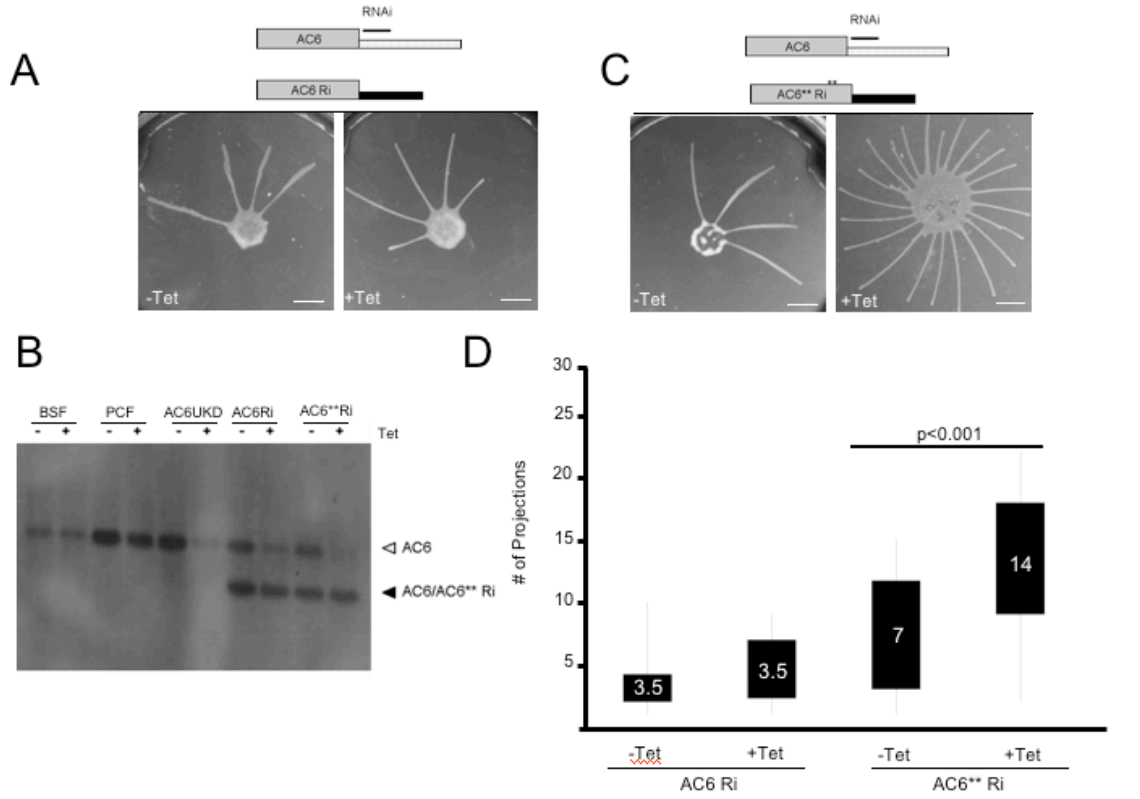

Figure 5-5. Hyper SoMo phenotype is rescued by the expression of an RNAi-Immune AC6 in the AC6UKD line but not by the expression of a catalytically inactive version. A) Expression of an RNAi-Immune AC6 in the AC6UKD parental line results in a rescue of the hyper SoMo phenotype (AC6Ri). AC6 KD was achieved in the AC6UKD line by targeting the endogenous 3' UTR of the gene (top). To generate an RNAi-Immune line, the 3' UTR was replaced on one allele of AC6 in the AC6UKD line with the Alpha/Beta tubulin intergenic region. In addition, the region downstream of the transmembrane domain was replaced with a version carrying point mutations to highly conserved

residues in the catalytic domain. B) A Northern Blot using a probe specific to the C-term end of AC6 was carried out to assess expression of AC6 and the AC6 RNAi-Immune versions in the various cell lines. Both RNAi-Immune versions of AC6 are smaller in size than endogenous AC6 and thus can be visualized based on their size difference. C) Expression of a mutated version of AC6 in the AC6UKD cell line (AC6**Ri) fails to rescue the hyper SoMo phenotype (Scale bar = 1cm). D) Box plot of the number of radial projections formed by AC6Ri and AC6**Ri cell lines in induced vs uninduced AC6KD conditions. The median value of total projections formed by each cell line is indicated in white. AC6Ri generates the same number of radial projections in induced or uninduced AC6KD conditions. AC6**Ri generates twice as many radial projections in induced AC6KD conditions versus uninduced conditions (AC6Ri n≥20, AC6**Ri n≥26).

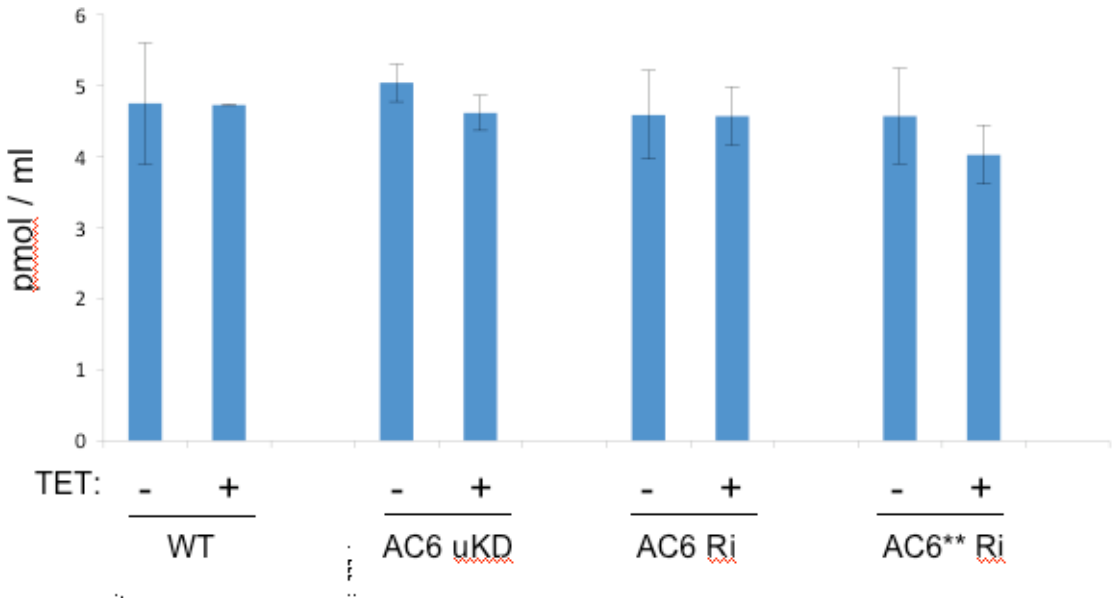

Figure 5-6. cAMP levels are reduced in AC6 uKD and AC6** Ri cells in the presence of tetracycline. Levels remain constant in WT and AC6 Ri cells -/+ tet.

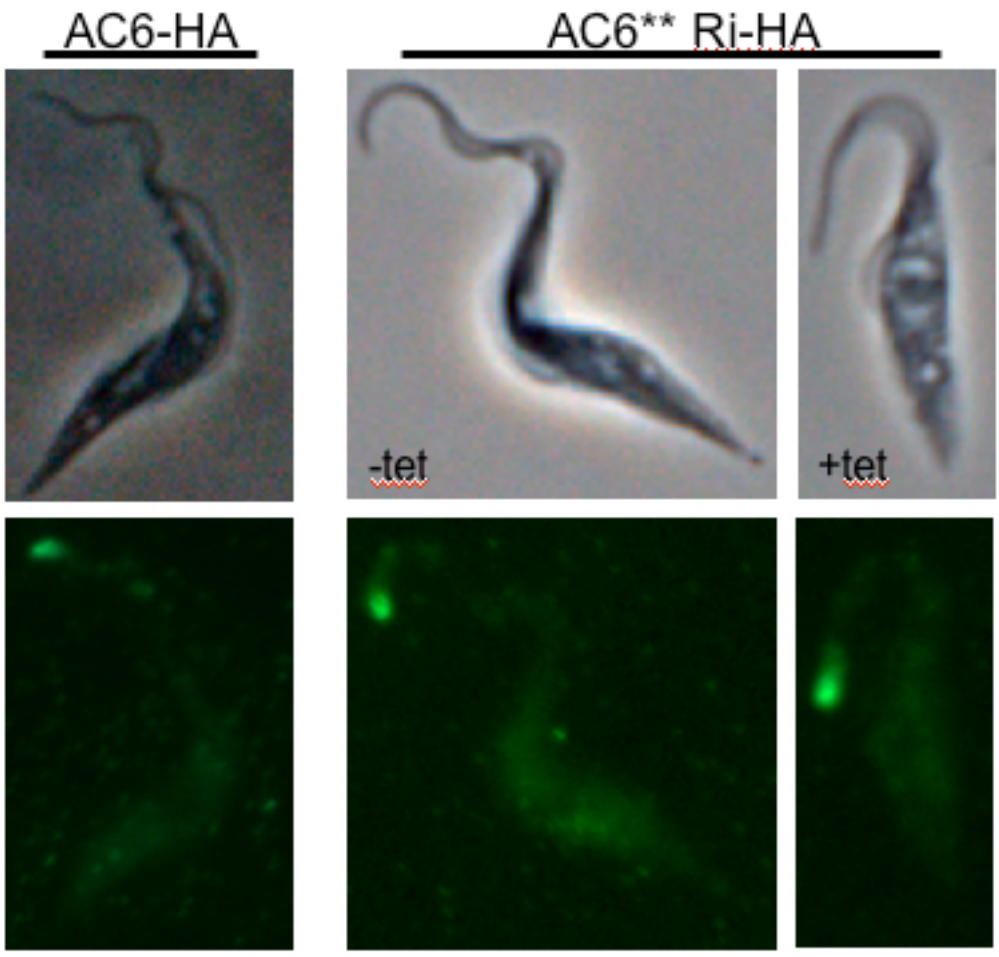

Figure 5-7. Localization of AC6** is unaffected. The localization of AC6HA and AC6**HA were compared. Epitope tagged AC6 (AC6-HA) localizes to the flagellar tip in wild type cells. In -/+ tet conditions, epitope tagged AC6** also localizes to the flagellar tip in the AC6**Ri cell line. Top panels are DIC images. Bottom panels are fluorescent images with HA-tagged protein in green.

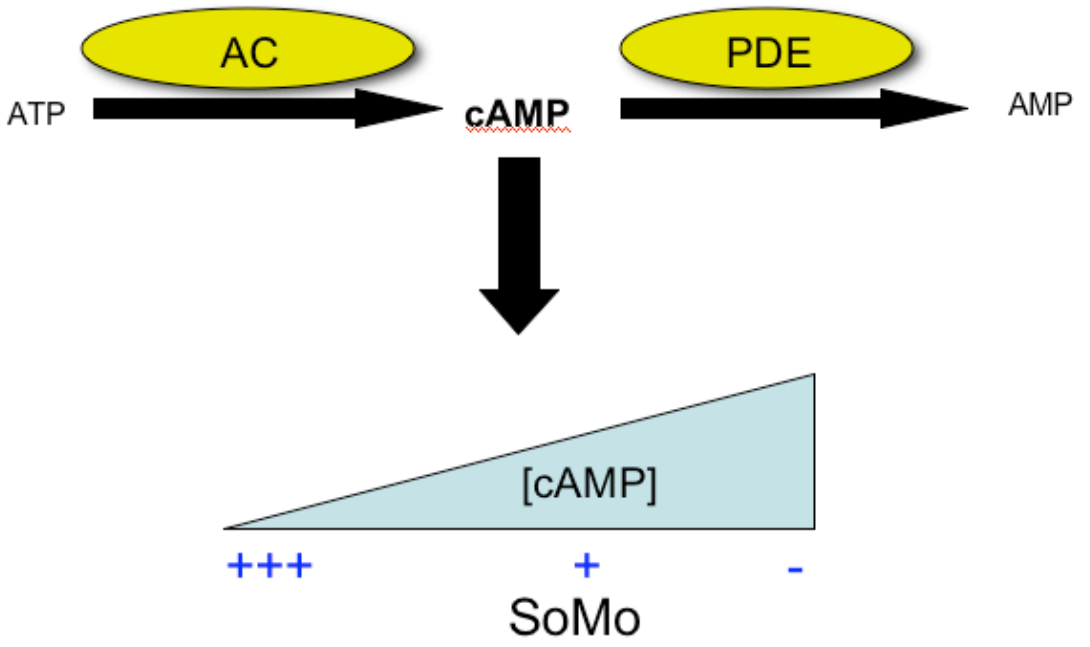

Figure 5-8. Model of cAMP involvement in SoMo regulation. SoMo is regulated by by local intracellular concentrations of cAMP. When cAMP levels drop below a certain level (as when AC6 function is reduced), *T. brucei* becomes more social. If local cAMP levels rise (such as when PDE function is inhibited), the parasite becomes less social.

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Chapter 6

T. brucei and Inter-Kingdom Signaling

Importance of Inter-kingdom Interactions

Although usually studied under sterile environments, microbes rarely exist in isolated cultures and are instead in environments comprised of varying agents and microbial life. Accordingly, a microbe's ability to communicate with surrounding life forms is vital for the execution of key biological processes. The study of this inter-kingdom signaling has led to several important discoveries pertaining to our understanding of how microbes interact with their hosts and environments. Furthermore, the study of how a host's own microbiome influences pathogens and diseases is revolutionizing how we consider, treat and prevent certain ailments.

Much of our understanding of how microbes communicate with their hosts comes from work done by bacteriologists. In the past years, a growing number of reports have shown that bacterial quorum sensing signals (known as autoinducers) signal to eukaryotic cells and can mimic hormones [1,2,3,4]. At the same time, hormones can signal to bacterial cells through converging pathways to autoinducer signaling. This cross-talk between bacterial signaling molecules and host-derived hormones is not surprising, given that bacteria and hosts have co-evolved for billions of years. The consideration of inter-kingdom signaling in the study of pathogen-host interactions has revealed much insight into how these microbes cause disease.

