G α 5 subunit-mediated signalling requires a D-motif and the MAPK ERK1 in *Dictyostelium*

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The *Dictyostelium* G α 5 subunit has been shown to reduce cell viability, inhibit folate chemotaxis and accelerate tip morphogenesis and gene expression during multicellular development.

Alteration of the D-motif (mitogen-activated protein kinase docking site) at the amino terminus of the G α 5 subunit or the loss of extracellular signal-regulated kinase (ERK)1 diminished the lethality associated with the overexpression or constitutive activation of the G α 5 subunit. The amino-terminal D-motif of the G α 5 subunit was also found to be necessary for the reduced cell size, small aggregate formation and precocious developmental gene expression associated with G α 5 subunit overexpression. This D-motif also contributed to the aggregation delay in cells expressing a constitutively active G α 5 subunit, but the D-motif was not necessary for the inhibition of folate chemotaxis. These results suggest that the amino-terminal D-motif is required for some but not all phenotypes associated with elevated G α 5 subunit functions during growth and development and that ERK1 can function in G α 5 subunit-mediated signal transduction.

INTRODUCTION

Signal transduction through G protein-coupled receptors activates many downstream regulatory proteins and leads to a wide variety of cellular responses (Hur & Kim, 2002; Landry & Gies, 2002; Milligan & Kostenis, 2006; Neves *et al.*, 2002; Offermanns & Simon, 1998; Simon *et al.*, 1991). Receptors, and sometimes G proteins, are pathway-specific, but many other signalling proteins function in multiple G protein-mediated pathways, suggesting that signal-specific responses involve receptor and/or G protein interactions with specific downstream signalling components (Albert & Robillard, 2002; Hildebrandt, 1997). The soil amoeba *Dictyostelium discoideum* uses several different G protein-mediated pathways during growth and development to regulate cell movement and differentiation; therefore, this organism offers a useful system for studying the interactions between pathway-specific and common signalling components during development (Firtel *et al.*, 1989; Firtel, 1996).

The *Dictyostelium* G α 5 subunit functions in a signalling pathway that inhibits folate chemotaxis, promotes a small aggregate size and accelerates tip formation and gene expression during development (Hadwiger *et al.*, 1996;

Natarajan *et al.*, 2000; Srinivasan *et al.*, 1999). The activator and receptor for this pathway remain to be determined but a recent study of chimeric G α subunits suggests that the functional specificity of the G α 5 subunit is not limited to the specificity of receptor coupling but requires interactions with other signalling components (Hadwiger, 2007). The G α 5 subunit primary sequence is closely related to the G α 4 subunit, a subunit required for folate chemotaxis, except for a region near the amino terminus of the G α 5 subunit where a putative mitogen-activated protein kinase (MAPK) docking site, known as a D-motif, exists, suggesting that interactions with MAPKs might be important for G α 5-specific responses (Hadwiger, 2007; Remenyi *et al.*, 2005). An interaction between the amino-terminal D-motif of a G α subunit and a MAPK has been reported previously in the budding yeast *Saccharomyces cerevisiae*, where the interaction of the Gpa1 G α subunit and the Fus3 MAPK facilitates the G α subunit-mediated adaptation to pheromone stimulation (Blackwell *et al.*, 2003; Metodiev *et al.*, 2002). In *Dictyostelium*, a D-motif in the central region of the G α 4 subunit has recently been shown to be important for interactions with the extracellular signal-regulated kinase (ERK)2 and for G α 4 function late in the culmination stage of development (Nguyen & Hadwiger, 2009).

The *Dictyostelium* genome contains genes for only two MAPKs, ERK1 and ERK2, and both kinases can be activated in G protein-mediated signalling pathways (Aubry & Firtel, 1999; Goldberg *et al.*, 2006; Maeda & Firtel, 1997; Miranda-Saavedra & Barton, 2007). ERK1 can be activated in response to cAMP and loss of ERK1 results

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Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

A supplementary table showing the selection of *g α 5* transformants is available with the online version of this paper.

in small aggregate size during development (Sobko *et al.*, 2002). ERK2 is rapidly phosphorylated and activated in response to either cAMP or folate, and the loss of ERK2 prevents aggregation and reduces prespore gene expression during development (Brzostowski & Kimmel, 2006; Knetsch *et al.*, 1996; Maeda *et al.*, 1996; Maeda & Firtel, 1997). Both ERK1 and ERK2 contain sequence similarity with MAPKs from other organisms in a common docking domain, suggesting that they also interact with proteins containing D-motifs (Gaskins *et al.*, 1994; Segall *et al.*, 1995).

