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A GPCR involved in post aggregation events in *Dictyostelium discoideum*

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Abstract

Dictyostelium has 55 genes encoding seven-transmembrane G-protein-coupled receptors (GPCR) that belong to five of the six GPCR families. GrlA is one of the 17 family 3 GPCRs in *Dictyostelium* all of which resemble $GABA_B$ receptors from higher eukaryotes. GrlA is a 90-kDa protein present on the plasma membrane and on membranes of the ER. It has a large extracellular domain with homology to bacterial periplasmic proteins. The GrlA message is present throughout development and shows increased levels during the post aggregation stages. Inactivation of the grlA gene does not severely affect the growth phase, however, it leads to a delay in the development at the post aggregation stage. GrlA deficient strains show an altered DIF-1 response specific to the prestalk-specific *ecmA* and *ecmB* gene, reduced *car2* and *pkaC* transcript levels and form a reduced number of spores. Germination of the spores was as in wild type. Transcriptional profiling supported the defect in the sporulation pathway as a large number of genes involved in the biogenesis and organization of the extracellular matrix and the sporulation process were significantly downregulated in the mutant.
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Keywords: GPCR; GrlA; Live dynamics of a GPCR; *Dictyostelium*; Spore formation; Microarray

Introduction

The existence of multicellular organisms greatly depends on the ability of individual cells to communicate with each other and the environment. Environmental cues are detected and transduced by a large and diverse repertoire of membrane spanning cell surface G-protein-coupled receptors (GPCRs) (Bockaert and Pin, 1999). GPCRs are characterized structurally by an amino-terminal extracellular domain, a heptahelical transmembrane domain (HTD or 7TMD) and a carboxy-terminal intracellular domain. Agonist binding at the N-terminal GPCR pocket leads to a conformational change in the 7TMD, which allows association with G-proteins and initiation of signalling cascade(s) within the cell eliciting a physiological response (Prinster et al., 2005). Since GPCRs are involved in a wide range of biological processes, they have relevance in many pathological conditions related to the immune system and neurological disorders, to inflammation, HIV infection, cardiovascular dysfunction, obesity, diabetes and cancer. GPCRs represent the target of more than 40% of today's best-selling drugs although only about 4% of the known GPCRs are targeted. As a large majority of human-derived GPCRs still remain 'orphans' with no identified natural ligands or functions, one key aspect in GPCR research is to identify new ligands for such orphan GPCRs (Kamps and Coffman, 2005; Tyndall and Sandilya, 2005; Moro et al., 2006). This widens the scope of various model organisms as experimental systems to understand and explore the diversity of signalling exhibited by GPCRs.

Dictyostelium discoideum is a unicellular soil-living amoeba feeding on yeast and bacteria. When deprived of nutrients, the cells undergo a developmental life cycle, aggregate towards cAMP and form mounds, wherein cells differentiate into prespore and prestalk cells (Manahan et al., 2004; Chisholm and Firtel, 2004). The mounds then form slugs. In the slug the prestalk cells are primarily present at the front and form the tip which acts as an organizer of the slug, whereas the prespore

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cells are located in the rear four-fifths. The slugs migrate towards light and culminate into mature fruiting bodies containing a mass of spores atop a stalk. The spores remain dormant until they come across favourable conditions, and unicellular amoebae emerge. The process of *Dictyostelium* development can be studied under laboratory conditions. It takes about 24 h and is thus a relatively simple and valuable tool to study various aspects of multicellularity involving adhesion, sorting, differentiation and patterning and signalling involved therein. The major signal that governs the *Dictyostelium* life cycle is cAMP. Further signals are, among others, PSF (prestarvation factor), CMF (conditioned medium factor), DIF (differentiation inducing factor) and SDFs (spore differentiation factors). PSF and CMF are important for early developmental steps. DIF becomes active in the mound stage and is released in response to saturating levels of cAMP (Thompson and Kay, 2000). It is an inducer of prestalk gene expression and causes prestalk cells to further differentiate into stalk cells. Prespore gene expression is inhibited by DIF (Aubry and Firtel, 1999; Kay et al., 1999; Williams, 2006), and SDFs are peptides that promote terminal spore differentiation (Anjard et al., 1998).

The *Dictyostelium* genome encodes 55 genes for GPCRs divided into 5 families of which only the receptors of the cAMP family have been addressed extensively, whereas the remaining ones are yet to be explored. 17 GPCRs belong to the family 3 GPCRs, the metabotropic glutamate or $GABA_B$-like receptor family (Eichinger et al., 2005; Glöckner et al., 2002). GABA is the principal neurotransmitter in the central nervous system acting on two types of receptors present on the peripheral nerve terminals: the ionotropic GABA types A and C ($GABA_{A/C}$); and the metabotropic GABA type B ($GABA_B$) receptors; each of which mediates synaptic inhibition (Blein et al., 2000). Dysfunction in the GABA system leads to several neuropsychiatric disorders including anxiety, depression, schizophrenia, loss of memory, loss of sleep, and epilepsy (Cryan and Kaupmann, 2005). Of the *Dictyostelium* family 3 GPCRs, only GrlE shows significant similarity over most of its length to the mammalian GABA receptors and was recently shown to be a true GABA receptor (Anjard and Loomis, 2006; Prabhu and Eichinger, 2006). In *Dictyostelium*, GABA acts via GrlE and induces sporulation by releasing AcbA (acyl-CoA binding protein). AcbA is processed to the peptide SDF-2 by a protease, which is present on the surface of prestalk cells and induces encapsulation. The human homolog of AcbA is the precursor of neuropeptides that bind to and modulate $GABA_A$ receptor function in the central nervous system. The agonist of such peptides is diazepam. This compound also acts on *D. discoideum* and induces sporulation (Anjard and Loomis, 2005, 2006).

We have investigated GrlA, a member of the *Dictyostelium* family 3 GPCRs. GrlA is expressed throughout growth and development and has important roles in the post aggregation stages of *Dictyostelium* development. Mutants lacking GrlA show altered expression of genes that trigger post aggregation events and sorting of prestalk cells. GrlA is further involved in controlling the formation of spores. Together with GrlE, GrlA is another GPCR important for terminal differentiation in *Dictyostelium*.

Materials and methods

Dictyostelium cell culture, vector generation, mutant screening, rescue and localisation studies

Wild type cells and mutants were cultured as described in Marko et al. (2006). The GrlA gene replacement vector was constructed by retrieving a Blasticidin resistance (Bsr) gene with the act15 promoter and act8 terminator sequences with *Eco*RV and *Xba*I from the pBs–Bsr vector (Sutoh, 1993) and insertion into the GrlA gene such that a 1.3 kb *Eco*RV–*Xba*I fragment of the GrlA gene was replaced by the 1.4 kb Bsr gene. A PCR-based approach was used for screening *grlA*$^-$ cells followed by Southern blotting (Khurana et al., 2002). Several independent mutant strains were isolated. To generate a GFP-tagged version of GrlA (GrlA-GFP, where GFP is fused to the C-terminus of GrlA), the full-length GrlA gene was amplified as a *Hind*III–*Bam*HI fragment, cloned into GFP-vector p1ABsr8 (Gräf et al., 2000) and transformed by electroporation into Ax2 cells and the *grlA*$^-$ mutant for rescue. The transformants were selected using 3 μg/ml of Blasticidin. The mutant strains were analysed as described in Khurana et al. (2002). Cytokinesis was analysed by determining the number of nuclei after staining with DAPI, slug phototaxis was carried out as described in Khaire et al. (2007). Folate chemotaxis was assayed in a two drop assay (Van Haastert et al., 1982).

The mutant and wild type cells were used for live cell microscopy, or fixed with methanol and stained with antibodies against various proteins that are present in different cellular compartments. For immunofluorescence analysis and Western blotting, the following antibodies were used: mAb 185-447-3 (Döring et al., 1995) recognized Annexin C1 as a plasma membrane marker, mAb 221-135-1 (Monnat et al., 1997) is specific for the ER-marker protein disulfide isomerase (PDI), mAb 260-60-10 (Rivero et al., 1998) for interaptin, a nuclear envelope protein, and mAb 221-35-2 (Jenne et al., 1998) for VatA, a subunit of the vacuolar ATPase. For visualisation, a secondary Alexa 568-coupled antibody was used. The specimens were examined using a LEICA confocal laser scan microscope.

RNA isolation and quantitation, Northern blotting, microarray analysis

Total RNA was extracted from Ax2 and *grlA*$^-$ at different developmental stages or different assay conditions using the Qiagen RNeasy Mini kit. The manufacturer's protocol for the isolation of RNA from animal cells was used for preparation. The RNA samples were used directly for Northern blot analysis or after reverse transcription for semi-quantitative RT–PCR, real-time PCR and microarray analysis. Microarray analysis was carried out with RNA harvested from growing (t0) and developing cells (t5, t16). cDNA was generated using the M-MLV reverse transcriptase, RNAse H minus (Roche), according to the manufacturer's protocol. Usually 1–5 μg of the respective total RNA was used for each RT reaction. cDNAs generated were used as a template to carry out PCR with the respective gene-specific primers. For the microarray analyses three independent RNA preparations were performed per time point and strain, cDNAs were generated for each preparartions and used for two arrays each. *Quantitative real-time PCR*: Primers were selected such that the expected product size was between 250 and 500 bp. Prior to use in real-time PCR experiments, the quality of the cDNA and the primers were tested by PCR. Real-time PCR was carried out with the QuantiTectSYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. For each sample, gene-specific primers (10 pmol) and 1 μl of cDNA were used. As a quantification standard, defined concentrations (10 ng, 1 ng, 100 pg, 10 pg and 1 pg) of GrlA C-terminal gene sequences in pGEMTeasy (Promega) were used. Actin-specific primers were used as positive controls to ensure comparable concentrations of cDNA in samples of wild type and mutant cells.