Of particular interest have been the advancements made in our understanding of how *Pseudomonas aeruginosa*, *Salmonella enterica* and *Vibrio parahemolyticus* use

inter-kingdom signaling to cause disease [4]. In the case of *P. aeruginosa*, factors secreted by the pathogen influence the host immune system so as to promote the growth of the bacteria. Not to be outdone, the host has developed ways to reduce the pathogenicity of the bacteria by developing means of breaking down some of the signals used by the bacteria to promote its virulence. Most notable are the mechanisms used by the host to disrupt the quorum sensing capabilities of this pathogen [3]. Rather than producing their own signals to promote virulence, other bacterial pathogens high-jack host-derived signals. In the case of *S. enterica* and *V. parahemolyticus* for instance, researchers found that detection of host-derived norepinephrine by the pathogens leads to increased expression of virulence genes [2,5].

Although the discussion above illustrates how organisms from two kingdoms can communicate with one another, microbes are often present in heterogeneous environments often containing cells from not multiple kingdoms. In these instances, it becomes increasingly important to study the cross talk between not just two different cell types, but also between all of the different cell types present. To this end, the study of microbial mats and microbiomes has unveiled interesting knowledge of how microbes carry out biological processes.

In microbial mats, for instance, researchers have found that organisms comprising these microbial superstructures are restricted to particular zones within the heterogeneous community based on chemical as well as physical properties [6]. Organisms within these communities are in constant communication with each other trhough the secretion and

detection of signals [6]. This communication results in competition, symbiosis and /or commensalisms between organisms. Whether by choice or demand, certain microbes within these communities take on certain roles. Ultimately, if enough interdependence arises, the health of the entire community depends upon the health of individual members. The study of microbial mats has proven crucial in our understanding of how microbes interact with one another in complex environments.

The study of host microbiomes has also revealed important aspects of biology. In recent years, this has become an exciting and promising new area of research and has led to many discoveries correlating the health of an organism's natural microbiome to the overall health of the organism. Indeed, this has led to the development of an entire industry in the production of probiotics geared at helping us maintain a healthy microbiota. In addition, the investigations directed at understanding how a host's native microbiome impacts the virulence of a pathogen have revealed novel findings about the immune system and the ways organisms combat foreign invaders [7]. It should come as no surprise then that researchers have discovered that an insect vector's native microbiome can greatly influence the transmission of pathogens [1].

Many insects have their own unique microbiota that help in their digestion of food and provide necessary nutrients. In the case of some blood-feeding insects, these insects can also act as vectors for many pathogens. In some of these insects, the native microbiota has been shown to impact transmission of the mammalian pathogen by the insect by preventing replication of the pathogen within the insect thus offering the insect

increased resistance to the mammalian pathogen [1]. Conversely, in other instances it has been shown that the insect's microbiota can aid in the replication and transmission of the pathogen [1]. Using this knowledge, investigators have begun developing methods to control transmission of pathogens by insects via the use of insect symbionts.

Thus microbes live in complex environments. The interactions microbes have with their environments and in particular with cells from other domains are key to their overall fitness. The study of this inter-kingdom cross-talk has uncovered many important findings of how microbes carry out biological processes. In addition, the study of pathogens in this context has greatly contributed to our knowledge of how they cause disease.

Inter-kingdom Interactions and *T. brucei*

Several aspects of the *T. brucei* life cycle would benefit from inter-kingdom cross-talk. In both the insect and the mammal, the parasite is exposed to many different tissues and extra cellular host-derived signals. The need for cross-talk between *T. brucei* and other domains is most readily apparent in the tsetse, where at least three bacteria can reside to make up the insect's microbiome (Figure 6-1) [8]. Although the details of any *T. brucei* and bacteria interaction are not known, it has been shown that there is a positive correlation between the presence of one of these bacteria and the success rate of a *T. brucei* infection within the fly (see below)[9,10].

We employed our Social Motility assay to investigate the possible cross-talk between *T. brucei* and bacteria. The Social Motility assay is an excellent platform on which to study the cross-talk that occurs between bacteria and trypanosomes because: 1) it is a controlled *in vitro* system amenable to manipulation, 2) *T. brucei* lends itself well to molecular genetic dissection and cellular biology studies (see prvious chapter(s) and 3) the bioassay provides a straightforward way to interpret micro and macroscopically.

To investigate cross-talk between *T. brucei* and bacteria, we tested the ability of *T. brucei* communities to respond to two common laboratory bacterial strains: the Gram-positve bacteria *Bacillus subtilis* and the Gram-negative bacteria *Escherichia coli*. Regardless of bacterial type, when colonies from agar plates were inoculated in the path of migrating *T. brucei* communities, the parasite communities diverged from their course of migration so as to avoid the bacterial colony (Figure 6-2). This avoidance behavior is similar to previous observations where at later stages of SoMo, parasite communities avoid one another (see Chapter 3). Inanimate objects (such as scratches on the agarose surface, silicon beads and filter discs) fail to elicit such a response from parasite communities, indicating that this avoidance is due to the active detection of factors derived from the living bacterial cells.

Conversely, we observed that *T. brucei* communities are attracted to stationary phase *E. coli* and *B. subtilis* suspension cultures and lysates (Figure 6-3 and not shown). In these studies, an aliquot of either a stationary phase *E. coli* or *B. subtilis* culture was

inoculated away from *T. brucei* cells at an early stage of SoMo. Interestingly, the parasite communities were strongly attracted to the inoculated bacterial suspensions as demonstrated by their biased migrations to the bacterial suspensions (Figure 6-3).

Although these experiments are still in their infancy, these findings mark the first time, to our knowledge that *T. brucei* has been shown to be capable of responding to bacteria and provide evidence of inter-kingdom signaling between Trypanosomes and bacteria. Very little is known of *T. brucei* chemotaxis and navigation strategies and our studies also provide one of very few examples of *T. brucei* directional movement being modified in response to a signaling substance, albeit a complex substance.

Conclusions and Future Directions

As discussed above, *T. brucei* would benefit from inter-kingdom signaling. In both the insect and the mammal, the parasite is exposed to many different tissues and extra cellular host-derived signals. The need for cross-talk between *T. brucei* and other domains is might be most readily apparent in the tsetse, where at least three bacteria can reside to make up the insect's microbiome [1].

The three distinct bacterial symbionts are maternally-inherited and have important influences on tsetse nutritional and reproductive biology [1]. *Wigglesworthia* are intracellular, mutualistic bacteria that reside in specialized cells called mycetocytes in the

tsetse midgut and aid in nutrient acquisition. *Wigglesworthia* also affects tsetse reproductive biology and eliminating the bacterium through antibiotic treatment causes tsetse sterility [11]. *Sodalis* is a reported mutualistic symbiont that resides extracellularly and intracellularly in several tsetse tissues, including the midgut. It should not go without mention that both, *Sodalis* and *Wigglesworthia* reside in the midgut where trypanosomes first reside during infection of the tsetse. Interestingly, the presence of *Sodalis* has been shown to potentiate susceptibility to trypanosome infection and selective elimination of it reduces trypanosome infection in the midgut [10]. *Wolbachia*, the third bacteria that can inhabit the tsetse, is a parasitic bacterium that affects reproduction in many insects and has been implicated in cytoplasmic incompatibility in the tsetse [8].

The complete impact of these bacteria on tsetse biology, including trypanosome transmission is currently the focus of study. In addition, the altering of tsetse microbiome holds potential for blocking parasite development and transmission as has been done in other systems [1]. Although the details of bacterial symbiont influence on *T. brucei* infection are unclear, the ability of bacteria to potentiate trypanosome infection of the tsetse fly [9,10]clearly points to the importance of trypanosmal and bacterial cross-talk and reemphasizes the relevance of inter-kingdom signaling. In addition, there also has been lateral gene transfer of *Sodalis* genes to *T. brucei [12,13]*, indicating an ancient and intimate interaction.

We employed our Social Motility assay to investigate the cross-talk between bacteria and *T. brucei*. To our surprise we found that bacteria could both repel and attract

the parasite depending on when inoculation of the bacteria took place. It is known that bacteria produce different signals depending on what stage of development they are in. Going forward, efforts in the lab will be focused on uncovering the nature of the compound(s) and mechanism(s) mediating this inter-kingdom interaction. To this effect, we have begun collaborations with Dr. Pieter Dorrestein of the University of California, San Diego. His lab has developed a novel method of imaging mass spectrometry through which researchers can view the 2 and/or 3 dimensional distributions of signals generated by cells within a matrix [14]. This technique has proven extremely useful in viewing and mapping the variety distribution of signals generated by microbes in a variety of surfaces including our own epidermis [15,16,17]. These techniques are proving to be essential in furthering our knowledge of inter-kingdom signaling and the human microbiota.

In addition to this collaboration, we are also planning to characterize the details of the *T. brucei* response to bacteria by video microscopy and evaluate the impact of *Sodalis* on this behavior. We have developed very comprehensive techniques to record *T. brucei* migrations across a surface [18]. Using these techniques we will detail the steps leading to a response to bacteria inoculation. The goal with these experiments is to determine how the community makes the decision to react to the stimulant. In addition, we will repeat our experiments from above using a more relevant bacterial strain, *Sodalis*. *Sodalis* has been successfuly cultured by other researchers and evaluation of its possible interaction with *T. brucei* is readily achievable given the reproducibility and macroscopic readout offered by our SoMo assay. These experiments will undoubtedly provide key insight as to how *T. brucei* interacts with bacteria in the tsetse and the information

obtained could also be used to begin understanding how the parasite interacts with its environment.

Figures

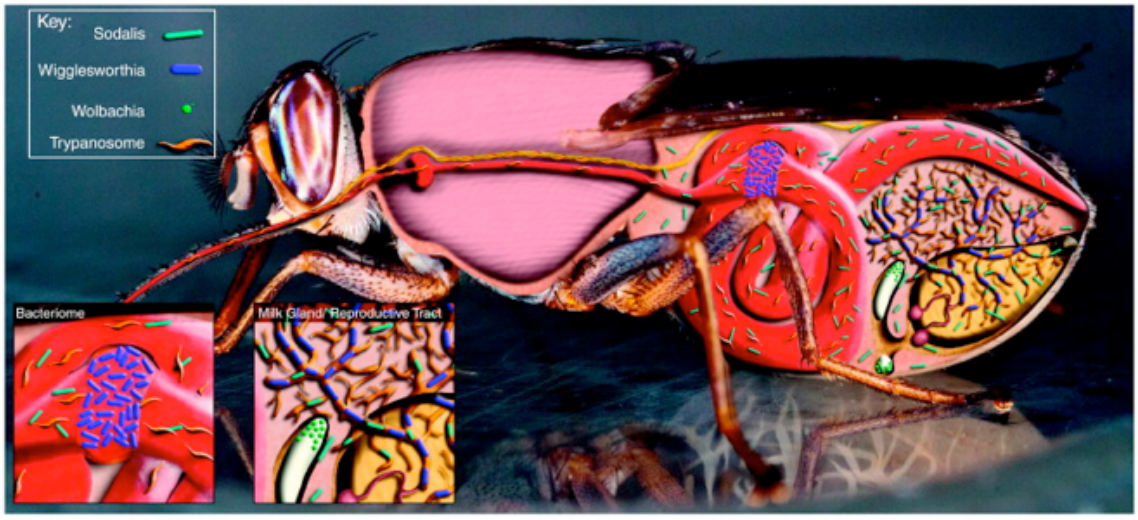

Figure 6-1. Cartoon illustration of a tsetse and 4 different microbes that can inhabit it: *Sodalis, Wigglesworthia, Wolbachia* and African trypanosomes. The 4 microbes are shown in the organs they typically reside in. During its transmission in the fly, the trypanosome resides in the midgut and then in the salivary glands of the fly. In the midgut the trypanosome is in close proximity to *Wigglesworthia* (housed in the bacteriome organ) and *Sodalis* found in the midgut. Thus anti-pathogenic products expressed by *Sodalis* or induced by *Wigglesworthia* can have an adverse effect on trypanosome transmission (shown by inset labeled bacteriome). Reproduced with permission from [1]

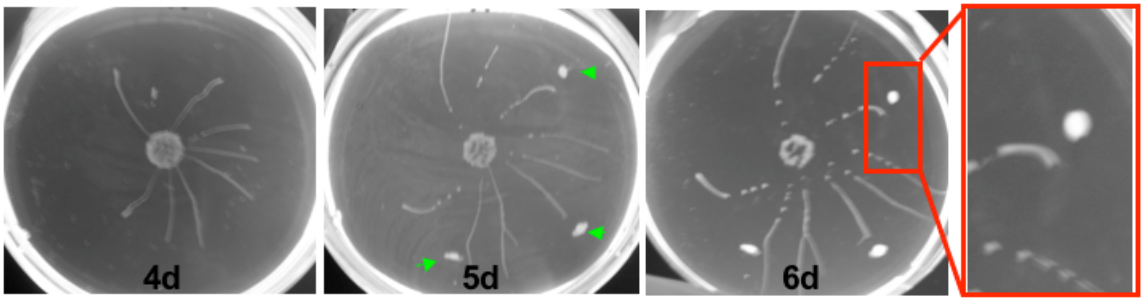

Figure 6-2. *T. brucei* communities avoid bacterial colonies. Bacterial colonies (in this case *E. coli*) were picked from agar plates and placed in the path of migrating *T. brucei* communities 4 days after inoculation of *T. brucei* on semi-solid agarose plates (indicated by the green arrows in panel 5d). Projections were then monitored for their avoidance at 4, 5 and 6 days post inoculation of *T. brucei* on plates (panels 4d, 5d and 6d respectively). As can be seen in the the right-most panel, *T. brucei* senses the bacterial colony and avoids it well before contacting it physically.