In this study, the role of the D-motif at the amino terminus of the G α 5 subunit was explored by analysing the phenotypes of G α 5 subunits with alterations in this D-motif. Growth and developmental phenotypes associated with G α 5 subunit overexpression or constitutive activation were found to depend on the amino-terminal D-motif. Sensitivity to elevated G α 5 subunit expression and function during vegetative growth was found to be absent in *erk1* but not *erk2* cells, indicating a specific requirement for ERK1 in G α 5 subunit-mediated signalling. The G α 5 subunit D-motif alteration did not eliminate the inhibition of folate chemotaxis, suggesting that this D-motif plays an important role in some but not all G α 5 functions during growth and development.

METHODS

Strains and media. All of the *Dictyostelium* strains were isogenic to the wild-type strain KAx3, except where noted in the mutants. The *g α 5* strain (JH257) has been described previously (Hadwiger *et al.*, 1996). The *erk2* strain (JH697) was created from the JH8 strain using the pAK240Cla gene disruption construct, as described previously (Segall *et al.*, 1995). The pAK240Cla gene construct was kindly provided by J. Segall (Albert Einstein College of Medicine, Bronx, NY) and the *Dictyostelium* Stock Center. Disruption of the *ERK2* (*erkB*) locus was verified by genomic DNA blot analysis (data not shown). The *erk1* strain (JH1058) was created in KAx3 cells by disrupting the *ERK1* (*erka*) gene by inserting the blasticidin S resistance gene, as described by Nguyen *et al.* (2010). Cells were grown in axenic HL5 medium or on bacterial lawns (Watts & Ashworth, 1970). DNA vectors were electroporated into cells as described previously (Hadwiger, 2007). Transformed cells were grown in medium containing G418, and drugs used for selection were removed several hours prior to analysis. Modelling of the G α 5 subunit structure was conducted using the SWISS-MODEL program and displayed using the PyMOL Molecular Graphics System 2008 (DeLano Scientific) (Arnold *et al.*, 2006).

Recombinant DNA constructs. The wild-type G α 5 gene, replacing the GFP gene, was expressed from the *act15* promoter (pJH510) in the pTX-GFP vector (Ddp1-based plasmid) as described previously (Hadwiger, 2007). Alterations to the G α 5 subunit D-motif, G α 5^{K10E,K11E} subunit (designated G α 5^{d-}), were created in a *HindIII/PstI* fragment of the G α 5 gene in the vector pT7T318U (Pharmacia) by PCR mutagenesis of the entire vector using the following overlapping oligonucleotides: sense strand, 5'-gcccgcATCGAtTATCAATTAAGAAAAGAAGGAAG, and anti-sense strand, 5'-gcccgcATCGATGTCTCTTGATTcCTcTGTTCAA-TTGTTAATATACAACCC (lower case nucleotides differ from those of the template sequence). These mutations were confirmed by

sequence analysis and this mutant segment of G α 5 was used to replace the same segment of the wild-type G α 5 gene in the expression vectors. The constitutively active G α 5^{Q198L} (designated G α 5*) subunit expressed from the *act15* promoter (pBR515) was created by replacing a *PstI/BclI* fragment of the wild-type G α 5 constructs with a fragment from a G α 5^{Q198L} gene previously created in a genomic fragment (Hadwiger *et al.*, 1996). This mutation was also combined with the mutations in the G α 5 D-motif to create the combined mutant G α 5^{d-*} subunit expression vector (pBR558). To create this gene, the *HindIII/PstI* fragment of the G α 5^{d-} gene was used to replace the beginning of the G α 5* ORF. All G α 5 expression constructs were also created in the pDXA-GFP2 vector (Ddp2-based plasmid) that integrates into the genome unless co-electroporated with the pREP vector, because plasmid copy number can sometimes influence the phenotypes of cells (Levi *et al.*, 2000). Similar results were obtained with both vector systems but only the results from the pTX vector are shown.