Microarray analysis was done as described (Farbrother et al., 2006). Northern blot analysis was carried out using total RNA isolated at different time points (Faix et al., 1990).

DIF induced gene expression by shaking suspension assay

Logarithmically growing Ax2 and *grlA*$^-$ cells were harvested, washed with Soerensen phosphate buffer (17 mM K^+/Na^+-phosphate buffer, pH 6.0, made

from KH_2PO_4 and Na_2HPO_4) and plated at a density of 5×10^6 cells/cm^2 and developed until mound stage. Cells were disaggregated with a syringe and a 22-gauge needle and were resuspended at 1×10^7 cells/ml and divided into four sets. One set each was treated with 5 mM cAMP, 100 nM DIF, and 5 mM cAMP and 100 nM DIF, respectively, and the last set without any additions as a control. Incubation was for 2 h at 21 °C on a shaker (160 rpm) (Berks and Kay, 1990). RNA from the respective samples was prepared using the RNAeasy kit and 10 μg from each sample was used for Northern blot analysis of *ecmA*, *ecmB* and *pspA* transcripts, respectively. cAMP was obtained from Fluka and DIF-1 from Biomol.

Spore formation assay

An equal number (5×10^7) of cells (Ax2 and *grlA*$^-$) was plated onto phosphate agar plates and spores formed at 48 h were harvested and counted, respectively. They were then treated with 0.5% Triton X-100 for 15 min and diluted with Soerensen buffer and 100 spores each were plated onto SM agar plates in association with *Klebsiella aerogenes* (Ennis and Sussman, 1975, modified). Efficiency of spore formation was calculated by counting the number of plaques that were formed by detergent-resistant spores. The experiments were carried out thrice in triplicates. Spore formation induced by 8-Br-cAMP (Fluka), a membrane-permeable cAMP analogue, was done according to published protocols and spore formation was followed by microscopy (Richardson et al., 1991). Early culminants were employed for this assay.

LacZ staining, neutral red staining

Ax2 cells and *grlA*$^-$ cells were transformed using electroporation with the ecmAO-Gal, ecmO-Gal, ecmB-Gal and pspA (D19)-Gal plasmids separately, which express β-galactosidase in pstA, pstO and pstB cells located in the tip and in prespore cells (psp) in the posterior region (Williams, 2006). The transformants were selected with 4 μg/ml G418. Single colonies were obtained by replica plating and they were developed until slug and culminant stages, transferred to nitrocellulose filter and then processed for LacZ expression (Dingermann et al., 1989). For staining with the vital dye Neutral red cells were harvested at a density of $2–3\times10^6$ cells/ml, washed twice with Soerensen phosphate buffer and treated with an equal volume of 0.1% Neutral red solution (Sigma) for 1 min. The cells were washed once with a large volume of buffer and plated onto phosphate agar plates for development.

Miscellaneous methods

Cell fractionation was done as described (Marko et al., 2006). mAb 47-19-2 was used to detect α-actinin in Western blots (Schleicher et al., 1984), mAb 190-68-1 detected comitin (Weiner et al., 1993), and K3-184-2 detected GFP-tagged proteins (Noegel et al., 2004).

Results

GrlA, a Dictyostelium GPCR of Family 3

GrlA (metabotropic *G*ABA$_B$/glutamate *r*eceptor-*l*ike) is a 798-amino-acid containing protein of the family 3 of *Dictyostelium* GPCRs and shares similarity with regard to its domain architecture with 13 peer members (Prabhu and Eichinger, 2006). GrlA possesses a predicted signal peptide followed by a Bmp (basic membrane protein) domain at the N-terminus, a well-distinguished 7 transmembrane domain and a coiled coil domain at its C-terminus (Figs. 1A, B). A detailed phylogenetic analysis placed GrlA closer to the GABA receptor orthologs with respect to its transmembrane segments (Fig. 1C). The intracellular loop 3 of the GPCRs has specific residues that help in determining the G-protein specificity whereas the intracellular loop 2 possesses enough molecular determinants for G-protein activation (Francesconi and Duvoisin, 1998; Duthey et al., 2004; Havlickova et al., 2002). As depicted in Fig. 1D, the intracellular loop 2 (il2) of the GABA$_B$R1 has highly conserved glutamate residues, represented as EE KK E motif, which are replaced by lysine in GABA$_B$R2 represented as KM KK K. The intracellular loop 2 in GrlA is related to the GABA$_B$R2 signature of mammals and harbours lysine and arginine as basic residues (KL KK R, Fig. 1D), probably allowing to couple to G-proteins as observed for GABA$_B$R2 (Robbins et al., 2001; Havlickova et al., 2002). The intracellular loop 3 (il3) between the TMD-5 and TMD-6 in GrlA contains lysine at the key position as in most GABA$_B$R1 (Fig. 1D).

Expression of GrlA

GrlA transcripts were detected throughout development. During growth and early development the levels were moderate. At 12 h, when cells aggregated they increased by a factor of 3 to 5 and stayed high in the later developmental stages as revealed by real-time PCR. The expression pattern was compared to the one of the actin transcript which is present throughout growth and development. The absolute message levels for GrlA were however very low ranging from 0.4 to 2.9 pg, whereas for actin we obtained amounts between 382 and 605 pg in these samples (Table 1, Fig. 2A).

Subcellular localization of GrlA

The subcellular localisation of GrlA was studied by expressing a full-length GrlA–GFP fusion protein in Ax2 wild type cells as the polyclonal antibodies which we had generated did not recognise the protein in Western blots containing cell homogenates nor in immunofluorescence analysis. In fixed cells GrlA–GFP was present at the plasma membrane, the majority of the protein was however found on internal structures. At the plasma membrane GrlA–GFP overlapped with Annexin C1 which localises to the plasma membrane and to internal membranes (Döring et al., 1995; Marko et al., 2006). The majority of the protein remained in the cytoplasm predominantly at the perinuclear region where it colocalised with the ER protein PDI and with interaptin, a nuclear envelope protein (Fig. 2B). We also monitored for colocalisation of GrlA–GFP with antibodies against Comitin (Weiner et al., 1993), a protein present on Golgi membranes, VatA (the A subunit of vacuolar ATPase) which is present on endosomal membranes and the contractile vacuole of *D. discoideum*, lysosomal antigen (mannose-6-sulfate containing carbohydrate epitope present in lysosomal enzymes) (Neuhaus et al., 1998), and Vacuolin (Rauchenberger et al., 1997), a protein on post lysosomal vacuoles. However, we did not note colocalisation with any of these proteins (data not shown). In Western blot analysis of subcellular fractions of GrlA–GFP expressing cells GrlA–GFP was present in the 100,000×*g* pellet supporting the membrane association (Fig. 2C).

