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Developmental Gene Regulation by an Ancient Intercellular Communication System in Social Amoebae

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The social amoebae (Dictyostelia) use quorum sensing-like communication systems to coordinate the periodic transition from uni- to multicellularity. The monophyletic descent of the Dictyostelia provides a unique opportunity to study the origin and adaptive evolution of such intercellular communication systems. We determined that the ability of aggregation-competent cells to respond to the intercellular messenger glorin occurred in the most ancient taxa of the Dictyostelia. We show using Illumina sequencing technology that glorin mediates rapid changes in gene expression at the transition from vegetative growth to aggregation. We conclude that peptide-based communication is the most ancient form of intercellular signaling in the evolution of multicellularity in the social amoebae, but has been repeatedly replaced by other communication systems during the monophyletic evolution of the social amoebae. Glorin communication has parallels with quorum sensing in that the molecule diffuses into the field, stimulates gene expression in receptive cells and coordinates a population-wide response.

Key words: Social amoebae; acrasin; RNA-seq; quorum sensing; *Dictyostelium*.

Introduction

Cell-cell communication is a fundamental cellular process, both in unicellular and multicellular organisms. Bacteria use cell-cell signaling to coordinate adaptive changes in cell behavior such as genetic competence, bioluminescence, virulence, and sporulation, in a population density-dependent manner. This phenomenon is referred to as quorum sensing (Dunny and Winans 1999; Fuqua et al. 2001; Platt and Fuqua 2010). A characteristic property of quorum sensing is that diffusible

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extracellular molecules ('cues') induce changes in gene expression in the receptive cells after reaching a threshold concentration (quorum level). Similar to quorum sensing systems, intensive intercellular communication and chemotactic movement towards gradients of diffusible molecules known as acrasins mediates aggregation of single cells into multicellular aggregates in the social amoebae (Dictyostelia) (Bonner 2009; Kessin 2001; Raper 1984). Because of the former taxonomy that grouped the Dictyostelia with the acrasid amoebae in the division of mycetozoans, the term acrasin was suggested by John Bonner to describe "a type of substance consisting either of one or numerous compounds which is responsible for stimulating and directing aggregation in certain members of the Acrasiales" (Bonner 1947).

It is unknown whether or not acrasin communication systems used by social amoebae have their roots in ancient quorum sensing systems or if they have instead evolved from signaling systems that mediate the chemotactic movement towards metabolites propagated by bacteria, the food source of amoebae. The recently determined molecular phylogeny of the social amoebae (Schaap et al. 2006) provides an exceptional opportunity to examine adaptive processes that educed mechanisms for transient cooperation of single cells in multicellular organisms that ensure survival of a population of cells under unfavorable environmental conditions.

The taxonomy of the social amoebae is traditionally based on morphological characters such as fruiting body architecture and spore appearance. Accordingly, Dictyostelia have been divided into the following three genera: *Dictyostelium*, *Polysphondylium* and *Acytostelium*. This classification is challenged by the molecular phylogeny of the Dictyostelia, which suggests that at least the genera *Dictyostelium* and *Polysphondylium* are polyphyletic (Schaap et al. 2006). Phylogenetic descent indicates four major groups of Dictyostelia, of which groups 1 and 2 contain the most ancient taxa. Relevant to this study, the molecular phylogeny of the social amoebae has recently been refined using internal transcribed spacer (ITS) ribosomal gene sequences, further dividing group 2 into the acytostelid group 2A and groups 2B1 and 2B2 containing all polysphondylids except *P. violaceum* (Romeralo et al. 2010).

The acrasin of *P. violaceum* is the unusual dipeptide N-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam-ethylester, known as glorin (Shimomura et al. 1982; Wurster et al. 1976) (Fig. 1). Phylogenetic analysis has indicated that *P. violaceum* is more related to the most derived group 4 dictyostelids than to the ancient group 2 polysphondylids such as *P. pallidum*. Nevertheless it has been reported that *P. pallidum* cells respond to glorin in chemotaxis assays (Shimomura et al. 1982; Wurster et al. 1976). This has led to the assumption that glorin-based communication may be the most ancient form of intercellular communication that Dictyostelia invented to organize early steps of multicellular development. In this study we add support to this hypothesis by showing that the ability of Dictyostelia to respond to glorin is a common trait of polysphondylids and also of the most ancient group 1 dictyostelids. Using the recently established reference genome of *P. pallidum* isolate PN500 (Heidel et al. unpubl. observ.) and Solexa/Illumina sequencing technology, we show that exposure of starving cells to glorin rapidly leads to changes in gene expression.

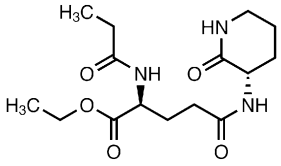

Figure 1. Structure of glorin.

Results

Intercellular Communication with Glorin is an Ancient Characteristic of Dictyostelia

The acrasin glorin was first isolated and characterized by Bonner and coworkers from *P. violaceum* using a simple chemotaxis assay and bioassay-guided fractionation (Shimomura et al. 1982; Wurster et al. 1976). In the chemotaxis assay, drops of aggregation-competent cells were placed on hydrophobic agar containing glorin. If cells were able to sense glorin and to remove the acrasin from their vicinity by means of an extracellular glorinase, they generated a local glorin gradient and, as a consequence, moved to the outside of the drop towards the higher glorin concentration. We adapted this assay to evaluate if other species of the social amoebae use glorin as acrasin (Figs 2, 3). The assay was validated by testing the response of *D. discoideum* to cAMP and *P. violaceum* to glorin (data not shown).

P. pallidum cells have been reported to respond to glorin in chemotaxis assays (Shimomura et al. 1983; Wurster et al. 1976). Chemotactic movement of *P. pallidum* cells to glorin gradients was