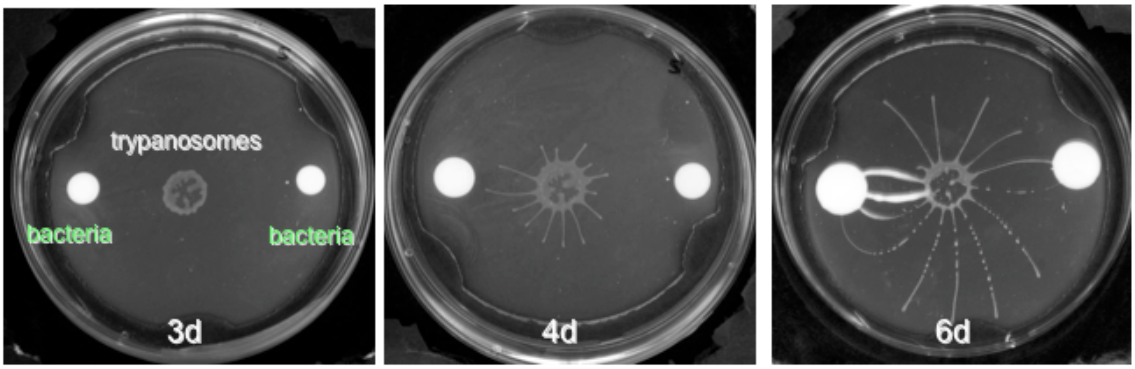

Figure 6-3. *T. brucei* communities are attracted to bacterial communities. Bacterial cells (in this case *E. coli*) were grown to stationary phase in LB over night. Bacteria and *T brucei* cultures were then inoculated onto semi-solid agarose plates at the same time. The

plates were monitored for several days; Images from days 3, 4 and 6 following inoculation are shown above (panels 3d, 4d and 6d respectively). As can be seen in panel 4d, *T. brucei* communities preferentially migrate towards the bacteria well before coming into physical contact with it indicating detection of a soluble or volatile signal.

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Chapter 7

Conclusions and Future Directions

This thesis has focused on investigating how *T. brucei* moves through fluids and on surfaces. By dissecting the movement of the parasite in media of varying viscosities with and without the use high-speed imaging, we uncovered previously unappreciated complexities of *T. brucei* movement. These observations have changed the way we perceive the parasite's motility. In addition, our studies on the effects of surface-exposure on parasite motility revealed that these parasites engage in social behavior: an unprecedented finding in protozoan parasite biology. Investigation of the mechanisms underlying this social behavior have revealed a novel role for a unique family of proteins that were previously mostly uncharacterized. Specifically, we showed that these proteins are not redundant in function nor constitutively expressed. In addition, the search for extracellular factors that regulate this social behavior have led us to discover an unexpected cross-kingdom interaction between *T. brucei* and bacteria. In short, by using classic as well as novel laboratory techniques, the investigations presented here have uncovered novel aspects of *T. brucei* behavior that have transformed our understanding of protozoan biology.

Analysis of T. brucei motility using high-speed imaging and increased viscosity

As discussed in the introduction, *T. brucei* is extra cellular at all stages of infection and depends on its own flagellum-mediated motility for dissemination. Indeed, this single flagellum is a hallmark feature of *T. brucei's* architecture and is essential for the parasite's motlity. Flagellar motility of *T. brucei* in various environments is believed to be central not only to host-parasite interaction, but to a variety of other processes.

Prior to our studies using high-speed video microscopy, it was widely believed and accepted that the *T. brucei* moved through fluids and tissues by rotating in one direction. This rotation was determined by the fact that the flagellum wraps around the parasite forming a left-handed helical. Through the use of millisecond DIC microscopy, supplemented by SEM and 3D confocal microscopy, we found that forward *T. brucei* motility is not as simple as previously believed and is instead characterized by (i) tip-to-base propagation of kinks separating LH and RH helical waves, (ii) very high motility of the flagellum tip in BSF cells, and (iii) a pronounced rotation-frequency gradient along the cell body, with the anterior end performing rapid full rotations while the posterior end rocks back and forth more slowly [1]. This specialized form of motility appears to reduce viscous dissipation by minimizing rotary motion of the large posterior end of the cell, while allowing *T. brucei* to negotiate complex viscous environments, such as mammalian blood. Furthermore, this rotation-based movement appears to offer the parasite an advantage for increased motility in these environments as was confirmed by analysis of the average speed traveled by *T. brucei* in viscous environments.

These results challenged the way the scientific community considered Trypanosome motility and demonstrate the analytical power available to scientists via the use of new technology. Through the use of cutting-edge visualization and computing technologies as well as molecular tools available to biologists, our interdepartmental collaboration is a shining example of how science in the new era can be used to shed light on unknown phenomena and dispel dogma.

Our results have definitely challenged the field's view of *T. brucei* motility and have sparked an interest in the further characterization of the parastie's motility. Indeed, recently Heddergott et al. employed high-speed fluorescence microscopy to characterize the parasite's motility in a variety of conditions [2]. Their work ultimately confirmed my findings that *T. brucei's* method of motility enables the parasite to move faster in cases of increased viscosity. In addition, the ability of the parasite to reverse its flagellar beat from tip-base to base-tip allows the parasite to maneuver through crowded spaces without getting stuck. We have seen similar behavior with trypanosomes moving backwards via base-tip beat propagation on the agarose surface and in media following knockdown of an axonemal protein [3]. Although in this study the authors proposed a method of *T. brucei* motility without a need for kinks, as we proposed, they were not able to disprove our model.

Thus even more than 150 years following the first description of *T. brucei* motility, we remain without a complete description of the parasite's motility, albeit more specifics are known to us now than before. It is only through rigorous investigations such as those we published and the work of Heddergott et al., that we will gain a complete understanding of how Trypanosomes move through their environment. These efforts will undoubtedly be facilitated by the continued advancement of visualization techniques and the large molecular toolkit available to researchers studying *T. brucei*.

T. brucei Social Motility

T. brucei lives a complex life cycle through two hosts, the tsetse and a mammal. Although it is known that in both hosts the parasite interacts with host tissue surfaces and is in constant intimate contact surfaces, not much is known about how the parasite's behavior is influenced by exposure to these surfaces. In other microbes, interaction with tissue surfaces results in the onset of social behaviors. These social behaviors come in a variety of types and provide organisms in a community with a wide variety of advantages over solitary life (see Chapter 3). In the case of pathogens, exposure to tissue surfaces results in social behaviors that increase the organism's virulence [4,5,6,7,8,9]. The study of these social behaviors has transformed our understanding of microbes and the diseases they cause. Since the concept of social behaviors had previously not been applied towards the study of *T. brucei* and it was unknown how surfaces influence its behavior, we decided to look at the effect of surface exposure on *T. brucei*.

In an attempt to better understand how surface-exposure affects T. brucei, we cultivated procyclic (insect-stage) *T. brucei* on semi-solid agarose plates [10]. We found that surface exposure induced profound changes in trypanosome behavior, causing individual cells to assemble into multicellular communities with properties that are not evident in single cells. We termed this behavior "social motility" (or SoMo), based on analogies with similar surface-induced social interactions in bacteria [11]. During initial stages of social motility, trypanosomes assemble into communities that grow through the active recruitment of nearby cells [12]. Continued recruitment, merger with other communities and replication results in the formation of macroscopic communities 3 to 4

days post inoculation. Parasites then undergo polarized migration outward from the site of inoculation, forming radial projections reminiscent of bacterial social motility. These projections are evenly spaced and consistent in number. They can also change their course of migration in response to extracellular factors, as projections avoid one another when they are in close proximity. Thus tyrpanosomes can sense and communicate with one another and coordinate their efforts to engage in a social behavior. These results represented a novel finding in protozoan biology and definitely show that trypanosomes engage in social behaviors.

Looking forward, the continued characterization of SoMo will be an important area of research. Several aspects of *T. brucei* life within the tsetse remain unknown and would benefit from social behavior. Social behaviors in other organisms assist with a wide variety of functions including increased tissue penetration, communal navigation, resistance to external antagonists and mating [13]. As discussed in Chapter 3, it is clear that *T. brucei* engages in *en masse* migrations, tissue penetrations and mating. In addition, the parasite is exposed to a variety of tsetse and bacteria-derived factors that can be detrimental to the parasite [14,15,16]. As such, given the role(s) of social behaviors in other organisms it is not unlikely that *T. brucei* would employ SoMo in the tsetse to establish a successful infection. In support of this, the Hill lab has recently observed that laboratory strains capable of establishing a tsetse infection display SoMo whereas those that do not are deficient in SoMo (unpublished). Also, future experiments assessing the ability of pleomorphic lines deficient in the expression of genes required for regulation of

SoMo to infect tsetse flies will be fundamental in evaluating the role of SoMo *in vivo* (see below).

As discussed in the introduction chapter, although the paradigm of social behavior has remained largely unapplied towards the study of parasitic protozoa, it is clear that several aspects of these organisms' biology would benefit from these behaviors. Thus although *T. brucei* is the first parasitic protozoan to definitively display social behavior, it will not be the last.

Identification of Stage-Specific Adenylate Cyclases in the T. brucei flagellum

As discussed in Chapter 4, because *T. brucei* remains extra cellular throughout its life cyle, it is exposed to a variety of host and parasite-derived factors. Sensation of these factors influences parasite biology and behavior. In addition, we recently reported that procyclic form (or insect-stage) *T. brucei* engages in social behavior when inoculated on semi-solid agarose plates (see above and Chapter 3). Throughout this process, the parasites sense and communicate with one another to form macroscopic communities capable of coordinated actions [12]. Specifically, these macroscopic communities can move *en masse* and can detect and avoid one another through unknown mechanisms.

Thus, although it is clear that *T. brucei* infection depends on navigation to different tissues and that the parasite senses extra cellular factors, there remains no identification of factors or receptors that could mediate navigation or sensation. When

evaluating how *T. brucei* might sense external factors, it is important to consider the parasite's flagellum given its role as a sensory organelle in other eukaryotic cells. Although much work has been done to characterize the proteins to that make up the *T. brucei* flagellum, until recently much remained unknown about the proteins that are found on the flagellum's surface and could play a role in the sensation of external factors. In an effort by Oberholzer et al., a proteome of the *T. brucei* blood stream form flagellar membrane was published and became the first to identify transmembrane, surface-exposed proteins that were specific to the flagellum [17].

In a similar effort, we decided to look for proteins that are specific to the procyclic form (insect-stage) *T. brucei* flagellum. Our investigation uncovered the presence of six adenylate cyclases (ACs) on the flagellum of the procyclic form of the parasite. Trypanosomal ACs are unique structures with a cytoplasmic catalytic domain near the C-terminus connected by a single transmembrane domain to a large, extracellular N-terminal domain [18]. The N-terminal domain varies among family members and exhibits homology to a superfamily of proteins involved in nutrient sensing in bacteria and ligand binding in mammalian receptors. Architecturally, these molecules resemble membrane-bound type I guanylyl cyclases which act as receptor molecules to generate cGMP in response to extra cellular signals [19]. Additionally, unlike related kinetoplastids, African trypanosomes show a remarkable expansion of receptor-like ACs [20]. The unique structure of trypanosomal ACs as well as their expansion within the genomes of African trypanosomes has lead to the tantalizing hypothesis that these molecules function as receptors to extracellular signals [18,19]. As discussed in Chapter

4, of the approximately 60 family members, very few *T. brucei* ACs have been previously studied or characterized and have largely been considered to be constitutively expressed and redundant in function [19]. We therefore sought to characterize the 6 ACs that we identified through different molecular and biochemical approaches and demonstrated that these proteins are not redundant in function nor constitutively expressed.

Differential expression in the bloodstream versus insect-stage form of the parasite as well as distinct localization to specific domains within the flagellum suggests that these ACs do not serve redundant functions. This thought is further supported by the widely held view that cAMP signaling operates within microdomains [21,22], thus making it possible for these proteins to operate and initiate cellular pathways independently of one another. Going forward it will be interesting to evaluate how and why these ACs localize to their specific regions within the flagellum. To this end, it will be important to consider the possible role(s) that known flagellar trafficking machinery has in the transport of these ACs to their destinations. In addition, it is important to consider the role(s) of signaling sequences in the localization of these proteins. AC1 and AC2 for instance, share 90% homology and yet localize to different regions. These proteins have been the subject of current Hill lab investigations and a signal sequence has been attributed to their differential localization (Hill lab unpublished and not shown).

Given that cAMP plays an important role in many *T. brucei* processes [23,24,25,26,27], it will be interesting to investigate the individual roles of these six ACs

within the parasite. As seen in Chapter 5, although knockdown of individual ACs does not cause defects in growth or motility of *T. brucei*, social behavior is affected. Identification of effector molecules that interact with these ACs will also be important in furthering our knowledge of what functions these ACs might serve and how cAMP signaling is employed by the parasite to mediate cellular processes.

SoMo is Regulated by Insect-Stage Specific Adenylyl Cyclases

As discussed above and in Chapter 3, the discovery of social behavior in trypanosomes was a novel finding. In addition, social behaviors in response to surface exposure are widespread and the study of these behaviors in pathogenic microbes has transformed the way we consider and treat diseases caused by them. Although the mechanisms that regulate these behaviors are diverse, they generally require organisms to be able to sense external signals, transduce the signal to generate a response and cooperate with surrounding organisms [13]. One particular class of molecules that play a major role in the regulation of social behaviors is cyclic nucleotides.

In trypanosomes, cyclic nucleotide signaling is important for a variety of processes including differentiation, cytokenesis and pathogenesis [18,24,25,26]. In *T. brucei,* the flagella is speculated to be a center for cAMP production and signaling [21]. Accordingly, adenylate cyclases (ACs) and phosphodiesterases (PDEs) have been localized to the parasite flagella [23,24,28]. *T. brucei* phosphodiesterases are fairly well studied and characterized and have been implicated in cell viability [23]. In addition, in

recent years they have emerged as candidates for targeting by therapeutics [29].

In contrast to PDEs, not as much is known about trypanosomal ACs (see above and Chapter 4). As discussed above, we identified 6 previously uncharacterized ACs that localize to distinct subdomains of the *T. brucei* flagella; 5 of which were preferentially expressed in the procyclic (insect-stage) form of the parasite. Given their increased expression in the procycylic form of the parasite, localization to the flagella (a known sensory organelle) and potential role as receptor molecules, I decided to investigate their possible role(s) in the regulation of SoMo.

The results presented in Chapter 5 are the first to demonstrate regulation of SoMo at the molecular level. In addition, these are the first studies showing that trypanosomal ACs do not serve redundant roles as previously thought. The generation of AC 1 and 2, 3, 4, 5, and 6 KD cell lines was important in evaluating the different contribution(s) of these molecules in the regulation of SoMo. The observation that only 2 of the 5 generated KD cell lines (AC1,2 KD and AC6 KD) produced a hyperSoMo phenotype points to a distinct role for *T. brucei* ACs in the regulation of cellular processes.