Cell growth and drug selection. Comparison of cell viability for cells expressing wild-type and mutant G α 5 subunits was assessed by either counting the number of transformants after electroporation or growing established strains in media containing different concentrations of drug selection. Briefly, cells were electroporated with equal amounts of wild-type and mutant G α 5 expression vectors, plated onto solid HL5 medium, and after 24 h, the cells were selected by using G418 from 1 to 10 μ g ml⁻¹. Drug-resistant colonies were counted 10–14 days after drug selection.

Cell sizes were compared by growing equal concentrations of different strains, harvesting cells by centrifugation, washing cells in phosphate buffer and weighing cell pellets. Protein content of cells treated with Triton X-100 (0.01% final concentration) was assessed using a Bradford protein assay (Bradford, 1976).

Development and chemotaxis assays. Cells were grown to mid-exponential phase (approx. $2\text{--}3 \times 10^6$ cells ml⁻¹), washed twice in phosphate buffer (12 mM NaH₂PO₄ adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10^8 or 2×10^7 cells ml⁻¹), before spotting on non-nutrient plates (phosphate buffer, 1.5% agar) for development. Alternatively, cells were spotted on to Whatman-50 filters soaked in phosphate buffer for developmental RNA isolation. Total RNA was isolated, separated on formaldehyde-denaturing agarose gels and blotted to membranes as described previously (Hadwiger *et al.*, 1996). Radioactive probes were created using the random primer method as described by Feinberg & Vogelstein (1983). Hybridized blots were detected using autoradiography. rRNA bands on blots were detected by UV light shadowing before hybridization to verify consistency in sample loading and blotting.

Chemotaxis assays were performed by spotting droplets (~0.5 μ l) of cell suspensions (10^8 cells ml⁻¹) on non-nutrient plates, allowing absorption of liquid into the agar, followed by the spotting of 1 μ l droplets of 1 mM folate solutions approximately 2–3 mm away from the cell droplet, as described previously (Hadwiger, 2007). Images of the cell droplet perimeter and the location of the leading edge of cells migrating toward the folate source were recorded after 3 h using a dissecting microscope and a digital camera. The early and late images were accurately aligned by scarring the agar surface with a needle.

RESULTS

Docking site motifs in G α subunits

A region near the amino terminus of the G α 5 subunit was identified as a potential site of functional specificity due to sequence diversity with respect to the G α 2 and G α 4

subunits; further inspection of this region revealed a D-motif (Hadwiger, 2007). This D-motif is located near a highly conserved guanine nucleotide interaction domain and at a position analogous to the previously characterized D-motif of the yeast mating response Gα subunit, Gpa1 (Holbrook & Kim, 1989; Metodiev *et al.*, 2002) (Fig. 1a). The sequence of this motif is similar to the consensus sequence [K/R]₁₋₃-X₁₋₆-[L/I]-X-[L/I] found in MAPK kinases and other proteins that dock with MAPKs (Grewal *et al.*, 2006). The Gα5 D-motif contains a tyrosine residue (Y20) at a position typically occupied by a leucine or isoleucine residue, but the aromatic ring of the tyrosine side group probably contributes to the hydrophobic nature of this region. A survey of the other 11 *Dictyostelium* Gα subunit sequences revealed putative D-motifs on the Gα2, Gα3 and Gα11 subunits in the same region, suggesting that MAPKs might interact with multiple Gα subunits in this organism. The human Gα16 (murine Gα15) subunit also has a D-motif near the amino terminus (Wilkie *et al.*, 1992). The *Dictyostelium* Gα4 subunit does not contain a D-motif near the amino terminus but does have a D-motif at a more internal site (residues 117–134) where there is a second possible D-motif in the Gα5 subunit. Both the amino-terminal and internal D-motifs of the Gα5 subunit are predicted to be located in exposed regions of the subunit based on structural modelling of the Gα5 subunit (Fig. 1b).