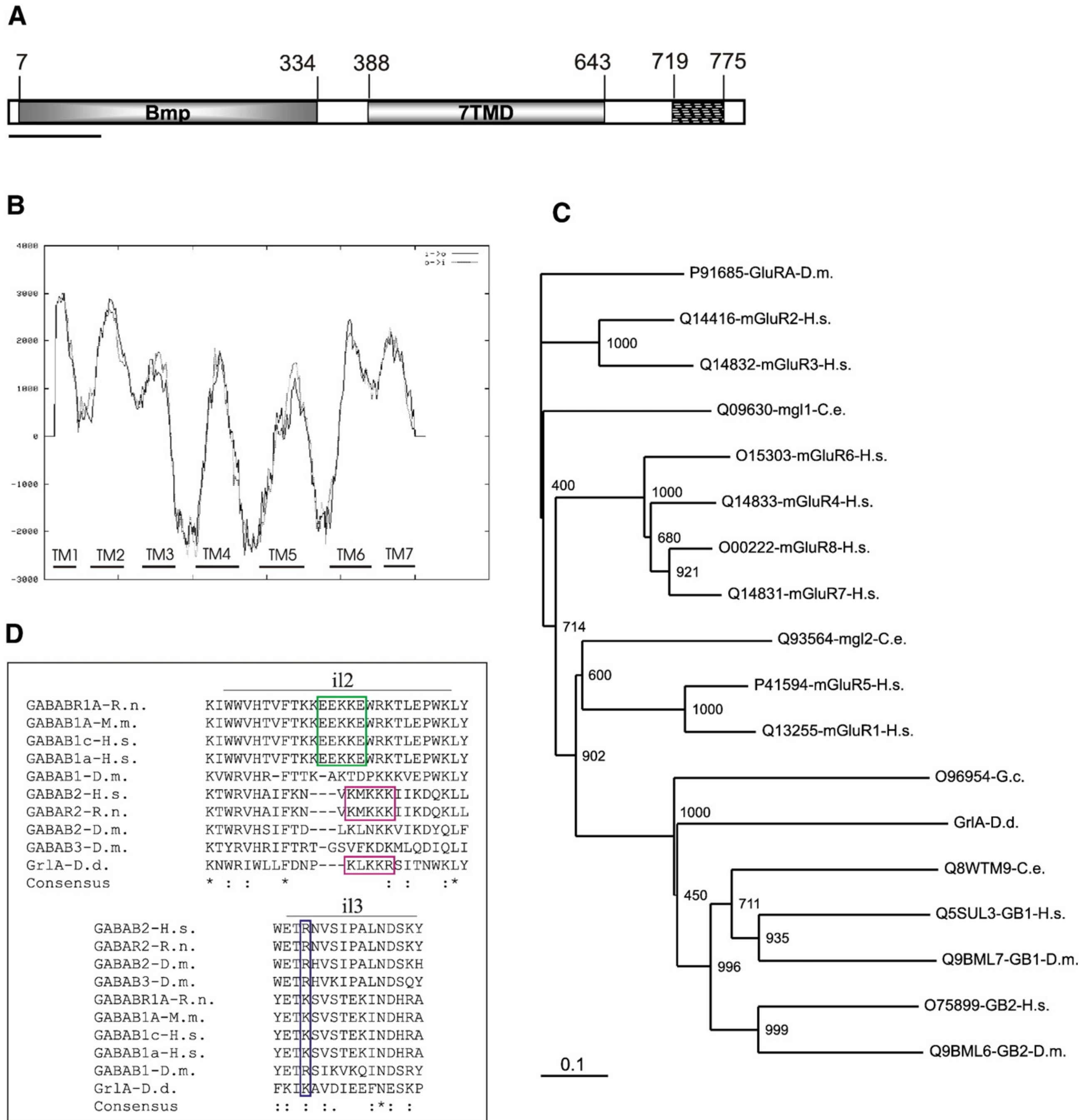

Fig. 1. (A) Schematic representation of GrlA. The conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and SMART (http://smart.embl-heidelberg.de/) were used to predict conserved domains and drawn to scale. The location within the protein is indicated according to the SMART output. Bar: 100 amino acids, Bmp (basic membrane protein), 7TMD (seven transmembrane domain). A predicted signal sequence is present at the extreme N-terminus (not shown). (B) Hydropathy analysis of GrlA. The putative transmembrane regions (amino acid residues 388–643) were used to deduce the hydrophobicity of GrlA using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). Positive scores indicate a membrane spanning potential which predicted the presence of 7 transmembrane regions (TM1, TM2, TM3, TM4, TM5, TM6, TM7). (C) Phylogenetic tree of *Dictyostelium* GrlA, $GABA_B$ and glutamate receptors from other eukaryotes. A CLUSTALX alignment of the transmembrane region of the *Dictyostelium* GrlA and selected $GABA_B$ and metabotropic Glutamate receptors from other organisms was applied to the TreeView program to obtain a phylogenetic tree. The scale bar indicates 10% divergence. The proposed name for the *Dictyostelium* GrlA and UniProt identifiers for the receptors from other organisms are given. C.e.: *Caenorhabditis elegans*; H.s.: *Homo sapiens*; D.m.: *Drosophila melanogaster*; G.c: *Geodia cydonium*. (D) Multiple alignment of the intracellular loops of GrlA with selected $GABA_B$ receptors. The loops in the intra- and extracellular regions were predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp). The intracellular loops 2 and 3 obtained from the sequences by aligning the TMDs of the receptors were used for alignment using ClustalW. The alignments of intracellular loops 2 (upper panel, il2) and 3 (lower panel, il3) are shown. Consensus residues are shown below both alignments. Star depicts completely conserved residues in all the proteins shown here, double dot signifies high similarity, whereas a single dot represents partial conservation. The boxes represent important residues for G-protein coupling or specificity.

Table 1
GrlA expression pattern as revealed by real-time PCR

Time points (h)	Receptor 1 (*GrlA*) (pg)	Actin control (pg)
0	1.2	502
4	0.7	412
8	0.4	382
12	2.7	539
16	2.3	605
20	2.9	487

Total RNA from different developmental time points was used as a template for the synthesis of cDNA. The Opticon Real-time PCR machine was used to quantify the receptor expression as compared to a standard (data not shown) and the control (actin). The experiments were carried out twice independently and in duplicate. The results from a representative experiment are shown.

GrlA gene disruption and mutant analysis

To inactivate the *grlA* gene we used a targeting vector that was constructed in such a way that a central 1.3 kb fragment of the *grlA* gene which encoded a large part of the N-terminus and nearly all of the 7TM region was replaced by the 1.4 kb blasticidin resistance cassette (Fig. 3A). The transformants were screened by a PCR-based approach followed by Southern blot analysis to confirm the gene replacement event (Figs. 3B, C). Loss of transcript was observed in the *grlA*$^-$ cells by RT–PCR, where a C-terminal region of *grlA* was only amplified from RNA isolated from the wild type Ax2 but not from *grlA*$^-$ cells (Fig. 3D). In contrast, unrelated sequences such as the one for *arpG* were amplified for both strains (data not shown). Two independent knockout strains, G1a3 and F1a2, were used for further characterisation. They behaved similar in all assays.

grlA$^-$ cells grew comparable to the wild type Ax2 cells in suspension culture in axenic medium as well as on a lawn of *K. aerogenes*. Cytokinesis and phototaxis were unaltered in the mutant as well. As folate, a long-known chemoattractant (Van Haastert et al., 1982; Segall, 1988), mediates its response via G-proteins by an unidentified GPCR, we tested folate chemotaxis by the two-drop assay and found that it was unaffected in *grlA*$^-$ at both stages during which folate signalling is active, i.e. in the growing cells and at the tipped aggregate stages (data not shown). Aggregation assays in monolayer with *grlA*$^-$ displayed timely formation of aggregation centres with thick streams comparable to the wild type cells (data not shown).

Role of grlA$^-$ *in post aggregation stages*

The *grlA*$^-$ strains aggregated well and formed mounds similar to the wild type cells. However, the tip formation was delayed by 3 to 4 h and *grlA*$^-$ cells completed development with normally shaped fruiting bodies only after 26–27 h (Fig. 4A). The expression pattern of cell-type-specific developmental markers confirmed these observations (Fig. 4B). Early aggregation genes (*acaA* and *pdeE*) were expressed timely and at comparable levels, but there was a delay in the expression of the prestalk-specific genes *ecmA*, *ecmB* and *car2* (Aubry and Firtel, 1999). Moreover, the transcript levels were strongly reduced in comparison to wild type. In contrast, the prespore-specific *pspA* mRNA appeared timely and the levels were unaltered when compared to wild type levels. This may be attributed to the induction of *pspA* which occurs much earlier in development via regulatory pathways that do not control post aggregation (Aubry and Firtel, 1999). The *ecmA* and *ecmB* genes are expressed in the tip of the slug, which is the morphological organizer during multicellular development and contains primarily prestalk cells (Weijer, 2004), *pspA* is expressed in the prespore cells located in the posterior region. Correct patterning of the cell types was tested by expression of β-galactosidase under the control of the ecmO, ecmAO and ecmB promoters and the *pspA* promoter. The ecmO promoter is a more distal promoter in the ecmA gene which directs reporter expression in the cells that localise to the rear of the prestalk region, the ecmAO–lacZ fusion is a reporter construct containing the ecmA and ecmO promoter regions, the ecmB promoter is active in the pstB and pstAB cells, two minor populations in the prestalk region. pstAB cells form a central core in the tip (Strmecki et al., 2005; Fukuzawa et al., 2006; Williams, 2006). The *pspA* promoter controls the expression of the prespore-specific gene *D19* (Early et al., 1988; Williams, 2006). The staining patterns were comparable in Ax2 and *grlA*$^-$ cells (Fig. 4C).

Changes were also noted for *car2* and *pkaC*, the catalytic subunit of protein kinase C, which had strongly downregulated transcript levels during development as compared to Ax2 (Fig. 4B). cAR2 (cAMP receptor 2) is a prestalk-specific marker and has been suggested to be involved in inducing competence for *ecmB* induction by DIF (Verkerke-Van Wijk et al., 1998), PKA plays a central role throughout development (Aubry and Firtel, 1999).

The developmental delay was rescued by expression of GFP-tagged GrlA full-length protein in the mutant (Fig. 4A). GrlA–GFP is expressed under the control of the actin15 promoter which is active throughout development and presumably leads to overexpression of the protein. The successful rescue suggests, that the protein is functional and also localises properly as the subcellular distribution of GrlA–GFP which we observed in *grlA*$^-$ cells resembled the one in Ax2 cells. GrlA–GFP was present in the perinuclear area, on membranous structures and at the plasma membrane. In living cells, GrlA–GFP was prominently present at the plasma membrane and on internal membranes. It showed a very dynamic behaviour and moved into protrusions that were newly formed by the cells. We also observed that extending filopodia carried the GFP–fusion protein (Fig. 1; Supplementary movies 1, 2 and 3).

