Quorum sensing in African Trypanosomes

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Many microbial eukaryotes exhibit cell-cell communication to co-ordinate group behaviours as a strategy to exploit a changed environment, adapt to adverse conditions or regulate developmental responses. Although best characterised in bacteria, eukaryotic microbes have also been revealed to cooperate to optimise their survival or dissemination. An excellent model for these processes are African trypanosomes, protozoa responsible for important human and animal disease in sub Saharan Africa. These unicellular parasites use density sensing in their mammalian host to prepare for transmission. Recently, the signal and signal transduction pathway underlying this activity have been elucidated, revealing that the parasite exploits oligopeptide signals generated by released peptidases to monitor cell density and so generate transmission stages. Here we review the evidence for this elegant quorum sensing mechanism and its parallels with similar mechanisms in other microbial systems. We also discuss its implications for disease spread in the context of coinfections involving different trypanosome species.

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Introduction

Quorum sensing (QS) is a mechanism of cell–cell communication *via* secreted, hormone-like signalling molecules that is most commonly associated with bacteria. It is a type of cell-cell signaling that triggers changes in gene expression in individual cells when the population reaches a critical density. Using these diverse signal-response systems, bacteria synchronise particular behaviours on a population-wide scale [1]. As well as bacterial QS systems, however, QS-like systems have also been reported in eukaryotic microbes (*e.g.* pathogenic fungi *Candida* and *Histoplasma*) [2], viruses [3], and even higher-order species such as ants [4], providing clear examples of sociality of individuals across the spectrum of life. Indeed, in certain environments, QS regulates the production of extracellular molecules that provide the social signals to co-ordinate group behaviours. The QS mechanism has been recently extended to signalling between phyla or interkingdom signalling [5] and can also form part of the extensive and emerging dialogue between microorganisms and their hosts. Each of these processes is discussed later in the light of mechanisms recently elucidated in African trypanosomes.

African trypanosomes, the causative agents of sleeping sickness in humans and 'nagana' in livestock, are protozoan parasites that have a developmental cycle involving a mammalian host and an insect vector, the tsetse fly. In the mammalian host, parasites exist as replicative "slender" forms, which establish the infection, and once a critical parasitaemia level is reached, differentiate first into "intermediate" forms and then into the non-dividing short "stumpy" form, which are arrested at the G0/G1 phase of the cell cycle [6,7]. This density-dependent differentiation, a form of QS, prolongs the survival of the host and also prepares the parasites for uptake by the tsetse fly.

Work on the trypanosome QS signal has shown that 'pleomorphs' (that respond to this density-mediated growth control to generate stumpy forms) and 'monomorphs' (unable to respond to the density signal through rapid passage either between animals or in culture), produce a soluble, low molecular weight, heat stable factor/s termed stumpy induction factor (SIF) [8]. SIF was reported to be of parasite origin and its accumulation with increasing parasite density was proposed to act as the trigger for the slender to stumpy differentiation. The chemical identity of SIF has remained elusive for over 20 years, as has the mechanism of its detection.

In this review we provide an insight into the history and recent advances in one of the longest enigmas in trypanosome biology: how African trypanosomes signal and sense their population in a mammalian infection, allowing them to differentiate to the transmissible stumpy form and so continue their life cycle.

Deciphering the QS signal in *T.brucei*

An insightful early study proposed several hypotheses on how trypanosomes could differentiate in the mammalian bloodstream [9]. Specifically, it was proposed that the

slender to stumpy differentiation could be i) a programmed event, where parasites divide a certain number of times, ii) a density-dependent phenomenon, which could involve inhibition by contact, iii) a response to the depletion of a critical nutrient or growth factor or iv) a response to the accumulation of a growth inhibitor or stumpy inducer. This exogenous factor could be either a host product, a trypanosome product, or a product of the host-parasite interaction. In this work, it was shown that in murine infections with varying number of trypanosomes, the peak parasitaemia always reached the same number of cells regardless of the inoculum (excluding a programmed event), and that the peak at which replication stopped and transformation occurred depended upon the line of inbred mouse used (excluding inhibition by contact, but involving a possible host product). Prior injection of irradiated mice with glucose, mouse plasma, foetal calf serum or polyamines did not alter the peak parasitaemia. These results argued against nutrient depletion as a contributor and supported the idea that a factor or factors accumulate in blood, initiating growth arrest and differentiation of slender into stumpy forms.