Further characterization of the hyper SoMo phenotype observed in the AC6 KD cell line demonstrates a clear role in the regulation of SoMo by AC6. First of all, generation of two independent KD lines targeting distinct regions of the AC6 mRNA for RNAi KD indicates that the hyper SoMo phenotype observed is the result of AC6 KD and not off-target effects; QRTPCR results demonstrating that expression of other ACs in

the AC6 KD and AC6 uKD lines is unaffected in addition to the ability to rescue the hyper SoMo phenotype via the introduction of an RNAi-Immune version of AC6 in the AC6 uKD line supports this hypothesis. The failure of a catalytically-inactivated version of AC6 to rescue the hyperSoMo phenotype demonstrates a clear role for the function of AC6 and cAMP signaling in the regulation of SoMo.

My work on AC6 in addition to recent work by the Hill lab demonstrating that disruption of TbrPDEB1 (a flagellum-localized phosphodiesterase) results in a null SoMo phenotype definitively implicates cAMP signaling in the regulation of SoMo. This conclusion is exciting and in line with other studies demonstrating the importance of cyclic nucleotides in the regulation of other social behaviors. The results from our work with AC6 and TbrPDEB1 have led us to develop a model to explain how cAMP signaling might mediate SoMo (see Chapter 5).

Looking forward, the next step in our work will be to further evaluate our model by looking for the signal(s) that mediate AC6 activity as well as identification of effectors that translate the cAMP signal intracellularly. As discussed previously, due to their distinct architecture and similarity to membrane-bound receptor-type guanylyl cyclases, *T. brucei* ACs have been speculated to be receptors for extracellular ligands [19]. Indeed, this thought is supported by the fact that these molecules possess a periplasmic binding protein domain and localize to the parasite's flagellum. However, to date no ligands have been identified that bind to *T. brucei* ACs. Periplasmic binding protein domains bind a variety of different ligands and are present in a variety of different receptors including

guanylyl cyclases. Thus, the search for ligands that might bind to *T. brucei* ACs is a complicated endeavor due to the possible number of potential ligands. These sorts of investigations have been further complicated by the lack of an assay to monitor the parasite's response to different stimuli. Our findings show that in the absence of AC6 activity, an increased amount of social behavior occurs. Thus under normal conditions, signal(s) detected by AC6 result in: activation of the enzyme, production of the appropriate amount of cAMP, and suppression of hyperSoMo. Given the reproducibility of the SoMo assay, the ease in interpreting the results due to the macroscopic nature of the assay, and the overall manipulability *T. brucei* as a model system, we are confident that this assay can be used as a readout to identify a ligand for AC6. Investigations looking for inhibitors and activators of different *T. brucei* ACs will be the focus of future experiments in our lab.

Identification of molecules downstream of AC6 will also be important in furthering the understanding of SoMo. cAMP exerts its influence on cellular processes via a variety of different effectors. One of the most common effectors used by cAMP in mammals is Protein Kinase A (PKA). In mammals, PKA is found in an inactive state, with two catalytic subunits (PKA-C) bound in a heterotetramer to two regulatory subunits (PKAR). cAMP binds to the regulatory subunits, resulting in release of PKA-C subunit. Although a putative regulatory subunit of PKA has been identified in *T. brucei*, this molecule was paradoxically found to be stimulated by cGMP as opposed to cAMP [30]. That being said, it would be interesting to test the role this protein might have in SoMo. In addition, the *T. brucei* genome contains several other proteins with putative cNMP

binding domains; these molecules are currently being evaluated in our lab for their role(s) in the regulation of SoMo.

In addition, although my work focused on characterization of the hyperSoMo phenotype as a function of AC6, it should not go without mention that the hyperSoMo phenotype was also observed when ACs 1 and 2 were knocked down in tandem. I have generated a line with specific KD of AC1. It will be interesting to see if the hyperSoMo phenotype is maintained in the AC1KD line.

Lastly, it will be interesting to evaluate the role of SoMo *in vivo* by testing the ability of pleomorpic lines to deficient in AC6 or TbrPDEB1 to infect tsetse flies. Members of the Hill lab have been recently trained in techniques to culture and manipulate pleomorphic lines. Generation of pleomorphic lines with AC6 or TbrPDEB1 knocked out is a relatively straightforward endeavor. Once these lines are generated we can assess their ability to infect tsetse flies with a collaborator.

In summary, the results presented in Chapter 5 are the first to show a mechanism for the regulation of SoMo and that *T. brucei* ACs are not redundant in function. This work, in addition to that described by the Hill lab regarding TbrPDEB1 point to a distinct role for cyclic nucleotide signaling in the regulation of SoMo. Furthermore, these findings will serve as a starting point from which we can begin dissecting the mechanisms underlying *T. brucei* social behavior. Given the parallels with other systems, work done to investigate SoMo will clearly further our understanding of microbes and

how they interact with their environments.

T. brucei and Interkingdom Signaling

Microbes live in complex environments. The interactions microbes have with their environments and in particular with cells from other domains are key to their overall fitness. The study of this inter-kingdom cross-talk has uncovered many important findings of how microbes carry out biological processes [31]. In addition, the study of pathogens in this context has greatly contributed to our knowledge of how they cause disease [13,32].

Several aspects of the *T. brucei* life cycle would benefit from inter-kingdom cross-talk. In both the insect and the mammal, the parasite is exposed to many different tissues and extra cellular host-derived signals. The need for cross-talk between *T. brucei* and other domains is most readily apparent in the tsetse, where at least three bacteria can reside to make up the insect's microbiome. Although the details of any *T. brucei* and bacteria interaction are not known, it has been shown that there is a positive correlation between the presence of Sodalis and the success rate of a *T. brucei* infection within the fly [15,33].

We employed our Social Motility assay to investigate the cross-talk between bacteria and *T. brucei*. To our surprise we found that bacteria could both repel and attract the *T. brucei* depending on when inoculation of the bacteria took place (Chapter 6). It is

known that bacteria produce different signals depending on what stage of development they are in. Going forward, efforts in the lab will be focused on uncovering the nature of the compound(s) and mechanism(s) mediating this inter-kingdom interaction. To this effect, we have begun collaborations with Dr. Pieter Dorrestein of the University of California, San Diego. His lab has developed a novel method of imaging mass spectrometry through which researchers can view the 2 and/or 3 dimensional distributions of signals generated by cells within a matrix [34]. This technique has proven extremely useful in viewing and mapping the variety distribution of signals generated by microbes in a variety of surfaces including our own epidermis [35,36]. These techniques are proving to be essential in furthering our knowledge of inter-kingdom signaling and the human microbiota.

In addition to this collaboration, we are also planning to characterize the details of the *T. brucei* response to bacteria by video microscopy and evaluate the impact of *Sodalis* on this behavior. We have developed very comprehensive techniques to record *T. brucei* migrations across a surface [12]. Using these techniques we will detail the steps leading to a response to bacteria inoculation. The goal with these experiments is to determine how the community makes the decision to react to the stimulant. In addition, we will repeat our experiments from above using a more relevant bacterial strain, *Sodalis*. *Sodalis* has been successfuly cultured by other researchers and evaluation of its possible interaction with *T. brucei* is readily achievable given the reproducibility and macroscopic readout offered by our SoMo assay. These experiments will undoubtedly provide key insight as to how *T. brucei* interacts with bacteria in the tsetse and the information

obtained could also be used to begin understanding how the parasite interacts with its environment.

Although these experiments are still in their infancy, these findings mark the first time, to our knowledge that *T. brucei* has been shown to be capable of responding to bacteria and provide evidence of inter-kingdom signaling between Trypanosomes and bacteria. Very little is known of *T. brucei* chemotaxis and navigation strategies and our studies also provide one of very few examples of *T. brucei* directional movement being modified in response to a signaling substance, albeit a complex substance.

Concluding Remarks

T. brucei is an important pathogen and an excellent model organism. Through the use of novel approaches, cutting edge technology and a wide array of molecular tools, my research has furthered knowledge in the field and dispelled long-held dogma. In addition, the identification of several new aspects of *T.brucei* biology and behavior has changed the way we view protozoan parasites and will serve as a starting point for the next series of investigations. I have been fortunate to be surrounded by many very supportive lab members and collaborators. It is only fitting that since this thesis is on the study of social behavior that I acknowledge the contributions of so many. It is as true in our society as it is in the microbial world that diversity and community bring much more prosperity than isolation. I am eternally indebted to the contributions of others. Thank you all.

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Appendix A

Social Parasites

Social parasites

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Protozoan parasites cause tremendous human suffering worldwide, but strategies for therapeutic intervention are limited. Recent studies illustrate that the paradigm of microbes as social organisms can be brought to bear on questions about parasite biology, transmission and pathogenesis. This review discusses recent work demonstrating adaptation of social behaviors by parasitic protozoa that cause African sleeping sickness and malaria. The recognition of social behavior and cell–cell communication as a ubiquitous property of bacteria has transformed our view of microbiology, but protozoan parasites have not generally been considered in this context. Works discussed illustrate the potential for concepts of sociomicrobiology to provide insight into parasite biology and should stimulate new approaches for thinking about parasites and parasite–host interactions.

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Current Opinion in Microbiology 2011, **14**:642–648

This review comes from a themed issue on
Growth and Development: Eukaryotes
Edited by Joseph Heitman

Available online 21st October 2011

1369-5274/$ – see front matter

DOI 10.1016/j.mib.2011.09.012

Introduction

Social behaviors are most widely recognized in communication and cooperation observed in metazoans, ranging from navigation strategies and group hierarchies in insect communities to complex social networking in humans and other primates. However, communication and cooperation among individuals in a group also occurs at the cellular level, as illustrated in collective motility of migrating cells during wound healing, tissue morphogenesis and tumor metastases. Moreover, cell–cell communication and cooperative behavior is not restricted to higher animals and recent years have seen a surge in the study and understanding of social interactions and their underlying mechanisms in microbial systems.

Social interactions among microbes give rise to multicellular groups having emergent behaviors that are not possible in single cells [1••,2•,3•,4–8]. For example, quorum sensing enables synchronization of gene expression and cellular activities to allow a population to act as a group [1••]. Surface-associated behaviors such as biofilm formation and swarming motility allow microbes to establish communities with enhanced protection against external agonists and promote colonization and penetration of biotic and abiotic surfaces [9,10,11•,12•,13]. Cell–cell signaling during sporulation in myxobacteria and slime molds directs group motility behaviors and developmental programs in which cellular differentiation gives rise to multicellular forms having distinct cell types with specialized functionalities, thereby enhancing survival through division of labor [14,15]. In extreme cases, multispecies biofilms and microbial mats constitute complex microbial ecosystems where numerous microbes communicate, cooperate and battle with each other [16]. Ultimately, the goal is to enhance survival and proliferation of the organism and when the microbe is a pathogen, this has dire consequences for the host [5,17,18].

In the bacterial world, cell–cell communication is the rule and considering social behavior as a ubiquitous property of bacteria has transformed our view and understanding of microbiology [1••,2•,3•]. Social behaviors are also well-documented in eukaryotic microbes [19,5,6,20]. However, despite the tremendous influence that the paradigm of 'sociomicrobiology' has had on our understanding of microbiology, one group of microbes, the parasitic protozoa, seem to have been left without an invitation to the party. Studies of these organisms generally consider them as individual cells in suspension cultures or animal models of infection, while social interactions are largely unstudied.

Parasitic protozoa are etiologic agents of several major human maladies, including malaria, epidemic dysentery, Leishmaniasis and African sleeping sickness, that affect over half a billion people worldwide. Parasites also limit economic development in some of the poorest regions on the planet and are thus major contributors to the global human health and economic burden. Parasites have complex life cycles requiring transmission through multiple hosts, survival in diverse environments and a wide variety of cellular differentiation events. Hence, there are numerous facets of parasite biology that may benefit from, or may even depend upon, social interactions. In this review, we highlight recent work on social behavior in two well-studied parasites, *Trypanosoma brucei* that causes sleeping sickness and *Plasmodium* parasites that cause malaria. In

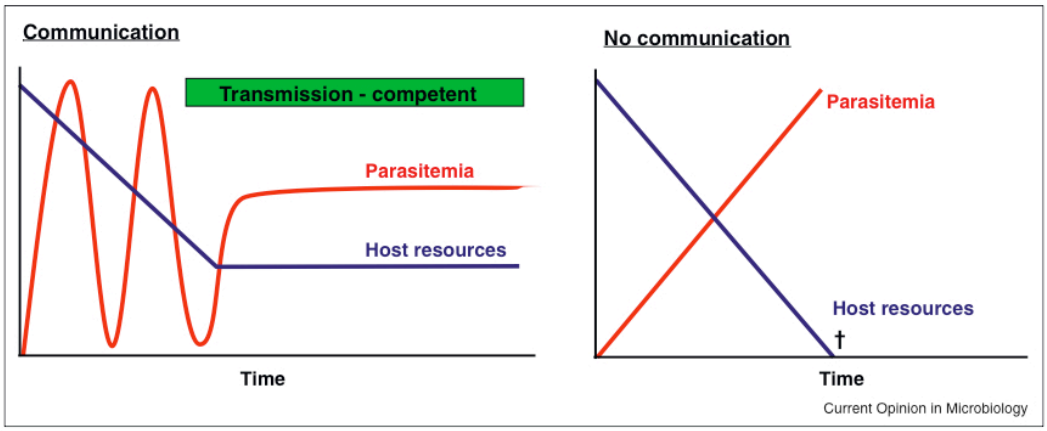
addition to uncovering underappreciated aspects of parasite biology, these studies illustrate the potential for sociomicrobiology concepts to advance understanding of the biology, transmission and pathogenesis of parasitic protozoa.

Cell–cell signaling and cell density-dependent behavior

The protozoan parasite *T. brucei* is the etiologic agent of African trypanosomiasis, which causes widespread mortality and morbidity of humans and livestock in subSaharan Africa. These parasites are transmitted to the bloodstream of a mammalian host through the bite of a tsetse fly vector. In the mammalian host, *T. brucei* must balance competing objectives of promoting parasite proliferation and limiting pathologic consequences to preserve the host as nutrient source (Figure 1). In addition, as a vector-borne pathogen, *T. brucei* must ready itself for survival in the tsetse vector and must maintain sufficient parasite density in the bloodstream to permit transmission during a tsetse blood meal [21••,22•]. Parasitemia is controlled partly via host immune defenses, but *T. brucei* is an expert at evading these defenses and thus benefits from differentiation of proliferating 'slender' form parasites into growth-arrested 'stumpy' forms [23•,24,25]. Differentiation into non-dividing stumpy forms is irreversible in the bloodstream and premature commitment to this pathway would jeopardize maintenance of the infection [23•,26]. Control is provided via a postulated quorum sensing-type system in which a soluble, parasite-derived 'stumpy induction factor' (SIF) accumulates as parasite cell density increases and triggers parasite differentiation only after a sufficient parasitemia has been achieved [23•,24]. The nature of SIF and the SIF signaling pathway are not known, but cyclic nucleotide signaling has been suggested to be involved [24,25]. Stumpy-form parasites are pre-adapted for survival in the tsetse midgut, while slender forms are not. Thus, SIF-dependent slender-to-stumpy differentiation limits maximum parasite density in the mammalian host and simultaneously modulates parasite preparation for survival in the next host, optimizing probability of transmission [22•,23•].