The *grlA*$^-$ strain expressing GrlA–GFP differed in one respect from wild type as tip formation was not as pronounced as in Ax2 and *grlA*$^-$ (Fig. 4A, 15 h time point). For further analysis, we used Neutral red staining. Neutral red is a vital dye staining strongly the prestalk cells in the tip as well as anterior like cells (ALC) in the back of a slug. ALCs express several genes that are also expressed in prestalk cells such as *ecmB*. They form, however, a distinct population of cells which have a different fate at culmination (Williams, 2006). In Ax2 and *grlA*$^-$ Neutral red-stained tips were easily seen after 14 h of starvation. The Neutral red staining was retained at the tip also at later stages of development when slugs had formed. In the slugs, Neutral red-stained cells were present at the rear of

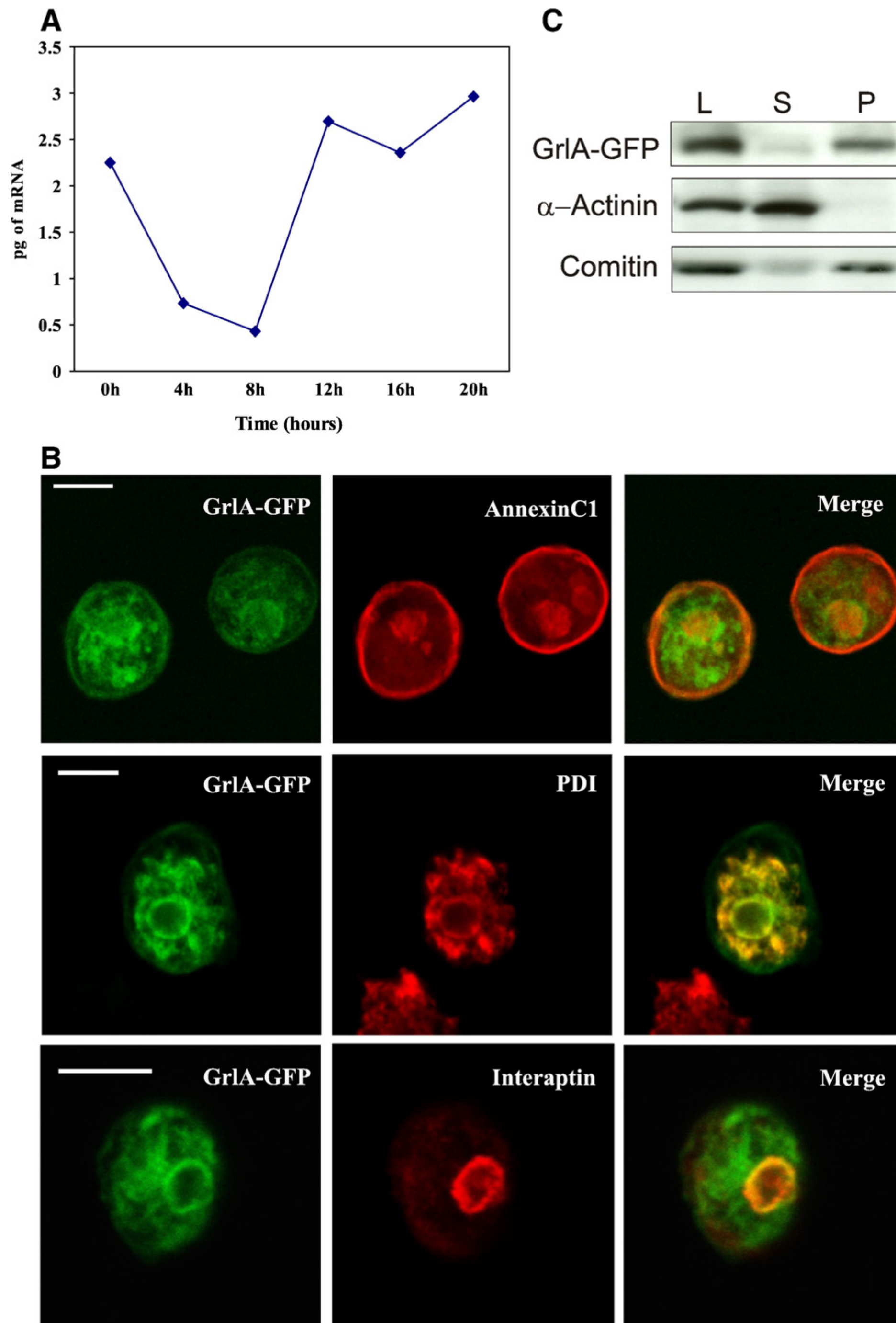

Fig. 2. (A) grlA expression pattern during development as revealed by Real time PCR. (B) Subcellular localisation of GrlA–GFP fusion protein in Ax2 cells. The full-length GrlA gene sequence was fused at its C-terminus to the EGFP gene and the protein expressed in wild type Ax2 cells. Co-staining was carried out with monoclonal antibodies for the plasma membrane marker Annexin C1 (mAb 185-447-3), the ER protein PDI (mAb 221-135-1), and for Interaptin (mAb 260-60-10) that is predominantly located at the nuclear envelope. A secondary antibody conjugated to Alexa 568 was used for detection. Images were captured using a confocal microscope. Bar: 5 μm. (C) Subcellular fractionation of GrlA–GFP fusion proteins. GrlA–GFP expressing Ax2 cells were harvested in the logarithmic phase of growth, resuspended in MES/sucrose buffer, pH 6.0, and lysed by sonication. The membrane fraction was separated from the cytosol by centrifugation at 100,000×*g*. Immunoblotting with anti-GFP antibody (mAb K3-184-2) was carried out to detect GrlA–GFP. The membrane marker Comitin (mAb 190-68-1), associated with the Golgi membrane, and α-actinin (mAb 47-19-2), present in the cytosolic fraction, were used for control. L, total cell lysate; S, 100,000×*g* supernatant and P, 100,000×*g* pellet.

the slug as well. By contrast, the GFP–GrlA expressing mutant did neither show Neutral red staining at the mound stage nor in the tip of the slugs. The rear of the slug was however stained (Fig. 4D).

Altered DIF-1 response in mutants lacking GrlA

DIF-1 is a chlorinated hexaphenone whose synthesis is induced at the mound stage by high cAMP concentrations when