Subsequently, it was reported that trypanosomes release a soluble activity of low relative molecular mass, termed stumpy induction factor (SIF), which accumulates in spent media harvested from cultured cells (conditioned medium) providing a potential QS signal that drives parasite differentiation [8,10] and restricts virulence [11]. Another important milestone was the discovery that trypanosomes exhibit a 3-fold increase of intracellular cyclic 3′,5′-adanosine monophosphate (cAMP) at peak parasitaemia, which decreased as the transition to intermediate and stumpy forms commenced [12]. Exposure of pleomorphic cells to a cell permeable cAMP analogue (8-pCPT-cAMP) was also found to be a potent growth inhibitor and to induce stumpy morphology [8], this response being linked to hydrolysis products of cAMP rather than conventional cAMP signalling [13]. Indeed, conventional cAMP signalling does not seem to operate in these parasites, with even protein kinase A (PKA) exhibiting alternative regulatory ligands [14••].

As with SIF-responsive pleomorphs, laboratory adapted monomorphs can also respond to cell permeable 8-pCPT-cAMP/AMP and undergo growth arrest, suggesting that 8-pCPT-cAMP intersects with the SIF signalling pathway at least to some extent. This has been exploited in a genome-wide RNAi library screen that sought to select parasites that were unresponsive to 8-pCPT-cAMP/AMP after gene knockdown [15••]. Thus, a monomorphic genome-wide RNAi library, capable of tetracycline-induced gene silencing, was exposed to 8-pCPT-cAMP/AMP, and growth compared with the uninduced set. Parasites that continued to proliferate were interrogated by ion torrent amplicon sequencing to identify enriched RNAi genome fragments anticipated to have silenced genes required for responsiveness to 8-pCPT-cAMP. This identified ~30 genes of which several were validated for their role in physiological SIF signalling *in vivo* by individual gene targeting. Analysis of the targets predicted a potential signal transduction pathway composed of protein kinases, protein phosphatases, gene regulators and hypothetical proteins of unknown function. Further analysis explored the hierarchical structure of these components through combinatorial overexpression and gene knock out analysis, predicting a potentially non-linear, or branched, organisation to the signalling pathway [15••]. Through independent studies, other key components of the pathway have also been identified. Central to these has been the involvement of the modified TOR and AMPK components of nutritional signalling pathways [16], with TOR acting as an inhibitor of the stumpy formation pathway [17].

Transporting the signal

Through its use of a cell permeable SIF mimic, 8-pCPT-cAMP, the genome-wide RNAi screen described above by-passed components of the pathway operating at the cell surface or in close functional relationship with a SIF signal receptor or transporter. In opistokhonts, external environmental signals are often perceived through G-protein coupled receptors (GPCRs); however these are absent in excavates such as kinetoplastids, as are their heteromeric G protein molecular partners [18]. Nonetheless, exploration of the trypanosome genome identified a gene encoding a member of the orphan GPCR-like protein (GPR89) family, TbGPR89 [19••]. In humans, a GPR89 member, GPHR, is a pH-sensitive chloride channel resident in the Golgi [20], whereas in *Arabidopsis*, two novel plasma membrane-located proteins with 45–68% sequence identity to human GPR89 were identified [21]. These *Arabidopsis* homologs, named GTG1 and GTG2 (for GPCR-type G protein) respond to Abscisic acid (ABA) at seed germination, seedling growth, and stomatal closure. In humans, plants and trypanosomes, GPR89 proteins have 9 predicted transmembrane domains (TMDs), distinct from the 7-TMDs conventionally found in GPCRs.

Functional analysis showed that TbGPR89 is an essential protein expressed on the proliferative slender form; as cells differentiate to stumpy forms, its levels decrease. Overexpression of the protein drives proliferating cells into a G0/1 arrest, a precondition for initiation of differentiation to stumpy forms, this occurring at unusually low parasite density *in vitro* and *in vivo*. RNAi-mediated silencing of either of two genes required for the response to SIF *in vivo* or 8-pCPT-cAMP/AMP *in vitro* (RBP7 and PPI; [15••]) prevented the differentiation phenotype generated by TbGPR9 overexpression, indicating that the signalling induced by TbGPR89 is acting through the SIF pathway.