Recent work has provided insight into slender-to-stumpy differentiation and its contribution to *T. brucei* disease progression and transmission. Previously, studies were limited by subjective parameters for distinguishing slender from stumpy-form parasites. MacGregor *et al.* [21••] used a stumpy-specific marker, PAD [27•] to conduct a quantitative analysis of trypanosome population dynamics during chronic infection in mice. They demonstrated that stumpy forms dominate the parasite population throughout late stages of infection. The quantitative nature of the approach enabled mathematical modeling, which provided overwhelming support for a quorum sensing mechanism. Moreover, the authors were able to make specific predictions for the cell types that produce SIF and define kinetic parameters for its production, activity and turnover. These data will facilitate efforts to identify the SIF molecule(s). Because SIF is produced only by a subset of cell types in the population, the system has the capacity to make qualitative as well as quantitative assessments of population dynamics. Interestingly, the findings also have implications for immune evasion strategies employed by *T. brucei*, because stumpy forms do not undergo antigenic variation [28]. Overall, the results emphasize the importance of parasite–parasite communication as a crucial element in disease progression and transmission.

Figure 1



Cell–cell communication benefits *T. brucei*. Parasite–parasite communication (chart on left) via cell density-dependent signaling controls *T. brucei* differentiation from proliferating forms that are adapted for survival in the bloodstream to growth-arrested, transmission competent forms that are adapted for survival in the tsetse vector. By linking differentiation to population density, the parasite avoids depletion of host nutrients and prevents premature commitment to a developmental form that is not optimized for survival in the mammalian host [21]. Without density-dependent cell–cell communication (chart on right), continued parasite proliferation would deplete host resources and thus reduce chances for transmission.

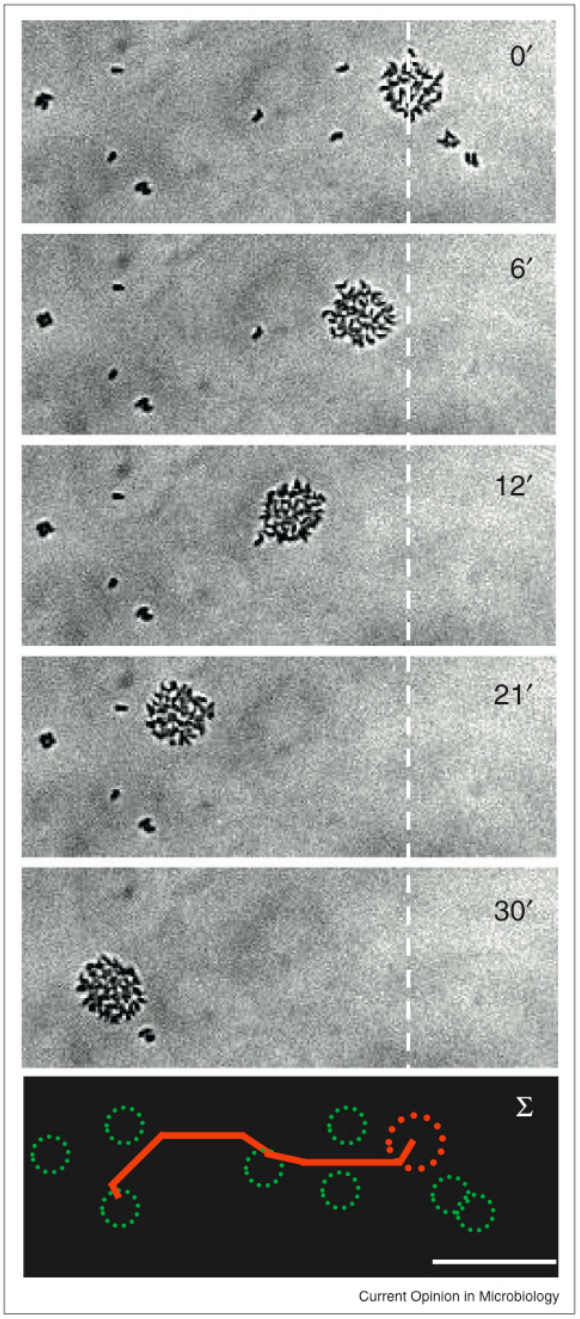
Another fascinating example of parasite surveillance of its own population during infection comes from studies of sex ratio adjustment in the malaria parasite *Plasmodium chabaudi* [29••]. Malaria affects an estimated 247 million people worldwide [30]. Malaria parasites are transmitted through the bite of an *Anopheles* mosquito. In the transmission cycle, male and female gametocytes are produced in the mammalian bloodstream and taken up during a mosquito blood meal. Within the mosquito, gametocytes mature, then fuse and complete their life cycle in a series of steps that culminate in formation of infectious parasites in the mosquito salivary gland. The ratio of female to male gametocytes varies and is biased toward females. This sex ratio distribution contributes to parasite fitness and influences parasite evolution, but the factors controlling it are unknown. In multicellular animals, gamete sex ratio distribution is governed by rules of social evolution theory, which predict that sex ratios are dictated by population diversity [31,32]. In essence, at low population diversity, female gametes outnumber males and as population diversity increases, the ratio of females to males decreases. In an elegant series of experiments, Pollitt *et al.* tested this theory in mixed *Plasmodium* infections using different numbers of *Plasmodium* genotype variants [33]. They found that the parasites adjusted their sex ratio in response to the presence of unrelated genotypes in the parasite population. Their results indicate that not only can malaria parasites sense population density during an infection, but they can also sense diversity in the population and adjust their behavior in response. In addition to resolving a long-standing question about *Plasmodium* biology, the studies offered a test of one of the basic tenets of social evolutionary theory, thus emphasizing another aspect of the value in applying social biology concepts to parasite biology.

Life on a surface and social motility in *T. brucei*

Most microbes are associated with surfaces in their natural environments and engage in surface-induced social behaviors, such as biofilm formation and various forms of social motility [5,6,8,12•,13]. These group activities facilitate surface colonization, defense and efficient use of nutrients [34,11•,13]. *T. brucei* is extracellular in both hosts and spends most of its lifecycle in direct contact with host tissue surfaces. Within the tsetse in particular, parasite movement across, and colonization of tissue surfaces are crucial for development and transmission [35–37]. Currently, *T. brucei* is studied almost exclusively in suspension cultures and little is known about how life on a surface influences parasite behavior.

With bacteria and fungi, cultivation on semisolid agarose matrices has proven valuable for studies of social behavior [12•,5,13]. Oberholzer *et al.* thus employed semisolid agarose matrices to study surface behavior of procyclic-form (insect life cycle stage) *T. brucei* [38••]. They discovered a novel group behavior, termed social motility, in

Figure 2

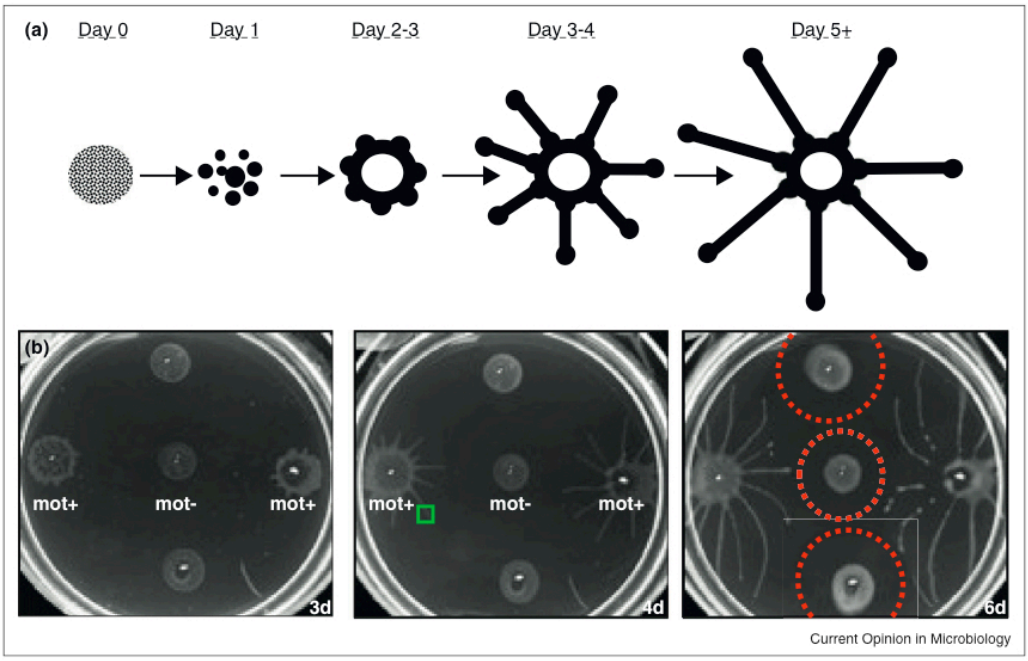


T. brucei social motility on a surface. *T. brucei* cells assemble into small groups that migrate en masse across the surface and enlarge through recruitment of other cells. Panels are time-lapse images (see movie M1) showing movement of a group of parasites (top right of top panel) across the surface of a semisolid agarose plate, with dashed white line indicating starting position of the group. Bottom panel shows summary. Elapsed time is indicated in minutes. Scale bar is 100 μm.

which parasites assembled into multicellular communities with emergent properties that are not evident in single cells. Initially, parasites collect into small groups that move en masse across the agarose surface and grow larger through recruitment of other cells (Figure 2). At the periphery of the inoculation site, groups of parasites collect in nodes of high cell density and then advance outward, forming radial projections (Figure 3). The number and spacing of radial projections is generally consistent from one group to the next and patterns formed resemble those generated during surface colonization by swarming bacteria [12•,13]. The events of *T. brucei* social motility occur in defined stages as summarized schematically in Figure 3A.

Several features of *T. brucei* social motility indicate cell–cell communication governs the behavior. First, coordination among individuals to enable group movement is striking, for example, Figure 2 and movie M1, and in some cases, group movements occur only when other parasites are detected nearby, suggesting cell–cell communication within and between groups. Additionally, individual cells within each radial projection are highly motile (movie M2) and can freely move out and back from lateral edges, yet the group advances only at its leading edge. This indicates that polarized migration of the group is governed by parasites 'choosing' to move in a specific direction and suggests that parasite-derived signals may govern spacing of adjacent projections. In support of this idea, radial projections continue to advance unless they encounter a separate group of parasites, in which case movement is halted or diverted to avoid contact (Figure 3B). Adjacent projections alter their course in parallel, indicating that signaling between groups controls group movement. The zone of avoidance is a direct function of parasite number, suggesting that a diffusible substance(s) is responsible, as has been reported for swarming motility in bacteria [39•,40]. Overall, the work demonstrates the capacity of protozoan parasites to engage in group activities and reveals a level of complexity and cooperativity to trypanosome behavior that was not previously recognized. The findings also offer a convenient assay for studying environmental sensing in these organisms, which is an understudied problem.

Figure 3



Social motility in *T. brucei*. When cultivated on semi-solid surfaces, *T. brucei* engages in complex social interactions that culminate in the formation of characteristic colony patterns. **(A)** Schematic diagram of the main steps of social motility in *T. brucei*, with parasites represented in black. Initially, individual parasites (Day 0) form small groups (Day 1). These groups move *en masse* across the surface and grow through recruitment of additional parasites. Groups assemble at the periphery of the inoculation site, concentrating in nodes (Days 2–3). From these nodes, parasites advance outward, forming radial projections (Days 3–5) that are regularly spaced and advance at the leading edge only (Days 5+). **(B)** Suspension cultures of wild type (mot +) or motility mutant (mot −) parasites were inoculated on semisolid agarose and imaged at 3, 4 or 6 days (3 d, 4 d, 6 d) post inoculation. Social motility requires active parasite motility, as motility mutants (mot-) fail to undergo social motility. Individual cells in each projection are highly motile (see movie M2, corresponding to a region represented by the green box in panel B4d). Projections can sense neighboring cells and halt or redirect their movements to avoid contact, resulting a zone of avoidance (dotted red circles in panel B6d).Adapted from [38••] with permission.