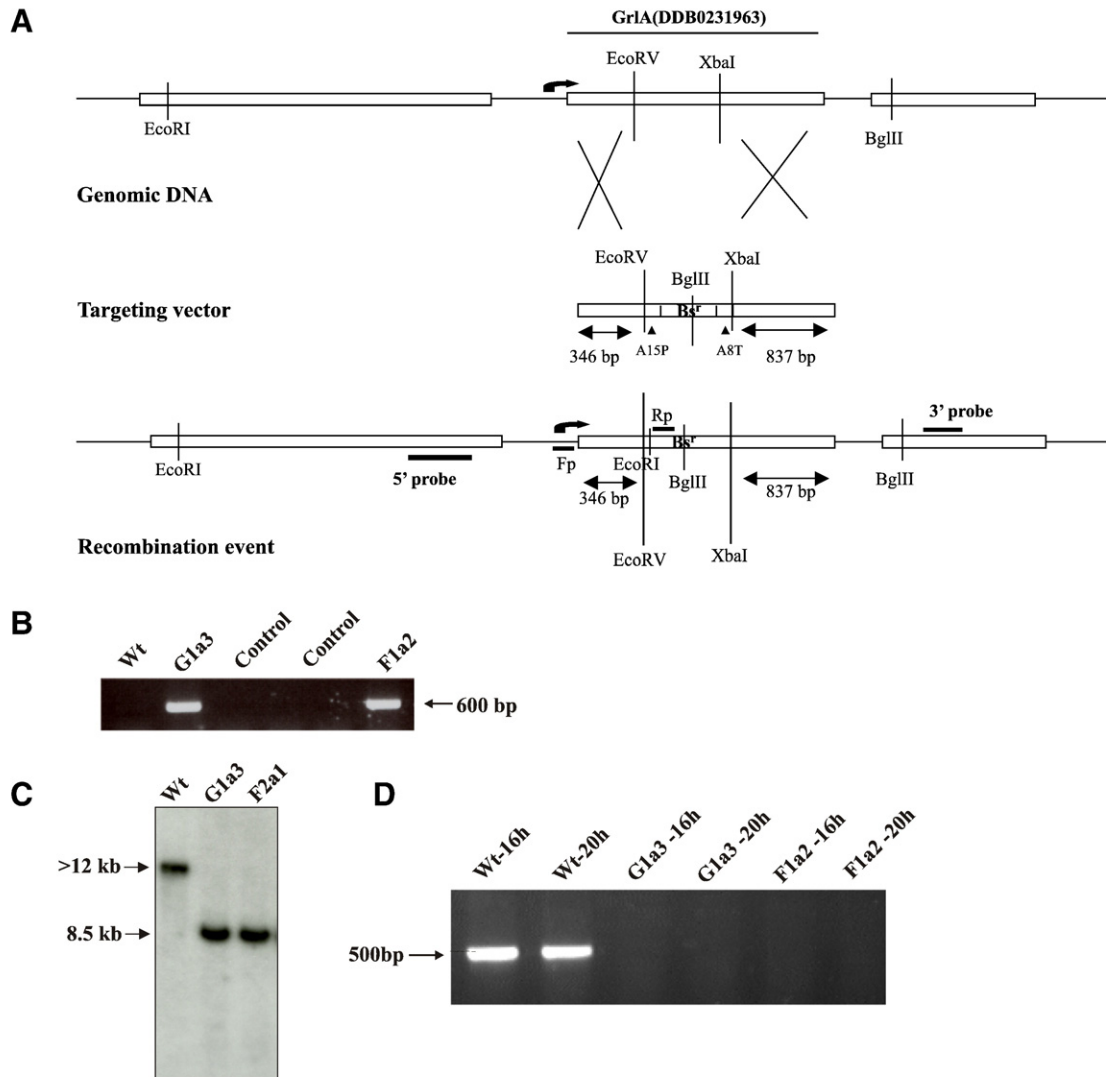

Fig. 3. (A) Targeting vector and recombination strategy of GrlA. The Blasticidin resistance gene with the act15 promoter and act8 terminator sequences were retrieved using *Eco*RV and *Xba*I from the pBs–Bsr vector and inserted into the GrlA gene such that a part of exon 1, the following intron (76 bp) and a part of the second exon which encompass half the N-terminal region and the first four TMDs were replaced by the blasticidin resistance cassette (1.4 kb). *GrlA* and two neighbouring genes are indicated by rectangles. (B, C) Confirmation of the recombination event. A PCR-based approach was used for screening the transformants followed by Southern blotting. Primers were designed so as to cover the genomic locus with a forward primer (Fp in panel A, Recombination event) 50 bp upstream of the ATG of the GrlA gene and a reverse primer (Rp in panel A) in the promoter region of the Bsr cassette so as to cover a region of ~500 bp across the N-terminal part of GrlA. Two independent mutants (G1a3 and F2a1) were selected and used to isolate genomic DNA for Southern blotting which was carried out after digestion of the genomic DNA with *Eco*RI using the 5′ probe as designated in the schematic in panel A and additionally with the *Bsr* gene (not shown). (D) The respective mutants (G1a3 and F2a1) were further confirmed by the loss of transcript in a RT–PCR. Primers specific for the C-terminal region of GrlA were able to amplify the transcript in the 16- and 20-h starved wild type cells but not in *grlA*$^{-}$ cells.

the prespore and prestalk cells are largely intermixed. It is a general inducer of prestalk cell differentiation and is produced by the prespore cells. It acts on specific prestalk cells and is degraded in the prestalk cells by a dechlorinase so as to maintain the DIF gradient in the slug (Thompson and Kay, 2000). As we had noted a delay in the expression of prestalk-specific genes in *grlA*$^{-}$, we further studied the effect of DIF on *ecmA*, *ecmB* and *pspA* in a shaking culture assay (Berks and Kay, 1990).

In Ax2 cells cAMP had some positive effect on ecmB expression and led to a strong increase of *pspA* transcripts. Addition of DIF and cAMP + DIF together induced ecmB expression, whereas the one of *pspA* was reduced. In the mutant, the pattern of *pspA* expression resembled the one in Ax2. cAMP treatment led to a strong increase in *pspA* levels and DIF and cAMP + DIF repressed *pspA*. For the ecmB expression we noticed an increase in mRNA in response to DIF and cAMP + DIF treatment, however it did not differ from the increase observed after cAMP treatment. The pattern of ecmA transcript accumulation also differed from wild type as cAMP + DIF produced a massive induction in Ax2, whereas in *grlA*$^{-}$ the induction was not as strong and only marginal (Fig. 5). The data suggest that in the *grlA*$^{-}$ strain, the DIF response is altered in the prestalk cells with regard to the *ecmA* and the *ecmB* gene.

Spore formation defects in grlA^{-}

To explore the development of the *grlA*$^{-}$ strains further, we analysed the efficiency of spore formation by plating

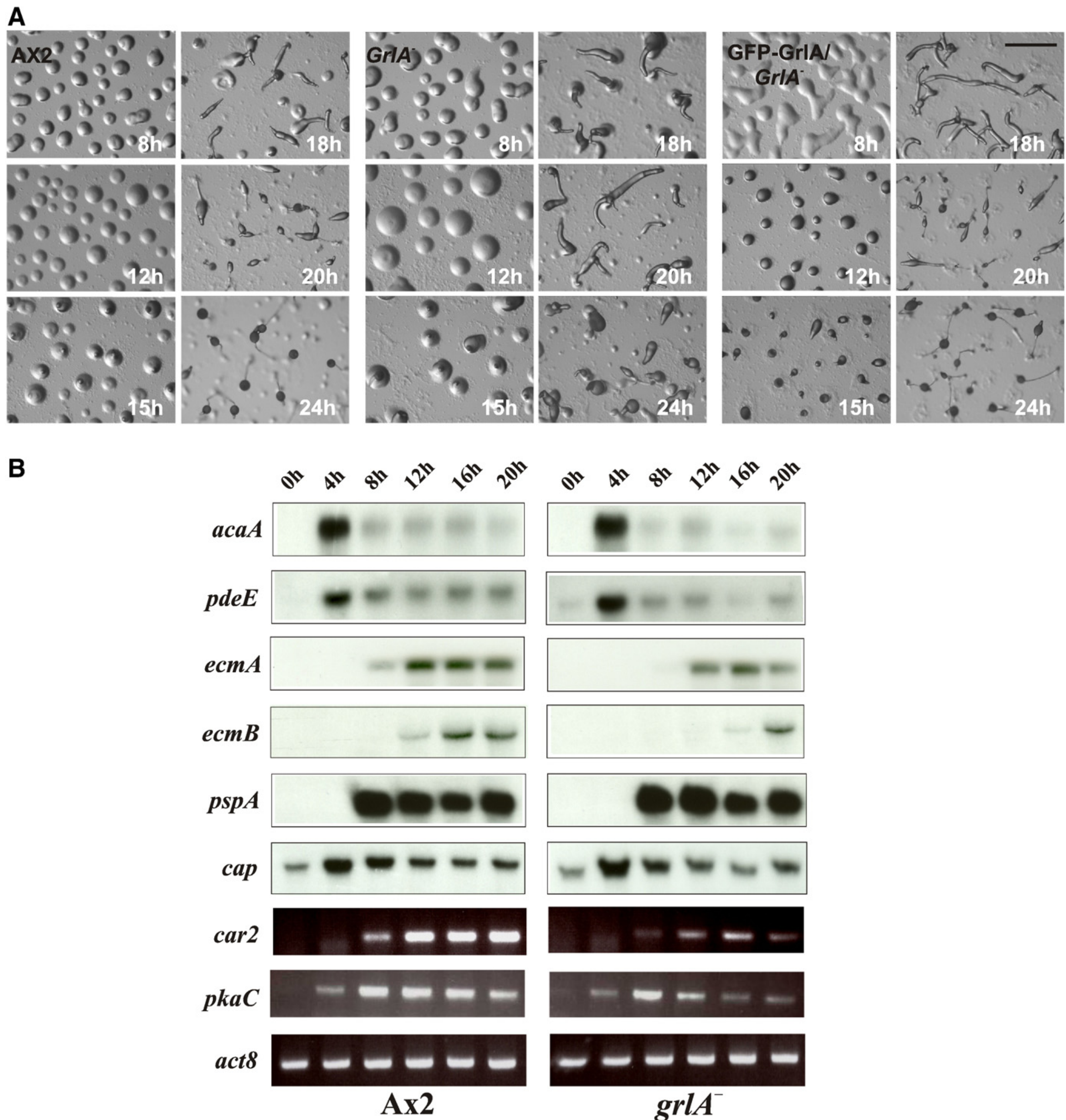

Fig. 4. (A) Development on phosphate agar plates. Axenically growing cells (Ax2, *grlA*⁻ and GrlA–GFP/*grlA*⁻) were plated at a density of 5×10^6 cells/cm^2 and were monitored throughout development. Images were captured at he indicated time points using a stereomicroscope. Bar: 1 mm. (B) Northern blot analysis of aggregation and post aggregation specific genes. Total RNA was isolated from the wild type Ax2 and *grlA*⁻ at the indicated time points. 20 μg RNA were separated on agarose gels (1.2% agarose) under denaturing conditions (6% formaldehyde) and transferred to membranes as described (Faix et al., 1990). cDNA probes specific for the indicated transcripts were used for hybridising the blots. A CAP cDNA probe was used as a control. CAP is upregulated at the onset of development and is present throughout development (Gottwald et al., 1996). Additionally, RNA from the above time course experiment was used to generate single stranded cDNA and RT–PCR was carried out for analysis of the expression of prestalk specific *car2* and of *pkaC*, the catalytic subunit of protein kinase A. The actin gene was amplified as a control. (C) Expression of β-galactosidase under control of the pstO, pstB and pspA promoters in Ax2 and *grlA*⁻. pspA (D19)-Gal, ecmB-Gal, ecmO-Gal and ecmAO-Gal plasmids were transformed into the respective strains separately, single colonies were assayed for β-galactosidase. Representative images are shown. (D) Neutral red staining in AX2, *grlA*⁻ and *grlA*⁻ expressing GrlA–GPF. Cells were incubated with Neutral red and deposited on phosphate agar plates for development. Images were taken at the indicated time points. *grlA*⁻ cells expressing GrlA–GPF exhibit Neutral red staining only at the base.