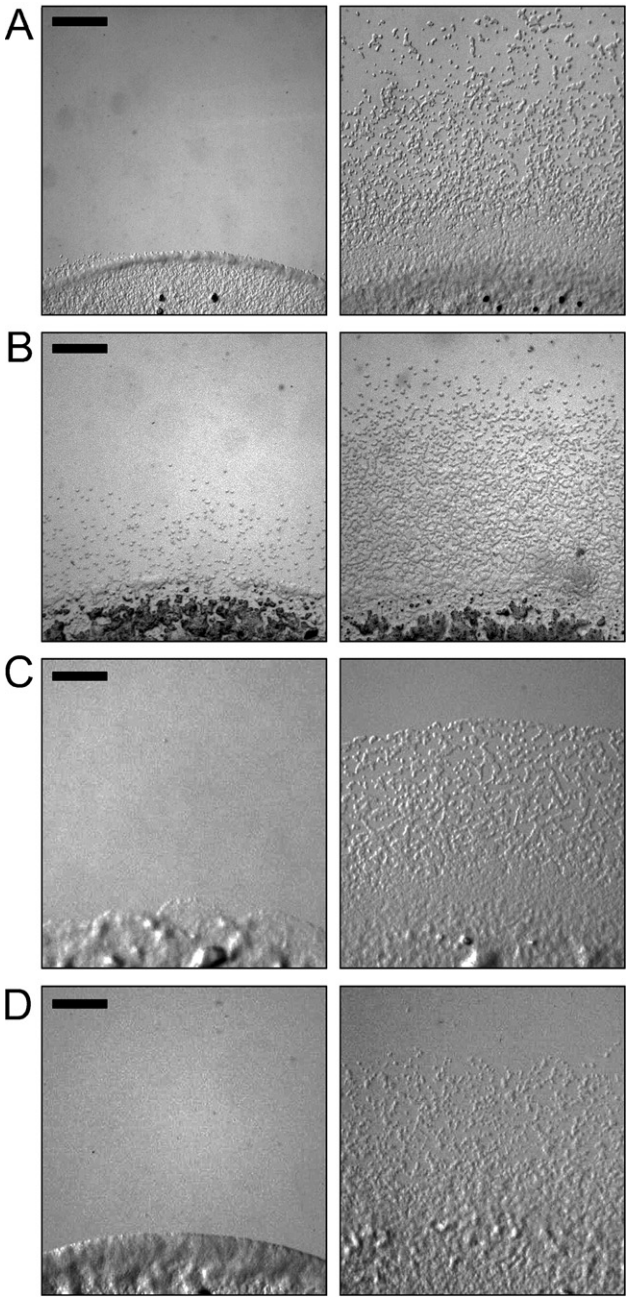

Figure 2. Glorin chemotaxis by group 2 species. Cells were starved for 3 hours in shaking cultures before 10 μl-drops containing $2x10^5$ cells were plated on agar without (left pictures) or with 1 μM glorin (right pictures). Pictures were taken 3 hours after plating. Scale bar indicates 0.5 mm. **A**: *Polysphondylium pallidum* PN500; **B**: *P. tenuissimum*; **C**: *P. asymmetricum*; **D**: *Dictyostelium gloeosporum*.

confirmed in our studies (Fig. 2A). *P. pallidum* cells were active on agar plates containing 5 nM to 20 μM glorin. *P. pallidum* is actually a species complex (Romeralo et al. 2010; Schaap et al. 2006). According to morphological differences between closely related strains, Kawakami and Hagiwara (Kawakami and Hagiwara 2008) suggested renaming of certain *P. pallidum* isolates as *P. album*. We confirmed by ITS DNA sequencing (Romeralo et al. 2010) that *P. pallidum* strain WS320 is closely related to PN500 and both may therefore be referred to as *P. album* isolates (data not shown). On the other hand, *P. pallidum* isolates CK8 and H168 are closely related, meaning that they should be considered as *P. pallidum* sensu strictu. By contrast, the ITS sequences of WS320/PN500 (group 2B1) and CK8/H168 (group 2B2) showed low conservation, except in the 5.8 rRNA gene sequence, demonstrating the relative phylogenetic distance between different isolates of the *P. pallidum* species complex (data not shown). Because it is not yet confirmed by the community that isolates such as PN500 or WS320 will be renamed *P. album*, we will refer to these strains as *P. pallidum*. In our hands all *P. pallidum* isolates responded equally well to glorin.

We tested eight other group 2 species for sensitivity to glorin. The results are summarized in Figure 2B-D and Table 1. We found that the group 2B1 polysphondylids *P. tenuissimum*, *P. pseudocandidum* and *P. asymmetricum* and the group 2B2 species *P. luridum* and *P. tikaliensis* responded to glorin. Qualitatively we did not see notable differences in the responses of these polysphondylids to glorin compared to the activity of *P. pallidum* isolates. Both group 2 dictyostelids, *D. gloeosporum* and *D. oculare*, responded to glorin, while none of the three tested *Acytostelium* species tested positive in this assay.

The group 1 species *D. fasciculatum*, *D. aureostipes*, *D. parvisporum*, *D. microsporum* and *D. bifurcatum* showed pronounced chemotaxis to glorin in our assay (Fig. 3), suggesting that group 1 species do generally use a glorin-based acrasin system.

We conclude that intercellular communication with glorin is a common property of species from the two most ancient clades 1 and 2 of the social amoebae. *D. caveatum*, a member of group 3 Dictyostelia, has been reported to respond to glorin (Waddell 1982); not tested by us). Likewise *P. violaceum*, which is more related to group 4 species than to other polysphondylids from group 2, uses a glorin acrasin system (Shimomura et al. 1982). Thus, we find that glorin communication has been

Table 1. Summary of glorin responses of Dictyostelia.

Species	Dictyostelia group[a]	Response to cAMP	Response to glorin
Dictyostelium discoideum NC4	4	+	–
Dictyostelium sphaerocephalum	4	+	–
Dictyostelium giganteum	4	+	–
Polysphondylium violaceum	(4)	(+)	+
Polysphondylium pallidum WS320	2B1	–	+
Polysphondylium pallidum PN500	2B1	–	+
Polysphondylium tenuissimum	2B1	–	+
Polysphondylium asymmetricum	2B1	–	+
Dictyostelium gloeosporum	2B1	–	+
Polysphondylium pallidum H168	2B2	–	+
Polysphondylium pallidum CK8	2B2	–	+
Polysphondylium luridum	2B2	–	+
Polysphondylium tikaliensis	2B2	–	+
Dictyostelium oculare	2B	–	+
Acytostelium ellipticum	2B	–	–
Acytostelium subglobosum	2A	–	–
Dictyostelium fasciculatum	1	–	+
Dictyostelium aureo-stipes	1	–	+
Dictyostelium parvisporum	1	–	+
Dictyostelium microsporum	1	–	+

[a]Group definition according to Schaap et al. (Schaap et al. 2006) and Romeralo et al. (Romeralo et al. 2010).

lost at least twice during the evolution of the social amoebae (Fig. 4).