Alteration of the amino-terminal D-motif reduces the lethal effects of Gα5 subunit overexpression

To determine if the amino-terminal D-motif in the Gα5 subunit plays a role in G protein-mediated signalling, the first two lysine residues of this motif were changed to glutamate because an analogous change in the yeast Gpa1 subunit was previously reported to reduce both Gpa1–Fus3 interactions and Gpa1-mediated downregulation of the pheromone response (Metodiev *et al.*, 2002). The Gα5 D-motif mutant subunit, Gα5^{K10E, K11E} (Gα5^{d−}) and the wild-type Gα5 subunit were electroporated into *gα5* and wild-type cells to examine phenotypes associated with D-motif alterations. Electroporation of the Gα5^{d−} subunit vector into cells typically resulted in at least a 10-fold higher number of viable transformants compared with the wild-type Gα5 subunit, unless the drug selection for the vectors was reduced to less than 2 μg G418 ml^{−1} (Fig. 2). Increasing the G418 concentration resulted in the death of clonal transformants expressing the Gα5 but not the Gα5^{d−} subunit, suggesting that the Gα5^{d−} subunit is less detrimental to vegetative growth than the wild-type Gα5 subunit (Supplementary Table S1, available in Microbiology Online).

Stimulation of the Gα5-mediated pathway with an exogenous signal has not been possible because the signal for this pathway remains unknown. However, the mutant

Fig. 1. Location of D-motifs in the Gα5 subunit. (a) Alignment of D-motifs in Gα subunits. Primary sequences of the yeast (Sc) Gpa1, *Dictyostelium* (Dd) Gα5, Gα3, Gα11, Gα2 and Gα4, and human (Hs) Gα16 subunits are shown, starting at the indicated residue number. The consensus sequence for D-motifs is indicated in the top row, with basic (+), hydrophobic (h) and nonconserved (x) residues. D-motif residues in Gα subunits are shaded. Sequence information for the *Dictyostelium* Gα subunits was obtained using dictyBase (<http://www.dictybase.org/>). (b) Modelling of Gα5 subunit structure and D-motifs. A structural model of the Gα5 subunit was created using the SWISS-MODEL workspace and the PyMOL program, and the structure was modelled using the determined structure of the mammalian heterotrimeric G protein consisting of Gα1, Gβ1 and Gγ2 subunits with bound GDP. The Gα5 (green), Gβ (blue) and Gγ (magenta) subunits are shown with respect to the membrane surface. The amino-terminal (N-term) and internal D-motifs of the Gα5 subunit are indicated (red) on the Gα5 subunit.

Fig. 2. Survival of wild-type, *erk1* and *erk2* cells transformed with *Gα5* subunit expression vectors. The strains were electroporated in parallel experiments with equal concentrations of *Gα5*, *Gα5^{d-}*, *Gα5^{*}* and *Gα5^{d-*}* expression vectors and transformants were selected at 3 µg G418 ml⁻¹. The number of viable transformants in different sections of the transformation plates was counted after 10–14 days of selection. Data are the mean ± SD from four different plate sections. Results are representative of three independent electroporations. Transformation results of *gx5* cells were similar to those of wild-type cells, and strains electroporated without DNA did not produce viable transformants (data not shown).

Gα5^{Q198L} (*Gα5^{*}*) subunit contains an alteration known to impair the GTPase activity of *Gα* subunits resulting in a constitutively active *Gα* subunit (Hadwiger *et al.*, 1996). Expression of the *Gα5^{*}* subunit typically resulted in 10-fold fewer viable transformants compared with the expression of the wild-type *Gα5* subunit, consistent with earlier studies

that suggest *Gα5* subunit activation is detrimental to vegetative growth (Fig. 2). Combining the D-motif mutations with the constitutively active mutation resulted in a *Gα5^{d-*}* subunit expression vector that typically generated 100-fold more viable transformants than the *Gα5^{*}* vector, suggesting that the D-motif is important for the lethal effect of the *Gα5^{*}* subunit on vegetative cells. The increased viability of cells expressing the *Gα5^{d-*}* subunit also correlated with the ability of clonal transformants to survive at higher G418 concentrations (Supplementary Table S1).