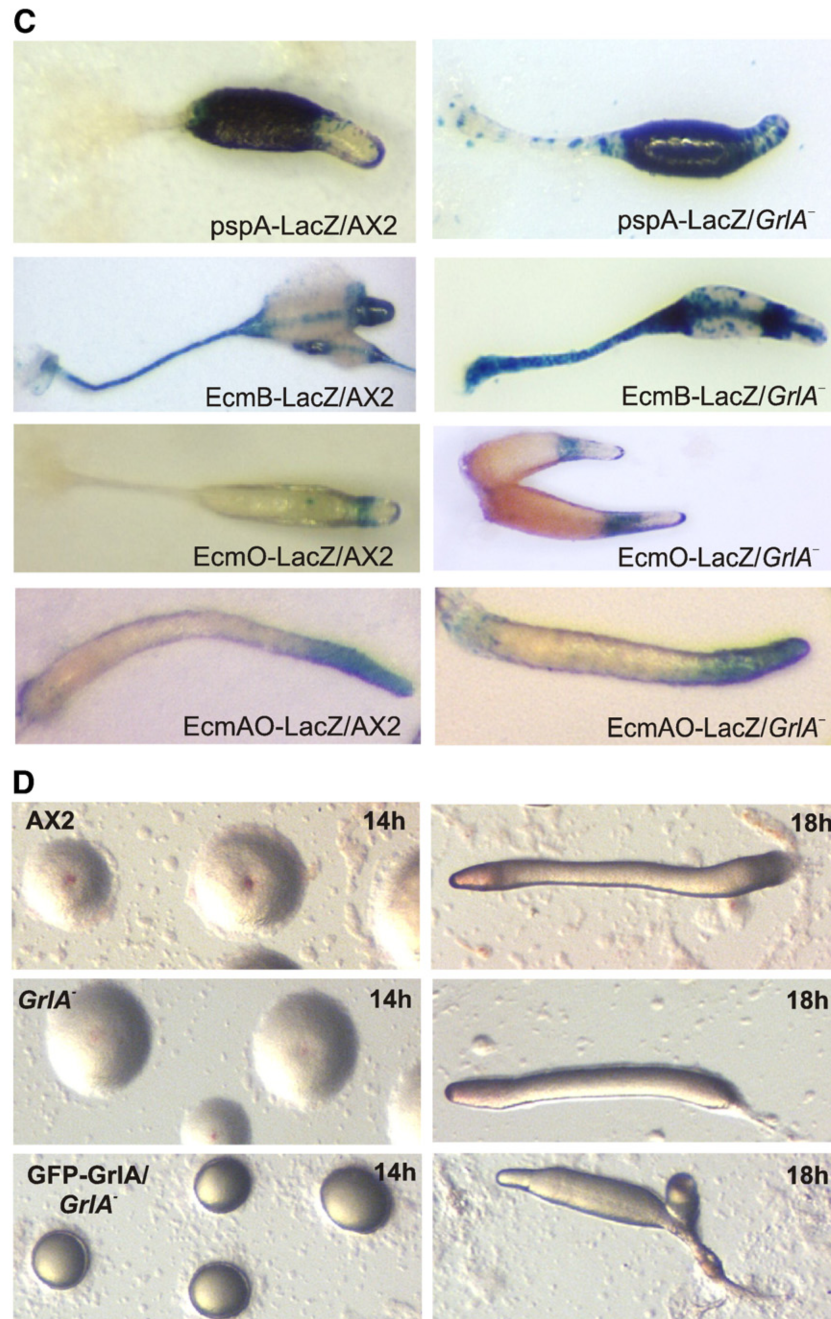

Fig. 4 (*continued*).

equal amounts of axenically growing cells on phosphate agar plates and allowing them to develop for 48 h until spore formation was complete. Spores were then harvested and counted to determine the number of spores produced compared to the initial number of cells plated. Additionally, detergent resistance was checked. The wild type cells showed recovery of 130% spores from the cells initially plated indicating that they were all viable and resistant to detergent treatment. *grlA*$^{-}$ produced a lower number of spores (80% from the initial input of cells) as compared to wild type (Fig. 6). The viability of spores with and without detergent treatment was comparable to the wild type strain. The reduced number of spores produced in *grlA*$^{-}$ was rescued by expressing GFP-tagged full-length GrlA in the mutant background (Fig. 6). The morphology of the spores was determined using calcofluor that stains the cellulose matter present on the spore coat. Ax2 cells and *grlA*$^{-}$ both produced normal oval-shaped spores (data not shown).

We also assayed induction of sporulation in response to 8-Br-cAMP. This cAMP analogue when present at high levels can trigger spore maturation in dissociated prespore cells through activation of protein kinase A (PKA). We found that 8-Br-cAMP induced sporulation in Ax2, *grlA*$^{-}$ cells and the rescue strain, whereas in untreated control cells no spore formation was

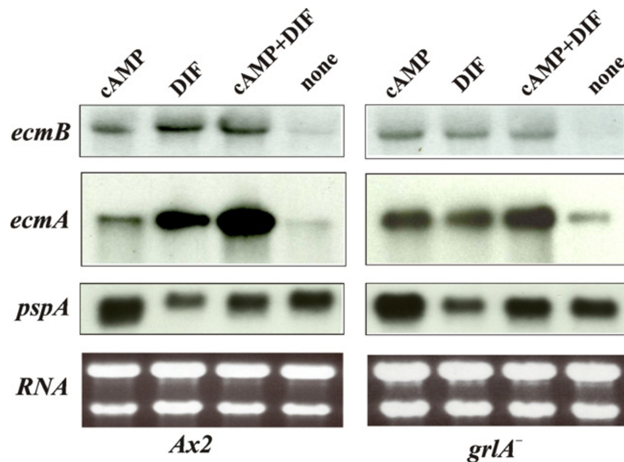

Fig. 5. DIF and cAMP induced induction/repression of developmental markers. Log-phase cells (Ax2 and *grlA*⁻ cells) were starved at a density of 5×10^6 cells on phosphate agar plates. After 12 h, the respective mounds or tipped mounds were harvested and treated with either 5 mM cAMP, 100 nM DIF, 5 mM cAMP plus 100 nM DIF or no addition and allowed to shake for 2 h at 160 rpm at 21 °C. RNA was extracted, followed by Northern blotting and the blot was hybridised with probes for the prestalk genes *ecmA*, *ecmB* and the prespore gene *pspA*. The ribosomal RNA (rRNA) is shown as loading control.

observed. The results suggest that this pathway is active in the *grlA*⁻ strain.

Transcriptional profile of grlA⁻ cells

Microarray analysis carried out with RNA from the *grlA*⁻ and wild type cells at 0, 5 and 16 h of starvation unraveled a global transcriptional profile of the genes differentially regulated in the grlA mutant at 16 h of starvation, whereas in growing cell (0 h) and during early development (5 h), no significant differences were noted (Tables 2 and 3 and ST 1 and 2 and data not shown). In our analysis of the 16 h time point, we obtained 179 genes that were at least 1.5-fold differentially regulated. This value constituted approximately 3.3% of the genes studied or approximately 1.4% of all *D. discoideum* genes. 56 genes were at least 2-fold differentially regulated in the *grlA*⁻ cells. Out of these 56 genes, only 16 were upregulated whereas a much larger proportion of the differentially regulated genes, more than 70%, was downregulated in the mutant. The differentially regulated genes were analysed for enriched functional categories using the GO (gene ontology) classification in conjunction with the program GOAT (http://www.godatabase.org/dev/database, Harris et al., 2004, Xu and Shaulsky, 2005, Farbrother et al., 2006). In the "biological process" category, the enrichment of downregulated genes involved in sporulation was most interesting. The "cellular component" category showed for the downregulated genes an enrichment of extracellular matrix genes and of genes of the SCF (**S**kp, **c**ullin, **F**-box) ubiquitin ligase complex, in particular *fbxA* and *culA* (Table 3 and Supplementary Table 2). One of the targets of the fbxA/SCF complex is RegA, an intracellular cAMP phosphodiesterase, which is ubiquitinated and degraded thereby increasing the cAMP levels (Mohanty et al., 2001; Tekinay et al., 2003). We also noted among the downregulated genes in *grlA*⁻ cells a large number of genes that are induced by the MADS-box transcription factor SrfA (Serum response factor) during later stages of development. Altogether 10 SrfA induced genes were downregulated at least 1.5-fold in *grlA*⁻ (Table 3 and Supplementary Table 2). For the hallmark gene *spiA*, we noted a 6-fold downregulation. Genes induced by SrfA are involved in spore maturation and spore coat formation in *D. discoideum* (Aubry and Firtel, 1999; Escalante et al., 2004). The SrfA mRNA is present from the loose mound stage onward at constant levels and at the onset of culmination a sharp increase was found (Escalante and Sastre, 1998). For SrfA expression itself, we did not note changes in the mutant. This result suggests that GrlA acts upstream of SrfA in *D. discoideum*. A significantly smaller number of genes were upregulated in the *grl*A mutant. Here an enrichment of genes encoding calcium binding proteins was observed (Table 2 and Supplementary Table 1).