Glorin Mediates Gene Expression Changes in Starving *P. pallidum* Cells

We hypothesized that glorin may induce changes in gene expression at the growth-to-development transition, thus rendering the cells competent for aggregation. We suspected that Illumina high-throughput sequencing technology (RNA-seq) may be the most effective means to address this question experimentally, taking advantage of the recently completed reference genome sequence of *P. pallidum* strain PN500. Having no clue about the optimal experimental conditions to evaluate glorin-mediated gene expression, we started by treating *P. pallidum* cells with glorin as suggested by Kopachik for *P. violaceum* cells (Kopachik 1990). The author noted that induction of a few proteins occurred after exposure of starving *P. violaceum* cells to 1 μM glorin applied every 30 minutes for 7 to 8 hours. We noticed that *P. pallidum* PN500 cells start to aggregate in petri dishes under buffer or on nutrient-free agar plates roughly 4 hours after plating. Accordingly, we starved *P. pallidum* PN500 cells in a phosphate buffer in shaking culture for 1 hour without adding glorin. After this initial treatment 1 μM glorin was added to the cells every 30 min for 1 or 2 additional hours (i.e., 2 or 4 pulses of glorin in total). From this approach we expected to gather information on genes that were consistently up- or downregulated for longer periods of time, but not transient gene expression changes. Cells were generally harvested for RNA preparation 30 minutes after the last glorin pulse.

RNA-seq was performed on a set of five cDNA preparations that were derived from growing cells, cells starved for 2 hours, cells starved for 3 hours, and cells pre-starved for 1 hour and then treated with 1 μM glorin for 1 and 2 hours, respectively. This set of cultures was generated twice to enable the comparison of two independent biological replicates. In the RNA-seq experiments each library was sequenced in one lane, resulting in about 35 million reads per sample. About two thirds of the obtained reads could be mapped uniquely to 12,657 gene models annotated from the *P. pallidum* genome and were therefore used for calculation of RPKM values. RPKM values were generated by standardization of data to 1 million reads and 1 kb of transcript length of the individual gene models. Experiment 1 yielded between 20,212,278 and 24,489,837 mappable reads and we obtained 16,943,246 to 20,182,623 mappable reads in experiment 2. We calculated Spearman correlation coefficients for all data sets. When comparing biological replicates 1 and 2 for each experimental condition, all Spearman correlation coefficients were >0.95, indicating a high quality of the data. Spearman correlation coeffi-

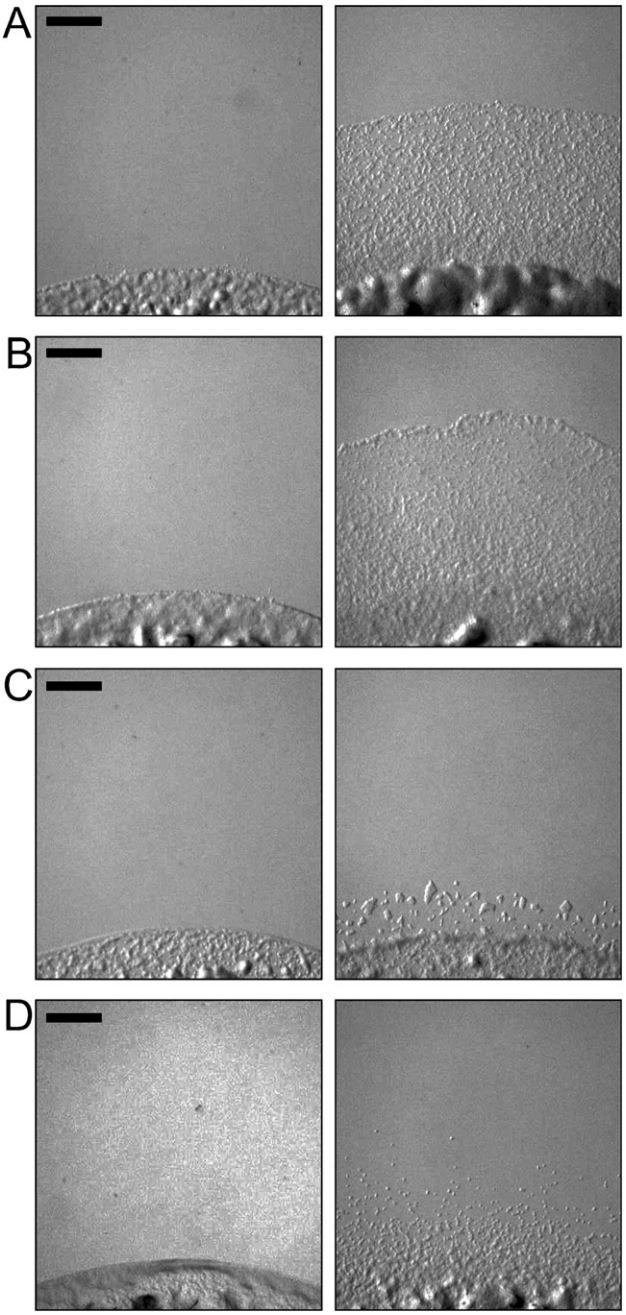

Figure 3. Glorin chemotaxis by group 1 species. Cells were starved for 3 hours in shaking cultures before 10 μl-drops containing $2x10^5$ cells were plated on agar without (left pictures) or with 1 μM glorin (right pictures). Pictures were taken 3 hours after plating. Scale bar indicates 0.5 mm. **A**: *Dictyostelium fasciculatum*; **B**: *D. aureo-stipes*; **C**: *D. parvisporum*; **D**: *D. microsporum*.

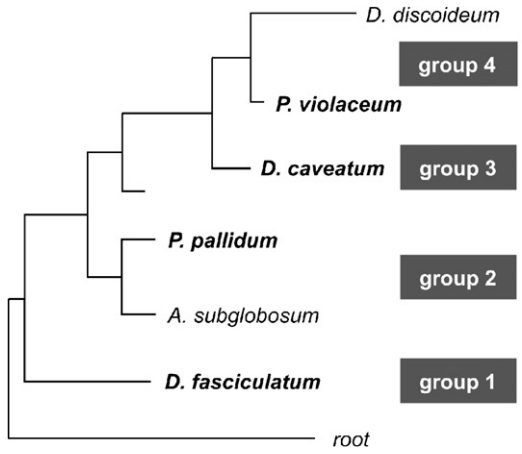

Figure 4. Summary of glorin chemotaxis by social amoebae. Shown is a simplified phylogenetic tree according to (Schaap et al. 2006). The tree is not drawn to scale. The four groups of social amoebae are indicated by a typical example investigated in this study (except for *Dictyostelium caveatum*, which was published elsewhere to respond to glorin (Waddell 1982)). Species that respond to glorin are written in bold.

cients were somewhat lower (ranging from 0.88 to 0.98) when comparing samples from growing and starving cells. This difference was taken as indicative of global changes in gene expression at the growth-to-development transition (see below).

