Gz5 subunit-mediated signalling requires a D-motif and the MAPK ERK1 in Dictyostelium

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The Dictyostelium Gz5 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 Gz5 subunit or the loss of extracellular signal-regulated kinase (ERK)1 diminished the lethality associated with the overexpression or constitutive activation of the Gz5 subunit. The amino-terminal D-motif of the Gz5 subunit was also found to be necessary for the reduced cell size, small aggregate formation and precocious developmental gene expression associated with Gz5 subunit overexpression. This D-motif also contributed to the aggregation delay in cells expressing a constitutively active Gz5 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 Gz5 subunit functions during growth and development and that ERK1 can function in Gz5 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 Gz5 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 Gz subunits suggests that the functional specificity of the Gz5 subunit is not limited to the specificity of receptor coupling but requires interactions with other signalling components (Hadwiger, 2007). The Gz5 subunit primary sequence is closely related to the Gz4 subunit, a subunit required for folate chemotaxis, except for a region near the amino terminus of the Gz5 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 Gz5-specific responses (Hadwiger, 2007; Remenyi et al., 2005). An interaction between the amino-terminal D-motif of a Gz subunit and a MAPK has been reported previously in the budding yeast Saccharomyces cerevisiae, where the interaction of the Gpa1 Gz subunit and the Fus3 MAPK facilitates the Gz 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 Gz4 subunit has recently been shown to be important for interactions with the extracellular signal-regulated kinase (ERK)2 and for Gz4 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...
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 Ga5 subunit was explored by analysing the phenotypes of Ga5 subunits with alterations in this D-motif. Growth and developmental phenotypes associated with Ga5 subunit overexpression or constitutive activation were found to depend on the amino-terminal D-motif. Sensitivity to elevated Ga5 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 Ga5 subunit-mediated signalling. The Ga5 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 Ga5 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 ga5 strain (JH257) has been described previously (Hadwiger et al., 1996). The erk2 strain (JH697) was created from the JHB strain using the pAK240Ci strain disruption construct, described as previously (Segall et al., 1995). The pAK240Ci 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 blastocidin S resistance gene, as described by Nguyen et al. (2010). Cells were grown in axenic HIL5 medium or on bacterial lawns (Watts & Ashworth, 1970). DNA vectors were electrotransferred 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 Ga5 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 Ga5 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 Ga5 subunit D-motif, Ga5N20E5H11E subunit (designated Ga5N20E5H11), were created in a HindIII/PstI fragment of the Ga5 gene in the vector pT7T318U (Pharmacia) by PCR mutagenesis of the entire vector using the following overlapping oligonucleotides: sense strand, 5'-gcgccagACTGAgATTAATAAAAGAAGAAAGAAG, and anti-sense strand, 5'-gcgcgctCGATGTCTTGGATCTCTGTTCAATTTGTAATACAAACGA (lower case nucleotides differ from those of the template sequence). These mutations were confirmed by sequence analysis and this mutant segment of Ga5 was used to replace the same segment of the wild-type Ga5 gene in the expression vectors. The constitutively active Ga5Q198L (designated Ga5*) subunit expressed from the act15 promoter (pBR515) was created by replacing a PstI/Bcl fragment of the wild-type Ga5 constructs with a fragment from a Ga5Q198L gene previously created in a genomic fragment (Hadwiger et al., 1996). This mutation was also combined with the mutations in the Ga5 D-motif to create the combined mutant Ga5N20E5H11* subunit expression vector (pBR538). To create this gene, the HindIII/PstI fragment of the Ga5* gene was used to replace the beginning of the Ga5 ORF. All Ga5 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 Ga5 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 Ga5 expression vectors, plated onto solid HIL5 medium, and after 24 h, the cells were selected by using G418 from 1 to 10 μg 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. 2–3×10⁶ cells ml⁻¹), washed twice in phosphate buffer (12 mM NaH₂PO₄, adjusted to pH 6.1 with KOH), and suspended in phosphate buffer (1×10⁶ or 2×10⁶ cells ml⁻¹), before spotting on non-nutrient plates (phosphate buffer, 1.5% agar) for development. Alternatively, cells were spotted on 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 μl) of cell suspensions (10⁶ cells ml⁻¹) on non-nutrient plates, allowing absorption of liquid into the agar, followed by the spotting of 1 μl 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 scoring the agar surface with a needle.