More components of spore formation pathways are affected in grlA⁻

Spore formation is controlled by two peptides, i.e. spore differentiation factors 1 and 2 (SDF-1 and SDF-2). SDF-1 accumulates during the slug stage and is released in a single burst at the onset of culmination, SDF-2, a proteolytic product of AcbA is released at the mid-culmination stage also in a single burst (Anjard et al., 1998; Anjard and Loomis, 2005). The cleavage of AcbA occurs through TagC, a Transporter/Serine protease present on the prestalk cells. SDF-2 triggers spore encapsulation in responsive cells as soon as they are exposed to the signal. This response is mediated by DhkA receptors, histidine kinase receptors present on the surface of both prestalk and prespore cells which in turn inhibit the phosphorelay via RdeA (phosphor-transfer protein) to RegA. The phosphodiesterase RegA is inactive in its unphosphorylated form which leads to an increase in cAMP concentration by the late adenylyl cyclase ACR. Thereby encapsulation of the spore cells is

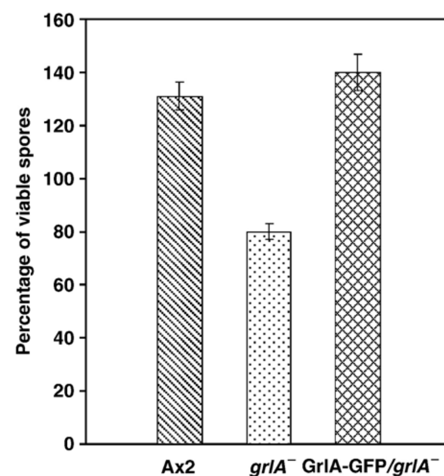

Fig. 6. Efficiency of spore formation in *grlA*⁻. Equal numbers (5×10^7 cells) of the respective strains were plated onto Soerensen phosphate agar plates and allowed to develop completely until 48 h. The spores were then harvested and counted. The percentage of spores was calculated in comparison to the initial input of cells.

Table 2
Significantly upregulated genes in *grlA*⁻ cells as compared to Ax2 cells at 16 h of development

DDB ID	Fold	Annotation	Score
DDB0217256	2.47	Similar to D. discoideum protein-tyrosine phosphatase 3	7.909
DDB0185515	2.24	Predicted nucleotide-binding protein containing TIR -like domain (Transcription)	6.057
DDB0191092	2.23	pefA, penta EF hand calcium binding protein	7.371
DDB0191175	2.18	cadA, calcium-dependent cell adhesion molecule-, glycoprotein gp24	9.727
DDB0167824	2.15	Similar to bacteriophage t4 lysozyme (P00720)	7.813
DDB0191502	2.11	Annexin 7, synexin, calcium-regulated phospholipid- and membrane-binding protein	7.012
DDB0231298	2.07	Probable methionyl-trna synthetase	8.737

Differentially regulated genes were identified using the SAM program (Tusher et al., 2001). The genes that are listed are the ones that were more than 2-fold upregulated in comparison to the wild type cells. DDB ID is the dictyBase sequence identification number (http://dictybase.org/), Fold represents the fold change in *grl*A⁻ cells as compared to the control Ax2 cells and Score represents the SAM value for reproducibility of the result between independent experiments. The higher the score the more reliable the result. The gene name and putative function was obtained from the *Dictyostelium* database and by performing BLAST searches with the protein sequences against the non-redundant database at NCBI. Genes for which no annotation was available have been deleted from the table. A full list of all genes that were more than 1.5-fold differentially regulated is available in Supplementary Table 1. Genes falling in the same category are indicated with a colour; Green: Ca^{2+} binding proteins.

induced by activating PKA which is present in both the prestalk and prespore cells (for review: Aubry and Firtel, 1999). SDF-1 also promotes spore induction, however, the signal transduction pathway involved is yet uncharacterized but it involves *de novo* protein synthesis which is not the case with SDF-2.

As GrlA is involved in spore formation pathways, we tested the expression profile of components of the SDF-2 response system i.e. *acbA*, *tagC* and *dhkA* by RT–PCR analysis. We observed downregulation of both *tagC* and *dhkA* transcripts during later development in the mutant compared to the wild

Table 3
Significantly downregulated genes in *grlA*⁻ cells at 16 h of starvation

DDB ID	Fold	Annotation	Score
DDB0231407	−7.05	Similar to spore coat protein PspB; expressed in prespore cell	9.20
DDB0191438	−6.08	spiA, regulated by the transcription factor SrfA during development; expressed in prespore cells	8.95
DDB0191248	−5.97	pspE, 1I prespore protein, expressed in prespore cells	16.25
DDB0229900	−4.26	Putative arabinofuranohydrolase, expressed in pstA cells and in pstAB and stalk cells during the mexican hat stage and culmination	8.38
DDB0218274	−4.11	Coiled-coil family protein, underexpressed in gskA-null strain	8.08
DDB0215373	−3.85	gluA, beta glucosidase	8.82
DDB0214855	−3.76	Similar to rickettsia rickettsii outer membrane protein a precursor (190 kda antigen) (cell surfaceantigen) (P15921)	5.99
DDB0189726	−3.64	Unknown, expressed in prespore cells	11.63
DDB0191269	−3.55	poxA, peroxinectin, regulated by the transcription factor SrfA during development	11.91
DDB0216219	−3.04	ecmB, extracellular matrix protein ST310	9.94
DDB0186921	−2.80	Putative quinone oxidoreductase (At5g16980)	7.67
DDB0201668	−2.76	sigB, leishmanolysin family protein, developmentally regulated gene whose expression is dependenton the transcription factors srfA and StkA	11.38
DDB0216182	−2.62	2C, coiled-coil family protein, cyclic AMP-regulated gene; underexpressed in gskA-null	12.64
DDB0219578	−2.59	Similar to anaeromyxobacter dehalogenans 2cp-c. short-chain dehydrogenase/reductase (Q4ny75)	9.32
DDB0191278	−2.46	sigF, regulated by the transcription factor SrfA during development	9.69
DDB0203213	−2.45	P90893 Putative serine protease F56F10.1 precursor	9.48
DDB0229992	−2.27	mitA, putative mitochondrial substrate carrier, expressed in pstAO cells and in upper and lower cupsduring culmination	
DDB0191392	−2.27	sigG, regulated by the transcription factor SrfA during development	7.34
DDB0230022	−2.25	rps5, 40S ribosomal protein S5, protein component of the small (40S) ribosomal subunit	4.15
DDB0191271	−2.23	aqpA, aquaporin, similar to aquaporin family of water-channel proteins; expressed in prespore cells	11.29
DDB0183918	−2.23	Esterase/lipase/thioesterase domain-containing protein, expressed in prespore cells	11.72
DDB0191486	−2.17	sigM, EGF-like domain-containing protein, regulated by the transcription factor SrfA during development	6.89
DDB0219701	−2.17	Unknown,expressed in prespore cells	7.84
DDB0218211	−2.10	Similar to thermobifida fusca intracellular protease/amidase (Q47l13)	11.46
DDB0231139	−2.06	ecmD, cellulose-binding domain-containing protein, sheathin, extracellular matrix protein	8.49
DDB0214915	−2.06	wacA, aquaporin-like, member of the major intrinsic protein (MIP) family of membrane transporters	11.59
DDB0231388	−2.05	Haloacid dehalogenase-like hydrolase, expressed in pstA and pstO cells and in pstAB cells during culmination	7.23
DDB0191094	−2.04	rpl3, 60S ribosomal protein L3, protein component of the large (60S) ribosomal subunit	6.07
DDB0230023	−2.03	rps6, 40S ribosomal protein S6, protein component of the small (40S) ribosomal subunit	4.49
DDB0186151	−2.00	Similar to arabidopsis thaliana putative gtpase activator protein (Q8lgj8)	6.38
DDB0230181	−2.00	masA, malate synthase ,expressed in pstAB and pstO cells and in upper cup during culmination	7.43

Differentially expressed genes were identified using the SAM program (Tusher et al., 2001) and processed the same way as mentioned in Table 1. Genes for which no annotation was available have been deleted from the table. A full list of all genes that were more than 1.5-fold differentially regulated is available in Supplementary Table 2. Genes belonging to the same categories are indicated with a colour; yellow: signalling components in GSK-related pathway; pink: srfA-induced genes; grey: prespore-specific genes; orange: extracellular matrix-encoding genes.

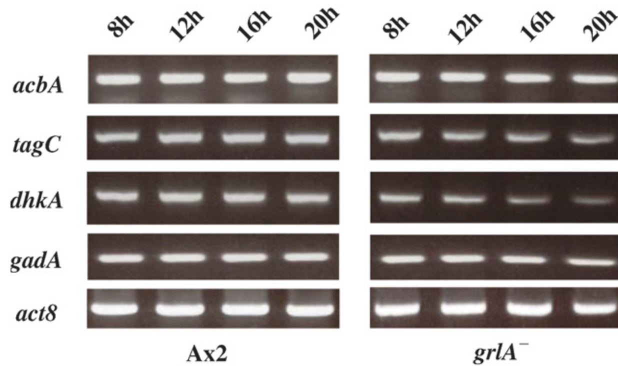

Fig. 7. Expression profile of components of late developmental pathways in *grlA*⁻ cells. RNA was isolated at different time points of the development and equal amounts of RNA were used to generate single-stranded cDNA (see Materials and methods). Specific primers were used to amplify *tagC*, *dhkA*, *acbA* and *gadA* sequences. Actin mRNA levels were determined for control (act8), as actin gene expression is nearly uniform throughout development.

type cells whereas the *acbA* levels were unaltered (Fig. 7). Downregulation of *tag*C but not *dhk*A was also seen in the microarray experiment. These results suggest an impairment of these regulatory pathways and indicate that GrlA might be involved in steps during sporulation that can control the release of SDF-2 from AcbA indirectly by controlling the protease TagC and the response in the prespore and prestalk cells by controlling the levels of DhkA receptor (Table 3).