In the first analysis of the RNA-seq data, we determined differential gene expression in cells starved in buffer for 2 or 3 hours with no glorin treatment, compared to growing cells. Genes were considered as being differentially regulated if they displayed at least 3-fold change in gene expression in each of the two biological replicates compared to the control condition. When growing cells were compared with cells starved for 2 hours, 1,299 genes were differentially regulated, 911 of which were upregulated and 388 downregulated. After 3 hours of starvation, 680 were upregulated and 376 were downregulated. Comparison of both lists of differentially regulated genes revealed that 553 of the upregulated genes and 283 of the downregulated genes matched, indicating that roughly 75% of the genes that were differentially regulated after 2 hours of starvation were similarly expressed one hour later. This set of genes obviously responded to the starvation conditions, i.e. represented the growth arrest of the cells.

We next compared gene expression in cells starved for 2 hours without glorin treatment with cells that were pre-starved for 1 hour and then treated with glorin (1 μM) for an additional hour in

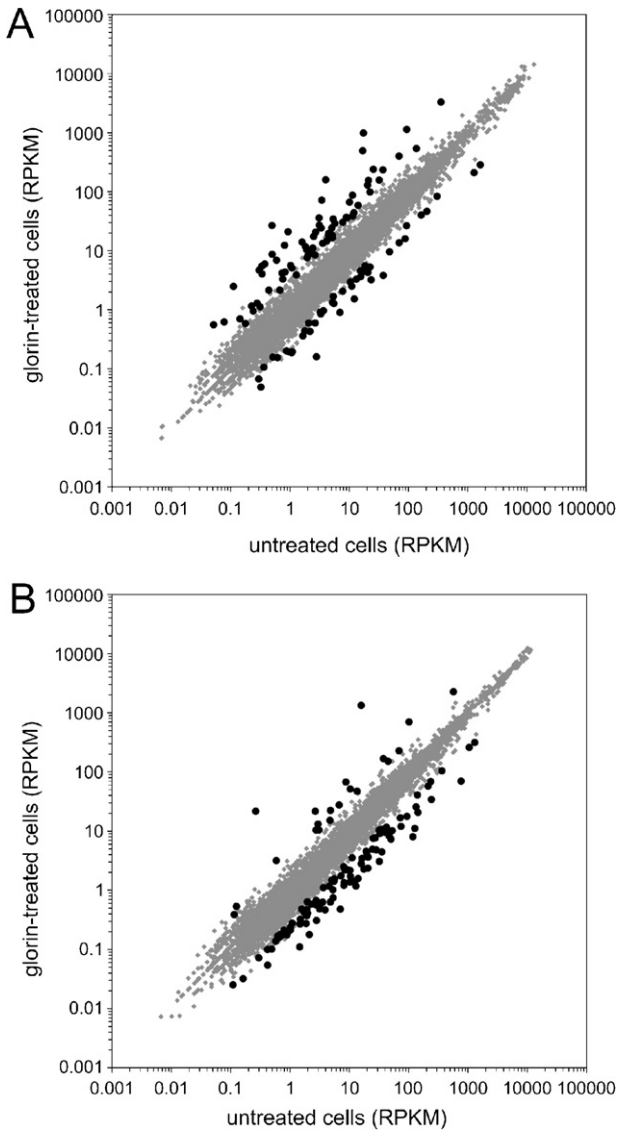

Figure 5. Effects of glorin treatment on global gene expression in *Polysphondylium pallidum*. RNA-seq experiments were performed comparing *P. pallidum* PN500 cells treated with glorin for 1 hour (**A**) or 2 hours (**B**) with untreated cells starved for the time period. All 12,627 gene models were plotted (grey symbols). Black dots indicate genes that were differentially regulated at least 3-fold.

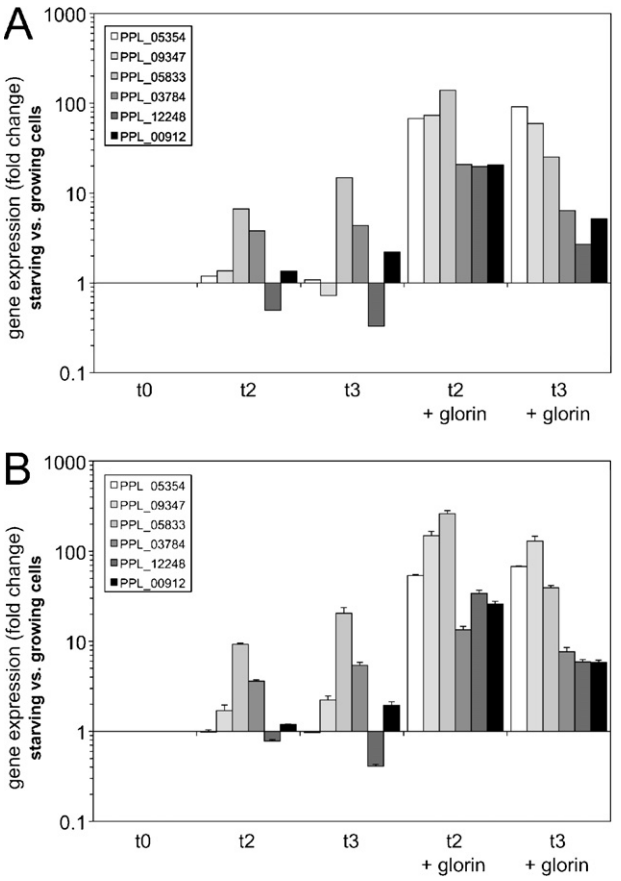

Figure 6. Model genes to characterize glorin-dependent gene induction. **A**. Representative genes reported to be upregulated by glorin in the first RNA-seq of the two biological replicates. All data were compared to growing *Polysphondylium pallidum* PN500 cells (t0, fold change set to 1). Cells were starved without glorin for 2 or 3 hours (t2, t3) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (t2 + glorin, t3 + glorin), respectively. The results from the second RNA-seq experiment were comparable to the result shown here. **B**. Real-time RT-PCR of the RNA preparation used in the first RNA-seq experiment and the same model genes shown in panel A. Shown is the mean value of triplicate measurements of the same cDNAs ± SD.

30-minute intervals, meaning that we applied only two pulses of glorin. Thus, we would be able to dissect the effects of starvation from those of the glorin treatment. As shown in Figure 5A, glorin treatment resulted in a more than 3-fold differential expression of 115 genes, 70 of which were upregulated by 3- to 57-fold. A somewhat different result was obtained when cells starved for 3 hours were compared with cells pre-starved for 1 hour and pulsed with glorin (1 μM every 30 minutes) for 2 hours (Fig. 5B). In this experiment 120 genes were more than 3-fold differentially regulated, but only 20 of them were induced. Of these, 11 genes were also induced more than 3-fold after 1 hour of glorin treatment. This unexpected difference between the two glorin pulsing experiments can be explained by comparing the absolute expression levels and kinetics of these genes under starving conditions (Fig. 6A).