**RESULTS**

**Docking site motifs in Ga5 subunits**

A region near the amino terminus of the Ga5 subunit was identified as a potential site of functional specificity due to sequence diversity with respect to the Ga2 and Ga4.
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 MAPKs 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, Gα5K10E, K11E (Gα5Δ–) 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 Gα5Δ– 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 Gα5Δ– subunit structure and D-motif alterations. Electroporation of the Gα5Δ– 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).

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...
that suggest Gz5 subunit activation is detrimental to vegetative growth (Fig. 2). Combining the D-motif mutations with the constitutively active mutation resulted in a Gz5D→* subunit expression vector that typically generated 100-fold more viable transformants than the Gz5* vector, suggesting that the D-motif is important for the lethal effect of the Gz5* subunit on vegetative cells. The increased viability of cells expressing the Gz5D→* subunit also correlated with the ability of clonal transformants to survive at higher G418 concentrations (Supplementary Table S1).

**Fig. 2.** Survival of wild-type, erk1 and erk2 cells transformed with Gz5 subunit expression vectors. The strains were electroporated in parallel experiments with equal concentrations of Gz5, Gz5D→, Gz5* and Gz5D→* 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 gz5 cells were similar to those of wild-type cells, and strains electroporated without DNA did not produce viable transformants (data not shown).

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

The increased yield in viable transformants with the Gz5D→ subunit expression vector in gz5 and wild-type cells could possibly result from decreased interactions with MAPKs. To test this possibility, the various Gz5 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 Gz5 or Gz5* subunit expression vectors gave very few viable transformants compared with those cells transformed with Gz5D→ or Gz5D→* subunits. In contrast, erk1 cells produced a similar number of transformants with all of the Gz5 subunit expression vectors, suggesting that these cells are insensitive to the detrimental effects of the Gz5 and Gz5* subunits. All erk1 transformants and the erk2 cells expressing the Gz5D→ or Gz5D→* 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 Gz5 or Gz5* subunits, implying that ERK1 functions downstream of the Gz5 subunit-mediated signalling pathway during vegetative growth.

Gz5 subunit inhibition of folate chemotaxis does not require the amino-terminal D-motif

Overexpression of the Gz5 subunit has been shown to inhibit chemotaxis to folate, indicated by the increased chemotactic movement of gz5 cells and the reduced chemotactic movement of cells overexpressing the Gz5 subunit (Natarajan et al., 2000). Clonal transformants overexpressing the Gz5 subunit were inhibited in their chemotactic movement to folate, similar to that previously reported; cells overexpressing the Gz5D→ subunit were also capable of inhibiting chemotaxis (Fig. 3). The level of chemotaxis inhibition correlates with Gz5 subunit expression level and so clonal transformants with similar levels of Gz5 and Gz5D→ subunit expression were compared in the chemotaxis assay and all subsequent analyses. Increasing the Gz5D→ subunit expression by using higher levels of drug selection did not significantly change the growth or developmental phenotypes. Cells expressing the Gz5* or Gz5D→* subunit were also inhibited with respect to folate
chemotaxis, indicating that the altered amino-terminal D-motif does not eliminate chemotaxis inhibition.

**Reduction of large cell phenotype by Gα5 subunit D-motif mutants**

Cells lacking the Gα5 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 Gα5 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 Gα5 subunit is overexpressed in these cells. Overexpression of the Gα5 subunit decreased cell size to near that of wild-type cells but not to the extremes observed for Gα5 subunit expression, indicating that the alteration to the amino-terminal D-motif reduces but does not eliminate the ability of the Gα5 subunit to decrease cell size. Expression of the Gα5* or Gα5d−* subunits in gα5 cells also reduced cell size to nearly that of wild-type cells.

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**Fig. 3.** Chemotaxis of wild-type and Gα5 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 Gα5 subunit expression vector, Gα5d− subunit expression vector, Gα5* subunit expression vector or Gα5d−* subunit expression vector is shown. The chemotaxis data are the mean ± SD of six individual assays of representative clones. RNA gel blots of Gα5 and Gα5d− expression in clones used for chemotaxis and other assays are shown (inset). Each lane was loaded with 8 µg total RNA and the blot was hybridized with a Gα5 probe (lanes are from the same blot).