***erk1* cells are not sensitive to *Gα5* expression vectors**

The increased yield in viable transformants with the *Gα5^{d-}* subunit expression vector in *gx5* and wild-type cells could possibly result from decreased interactions with MAPKs. To test this possibility, the various *Gα5* subunit mutants were transformed into *erk1* and *erk2* cells, and the numbers of viable transformants were determined after selection with 3 µg G418 ml⁻¹ (Fig. 2). *erk2* cells transformed with either the *Gα5* or *Gα5^{*}* subunit expression vectors gave very few viable transformants compared with those cells transformed with *Gα5^{d-}* or *Gα5^{d-*}* subunits. In contrast, *erk1* cells produced a similar number of transformants with all of the *Gα5* subunit expression vectors, suggesting that these cells are insensitive to the detrimental effects of the *Gα5* and *Gα5^{*}* subunits. All *erk1* transformants and the *erk2* cells expressing the *Gα5^{d-}* or *Gα5^{d-*}* subunits were also resistant to higher drug selection (>10 µg G418 ml⁻¹). These results indicate that ERK1 but not ERK2 is required for the lethality associated with overexpression of the *Gα5* or *Gα5^{*}* subunits, implying that ERK1 functions downstream of the *Gα5* subunit-mediated signalling pathway during vegetative growth.

***Gα5* subunit inhibition of folate chemotaxis does not require the amino-terminal D-motif**

Overexpression of the *Gα5* subunit has been shown to inhibit chemotaxis to folate, indicated by the increased chemotactic movement of *gx5* cells and the reduced chemotactic movement of cells overexpressing the *Gα5* subunit (Natarajan *et al.*, 2000). Clonal transformants overexpressing the *Gα5* subunit were inhibited in their chemotactic movement to folate, similar to that previously reported; cells overexpressing the *Gα5^{d-}* subunit were also capable of inhibiting chemotaxis (Fig. 3). The level of chemotaxis inhibition correlates with *Gα5* subunit expression level and so clonal transformants with similar levels of *Gα5* and *Gα5^{d-}* subunit expression were compared in the chemotaxis assay and all subsequent analyses. Increasing the *Gα5^{d-}* subunit expression by using higher levels of drug selection did not significantly change the growth or developmental phenotypes. Cells expressing the *Gα5^{*}* or *Gα5^{d-*}* subunit were also inhibited with respect to folate

Fig. 3. Chemotaxis of wild-type and G α 5 mutant cells to folate. Chemotaxis assays were conducted as described in Methods and measurements were determined for the distance travelled by the leading edge of cells. Measurements are the maximum distance of cell migration from the edge of the original cell droplet to the leading edge of cells migrating toward the source of folate after 3 h. Chemotaxis of wild-type cells with no expression vector (WT) and *gα5* cells with no expression vector, wild-type G α 5 subunit expression vector, G α 5^{d-} subunit expression vector, G α 5* subunit expression vector or G α 5^{d-,*} subunit expression vector is shown. The chemotaxis data are the mean $\pm$ SD of six individual assays of representative clones. RNA gel blots of G α 5 and G α 5^{d-} expression in clones used for chemotaxis and other assays are shown (inset). Each lane was loaded with 8 μ g total RNA and the blot was hybridized with a G α 5 probe (lanes are from the same blot).

chemotaxis, indicating that the altered amino-terminal D-motif does not eliminate chemotaxis inhibition.

Reduction of large cell phenotype by G α 5 subunit D-motif mutants

Cells lacking the G α 5 gene typically appear larger than wild-type cells when attached to culture plates, suggesting that the mutant cells are either larger or more capable of extensive spreading (Fig. 4). To quantify cell size differences, wild-type and *gα5* cells carrying various G α 5 subunit expression vectors were counted and then analysed for cell weight and protein content. The large cell phenotype of *gα5* cells is reversed when the G α 5 subunit is overexpressed in these cells. Overexpression of the G α 5^{d-} subunit decreased cell size to near that of wild-type cells but not to the extremes observed for G α 5 subunit expression, indicating that the alteration to the amino-terminal D-motif reduces but does not eliminate the ability of the G α 5 subunit to decrease cell size. Expression of the G α 5* or G α 5^{d-,*} subunits in *gα5* cells also reduced cell size to nearly that of wild-type cells.