Few genes, of which PPL_05354 and PPL_09347 are prominent examples, retained almost constant expression levels in the first hours after growth-to-starvation transition. Instead, the majority of genes (43 of 70) showed a moderate increase of expression under starvation, yet their expression was further increased by glorin (examples in Fig. 6A). While the expression levels of these genes increased over time under starvation, the effect of glorin treatment diminished. As a consequence, many glorin-induced genes were more than 3-fold induced after 1 hour of glorin treatment, but less than 3-fold one hour later. Thus, externally applied glorin enhanced the expression and possibly shifted expression induction towards earlier time points in the developmental cycle than under natural conditions.

Another fraction of glorin-induced genes was actually downregulated to various extents by starvation alone, and glorin treatment increased the expression of these genes significantly above transcription levels seen in growing cells (see Fig. 6A). These genes may represent background noise of glorin-mediated gene expression or normally play a role in the later phases of the developmental cycle.

The expression profile of six glorin-induced genes was analyzed by quantitative RT-PCR with results confirming the RNA-seq data (Fig. 6B). In many experiments we observed that the extent of gene induction under starvation depended on the conditions of cell preparation. Although we bestowed great care on harvesting the cells before complete consumption of food bacteria, batches of cells showed different degrees of starvation-induced gene expression, often resulting in more or less response of the investigated model genes to starvation (e.g., compare PPL_05833 in Fig. 6B and Fig. 7A). However, in each experiment glorin strongly induced these genes above expression levels seen in growing cells within the first hours of starvation.

In conclusion, glorin effects on gene expression occurred rather early in development. The results supported our initial hypothesis that a primary function of glorin-mediated gene expression may be to bring about aggregation competence of the cells.

To gain insight into possible functions of glorin-induced genes we searched putative molecular functions based on homologies to annotated *D. discoideum* genes. A list of gene ontology (GO) terms assigned to glorin-regulated genes is presented in Table 2 for the top 25 glorin-induced genes. Interestingly, several glorin-induced genes may function in signal transduction pathways. For example, the list contains three G protein-coupled

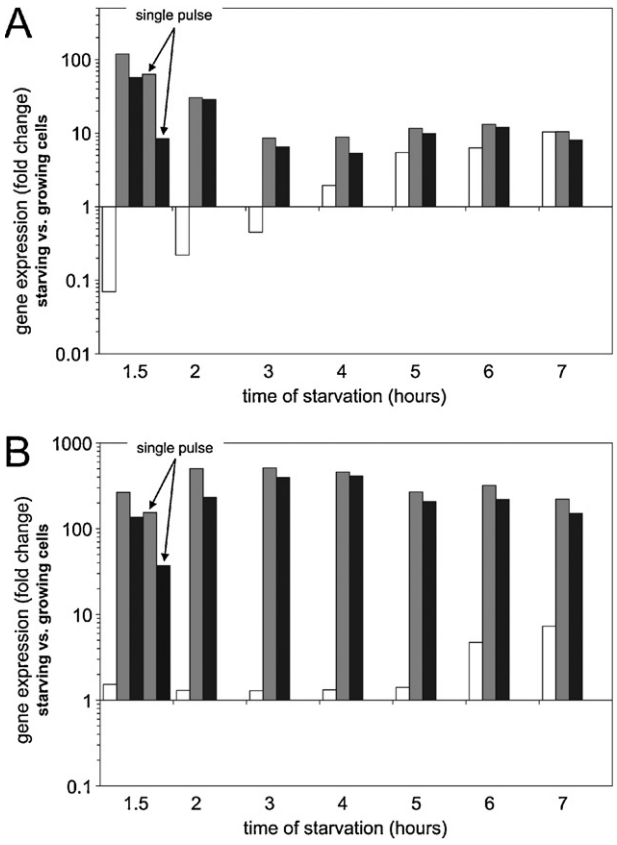

Figure 7. Time course of glorin effects on gene expression. Quantitative RT-PCR analysis of model genes PPL_05833 (**A**) and PPL_09347 (**B**). *Polysphondylium pallidum* PN500 cells were starved in buffer without addition of glorin (white bars) or with pulsing at 10-minute intervals for the indicated time periods with 1 μM glorin (grey bars) or 100 nM glorin (black bars). Pulsing was started after 1 hour of pre-starvation. Gene expression after application of a single pulse of 1 μM glorin (grey bar) or 100 nM glorin (black bar) is plotted separately and indicated by arrows.

receptors. Moreover glorin-induced genes include several putative serine/threonine kinases, among which are three alpha kinases and an ERK2 ortholog. Some of these proteins also contain von Willebrand factor motifs that may facilitate protein-protein interactions. Furthermore, glorin induced a Ras-GTPase-containing protein, a putative carbohydrate-binding, PA14 domain-containing protein with similarity to extracellular matrix proteins such as EcmB and a profilin I-like protein that may be required to modulate the F-actin distribution in the cell during chemotaxis.

When cells were treated for 1 hour with glorin, 45 genes were downregulated between 3- and 17-fold (Fig. 5A). After 2 hours of glorin treatment,

Table 2. Summary of top 25 glorin-induced genes in *Polysphondylium pallidum*.

Gene	Expression (fold change)[a]	Gene description
PPL_05354	56.9	putative extracellular matrix protein, PA14 domain-containing protein
PPL_09347	53.9	profilin I
PPL_12248	39.7	protein serine/threonine kinase activity (alpha kinase superfamily)
PPL_12249	29.2	protein serine/threonine kinase activity (alpha kinase superfamily)
PPL_11763	22.7	expansin-like protein
PPL_03541	22.3	Unknown
PPL_05833	20.9	Unknown
PPL_06644	17.4	Unknown
PPL_07811	16.4	Unknown
PPL_08454	15.9	G-protein coupled receptor activity (family 3)
PPL_07812	15.5	protein binding
PPL_00912	15.2	Unknown
PPL_00117	12.1	Unknown
PPL_08455	12.0	G-protein coupled receptor activity (family 3)
PPL_00861	11.6	TKL-like protein kinase, tyrosine kinase-like protein
PPL_10324	11.6	Unknown
PPL_04784	10.8	IPT/TIG domain-containing protein; immunoglobulin E-set domain-containing protein
PPL_02621	9.4	Unknown
PPL_02620	9.3	Unknown
PPL_12251	8.8	protein serine/threonine kinase activity (alpha kinase superfamily); type A von Willebrand factor (VWFA) domain-containing protein
PPL_03564	8.7	G-protein coupled receptor activity (family 3)
PPL_07801	8.0	Unknown
PPL_12271	7.7	ERK subfamily protein kinase
PPL_07296	7.4	Ras GTPase domain-containing protein; type A von Willebrand factor (VWFA) domain-containing protein
PPL_07818	7.2	Unknown

[a]Fold change calculated for gene expression in cells prestarved for 1 hour and then treated with glorin for another hour, compared to cells starved for 2 hrs without glorin treatment.