**Fig. 4.** Wild-type and Gα5 mutant cell sizes. Cells were grown in HL5 medium on plastic Petri dishes and photographed using an inverted phase-contrast microscope at ×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 Gα5 subunit expression vector, (iv) gα5 cells with Gα5d− subunit expression vector, (v) gα5 cells with Gα5* subunit expression vector and (vi) gα5 cells with Gα5d−* 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 Gα5 subunits (Gα5, Gα5d−, Gα5* or Gα5d−*) were analysed to determine cell density and then assayed for pellet weight (wet) and protein content. Data are the mean ± SD of three different measurements.
**Gz5 subunit amino-terminal D-motif is important for early tip formation**

Expression of the wild-type Gz5 subunit in gz5 cells resulted in noticeably smaller aggregates with precocious tip formation (9 h into development) compared with the aggregates formed by wild-type or gz5 cells (Fig. 5). This same level of accelerated development was not observed for gz5 cells expressing the Gz5d* subunit, but expression of the Gz5d* subunit does correct for the delayed development observed for gz5 cells. Expression of the Gz5* subunit in gz5 cells resulted in a greater delay in aggregate formation than the delay observed for gz5 cells with no expression vectors. This phenotype is probably due to an inhibition of cAMP chemotaxis previously observed for cells expressing constitutively active Gz5*, Gz4* or Gz2* subunits (Srinivasan et al., 1999). The expression of the Gz5d* subunit did not delay the formation of aggregates by gz5 cells, suggesting that the altered D-motif reduces the aggregation delay associated with the Gz5* subunit. Therefore, the developmental morphologies associated with elevated and constitutive Gz5 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.

**Gz5 subunit amino-terminal D-motif affects the timing of gene expression during development**

The importance of the Gz5 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 gz5 cells that express wild-type or mutant Gz5 subunits during tip formation (9–15 h after the onset of starvation) (Fig. 6). Expression of the prespore-specific gene cotC was delayed in gz5 cells and slightly accelerated in cells expressing the wild-type Gz5 subunit compared with wild-type cells, consistent with the results from previous studies (Hadhiger et al., 1996). Cells containing the Gz5d− or Gz5d−* subunit expressed cotC with kinetics similar to gz5 cells, suggesting that the Gz5 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 Gz5* 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 Gz5 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 Gz5 subunit that impacts vegetative and developmental functions suggests that Gz5 subunit–MAPK interactions contribute to signalling pathways in Dictyostelium. The molecular modelling of the Gz5 subunit predicts that the position of the amino terminal D-motif is near the Gβγ dimer and receptor binding sites, suggesting that this motif...
might not be available for MAPK interactions when the Gα subunit is inactive and bound by the Gβγ dimer and receptor. Support for this concept is provided by the increase in Gpa1–Fus3 interactions when yeast cells are stimulated by mating pheromone (Metodiev et al., 2002). However, molecular modelling of the Gα subunit structure predicts that the internal D-motifs of Gα might be available in the inactive state, and this is supported by the interactions of the internal D-motif of the Gα4 subunit with ERK2 in the absence of pathway activation (Nguyen & Hadwiger, 2009). Attempts to analyse the Gα5 subunit and ERK1 interactions in Dictyostelium have been hampered by the inability to express sufficient levels of epitope-tagged wild-type and constitutively active Gα5 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 Gα5 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 Gα5 or ERK1 function remains to be determined but this mechanism could possibly involve a loss of nutrient uptake similar to that accompanying the transition from vegetative growth to multicellular development.

The amino-terminal D-motif of the Gα5 subunit is important for the formation of small aggregates with precocious tip development and cell-type-specific gene expression. The Gα5 subunit-mediated tip development might also require ERK1 function because erkl aggregates are often delayed or blocked in tip development, and overexpression of the Gα5 subunit in erkl cells does not accelerate tip development (Nguyen et al., 2010). The spatial expression patterns of the Gα5 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 Gα5 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 Gα5 subunit signalling through ERK2 or other regulatory components cannot be excluded.

The presence of D-motifs in the amino terminus of other Gα subunits in Dictyostelium, yeast and mammalian cells implies that MAPK interactions with Gα subunits might be widespread among eukaryotes. The requirement of ERK1 for Gα5 subunit-mediated phenotypes and the previously determined association of ERK2 and the Gα4 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 Gα 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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