An analysis of the predicted similarity of TbGPR89 with proteins of known structure using iTASSER (Iterative Threading ASSEmbly Refinement) software [22] revealed that TbGPR89 showed similarity to proton-oligopeptide transporters (POT), present from bacteria to humans, over the substrate interacting region. Proton-dependent transmembrane transport of short-chain peptides occurs in all living organisms and provides an efficient and energy-saving route for the uptake of bulk quantities of amino acids in peptide form [23]. Peptide transport in the mammalian intestine and kidney is energised, like in prokaryotes and lower eukaryotes, by an inwardly directed electrochemical proton gradient [24].

By expressing TbGPR89 in *Escherichia coli* and using a fluorescent dipeptide substrate, it was demonstrated that TbGPR89 can indeed act as an oligopeptide transporter. Further, by comparing the predicted structure of TbGPR89 with *Geobacillus kaustophilus* POT [25], centered on the binding pocket of the dipeptide analogue, alafosfalin, it was possible to identify Y48 in TbGPR89 as a potentially important residue for substrate interaction. By mutating this amino-acid, dipeptide uptake was reduced by 40% compared to the wild type protein. Interestingly, African trypanosomes (unlike other kinetoplastids) lack conventional POTs in their genome, suggesting that TbGPR89 may replace this activity.

To explore the contribution of oligopeptides to stumpy differentiation, parasites were incubated with different concentrations of broths as a source of oligopeptides, this generating reduced cell proliferation and stumpy marker expression. Further, chemically synthesised di-and tripeptide mixtures also provoked differentiation, with tripeptides being more potent in generating stumpy forms, particularly those with particular N-terminal amino acids (namely, Asn, Gln, His, Phe, Asp, and Trp).

These results invoke environmental oligopeptide transport through TbGPR89 as a signalling mechanism responsible for parasite differentiation. This scenario is reminiscent of other systems, where the QS phenomenon is driven by the involvement of signaling molecules comprising short peptides (or autoinducing peptides, AIPs) [26] or Quorum Sensing Peptides (QSPs) in Gram-positive bacteria and Acylated Homoserine Lactone (AHL) in Gram-negative bacteria. Cyclic dipeptides are also used in QS to communicate information about population size and to regulate a behavioural switch from symbiosis with their host to virulence [27]. They are the most common peptide derivatives found in nature and are synthesised by proteobacterial species as well as by humans.

Secreted molecules by trypanosomes

The pathogenic effects of a trypanosome infection have been attributed to bioactive substances produced by the parasites, such as peptidases, released by dead and dying trypanosomes. The first report of such an activity was that trypanosomal peptidases were released into the host circulation, and were able to cleave GnRH (gonadotropin releasing hormone) generating a tetrapeptide, pGlul-His-Trp-Ser [28]. The activity of these enzymes could explain the hormonal imbalance seen in infected hosts. Further, trypanosome-derived Oligopeptidase B has been reported to be responsible for up to 80% of the trypsin-like hydrolytic activity observed in the plasma of rats infected with *T. b. brucei* and this activity was not inactivated by host inhibitors [29]. Highly catalytic parasite derived peptidases accumulating in the host plasma during an infection are in an ideal position to generate biologically active peptides in the bloodstream. Other peptidases released by parasites have been characterised, including pyroglutamyl peptidase type I (PGP) and Prolyl oligopeptidase (POP), which are able to hydrolyse peptide hormones and collagen from the host. Similarly, host derived peptidases produced in response to infection, can provide a further source of active peptides.