100 genes were downregulated (Fig. 5B). Only 18 genes were identified as glorin-dependently regulated under both conditions. A possible explanation for this difference may be the rapid kinetics of glorin effects. We found that 80% of the genes downregulated after two hours of exposure to glorin were already downregulated one hour earlier, but they did not reach the 3-fold change threshold. Annotation of GO terms to glorin-repressed genes was not informative as most of the genes have not yet been assigned to any particular functions. We speculate that repression of certain genes in the presence of glorin may have two effects: (i) the enhancement of the starvation response for a better induction of the developmental cycle and (ii) the repression of early developmental genes required for proceeding to post-aggregation.

Using the collection of all genes that were differentially expressed after either 1 or 2 hours of glorin exposure, we surveyed the genomes of *D. discoideum* and *D. fasciculatum* for orthologs of these genes. Out of 234 genes that could be subjected to this analysis, we found that 104 *P. pallidum* genes did not have any detectable ortholog in either of the two other species (orthologs are defined by at least 20% amino acid identity). On the other hand, 82 glorin-regulated *P. pallidum* genes had orthologs in both *D. discoideum* and *D. fasciculatum*, and 14 and 34 *P. pallidum* genes had orthologs only in *D. discoideum* and *D. fasciculatum*, respectively. When the analysis was performed on the set of 70 genes that were induced after 1 hour of glorin treatment, 44 *P. pallidum* genes had orthologs in *D. discoideum* and 48 in *D. fasciculatum*, respectively.

As mentioned before, the experiments aimed to investigate glorin-regulated gene expression were started without exact knowledge of the optimal glorin concentration and pulsing frequency required

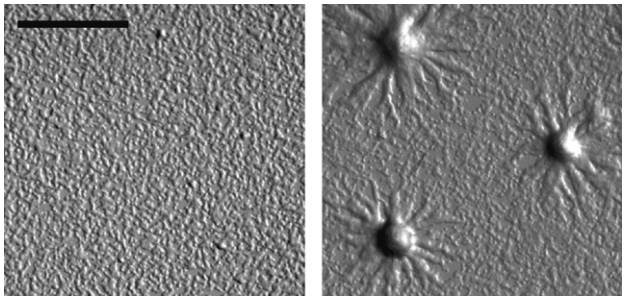

Figure 8. Glorin accelerates cell aggregation. *Polysphondylium pallidum* PN500 cells first pre-starved for 1 hour and then treated with 1 μM glorin every 10 min for 2 hours. Cells were then plated on non-nutrient agar plates and photographed when glorin-treated cells reached aggregation stage (right picture). Untreated cells were photographed at the same time, shown in the left picture. Scale bar indicates 0.5 mm.

to observe optimal response in *P. pallidum* cells. Having acquired a list of glorin-regulated genes, we could define model genes to study glorin effects on gene expression in more detail by using quantitative RT-PCR. When we treated *P. pallidum* cells with 10 nM, 100 nM or 1 μM glorin every 30 minutes for 1 hour, we found that exposure of cells to 10 nM glorin was sufficient to induce model genes, even though induction was significantly stronger at 100 nM and most pronounced at 1 μM glorin (data not shown). Interestingly, we found that even a single pulse of glorin induced model genes like PPL_05833 and PPL_09347 to almost the same level as observed after repeated pulsing (Fig. 7). Although investigating if more frequent pulsing with 1 μM glorin, for example every 10 minutes instead of 30 minutes, would be more effective, we found no pronounced differences (data not shown). When pulsing cells with 100 nM or 1 μM of glorin every 10 minutes, we found that model genes such as PPL_05833 (Fig. 7A) or PPL_09347 (Fig. 7B) were equally well induced at both glorin concentrations tested, and expression of the glorin-responsive genes stayed high for 8 hours of pulsing in the shaking cultures. In these RT-PCR experiments we confirmed observations made in the RNA-seq analyses indicating that glorin effects decreased with prolonged starvation because the investigated genes were moderately induced by starvation alone. Thus, glorin affects were most pronounced in the first 1-3 hours after starvation.

Exposure of *P. pallidum* cells to glorin conditioned starving cells for aggregation. In the experiment depicted in Figure 8, cells were pre-starved for 1 hour followed by pulsing with 1 μM glorin every 10 minutes for 2 hours. Then cells were plated on non-nutrient agar plates. The glorin-treated cells collected more efficiently into aggregates, indicating that pre-treatment with glorin shifts aggregation competence to earlier time points.

Discussion

Glorin Communication is an Ancient Characteristic of Social Amoebae

Aggregating *P. violaceum* amoebae secrete glorin and move chemotactically toward glorin gradients. The cells are able to sense glorin by specific cell surface receptors and degrade the signal by means of a membrane-bound and soluble extracellular glorinase (De Wit et al. 1988; Wurster et al. 1976). Thus, glorin meets the criteria of being the authentic acrasin in this species. However, it has not been formally proven that glorin secretion and sensing is indeed essential for aggregation of *P. violaceum* cells because mutants defective in glorin signaling do not exist. As a first step toward the analysis of the glorin communication system at the molecular level, we have shown using a chemotaxis assay that cells of the genetically tractable species *P. pallidum* are able to remove glorin from their neighborhood and move toward a self-generated chemotactic gradient produced by their glorinase activity. Thus, it is very likely that *P. pallidum* cells use the same communication system to mediate aggregation as *P. violaceum* cells despite the surprisingly large phylogenetic distance between the two species. Although it has to be shown that *P. pallidum* cells actively secrete glorin, the availability of transformation techniques and of a complete genome sequence now establishes *P. pallidum* as a new model to study glorin communication in detail.

We have shown data in support of the hypothesis that the glorin acrasin system is the oldest form of intercellular communication used at the transition from growth to multicellular development of social amoebae. Not only have we seen good response to glorin from six different Polysphondylia, but we have also shown for the first time that group 1 dictyostelids respond to glorin in a very similar manner as the polysphondylids. The molecular phylogeny presented by Schaap et al. (Schaap et al. 2006) predicts that group 1 organisms preceded group 2 taxa, although an alternative root placing groups 1/2 and 3/4 in paraphyletic clades cannot be entirely excluded. The latter seems to be supported by new phylogenetic analyses based on the complete nuclear genomes of *D. discoideum*, *P. pal-*

lidum, and *D. fasciculatum* (Heidel et al. unpubl. observ.) and the mitochondrial genomes of these organisms (Heidel and Glöckner 2008). Whatever the origin of the social amoebae is, our data support the assumption that the unknown common ancestor of all social amoebae used glorin to achieve multicellularity and that glorin-based intercellular communication was lost in group 2 acytostelia, some group 3 species and all group 4 organisms. In these taxa glorin-based communication was replaced by signaling systems based on cellular metabolites such as folic acid derivatives and cAMP.