Conflict, competition and cross-kingdom interactions

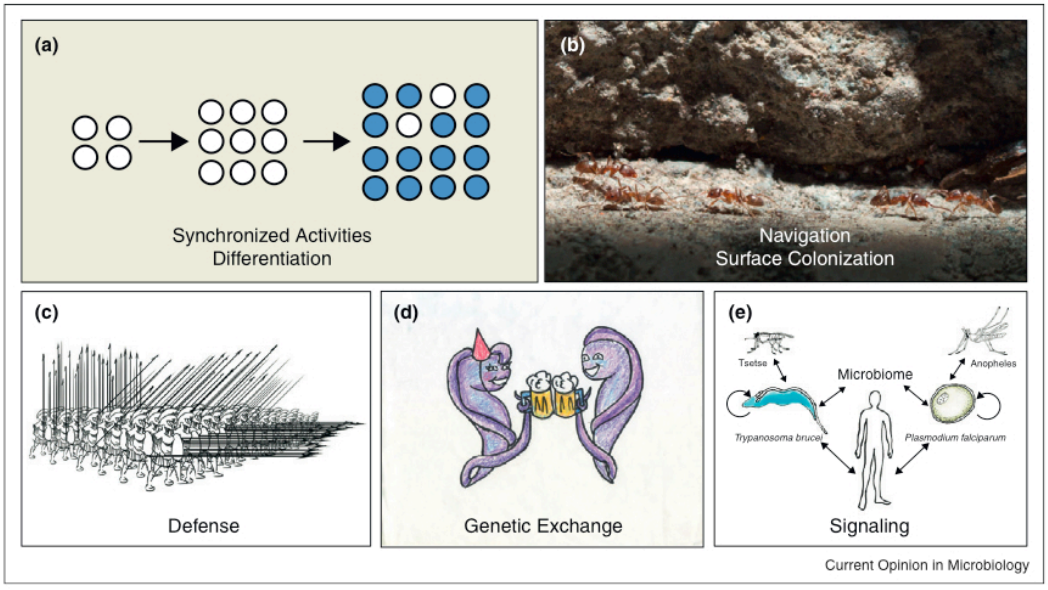
Wherever there is interaction among individuals, there is potential for conflict and competition. Bacteria engage in all manner of intercellular warfare and competition, ranging from growth inhibition and cytolysis of competing species, to bacterial cannibalism [1••,4,8,39•,41]. In an interesting case of sibling rivalry, neighboring colonies of *Paenibacillus dendritiformis* mutually inhibit each other's growth through secreted signaling molecules while growth inhibition does not occur in a single colony [39•]. The behavior bears strong resemblance to the avoidance behavior observed in *T. brucei* social motility (Figure 3), suggesting that procyclic form trypanosomes produce secreted factors that affect neighboring cells. Another instance of parasite–parasite competition has been reported for mixed *T. brucei* infections in mice [42] in which mutual competitive suppression was observed between co-infecting *T. brucei* strains of varying virulence. The authors report that mutual suppression of parasite growth in the host is correlated with extended host survival, suggesting that the less virulent strain reduces the pathogenic impact of the more virulent strain. The extent of mixed infections for *T. brucei* in the field is not known, but for some parasites, such as *Plasmodium*, the majority of natural infections are expected to involve multiple strains [43].

It is taken as de-facto knowledge that host–parasite interactions influence infection outcome. However, parasites are not the only microbes present in their hosts. The influence that the microbial flora of the mammalian host or insect vector exerts on parasite biology, transmission and pathogenesis is mostly unknown. For evolutionary ecologists, the influence of an organism's microbial flora on infection is well-known [44]. Recent work has demonstrated for both *T. brucei* and *Plasmodium*, that the presence or absence of specific bacterial symbionts in the insect vector is associated with refractoriness to parasite infection [45,46]. Thus, as is the case for bacterial pathogens [18], cross-kingdom social interactions exert significant influence on the biology of pathogenic protozoa.

Summary and perspective

Protozoan parasites cause tremendous human suffering worldwide, but strategies for therapeutic intervention are limited. Recent studies illustrate that the paradigm of microbes as social organisms can be brought to bear on questions about parasite biology, transmission and pathogenesis. In addition to uncovering novel aspects of parasite biology, these studies suggest alternative strategies for therapeutic intervention may include targeting parasite–parasite communication. Experimentally tract-

Figure 4



Benefits of social behavior. **(A)** Cell density signaling mechanisms enable synchronization of cellular activities thus preserving group level behaviors (blue circles) for when they are most advantageous. Additionally, not all individuals are equally receptive to the signals thus allowing for differentiation within a population (white among blue circles). **(B)** Cell–cell communication and cooperative motility facilitate colonization of tissue surfaces and navigation through specific host compartments. **(C)** Group defensive strategies protect against environmental antagonists. **(D)** Social interactions facilitate genetic exchange. **(E)** While the current review has primarily considered social behavior in the context of parasite–parasite signaling, cell–cell communication also occurs between the parasite and vector, host, and host microbiome, all of which will impact parasite transmission and pathogenesis. Studying these interactions is also expected to provide insight into the signal transduction pathways utilized by parasites.

able parasite systems also provide opportunities for empirically testing rules that govern social behavior.

Microbes derive a variety of benefits from social interactions and group behaviors (Figure 4). A focus of future efforts should be to determine which of these benefits apply in specific parasite systems. It will also be important to elucidate the underlying mechanisms. At a minimum, systems are required for production, perception and transduction of extracellular signals, whether diffusible or cell contact-mediated. Proteomic analyses of parasite surface proteins will facilitate efforts to define these systems [47,48•,49]. Exolipids are used as surfactants in bacterial surface motility [13] and parasites express abundant glycolipids and glycoproteins on their surface. Cyclic nucleotide signaling plays a major role in the regulation of social behaviors in other organisms [6,24,50] and has been implicated in *T. brucei* SIF signaling [24] and social motility (unpublished observation). Combined with other similarities discussed above, these observations indicate that mechanistic insights may come from comparing social behaviors in bacteria and parasitic protozoa.

Microbial social behavior was once considered to be a cottage industry of only a few species, but is now recognized to be ubiquitous among bacteria. Likewise, the few examples of social behavior in parasites discussed here may be just the tip of the iceberg and much more lies beneath the surface that is yet to be explored.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Conflicts of interest

The authors would like to declare no conflicts of interest regarding this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mib.2011.09.012.

Acknowledgements

Funding for the work was provided by grants from the NIH-NIAID (AI052348), Burroughs Wellcome Fund and the Beckman Young Investigator program to KLH. MAL is the recipient of an NIH-NRSA (F31AI085961) fellowship. HTN is the recipient of an NIH-NRSA (GM007185) fellowship. MO is the recipient of a Swiss National Science Foundation Fellowship. We would also like to thank Eric Nguyen and Anthony Nguyen for their artistic contribution to Figure 4.

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Appendix B

Propulsion of African Trypanosomes is driven by bihelical waves with alternating chirality separated by kinks

Propulsion of African trypanosomes is driven by bihelical waves with alternating chirality separated by kinks

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Edited by Howard C. Berg, Harvard University, Cambridge, MA, and approved September 16, 2009 (received for review June 23, 2009)

***Trypanosoma brucei*, a parasitic protist with a single flagellum, is the causative agent of African sleeping sickness. Propulsion of *T. brucei* was long believed to be by a drill-like, helical motion. Using millisecond differential interference-contrast microscopy and analyzing image sequences of cultured procyclic-form and bloodstream-form parasites, as well as bloodstream-form cells in infected mouse blood, we find that, instead, motility of *T. brucei* is by the propagation of kinks, separating left-handed and right-handed helical waves. Kink-driven motility, previously encountered in prokaryotes, permits *T. brucei* a helical propagation mechanism while avoiding the large viscous drag associated with a net rotation of the broad end of its tapering body. Our study demonstrates that millisecond differential interference-contrast microscopy can be a useful tool for uncovering important short-time features of microorganism locomotion.**

millisecond differential interference-contrast microscopy | *Trypanosoma brucei* | cilium | flagellum

The protozoan parasite *Trypanosoma brucei* is the causative pathogen of African sleeping sickness, a fatal disease indigenous to subSaharan Africa where 60 million people are at risk of infection (1, 2). *T. brucei* is transmitted between human hosts by a tsetse fly vector, and parasite motility is important in both hosts. In the tsetse fly, procyclic-form (PCF) parasites colonize the midgut and then migrate through the alimentary canal to the salivary glands, where maturation into human infectious forms occurs (3, 4). From the salivary gland, mature parasites can be injected into the blood of a mammalian host that has been bitten by the fly. In the mammalian host, migration of bloodstream-form (BSF) parasites through the blood-brain barrier initiates onset of the fatal course of the disease (5, 6). *T. brucei* is extracellular at all stages of infection and depends on its own flagellum-mediated motility for dissemination. Flagellar motility of *T. brucei* in various environments is believed to be central not only to host-parasite interaction, but also to cell division, morphogenesis, and development (3, 6–15).

The *T. brucei* cell body is roughly 20-μm long, with a relatively large posterior section tapering off into a long, narrow anterior section. It has a single flagellum, with the classic "9 + 2" microtubule axoneme architecture that is attached to the cell body along its length. Based on microscopy studies, it is believed that propulsion of *T. brucei* proceeds by left-hand (LH) helical waves propagating along the flagellum, from tip to base, driving the cell forward in a drill-like motion (see Fig. 1*A*) (15–17). The genus name of the parasite actually derives from this distinctive motility (from the Greek *trypanon* or auger, and *soma* or body), first described in 1843 (18). Here, we use millisecond resolution differential interference-contrast (DIC) microscopy, combined with other microscopy methods, to provide a quantitative analysis of *T. brucei* cell propulsion. Our results reveal that *T. brucei* forward motility is characterized by the propagation of kinks separating helical waves of alternate chirality.

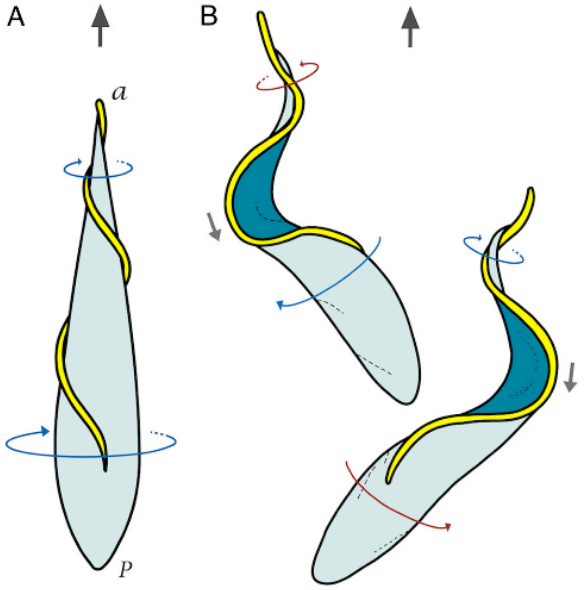

Fig. 1. Models of forward motility for *T. brucei* in aqueous media. (*A*) The traditional model: propulsion is caused by helical waves propagating from the tip to the base of the flagellum with LH chirality, resulting in a drill-like motion of the cell body ("*a*" and "*p*" represent the anterior and posterior end of the cell). (*B*) The bihelical model in which alternating LH and right hand (RH) helical waves propagate down the flagellum separated by a kink. The flagellum of the bottom cell exhibits a LH helical wave (*blue arrow*) at the tip and a RH helical wave (*red arrow*) near the base, separated by a "minus" kink (*gray arrow*). The flagellum of the top cell shows a RH helical wave at the tip and a LH helical wave near the base, separated by a "plus" kink. Kinks propagate in a direction opposite to that of cell propulsion. Two dominant cell-body configurations are associated with the propagation of kinks, with the cell body rocking back and forth between the two alternating configurations.

Results

Helical Waves with Alternating Chirality. To investigate *T. brucei* propulsion in standard culture conditions, we used high-speed

Author contributions: J.A.R., R.B., K.L.H., and J.M. designed research; J.A.R., M.A.L., M.C.T., Y.Z., M.O., D.D.C., N.K.K., and J.M. performed research; J.A.R., M.A.L., M.C.T., Y.Z., M.O., D.D.C., M.L.P., G.H., R.B., K.L.H., and J.M. analyzed data; and J.A.R., R.B., K.L.H., and J.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0907001106/DCSupplemental.

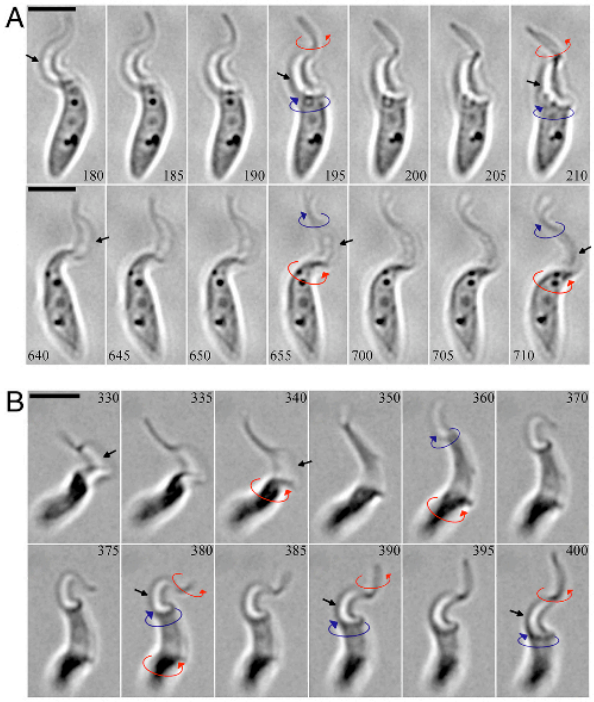

Fig. 2. Millisecond DIC microscopy imaging of PCF and BSF cell motility. (*A*) (*Top*) Image sequence of a PCF cell showing a RH (*red arrow*) helical wave at the tip and a LH (*blue arrow*) helical wave near the base of the flagellum, separated by a plus kink (*black arrow*). (*Bottom*) Image sequence of the same cell showing a LH helical wave at the tip and a RH helical wave near the base separated by a minus kink. The numerical values are in milliseconds. (Scale bars, 5 μm.) (*B*) Image sequence of a BSF cell showing bihelical waves that are separated by a minus kink (*Top*) immediately followed by a plus kink (*Bottom*).

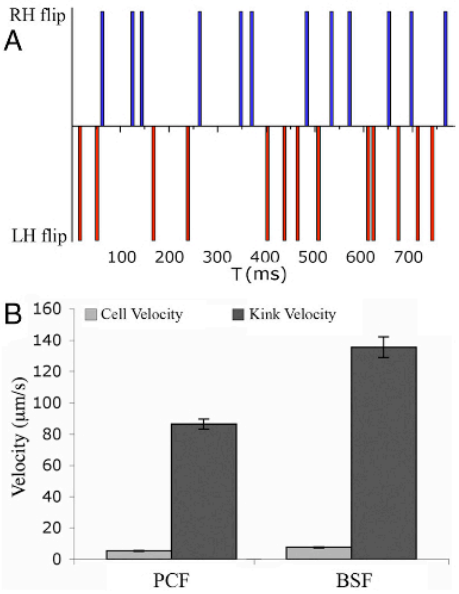

Fig. 3. Analysis of cell propulsion in PCF and BSF *T. brucei*. (*A*) Representative plot showing the distribution of the LH and RH helical waves at the anterior end of a PCF cell within a time interval of about 800 ms. The number of waves was determined by monitoring how many times the flagellum flipped to initiate a RH or LH wave, as indicated with blue and red vertical bars, respectively. Each flip represents a rotation of ≈180°. No more than three successive flips in the same direction are generated at the anterior end. Note that the rotation frequency in this example is somewhat higher than the average frequency of 19 ± 3 flips per second. (*B*) Cell velocities were calculated from 50 PCF (5 ± 2 μm/s) and 50 BSF (8 ± 2 μm/s) cells undergoing directional motion. The kink velocities were obtained from 27 kinks in five PCF cells (86 ± 3 μm/s) and 24 kinks in eight BSF cells (136 ± 7 μm/s), respectively. The ratio of the kink velocity to the cell velocity is about 16 for PCF and 18 for BSF cells.