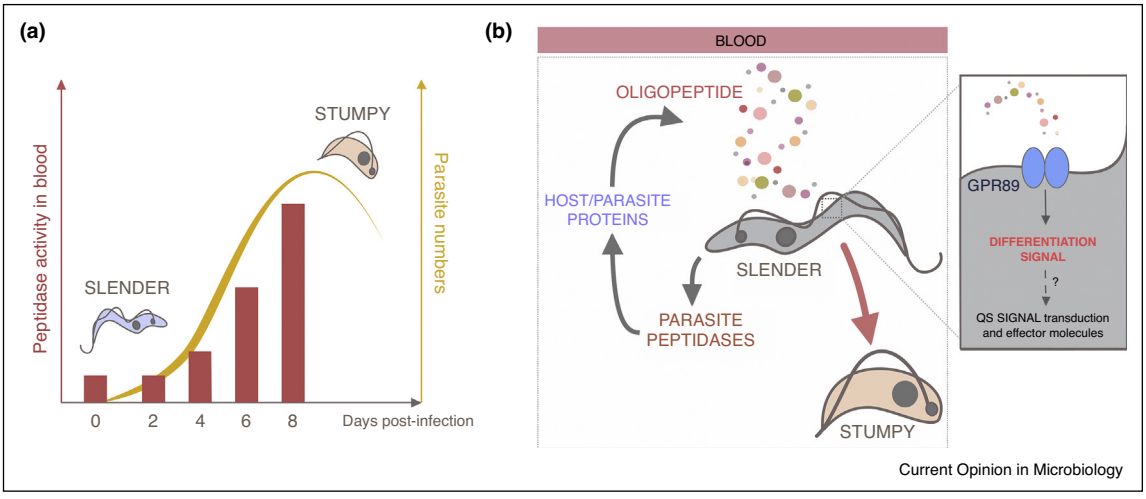
To experimentally determine if released peptidases could generate a signal for stumpy formation, TbPGP and TbPOP have been overexpressed in pleomorphic trypanosome cells under doxycycline regulated induction [19••]. Strikingly, parasites induced to express either oligopeptidase *in vivo* arrested and differentiated to stumpy cells at lower parasitaemia than uninduced cells. This indicated that the released oligopeptidases were generating a signal in the bloodstream by degrading host or trypanosome proteins. Moreover, the released peptidases generated an inter-cellular paracrine signal, this being demonstrated by the accelerated differentiation of parasites coinfected with peptidase secreting parasites *in vivo*. These results generated a model where density sensing operates through the activity of peptidases that are released by parasites during an infection. These degrade host substrates, generating small oligopeptides sensed by the growing population of trypanosomes, this ultimately providing a cue to stimulate differentiation into stumpy forms (Figure 1).

Oligopeptide-based sensing of the environment seems to be present in various organisms, albeit with some variations. For example, it has been shown recently that *Listeria monocytogenes* virulence is regulated through the balance of antagonistic effects of inducing and inhibitory peptides scavenged from the medium, which enter the cell through an oligopeptide transporter, and control the activity of its virulence transcriptional activator [30]. In the pathogenic fungus, *Cryptococcus neoformans*, a secreted oligopeptidase processes a secreted peptide, a central signaling molecule that regulates virulence, and sensing of this molecule requires an oligopeptide transporter [31].

Interspecies signalling

The QS mechanism has been recently extended to between-phyla or inter-kingdom signalling and forms