Glorin Mediates Adaptation of Gene Expression in Early Development of *P. pallidum*

We have seen pronounced gene induction and repression by glorin in *P. pallidum* cells during the first few hours of starvation. The characteristics of gene regulation by glorin implies that all components of an intracellular glorin signaling cascade are latently present in growing *P. pallidum* cells because we observed that even freshly washed cells, without any pre-starvation period, significantly responded to glorin pulses (data not shown). However, responses in chemotaxis assays and gene induction were stronger after prolonged pre-starvation of cells in buffer.

Applying external glorin pulses to starving cells is an artificial experimental setting. Using this approach we certainly disturbed the normal developmental cycle and the accompanying gene expression changes. In this sense physiologically normal concentrations and timings do not exist if applied to wild type cells. By applying glorin we could enhance the response of signaling-competent cells or shift this response to earlier time points. Thus, what we observed was a mixture of the above mentioned effects. In future experiments, if the glorin synthesis pathway is identified, mutants in these components could be used to study glorin effects in more detail.

Although the glorin signaling system has many parallels with the cAMP acrasin system of *D. discoideum*, there are also pronounced differences. The cAMP signaling system is expressed at very low levels in growing *D. discoideum* cells, but the genes involved in cAMP signal transduction are strongly induced by cAMP itself when starving cells are periodically exposed to nanomolar cAMP concentrations in 6-minute intervals (Aubry and Firtel 1999; Loomis 2008; McMains et al. 2008; Saran et al. 2002). *D. discoideum* cells secrete cAMP to the extracellular space in response to cAMP (known as signal relay) and cells move rhythmically and in streams into aggregation centers. Studies of *P. violaceum* cells have suggested that the cells express considerable amounts of glorin receptor during the growth phase with only a slight increase of receptor density during early aggregation (De Wit et al. 1988). The same authors noted that there is no glorin-induced glorin secretion in *P. violaceum*. Thus, it remains unknown whether or not aggregation of *P. violaceum* or *P. pallidum* cells is accompanied by any kind of signal relay. Although glorin may induce the release of a signal molecule other than glorin itself, one may also speculate that signal relay may be only required to ensure the formation of the large fruiting bodies typical for group 4 species, while the formation of smaller fruiting bodies may be accomplished without signal relay because it requires aggregation of less cells and therefore less extensive signal propagation into the field.

How did the Glorin-based Acrasin System Emerge?

The most ancient form of intercellular communication is quorum sensing used by bacteria. Quorum sensing is defined as a phenomenon by which cells regulate their gene expression in response to the concentration of diffusible molecules ('cues') produced by themselves or other bacteria of the same or different species (Platt and Fuqua 2010). Effective quorum sensing requires a secreted, diffusible molecule that is sensed by cells by means of (intra)cellular targets (receptors) that mediate differential gene expression in response to a threshold level of that cue molecule. The glorin-based acrasin system used by ancient social amoebae reflects many of the properties of a quorum sensing system. The diffusible acrasin is secreted to the extracellular space, where it binds to specific receptors; this provokes changes in gene expression in the receptive cells. Considering the possibility that the glorin-based acrasin system that mediates multicellular development of social amoebae is an adaptation of a quorum sensing process, two alternative hypotheses can be proposed. Firstly, glorin may be secreted by certain bacteria, whereas amoebae have invented the sensing of glorin to track down the glorin-producing bacteria as a food source. Amoebae may then have converted this food-sensing system into an acrasin system to facilitate their own aggregation. Secondly, glorin may have been (or may still be) a quorum-sensing cue of the amoebae themselves. In this case compo-

nents of the quorum-sensing system may have been adapted to create an acrasin system for the purpose of coordinating the first steps of multicellular development.

In favor of the first hypothesis, one could argue that during the evolution of the social amoebae, pre-existing food-seeking systems capable of detecting bacterial metabolites such as folic acid or cAMP have probably been converted into acrasin systems. Sensing of such primary metabolites requires the invention of only few components to be converted into an acrasin system. Although this hypothesis cannot be completely refuted, it is challenged by the consideration that, although biosynthetic pathways to generate folic acid and cAMP are common to both bacteria and social amoebae, glorin is a presumed product of secondary metabolism. It seems unlikely that social amoebae have adapted a glorin-based food-seeking system into an acrasin system because the synthesis of glorin by the amoebae themselves would have required the invention of an entirely new biosynthetic pathway.

Hence, glorin-based communication could be presumed to act as a quorum-sensing cue in modern Dictyostelia. This would mean a switch from a pure quorum sensing to an acrasin function, i.e., transition from unicellularity to multicellularity, using the same intercellular communication system. The obvious argument against this assumption would be the fact that the cells encode a glorinase that would prevent the quorum cue from accumulating to a threshold level required to provoke action. However, it is not yet clear if glorinase is expressed in growing amoebae or only after starvation. The answer to this question will await the cloning of the glorinase-encoding gene. It will be interesting to determine if some species of the social amoebae that have abandoned the use of glorin as an acrasin system still use it as a quorum-sensing cue. This observation may have been missed in previous studies because it does not require the chemotactic movement of cells in glorin gradients.

In conclusion, current data support the hypothesis that a peptide-based communication system is the most ancient form of intercellular signaling in the evolution of multicellularity in the social amoebae. Glorin mediates global rearrangements of gene expression in cells that proceed from unicellular growth to multicellular development. Our study provides the basis to further investigate the adaptive evolution of intercellular communication systems in a monophyletic group of species that freely transitions from unicellularity to multicellularity.

Methods

Cell culture: Dictyostelia were obtained from the Dictyostelium Stock Center (http://dictybase.org/StockCenter/StockCenter.html). Cells were cultured in association with *Klebsiella planticola* on 1/5 SM plates (Raper 1984). Cells were harvested before the first signs of aggregation were observed on the plates, washed four times in 17 mM phosphate buffer (pH 6.2) to remove bacteria and cell pellets were stored at -80 °C for preparation of genomic DNA or total RNA.