DIC microscopy with a millisecond timescale [supporting information (SI) Fig. S1] (see *SI Text* for details]. The millisecond frame-by-frame analysis revealed that cell propulsion of *T. brucei* is characterized by repeated reversals in the rotation direction of the flagellum tip, which produced helical waves of alternating chirality propagating tip to base (Figs. 1*B* and Fig. S2, Movies S1 and S2). The image sequence in Fig. 2*A* (*Top*) shows such a bihelical wave in a PCF cell having RH chirality at the tip and LH chirality near the base of the flagellum (see Movie S1). At a later time, this same cell initiated a beat with opposite chirality (see Fig. 2*A Bottom*): that is, exhibiting LH chirality at the tip and RH chirality near the base of the flagellum. We quantified the frequency of LH and RH helical waves by tabulating how many times the flagellum tip flipped to initiate a wave in either direction. The average frequency, calculated from five cells, was 19 ± 3 flips per second, with each flip representing a rotation of ≈180°. This was split approximately equally between LH (9.7 ± 1.3 flips/s) and RH waves (9.0 ± 2.0 flips/s), as illustrated by the representative example shown in Fig. 3*A*. Typically, no more than three successive waves with the same chirality are generated at the flagellar tip. Thus, there appears to be no systematic bias for LH or RH chirality in the motility dynamics.

Next, we examined BSF *T. brucei* cells to determine whether helical waves with alternating chirality are a shared feature of both life-cycle stages. The millisecond DIC images clearly demonstrate bihelical waves in the flagellum of BSF cells (Movies S3 and S4). The image sequence in Fig. 2*B* shows a BSF cell monitored over a 70-ms time period. The top panels show a bihelical wave with LH chirality at the tip and RH chirality near the base of the flagellum. As this wave propagates toward the flagellum base, a new RH helical turn is initiated at the flagellum tip, producing a wave with RH, LH, RH chirality from tip to base (*Bottom*).

Kinks. A segment of a filament connecting two helical segments of opposite chirality is known as a "kink." We define a "plus" kink as one separating an anterior RH helical wave from a posterior LH helical wave, while a "minus" kink separates an anterior LH helical wave from a posterior RH helical wave. A kink separating two traveling helical waves, as in Fig. 2*A*, will itself travel along the filament. Traveling kinks have been encountered earlier in motility studies of prokaryotes, such as *Escherichia coli* (19, 20), where they appear to be associated with changes in course. In *Spiroplasma*, which do not have flagella (21), pairs of kinks traveling along the helical cell body cause the cell to swim in a zig-zag path. A theoretical study of *Spiroplasma* motility (22) proposed that recoil against the motion of fluid carried backwards by traveling kinks actually is the propulsive mechanism of *Spiroplasma*.

Well-defined kinks could be observed in the millisecond DIC images of both PCF and BSF *T. brucei* cells (see the *gray arrow* in Fig. 1*B*, Fig. 2, and Movies S1–S4). The kinks propagated down the flagellum along the cell body from tip to base (see Fig. 2, and Movies S1–S4), opposite to the direction of cell propulsion. Typical kink propagation velocities in *T. brucei* were 85 ± 18 μm/s in PCF cells and 136 ± 7 μm/s in BSF cells, more than an order of magnitude higher than the center-of-mass velocity of the cells (Fig. 3*B* and details in the *SI Text*). The observation that a 1.6-fold increase of kink velocity in BSF cells versus PCF cells correlates with a 1.6-fold increase in the center-of-mass velocity of BSF versus

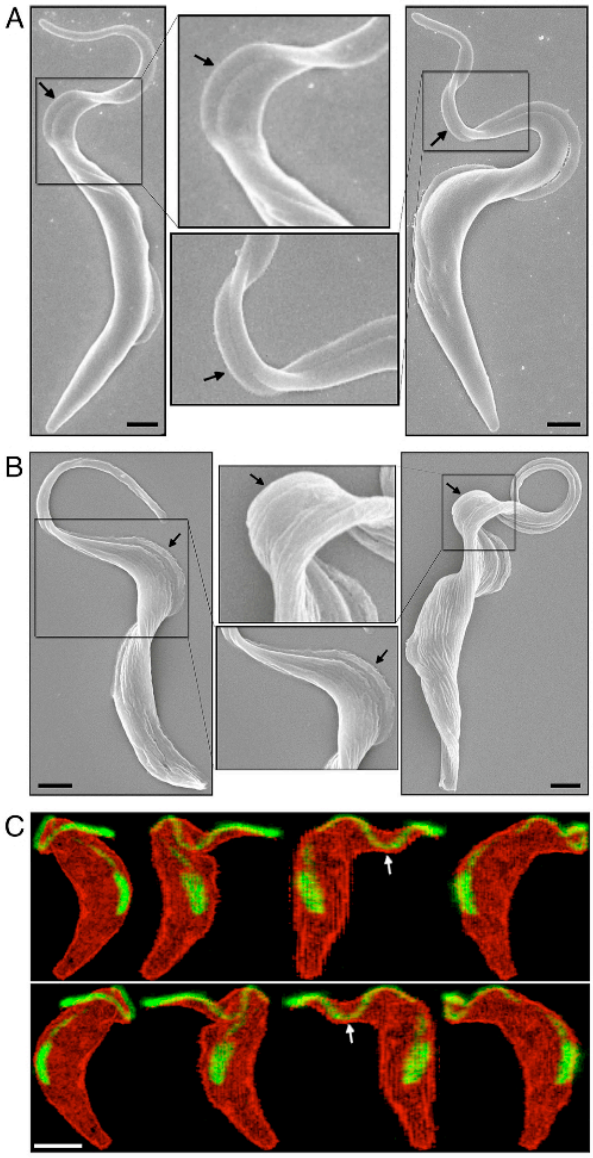

Fig. 4. SEM and confocal microscopy imaging of rapid-fixed PCF and BSF cells. (*A*) SEM images of PCF cells with bihelical waves separated by a plus (*Left*) or a minus (*Right*) kink. (*B*) SEM images of BSF cells with bihelical waves separated by a plus (*Left*) or a minus (*Right*) kink. The zoom-in views illustrate torsion of the cell body induced by the bihelical waves and kinks. (Scale bars, 1 μm.) (*C*) Confocal microscopy images of a GFP-labeled flagellum in a single PCF cell (see Movie S5). The cell is rotated along the vertical axis with ≈45° per image. The flagellum (*green*) wrapping around the surface of the cell body (*red*) exhibits RH chirality near the base and LH chirality near the tip, forming a minus kink (*white arrows*). (Scale bar, 3 μm.)

PCF cells (see Fig. 3*B*) suggests that kink motion is intrinsic to the propagation mechanism.

To further confirm the existence of helical waves and kinks in *T. brucei*, we used scanning electron microscopy (SEM) combined with a rapid-fixation technique that was optimized to preserve flagellar waveforms (23). SEM images of rapid-fixed cells indeed revealed bihelical waves and kinks in both PCF and BSF cells (Fig. 4 *A* and *B*). Interestingly, SEM images indicate that the cell body is subject to torsional strain, possibly generated by the flagellum. At the flagellar pocket where the flagellum emerges from the cytoplasm, there is a preferred LH chirality for the flagellum, consistent with earlier studies (24). As an independent test, we labeled the flagellum of PCF cells with a PFR2-GFP fusion protein and imaged these cells by three-dimensional (3D) confocal microscopy. In a representative 3D image (Fig. 4*C* and Movie S5), the flagellum (*green*) wraps around the surface of the cell body (*red*) and exhibits RH chirality near the base and LH chirality near the tip, forming a minus kink (*white arrows*).

Cell Body: Configurational Changes, Viscous Drag, and Torsional Stress. Arrival of kinks at the posterior end of the cell appears to correlate with transitions of the main body of the cell between two dominant configurations (see Figs. 1*B* and 2, Movies S1–S4). The configurational changes take place through alternating clockwise and counterclockwise rotations of the posterior end. In other words, the posterior end rocks back and forth about its own axis rather than completing full 360° rotations. The average frequency of the rocking motion of the posterior end was 5 ± 3 flips per second (calculated from five cells and 34 individual flips), compared to 19 ± 3 flips per second at the anterior end. The fact that the rotation frequency decreases significantly along the body of *T. brucei* is interesting. Different sections of a filament that supported a helical wave with a frequency gradient would, over time, be rotated with respect to each other over arbitrarily large angles, which for *T. brucei* would not be consistent with the mechanical integrity of the cell body. The reversals in rotation observed in *T. brucei* are thus necessary to maintain the frequency gradient.

The observation that the smaller anterior end of the body performs high-frequency complete rotations, while the larger posterior end only performs a low-frequency rocking motion, suggests a rationale why this may be an efficient mode of propagation for a microorganism with the asymmetric cell structure of *T. brucei*, given that at low Reynolds number viscous forces dominate (25). Note that a purely reciprocal motion (i.e., one that is symmetric under time reversal) cannot provide a net propulsive force to a microorganism, so the reciprocal rocking motion of the posterior end of *T. brucei* could not contribute a net propulsive force. On the other hand, the sequence of kinks traveling from the anterior to the posterior end separating helical sections with opposite chirality and opposite rotation direction obviously is nonreciprocal, and could therefore contribute a net propulsive force. Next, it follows from elementary hydrodynamics that a tapering cylindrical body rotating around its axis in a fluid is subject to a retarding viscous torque-per-unit length (τ_R) exerted by the surrounding medium that resists the rotation. This torque-per-unit length at a given point along the body is proportional to the local cross-section:

$$\tau_R = -4\pi\eta R_B^2\omega_B \qquad [1]$$

where η is the viscosity of the surrounding medium, R_B the radius of the cross section of the cell body at that point, and ω_B the rotation rate of the body. If, for example, we model the main body of *T. brucei* as a cylinder with a length L of ≈20 μm and a (constant) radius of ≈1 μm, then the power dissipated by viscous loss in water at the 20-Hz rotation rate of the tip would be substantial: about 10^4 times the ATP hydrolysis energy per second. Reducing this rotation frequency by a factor of four—the typical frequency reduction factor between posterior and anterior ends of *T. brucei*—reduces the power dissipation by more than an order of magnitude because power is proportional to the square of the rotation frequency. For a case in which the radius of the cell body near the anterior end is about five times less than that near the posterior end, a reasonable estimate for *T. brucei*, the viscous torque-per-unit length near the anterior end is about 25 times less than that near the posterior end, thus roughly compensating for the frequency increase of the anterior end. If the flagellum produced helical waves of uniform

chirality, then the posterior and anterior ends of the cell would necessarily rotate at the same "global" rate, which would be slowed greatly by the viscous torque on the cell body near its larger posterior end. The frequency gradient thus allows for high rotation rates at the smaller anterior end, which provides the traction force that pulls the cell body along, combined with reduced rotation rates toward the larger posterior end, which reduces viscous drag. Additionally, because the flagellum is attached laterally along the length of the cell body, a helical wave of the flagellum necessarily applies a torsional stress to the cell body, as also suggested by SEM images (see Fig. 4 *A* and *B*). Depending on the degree of viscoelasticity of the cell body, the cell body may well be able to store a significant amount of torsional elastic energy in reversible deformation of the microtubule cytoskeleton. This elastic energy could then be released when the flagellum started to rotate in the opposite direction, much like the rubber motor of a toy plane.

Rapid Swings of the Flagellar Tip of BSF Cells. Despite overall similarity, there is an interesting difference between the motilities of BSF and PCF cells. The anterior end of BSF cells exhibits more frequent and more pronounced movements than that of PCF cells under similar conditions (Fig. 5 *A* and *B*). Rapid swings of BSF flagellar tips were observed following large angle bends of this portion of the flagellum (Fig. 5*C*), a feature absent from PCF cells. To quantitatively analyze the rapid swings of the flagellar tip, we extracted 12 image sequences from two BSF cells undergoing fast forward locomotion. Each of the 12 trajectories (Fig. 5*D*) represents the distance traveled by the flagellar tip in each image sequence plotted as a function of the time elapsed (a linear fit of the average trajectories is shown as a *dashed line*). The slope of each trajectory corresponds to the velocity. The highest recorded velocity of the flagellar tip is 673 μm/s, while the average velocity (i.e., the slope of the dashed line) is 510 μm/s (see the *SI Text* for details). Note that these observations could not have been made with conventional video frame rates, suggesting that millisecond DIC microscopy could be a useful tool for exploring flagellar and ciliary motility in other organisms (26–29).

BSF Motility in Infected Mouse Blood. Our studies of *T. brucei* motility so far were carried out in standard culture medium used for most studies of trypanosome motility, which has a measured viscosity close to that of water (0.95cSt). In contrast, the macroscopic viscosity of human blood is about 35 times higher than that of water. What is perhaps most relevant is that in blood, the dense distribution of erythrocytes presents to *T. brucei* a highly inhomogeneous, although deformable, labyrinth that it must negotiate. Typical sizes of the free spaces of this labyrinth can be small compared to the length of a single BSF cell. To examine *T. brucei* motility in a more native medium, we investigated BSF cells in whole blood obtained from infected mice, 3 to 5 days after infection. Millisecond DIC images of actively swimming BSF *T. brucei* in infected mouse blood revealed the same motility motifs observed with cultured parasites (Fig. 6 *A* and *B*, Movie S6). Rapid swings of the flagellar tip were particularly striking, as they can be observed initiating contact with host blood cells and deforming them significantly (Fig. 6*C*). Comparison with the measured force-deformation curves of erythrocytes (30) indicates that the flagellum is capable of exerting forces in the 300 pN range (see the *SI Text* and Table S1).