Fig. 4. Wild-type and G α 5 mutant cell sizes. Cells were grown in HL5 medium on plastic Petri dishes and photographed using an inverted phase-contrast microscope at $\times 200$ magnification (a). Sizes of (i) wild-type cells with no expression vector, (ii) *gα5* cells with no expression vector, (iii) *gα5* cells with wild-type G α 5 subunit expression vector, (iv) *gα5* cells with G α 5^{d-} subunit expression vector, (v) *gα5* cells with G α 5* subunit expression vector and (vi) *gα5* cells with G α 5^{d-,*} subunit expression vector are shown. (b) Comparisons of cell pellet weights (open bars) and protein content (closed bars) were conducted as described in Methods to assess differences in cell size. Cultures of vegetative wild-type (WT) and *gα5* cells with no vector (none) or a vector expressing one of the various G α 5 subunits (G α 5, G α 5^{d-}, G α 5* or G α 5^{d-,*}) were analysed to determine cell density and then assayed for pellet weight (wet) and protein content. Data are the mean $\pm$ SD of three different measurements.

Gα5 subunit amino-terminal D-motif is important for early tip formation

Expression of the wild-type Gα5 subunit in *gα5* cells resulted in noticeably smaller aggregates with precocious tip formation (9 h into development) compared with the aggregates formed by wild-type or *gα5* cells (Fig. 5). This same level of accelerated development was not observed for *gα5* cells expressing the Gα5^{d-} subunit, but expression of the Gα5^{d-} subunit does correct for the delayed development observed for *gα5* cells. Expression of the Gα5^{*} subunit in *gα5* cells resulted in a greater delay in aggregate formation than the delay observed for *gα5* cells with no expression vectors. This phenotype is probably due to an inhibition of cAMP chemotaxis previously observed for cells expressing constitutively active Gα5^{*}, Gα4^{*} or Gα2^{*} subunits (Srinivasan *et al.*, 1999). The expression of the Gα5^{d-*} subunit did not delay the formation of aggregates by *gα5* cells, suggesting that the altered D-motif reduces the aggregation delay associated with the Gα5^{*} subunit. Therefore, the developmental morphologies associated with elevated and constitutive Gα5 subunit activity were diminished when the amino-terminal D-motif was altered, suggesting MAPK interactions might be important for the timing of these developmental processes.

Gα5 subunit amino-terminal D-motif affects the timing of gene expression during development

The importance of the Gα5 subunit D-motif in developmental morphology and the established role of MAPKs in gene regulation suggest that the D-motif might also contribute to the regulation of developmental gene expression. To examine developmental gene expression, RNA was isolated from *gα5* cells that express wild-type or mutant Gα5 subunits during tip formation (9–15 h after the onset of starvation) (Fig. 6). Expression of the prespore-specific gene *cotC* was delayed in *gα5* cells and slightly accelerated in cells expressing the wild-type Gα5 subunit compared with wild-type cells, consistent with the results from previous studies (Hadwiger *et al.*, 1996). Cells containing the Gα5^{d-} or Gα5^{d-*} subunit expressed *cotC* with kinetics similar to *gα5* cells, suggesting that the Gα5 amino-terminal D-motif is important for the early onset of prespore-specific gene expression. The expression of the *cotC* gene in cells expressing the Gα5^{*} subunit was similar to that of wild-type cells, even though these cells have a delay in aggregation. The effect of wild-type and mutant Gα5 subunits on the expression of the prestalk-specific gene, *ecmA*, was much more subtle than that observed for the prespore gene. Only slight differences were observed in the timing and level of *ecmA* expression.

DISCUSSION

The identification of a D-motif in the amino terminus of the Gα5 subunit that impacts vegetative and developmental functions suggests that Gα5 subunit–MAPK interactions

Fig. 5. Developmental morphology of wild-type and *gα5* cells with or without Gα5 subunit expression vectors. Cells were grown in axenic medium and washed with phosphate buffer and then cell suspensions (2×10^7 cells ml^{-1}) were plated for development as described in Methods. Developing cell aggregates of wild-type cells (WT) and *gα5* cells with no expression vector (none) or a vector expressing the Gα5, Gα5^{d-}, Gα5^{*} or Gα5^{d-*} subunit were photographed 9 and 11 h after initial plating using a digital camera and dissecting microscope ($\times 20$ magnification). The inset in the 9 h image of Gα5-expressing cells is a portion of this image magnified $\times 2$. Arrows indicate representative mounds with tip development.