Figure 1

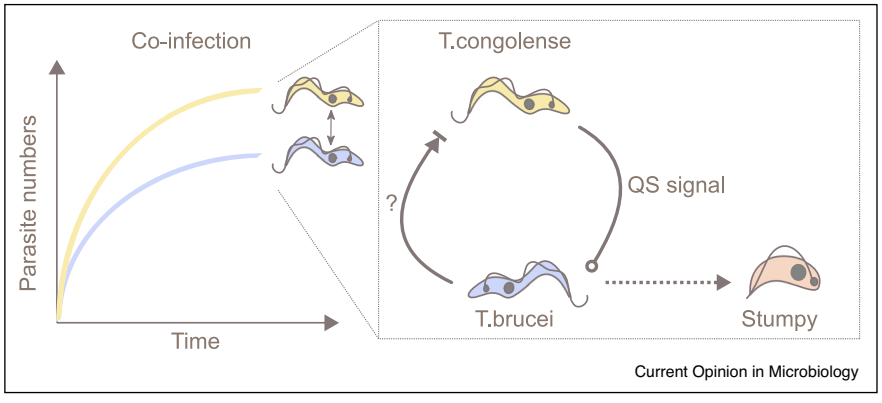


A. Time course for the appearance of peptidase activity in infected blood (red bars) and number of parasites in blood (yellow line). As the activity of trypanosome peptidases increases, the developmental transition of slender to stumpy forms starts to occur.
B. Proposed model for oligopeptide generation and differentiation in *T. brucei*. The released peptidases by slender forms hydrolyses host or trypanosome proteins, generating small peptides. These peptides are then sensed by the parasites following uptake by the oligopeptide transporter GPR89 expressed on their surface, initiating a differentiation signal, driving them to the cell cycle arrested stumpy form. How these oligopeptides initiate the signalling cascade is so far undetermined.

part of the extensive communication between microorganisms and their hosts [27]. For example, bacterial products can modulate mammalian cell-signal transduction, while host hormones can cross-signal with QS signals to modulate bacterial gene expression, suggesting a complex connection between host stress signalling, bacterial QS, and pathogenesis.

Cross species QS also operates in trypanosomes (Figure 2). *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma vivax* can infect game animals and livestock [32] with a co-infection frequency of up to 25% where analysed. Although *T. brucei* is the only trypanosome described to undergo developmental transformation, the potential for the QS signal to be sensed between co-infecting parasites has been recently investigated [33••]. This has shown that *T. congolense* exhibits density-dependent growth control *in vivo* despite its lack of morphological development in the bloodstream. Moreover, analysis of its genome has demonstrated the presence of QS regulatory genes, of which one can

Figure 2



T. brucei co-infection with *T. congolense* induces *T. brucei* to generate stumpy forms at low relative parasitaemia (compared to the *T. brucei* mono-infection) with dependence on the integrity of the *T. brucei* QS signalling pathway showing that this is mediated through QS cross-talk between the species *in vivo*. However, *T. congolense* does not seem to be affected by *T. brucei* co-infection. This could be because the signalling is unidirectional, that the *T. congolense* line used is less sensitive to the QS signal from *T. brucei* or there are insufficient *T. brucei* to stimulate the response.

complement a *T. brucei* QS signal-blind mutant to restore stumpy formation [33••]. Importantly, conditioned medium from a *T. congolense* culture is able to accelerate *T. brucei* stumpy formation and, in a co-infection, the presence of *T. congolense* causes *T. brucei* to differentiate at lower parasitaemia. Both observations highlight the existence of interspecies communication, this being confirmed to be mediated through QS signals by the loss of the response when QS regulatory genes are silenced in *T. brucei*. It is not clear if the communication is bilateral and whether *T. congolense* also responds to the presence of *T. brucei*. However, with the identification of SIF as oligopeptides, analysis of the mechanisms and extent of cross talk is now feasible, for example, by the manipulation of peptidase release by either *T. brucei* or *T. congolense*.

Conclusions and prospects

The recent advances in the identification of a paracrine signal for *T. brucei* differentiation generated by the release of peptidases into the host blood provide a new understanding of the QS system of these parasites. The involvement of oligopeptides is consistent with the reported properties of SIF (<500 Da, heat stable) but was unexpected: SIF had been assumed to be a directly released metabolite or other small molecule, rather than a signal generated outside the cell by the activity of parasite produced peptidases. The source of the protein substrates that provide the peptide signal are likely host derived – either host serum proteins or potentially locally available tissue substrates when parasites are constrained in either the skin or the adipose tissue [34••,35•,36]. Host derived peptidases could also contribute to the production of transmission stages in a density independent manner (*e.g.* host inflammatory responses) as could intracellular parasite peptidases released during immune lysis of successive antigenic variants. In combination, this could result in transmission stages being dynamically generated with different kinetics in different hosts, at different phases of each parasitaemia and in different body niches, explaining the complexity of stumpy formation observed *in vivo*.

Future challenges in the characterisation of the QS system in trypanosomes will involve determining if there are specific oligopeptide sequences that trigger differentiation and, if so, how that specificity is received and acted upon in the parasite in molecular terms. The enigmatic QS signal has been detected, but how that signal is decoded by the parasite to activate its developmental pathway remains to be elucidated.

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