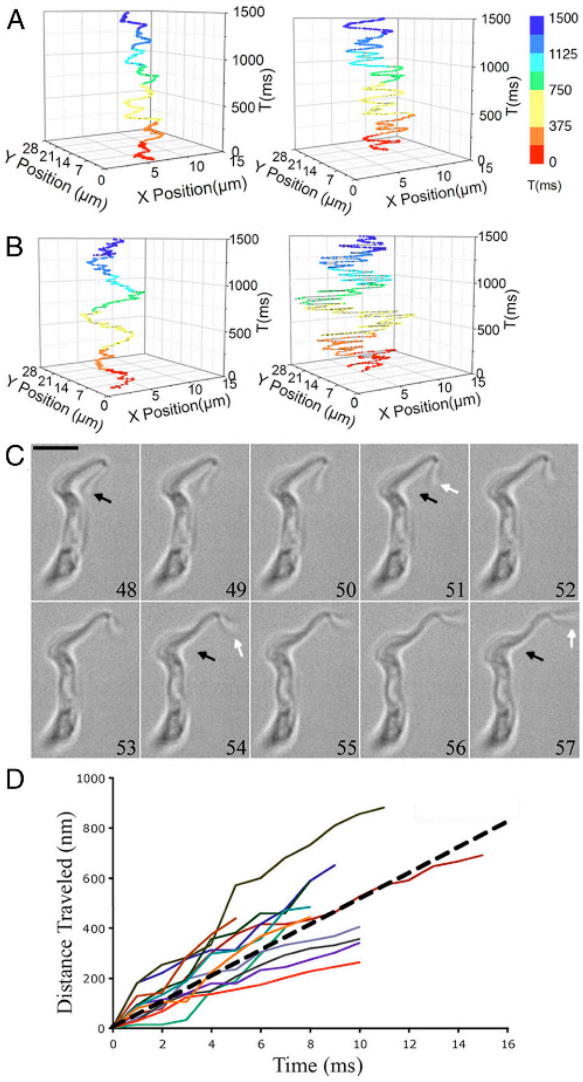

Fig. 5. Quantification of the motility of *T. brucei* cells. (*A*) Trajectory of the anterior (*Right*) and posterior (*Left*) end of a PCF cell as a function of time, where each color represents a different time period. (*B*) Trajectory of the motion of the anterior (*Right*) and posterior (*Left*) end of a BSF cell as a function of time. (*C*) Rapid motion of the BSF flagellar tip with a speed of 673 μm/s. White arrows show motion of the flagellar tip and the black arrows point to the initial tip position. The numerical numbers are in millisecond. (Scale bar, 5 μm.) (*D*) Twelve trajectories of rapid swings of the flagellar tip from two BSF cells of which the slopes represent the velocities of the flagellar tip swings. The average velocity (i.e., the slope of the dashed line) is 510 μm/s.

Discussion

Using millisecond DIC microscopy, supplemented by SEM and 3D confocal microscopy, we have found that forward *T. brucei* motility is characterized by (*i*) tip-to-base propagation of kinks separating LH and RH helical waves, (*ii*) very high motility of the flagellum tip in BSF cells, and (*iii*) a pronounced rotation-frequency gradient along the cell body, with the anterior end performing rapid full rotations while the posterior end rocks back and forth more slowly. This specialized form of motility appears to reduce viscous dissipation by minimizing rotary motion of the large posterior end of the cell, while allowing *T. brucei* to negotiate complex viscous environments, such as mammalian blood.

As noted earlier, the closest resemblance between the motility of *T. brucei* and that of other microorganisms is the prokaryote *Spiroplasma* (21, 31, 32). A comparison between these two widely divergent organisms is instructive. Both move in a zig-zag pattern, as kinks between waves of opposite chirality travel from the anterior to the posterior end. One can apply the theoretical analysis of kink motion in *Spiroplasma* (22) to *T. brucei* to argue that, also for *T.*

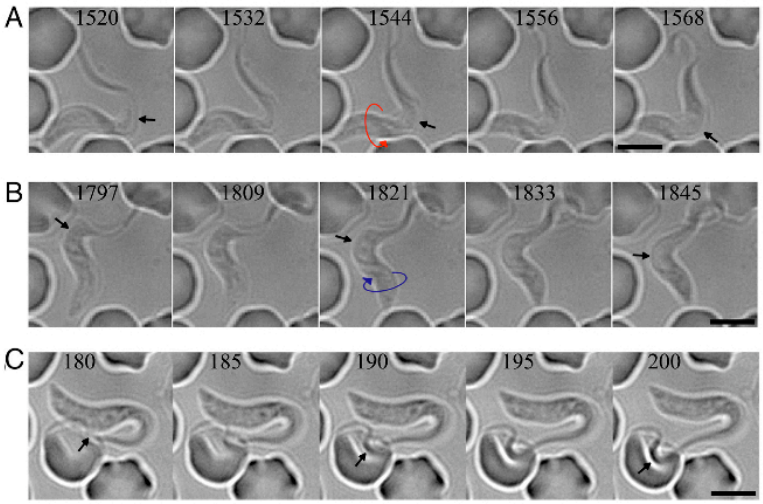

Fig. 6. Millisecond DIC microscopy imaging and analysis of BSF cell motility in infected mouse blood. (*A*) Image sequence of a BSF cell exhibiting bihelical waves separated by a plus kink, where the posterior end rotates clockwise, as indicated by the visible flagellum. The arrows pointing to the center of the kink show the kink propagation from the flagellar tip to base. The numerical values are in milliseconds. (Scale bars, 5 μm.) (*B*) Image sequence of the same BSF cell showing a minus kink where the posterior end rotates counterclockwise. (*C*) Rapid swing of the flagellum tip of the BSF cell was observed initiating contact with a host red blood cell over a time period of 20 ms.

brucei, the propulsive mechanism should be recoil against the motion of fluid carried backwards by the traveling kinks. However, whereas in *Spiroplasma* there is a preferred timing difference between kinks, which is consistent with the theoretical analysis, we encountered a broad distribution of timing differences, ranging between 150 and 300 ms. In addition, typical kink velocities of *T. brucei* (see Fig. 3*B*) are about an order of magnitude larger than that of *Spiroplasma* (about 10 μm/s), which may be because of the fact that *T. brucei* can recruit the efforts of large numbers of dynein molecular motors distributed along the axoneme (see below), whereas *Spiroplasma* presumably can rely on only a few motors. Interestingly, despite the higher kink velocity in *T. brucei*, the center of mass velocities of the two organisms is similar (in the range of 10 μm/s). This presumably reflects the larger viscous dissipative losses and also the larger mass of *T. brucei*, which by momentum conservation reduces the forward recoil velocity against the backward movement of fluid carried by the kinks. A key difference between *T. brucei* and *Spiroplasma* morphology is that *T. brucei* has a pronounced gradient in its body plan, while the body plan of *Spiroplasma* is so well described by a uniform helix (in the absence of kinks) that it is difficult to distinguish the anterior and posterior ends. In terms of motility, this translates into a uniform rotation frequency for *Spiroplasma* but a pronounced frequency gradient for *T. brucei*.

The eukaryotic axoneme is one of the most conserved structures in biology and was likely present in the last common ancestor of all extant eukaryotes (33). Axoneme motility is mediated by thousands of dynein motors that drive sliding and, ultimately, bending of microtubule doublets in the axoneme (34, 35). The switch point hypothesis is a generally accepted paradigm for wave propagation along the axoneme (36, 37). At its most basic, this hypothesis posits that axonemal dyneins are divided into two opposing groups, on either side of the axoneme, and that these groups are alternately activated or inactivated to cause axoneme bending in one direction or the other, thereby producing a plane wave. It has been demonstrated theoretically that arrays of coupled motor proteins subject to an external load can indeed switch collectively between two alternate directions of motion (38). Similarly, helical waves could be generated by assuming that the dynein motors also apply a rotary twist on each pair of microtubules of either chirality, thereby imposing a net twist on the cylindrical array of all nine outer doublet microtubules that would turn the plane wave into a helical wave, as has been proposed for waves in cilia (39). In such a model, the frequency of rotation would not be fixed but determined by the local, external load on the flagellum, determined in turn by the local radius of the flagellum. Because either helicity would be possible, there would now be dynamic instead of structural stability. Collective switching between these two helicity states, similar to the switch-point hypothesis, could then produce an array of moving kinks.

In summary, through quantitative and theoretical analysis of *T. brucei* motility, our results offer insights for considering propulsive mechanisms of microorganisms and provide new detail on an important, yet poorly understood, feature of trypanosome biology.

Materials and Methods

Millisecond DIC microscopy. A light microscope with DIC optics was assembled as follows. A Nikon Eclipse TE 2000-U inverted microscope was equipped with a Nikon Plan Apochromat 100× infinity-corrected oil DIC objective, which has a numerical aperture of 1.40 and a working distance of 0.13 mm. An open air motility chamber was placed on the microscope consisting of a glass slide, separated from a cover glass by an ≈200-μm spacer (40), placed onto the objective immersion oil. Cells were placed into the chamber in a volume of ≈100 μl in log phase. Illumination of the cells was achieved by using a Nikon 100W mercury lamp powered by an 18V to 40V source (Chiu Technical Instruments). Images were acquired using a latest generation CMOS-based Photron SA1.1 camera (Photron USA, Inc.) with 8-Gb onboard memory and a millisecond timescale.

Trypanosome Cell Maintenance and Motility Assays. PCF 29–13 and BSF-SM cells (41) were used throughout these experiments and maintained as previously described (9, 42). a Z1 Coulter Particle Counter (Beckman Coulter) to monitor cell doubling. For motility assays, cells were taken from mid-logarithmic phase cultures and placed in poly(L-glutamate)-treated glass motility chambers, described above, then imaged by using the millisecond DIC microscope. PCF cells were assayed at 25 °C, while BSF cells were assayed at 37 °C. Cells were not assayed for more than 15 min, and analyses were restricted to forward migrating cells. Image sequence acquisition, analysis, and quantification were performed as described in the *SI Text*.

Generation of GFP-PFR2 Cell Lines. The ORF of PFR2 (GenDB ID Tb927.8.4970) was PCR amplified from genomic DNA using Platinum Pfx polymerase (Invitrogen) according to the manufacturer's instructions. The forward and reverse primers used were as follows: PFR2-f 5′-TCTAGAATGAGCGGAAAGGAAGTTGAA-3′, forward. PFR2-r 5′-GGATCCCTACTGAGTGATCTGCGGC-3′, reverse (underlined are the 5′ XbaI and BamHI sites). The PCR product was ligated into a Zero Blunt TOPO PCR Cloning Kit vector (Invitrogen). The internal NotI site of the *PFR2* gene was destroyed by site-directed mutagenesis (changing C1773 into G) using a QuikChange Site-directed Mutagenesis Kit (Stratagene). The sequence was verified by sequencing at the University of California at Los Angeles genomics center. The gene encoding the PFR2 protein was subcloned using XbaI and BamHI sites into pKH10 (40, 41, 43). The construct was linearized using NotI, ethanol precipitated and transfected into PCF 29–13 cell line, as previously described (10, 42).

Transfectants were selected with 2.5 μg/ml Phleomycine (Cayla) and clonal lines were obtained by limited dilution. Individual clones were analyzed for the expression of the GFP-PFR2 fusion protein 48 h after induction with 1 μg/ml tetracycline.

Fluorescent Labeling and Confocal Microscopy Imaging of GFP-PFR2 Cells. For 3D confocal microscopy, cells expressing GFP-PFR2 (described above) were induced for 48 h with 1 μg/ml tetracycline, then washed once in prewarmed PBS and labeled with 0.5 μM CellTracker red, CMTPX (Molecular Probes, Invitrogen), in PBS for 15 min at 27 °C. Labeled cells were washed three times in prewarmed culture media. Cells were recovered for 5 min in culture media and fixed by adding paraformaldehyde (in PBS) to final concentration of 4% for 15 min directly in the labeled culture (23). Fixed cells were adhered to polyL-Lysine coated slides for 20 min. Slides were washed once with PBS, blocked in PBS containing 0.1-mM glycine for 10 min, and mounted with Vectashield mounting medium (Vector Laboratories). Slides were then imaged using a Leica TCS-SP2-AOBS Multiphoton-FLIM confocal microscope using a 63× oil immersion objective. Images were acquired using the Leica confocal software supplied with the microscope. For 3D reconstruction of fixed cells, a series of images was acquired for the red (605 nm) and green (535 nm) emission channels in 0.35-μm increments for a distance of 12.2 μm. Processing and 3D rendering of images were carried out using the National Institutes of Health ImageJ software.

Millisecond DIC Imaging of BSF *T. brucei* in Infected Mouse Blood. Bloodstream form single marker cells were maintained in vitro as previously described (9). BALB/c mice (Jackson Laboratories) were inoculated with 100 mid-log phase cells intraperitonealy in warm sterile PBS pH 7.35 (Gibco) containing 1% glucose (44). Blood samples, 3 to 5 days after infection, were collected in heparinized capillary tubes (Fisher Scientific), monitored for parasitemia by counting in a hemocytometer, and imaged by the millisecond DIC microscope.

SEM Imaging of Rapid-Fixed PCF and BSF Cells. We adopted a rapid-fixation technique optimized to preserve flagellar waveforms (23). Cells were harvested by centrifugation, resuspended in fresh medium, recovered for 30 min, and then fixed by adding paraformaldehyde/glutaraldehyde to final concentration of 3% directly in the culture medium, fixed for 5 min, diluted to 1% fixative with 0.2M sodium cacodylate buffer (pH 7.4), and allowed to settle onto cover slips for 1 h. Fixative was removed and samples were dehydrated in ethanol. Samples were then dried overnight, sputter-coated with gold and imaged using a JEOL JSM-6700F FESEM.

Further details about image acquisition and analysis, measurements of cell and kink velocity, and determination of flagellum tip motion are provided in the *SI Text*.

ACKNOWLEDGMENTS. We thank W. Shi, C. J. Brokaw, and L. Simpson for constructive comments, H. Jiang for assistance with the SEM images, as well as L. Bentolila and M. Schibler of the advanced light microscopy facility at the California NanoSystems Institute. This work was supported in part by the National Science Foundation (Grant DMR-0520894), National Institutes of Health/National Institute of Allergy and Infectious Diseases (Grant AI052348), the Alfred P. Sloan foundation, the Howard Hughes Medical Institute Gilliam fellowship for advanced studies, the Ruth L. Kirschstein National Research Service Award (GM07185), the Burroughs Wellcome Fund, and a University of California at Los Angeles Molecular Biology Interdepartmental Whitcome fellowship.

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