The following species were used in this study, and DBS strain numbers from the *Dictyostelium* Stock Center (http://dictybase.org/StockCenter/StockCenter.html) are given in parentheses: *D. discoideum* NC4 (DBS0304666), *D. giganteum* WS589 (DBS0235820), *D. sphaerocephalum* GR11 (DBS0235889), *D. oculare* DB4B holotype (DBS0235852), *D. gloeosporum* TC-52 (DBS0235825), *D. fasciculatum* SH3 (DBS0235810), *D. bifurcatum* UK5 8(DBS0235731), *D. aureostipes* JKS5150 (DBS0235725), *D. parvisporum* OS126 (DBS0235853), *D. deminutivum* M19A (DBS0235744), *D. microsporum* Hagiwara 143 (DBS0235841), *P. pallidum* PN500 (DBS0236807), *P. pallidum* PN500 *tasA*$^{-}$/*tasB*$^{-}$ (DBS0306505) (Kawabe et al. 2009), *P. pallidum* CK8 (DBS0236805), *P. tenuissimum* TNS-C-97 (DBS0266526), *P. tikaliensis* OH595 holotype (DBS0238791), *P. luridum* LR-2 (DBS0236804), *P. pseudo-candidum* Hagi-66-TNS-C-91 (DBS0235858), *P. asymmetricum* OH567 holotype (DBS0235724), *A. ellipticum* AE2 holotype (DBS0235447), *A. leptosonum* FG12A (DBS0235449) and *A. subglobosum* LB1 (DBS0235452). Strains *P. violaceum* (ATCC34156) and *P. pallidum* WS320 (ATCC44843) were obtained from the American Type Culture Collection (ATCC). *P. pallidum* H168 was a gift from M. Romeralo (University of Uppsala).

Chemotaxis assay for glorin and cAMP: Glorin was synthesized at the Institute of Pharmacy of the University of Jena using the protocol of Ball et al. (Ball et al. 1989) minor modifications. Alternatively, glorin was purchased from Phoenix Pharmaceuticals (Burlingame CA, USA). We did not observe notable differences in the biological activities of the two glorin preparations. Glorin was dissolved in 17 mM phosphate buffer (pH 6.2) and stored as a 3 mM stock solution at −20 °C.

Chemotaxis assays were performed essentially as described by Shimomura et al. (Shimomura et al. 1982). Cells were washed free of bacteria, adjusted to $2x10^6$ cells/ml in 17 mM phosphate buffer (pH 6.0) and shaken for 1-5 hours at 150 rpm at 22 °C to induce aggregation competence. Cells were concentrated to $2x10^7$ cells/ml and 10 μl drops of cell suspension were placed on 1% hydrophobic agar prepared in 17 mM phosphate buffer (pH 6.2). If the agar did not contain acrasin, cells stayed inside the drops over the complete period of observation. Instead, if acrasin was dispersed in the agar, extracellular acrasinase degraded the acrasin, thereby generating local acrasin gradients. Chemotaxis toward the acrasin caused the cells to move out of the drop over a certain distance. Pictures were generally taken 3 hours after plating of the cells.

RNA-seq and glorin pulsing experiments: *P. pallidum* PN500 cells were washed free of bacteria and adjusted to $2x10^6$ cells/ml in 17 mM phosphate buffer (pH 6.2). Growing cells were pelleted and stored frozen immediately after washing. They served as a reference for differential gene expression in developing cells. Cells were shaken at 150 rpm at 22 °C. After 1 hour of starvation, cells were pulsed with 1 μM glorin every 30 min for 1 or 2 hours as suggested by Kopachik (Kopachik 1990). Parallel cultures were left untreated. Total RNA was pre-

pared from frozen cell pellets as described previously (Lucas et al. 2009).

Transcriptome-wide expression levels were determined using Illumina/Solexa next-generation sequencing (Bentley et al. 2008). For library preparation an amount of 5 μg total RNA per sample was processed using Illumina's mRNA-Seq sample prep kit following the manufacturer's instructions. The libraries were sequenced using a Genome Analyzer (GAIIx) in a single read approach with 76 cycles resulting in reads with a length of 76 nucleotides. Reads were mapped to the *P. pallidum* reference transcriptome downloaded from the Social Amoeba Comparative Genome Browser (SACGB; http://sacgb.fli-leibniz.de/cgi/index.pl) using the Illumina-supplied tool ELAND (Bentley et al. 2008) with standard parameters. Expression values were calculated by counting the number of unique mappable reads per transcript and normalizing these to the total number of mappable reads and length of the respective transcript (adapted from Mortazavi et al. 2008). This resulted in RPKM values (Reads Per Kilobase transcript and Million mappable reads) representing the expression level of the respective transcript. RNA-seq was performed using RNA preparations from two independent experiments. Average RPKM values derived from both experiements were used to calculate differences in gene expression. However, genes were considered as being glorin-dependently expressed only if differences in RPKM values in glorin-treated versus untreated cells were greater than 3-fold in each of the two individual RNA-seq experiments. The RNA-seq data have been deposited in the Gene Expression Omnibus data base under accession number GSE24911.

Quantitative RT-PCR: Quantitative RT-PCR was performed as described previously (Lucas et al. 2009). Gene expression levels were standardized to the gene encoding glyceraldehyde-3-phosphate dehydrogenase (*gpdA*, SACGB accession number PPL_12017). Values are given as means of triplicate measurements. The following primers were used: PPL_00912: 5'-GATACATCGATCCAAGAGATCCAGCTC-3' and 5'-CAACAACATACTATCAGAATTATTCGG-3'; PPL_03784: 5'-CCACTACACAATACCATTGAACCTAAC-3' and 5'-GAAATTTCAACCATCTTATTGTGTTGG-3'; PPL_05354: 5'-GTCAACACCACCACCGGACAATGTG-3' and 5'-GTTTGGACATTACATTGGTCGTAGGTG-3'; PPL_05833: 5'-CAAACCTCCACAAGAAGGCAGCTCGTC-3' and 5'-CTGTCCAAGTTGGTCTCTCTGAAAGCG-3'; PPL_09347: 5'-GGTGTATGGGCATGTAGTCCAATAAAC-3' and 5'-CAGTCTCCACCTTGTCTACCATAGATAC-3'; PPL_12248: 5'-GGCACCACAACCATTTTCTCAAGGAG-3' and 5'-CCTCTGAGGTGATTGAAATCAAATGC3'; PPL_12017,5'- GTTGTTCTCATCACTACTGTTTTCGGAGG-3' and 5'-GGTGGATAAATTGATATTGAAGTCATAG-3'.

ITS sequencing: Sequencing of internal transcribed spacer (ITS) rDNA sequences was performed as described by Romeralo et al. (Romeralo et al. 2010) with the *P. pallidum* specific primers listed in (Romeralo et al. 2007). The following ITS sequences have been deposited in GenBank: *P. pallidum* PN500 (HQ213802), *P. pallidum* WS320 (HQ213803), *P. pallidum* H168 (HQ213801) and *P. pallidum* CK8 (HQ213800).

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