contribute to signalling pathways in *Dictyostelium*. The molecular modelling of the Gα5 subunit predicts that the position of the amino terminal D-motif is near the Gβγ dimer and receptor binding sites, suggesting that this motif

Fig. 6. Developmental gene expression in wild-type and *ga5* cells with or without G α 5 subunit expression vectors. Cells were grown in axenic medium and washed with phosphate buffer and then cell suspensions (1×10^8 cells ml^{-1}) were plated for development as described in Methods. Total RNA was isolated from cells at the times indicated (h after starvation) and subjected to RNA blot analysis using *cotC* (prespore-specific) or *ecmA* (pre-stalk-specific) probes as described in Methods. Each lane was loaded with 8 μg total RNA. UV shadowing of an rRNA band shown as loading control (under each blot). All lanes from each panel were run on the same gel and then blotted and hybridized together.

might not be available for MAPK interactions when the G α subunit is inactive and bound by the G $\beta\gamma$ dimer and receptor. Support for this concept is provided by the increase in Gpa1–Fus3 interactions when yeast cells are stimulated by mating pheromone (Metodieff *et al.*, 2002). However, molecular modelling of the G α 5 subunit structure predicts that the internal D-motifs of G α 5 might be available in the inactive state, and this is supported by the interactions of the internal D-motif of the G α 4 subunit with ERK2 in the absence of pathway activation (Nguyen & Hadwiger, 2009). Attempts to analyse the G α 5 subunit and ERK1 interactions in *Dictyostelium* have been hampered by the inability to express sufficient levels of epitope-tagged wild-type and constitutively active G α 5 subunits in *Dictyostelium*, presumably due to the lethal effects of these subunits (H.-N. Nguyen and J. A. Hadwiger, data not shown).

The requirement of the amino-terminal D-motif for the lethality associated with G α 5 subunit suggests that MAPK interactions are important for this phenotype; this concept is further supported by the specific requirement of ERK1 for this phenotype. In addition, ERK1 expression vectors can also affect cell viability (Nguyen *et al.*, 2010). The mechanism(s) responsible for the loss of viability due to G α 5 or ERK1 function remains to be determined but this mechanism could possibly involve a loss in nutrient uptake similar to that accompanying the transition from vegetative growth to multicellular development.

The amino-terminal D-motif of the G α 5 subunit is important for the formation of small aggregates with precocious tip development and cell-type-specific gene expression. The G α 5 subunit-mediated tip development might also require ERK1 function because *erk1* aggregates are often delayed or blocked in tip development, and overexpression of the G α 5 subunit in *erk1* cells does not accelerate tip development (Nguyen *et al.*, 2010). The spatial expression patterns of the G α 5 subunit and ERK1 appear to be very similar, with both proteins expressed

from anterior-like cells found near the posterior of slugs (Gaskins *et al.*, 1994; Hadwiger *et al.*, 1996). However, the small aggregate size phenotype cannot be readily attributed to the activation of ERK1 function because the loss of ERK1 results in a similar phenotype. How the G α 5 subunit D-motif affects the timing of prespore-specific gene expression is also not clear, but MAPKs often contribute to the regulation of gene expression through the phosphorylation of cytoplasmic or nuclear substrates (Caunt *et al.*, 2006; Raman *et al.*, 2007). Previous studies implicate *Dictyostelium* ERK2 function in prespore gene expression and the indirect activation of cAMP-dependent protein kinase, a regulator of prespore gene expression (Hopper *et al.*, 1995; Maeda *et al.*, 2004; Mann & Firtel, 1993; Mann *et al.*, 1994). Therefore, the contribution of G α 5 subunit signalling through ERK2 or other regulatory components cannot be excluded.

The presence of D-motifs in the amino terminus of other G α subunits in *Dictyostelium*, yeast and mammalian cells implies that MAPK interactions with G α subunits might be widespread among eukaryotes. The requirement of ERK1 for G α 5 subunit-mediated phenotypes and the previously determined association of ERK2 and the G α 4 subunit suggests that interactions between G proteins and MAPKs might be specific, but we cannot exclude the possibility of other signalling combinations (Nguyen & Hadwiger, 2009). Variations in D-motif sequence and location might contribute to the specificity of G α subunit–MAPK interactions, and these interactions are likely to play an important role in signalling specificity downstream of G protein-coupled receptors.

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