Discussion

This report deals with GrlA, one of the 17 known metabotropic Grls (metabotropic glutamate or $GABA_B$-like receptors) in *D. discoideum* that is expressed at increasing levels during late developmental stages (12 h onwards). GrlA expressed under the control of the constitutively active actin promoter is enriched in the membrane fractions and localises to the plasma membrane and to internal membranes. In fact, a major part of the protein decorates the internal membranes like the ER. The strong ER staining may reflect inefficient trafficking due to overexpression or the presence of incorrectly folded protein. GPCRs frequently form heterodimers with another GPCR and dimerisation is required for trafficking to the plasma membrane (Prinster et al., 2005). In the case of overexpression of GrlA as GrlA–GFP fusion protein, the counterpart of GrlA, if any, may not be available in sufficient quantities leading to the accumulation of GrlA–GFP in the ER membranes. GrlA–GFP did not accumulate in other intracellular membranes which it might pass on its way to the plasma membrane. Our rescue experiments show however, that GrlA–GFP is functional.

GrlA in morphogenesis

Strains lacking GrlA display a developmental delay from the mound stage onwards which coincides with the time when the grlA transcript levels increase strongly in the wild type cells. The tip is formed only after a delay of 3 to 4 h in the mutant cells and then they complete development with normally shaped fruiting bodies. Northern blot analysis and RT–PCR indicated a delay in the expression of the prestalk-specific genes, *ecmA*, *ecmB* and *car2*, and slightly reduced levels of the *ecmA* transcript at 12 h. These changes, however, did not lead to changes in patterning as the cell-type-specific markers studied showed unaltered localisation of LacZ driven by the *ecmAO*, *ecmO*, *ecmB* and *pspA* promoter in *grlA*⁻. These findings were also supported by staining with the vital dye Neutral red which specifically accumulates in the vacuoles of the prestalk and anterior like cells. In contrast, the induction of the prestalk-specific gene *ecmB* was altered in the mutant in response to exogenous DIF-1 in an induction assay and *ecmA* induction was less pronounced than in wild type. These data might be explained by the observed downregulation of cAR2 which was proposed to be required for DIF induction of *ecmB* (Verkerke-Van Wijk et al., 1998). However, there might be alternative explanations as the role of DIF and its derivatives is complex and not completely understood (Serafimidis and Kay, 2005).

GrlA is a player in the sporulation pathway

grlA⁻ formed normally shaped mature fruiting bodies, however the number of spores produced was less in *grlA*⁻ (~60% in comparison to wild type cells). The spores formed were however viable. The defect in spore numbers was reverted by re-expression of GrlA–GFP. To pinpoint the defect in *grlA*⁻, we carried out a microarray analysis and further tested the presence of components of signal transduction pathways that are specifically involved in spore differentiation. The results show that specific pathways are impaired in the mutant and many genes that are normally expressed during late development in prespore cells, such as several SrfA-dependent genes, show a strongly reduced expression. However, the *grlA*⁻ mutant still formed reasonable amounts of spores despite of its apparent deficiencies in various spore pathways. Also, *grlA*⁻ cells formed spores in the presence of 8-Br-cAMP, putting GrlA upstream of PKA. Although the 16 h time point is the one where a developmental delay was observed in the mutant which may lead to misinterpretation of the data, further analysis of selected genes in RT–PCR experiments in which RNA from all developmental stages was used underlined the validity of the results.

By combining our data from the mutant analysis with the current knowledge of signalling pathways active during post aggregative development (Aubry and Firtel, 1999), we developed a model on how GrlA can function at this stage of development (Fig. 8). GrlA is a plasma membrane protein and is activated by a so far unidentified ligand. Upon activation, GrlA promotes sporulation by further activating and/or increasing TagC on the surface of prestalk cells. As a consequence, more AcbA is processed to yield mature sporulation factor SDF-2 which in turn binds to the histidine kinase receptor DhkA on the surface of both prestalk and prespore cells and inhibits phosphorelay via RdeA to cAMP phosphodiesterase RegA. This leads to an increase in cAMP levels: PKA is activated and triggers sporulation in prespore cells, whereas in prestalk cells it is involved in a feedback loop increasing the activity of TagC.

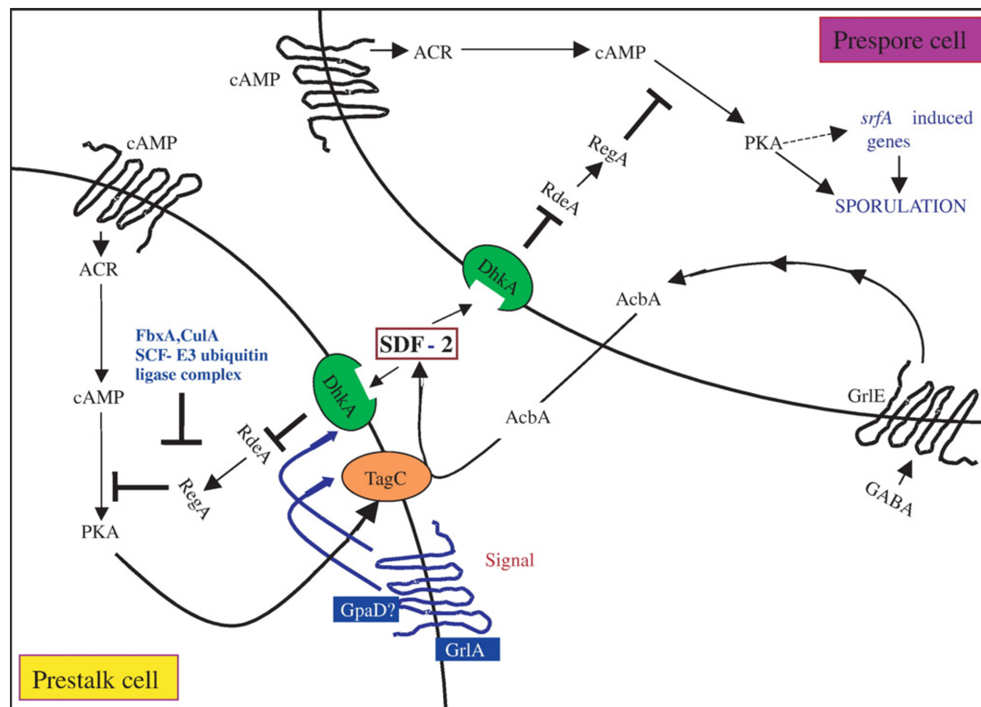

Fig. 8. Model depicting the role of GrlA in sporulation. This cartoon depicts the putative function of GrlA in prestalk cells. Upon activation, GrlA promotes sporulation by increasing extracellular exposure of TagC possibly via a G-protein, GpaD. The AcbA, which is released from prespore cells as a result of GABA signalling via GrlE, is cleaved by TagC to form SDF-2, which then binds to the DhkA receptor present on the surface of both prestalk and prespore cells. Activated DhkA inhibits the phosphorelay via RdeA to RegA, a cAMP phosphodiesterase, thereby increasing the cAMP levels. This activates PKA, which triggers encapsulation either directly or indirectly by inducing the expression of *srfA* in the prespore cells, whereas in prestalk cells it acts as a feedback loop to expose the protease domain of TagC. Additionally, in the prestalk cells, this feedback loop is complemented by the activity of SCF ubiquitin ligase complex and an interplay between *fbxA*, *regA* and *dhkA* to direct degradation of RegA thus increasing the intracellular cAMP levels (Tekinay et al., 2003). The dotted arrow indicates indirect regulation (transcriptional regulation of *srfA*-inducible genes). The present findings and assumptions are given in blue, whereas previous findings are indicated in black.

Histidine kinase receptors acting via the two-component system carry out a variety of functions in bacteria. In *Saccharomyces cerevisiae*, *Neurospora crassa* and in *D. discoideum*, they function in osmosensing as well as in morphogenesis (Schuster et al., 1996; Loomis et al., 1997). A similar kind of histidine kinase signalling pathway was reported to be functional in mammals that involves G-proteins (Besant and Attwood, 2005; Steeg et al., 2003). It is therefore possible that the $GABA_B$-like GrlA may control the activity of TagC/DhkA via involvement of G-proteins. Gα4 acts in the late folate and pterin-mediated response to regulate morphogenesis and spore production (Hadwiger et al., 1994, 1996). However, the folate response in the grlA mutant was unaltered at both stages when folate signalling is functional. GrlA may signal to control sporulation independently or along with GrlE in the prestalk cells and control the extracellular exposure of the protease domain of TagC via Gα4, which could mediate this response independent of its role as a G-protein in folate signalling. How exactly the extracellular activity of TagC (exposure of the protease domain) in prestalk cells is affected by GrlA needs to be explored and so do the ligands.

There are many unexplored molecules and secreted factors such as cell density factors, folate and the related pterins, LPA (lysophosphatidic acid), PSF, CF, small metabolites and numerous small peptides that may act via GPCRs (Hadwiger et al., 1994; Roisin-Bouffay et al., 2000) and may represent putative ligands. The role of GrlA in sporulation involving an ABC type of transporter/protease as well as a hybrid kinase points to an additional role for a GABA-like GPCR in a two-component signalling and also makes it a good target to explore multidrug resistance transporters.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.08.055.

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