



Published in final edited form as:

Dev Biol. 2007 December 1; 312(1): 1–12.

Developmental morphology and chemotactic responses are dependent on G α subunit specificity in *Dictyostelium*

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Abstract

Dictyostelium discoideum expresses multiple G α subunits but only a single G β and G γ subunit suggesting the specific response to an external signal depends largely on G α subunit function or G protein-independent signaling from the receptor. To test the contribution of G α subunit functional specificity, the chimeric G α subunits, G α 2/4 and G α 5/4, were created and analyzed along with wild-type subunits for the ability to substitute for the G α 4 subunit in mediating responses from folate receptors. The G α 2/4 subunit, but not the G α 2 or G α 5/4 subunits, partly rescued chemotaxis and cGMP accumulation in folate-stimulated *ga4*⁻ cells. Expression of the G α 5/4 or G α 5 subunits resulted in an inhibition of *ga4*⁻ and wild-type cell movement and a reduced aggregate size in developing wild-type and *ga5*⁻ cells suggesting these subunits mediate similar responses. Only the G α 4 subunit was capable of correcting developmental morphology in *ga4*⁻ multicellular aggregates suggesting the chimeric G α 2/4 or G α 5/4 subunits were insufficient to provide the G α 4 function necessary for proper development. These results indicate *Dictyostelium* G α subunit specificity is not limited to receptor coupling and that G α subunit sequences outside of the carboxyl terminus are important for cell movement and developmental processes.

Keywords

signal transduction; G protein; receptor; *Dictyostelium*; development; chemotaxis; folate

Introduction

G protein-coupled receptors are capable of detecting a wide variety of extracellular signals that regulate many cellular activities, including metabolism, growth, differentiation and migration (Neves et al., 2002). Many responses are mediated through receptor activation of heterotrimeric G proteins allowing the G α subunit and G $\beta\gamma$ dimer to separate and interact with other signaling components. The defining roles for specific G protein subunits is complicated in many eukaryotes by the different potential combinations of G protein subunits that can form a heterotrimeric unit and the diversity of interactions between specific G protein subunits and other signaling components such as receptors and downstream effectors (Albert and Robillard, 2002). Furthermore, some receptor-stimulated responses can occur in the absence of G protein subunits, indicating that some signaling occurs independently of G proteins (Brzostowski and Kimmel, 2001; Luttrell, 2005). While seemingly complex, the assessment of G protein subunit

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specificity can benefit from the analysis of signal transduction in cells with a relatively low number of potential G protein subunit combinations.

In many systems, $G\alpha$ subunits have been demonstrated to be the critical determinants for receptor specificity whereas the $G\beta\gamma$ dimers often serve as the primary mediators of downstream signal transduction events that regulate cell division, chemotaxis, and gene regulation (Conklin et al., 1993; Hur and Kim, 2002). For instance, specific $G\alpha$ subunits are necessary for mammalian cell chemotaxis but the role of these subunits appears to be primarily for the release of $G\beta\gamma$ dimers from chemoattractant receptors (Neptune and Bourne, 1997; Neptune et al., 1999). Likewise, the over-expression of the $G\beta$ subunit in yeast activates cell cycle arrest and gene expression in the absence of mating pheromone and $G\alpha$ subunit function (Cole et al., 1990). These observations highlight the importance of $G\beta\gamma$ dimer release but question the significance of $G\alpha$ subunits in downstream signaling. However, some $G\alpha$ subunits are known to interact with downstream signaling components (e.g., adenylyl cyclases, phosphodiesterases, phospholipases, etc.) other than $G\beta\gamma$ dimers implying additional roles for $G\alpha$ subunits in at least some cellular responses (Offermanns and Simon, 1998).

The microbe *Dictyostelium discoideum* offers an excellent system for the genetic analysis of $G\alpha$ subunit specificity and function because the genome contains 12 different genes for $G\alpha$ subunits but only single genes for the $G\beta$ and $G\gamma$ subunits (Brzostowski and Kimmel, 2001; Wu et al., 1995; Zhang et al., 2001). Therefore, all the heterotrimeric G proteins in *Dictyostelium* are predicted to contain the same $G\beta\gamma$ dimer and this prediction is supported by phenotypic analyses of $g\beta^-$ mutants (Wu et al., 1995). Loss of some individual $G\alpha$ subunits (e.g., $G\alpha 2$, $G\alpha 4$, or $G\alpha 5$) results in a wide variety of developmental phenotypes indicating that the respective signal transduction pathways can regulate different cellular functions for the developmental life cycle of this organism (Brandon et al., 1997; Brzostowski et al., 2002; Hadwiger and Firtel, 1992; Hadwiger et al., 1996; Kumagai et al., 1991). The $G\alpha 4$ subunit couples with folate receptors, mediates the chemotactic movement to folate but not to cAMP, and promotes spore development (Hadwiger et al., 1994). Cells lacking the $G\alpha 4$ subunit can aggregate when starved but then terminate development prior to fruiting body formation with deficiencies in spore development (Hadwiger and Firtel, 1992). In contrast, the loss of the $G\alpha 5$ subunit results in enhanced chemotactic responses to folate and large aggregates with delayed anterior tip formation (Hadwiger et al., 1996; Natarajan et al., 2000). Over-expression of the $G\alpha 5$ subunit mildly inhibits responses to folate responses, decreases aggregate size, and accelerates tip formation whereas over-expression of the $G\alpha 4$ subunit increases aggregate size and inhibits anterior tip formation. While the signal that stimulates the $G\alpha 5$ -mediated pathway remains to be identified it appears to promote developmental processes opposite of those promoted by the $G\alpha 4$ -mediated pathway. The $G\alpha 2$ subunit couples to cAMP receptors, mediates chemotaxis to cAMP but not to folate, and allows for chemotactic aggregation of starved cells (Kumagai et al., 1991). The $G\alpha 2$ subunit is also likely to function after aggregation as suggested by the developmental expression of this subunit and the requirement for cAMP receptors in later stages of development (Firtel, 1996). The $G\alpha 2$ and $G\alpha 4$ subunits are required for the accumulation of both cGMP and cAMP in response to external signals but the accumulation of at least cAMP is thought to be mediated through the release of $G\beta\gamma$ dimers (Hadwiger et al., 1994; Kumagai et al., 1991; Wu et al., 1995). The mutant phenotypes resulting from the absence or over-expression of $G\alpha$ subunits indicate differences between signaling pathways but do not determine a specific role for $G\alpha$ subunits in downstream signaling because the functions of the receptor and $G\beta\gamma$ dimer can be dependent on specific $G\alpha$ subunit-receptor coupling. Direct interactions of between G protein subunits with downstream signaling components have not been established in *Dictyostelium*.

Many previous studies have shown that the carboxyl terminus of $G\alpha$ subunits is the primary determinant of receptor coupling (Brown et al., 2000; Conklin et al., 1993; Conklin et al.,

1996; Coward et al., 1999; Neptune et al., 1999). Chimeric $G\alpha$ subunits with changes in the last several amino acid residues at the carboxyl terminus (typically within the last 12 residues) are sufficient to alter receptor specificity allowing $G\alpha$ subunits to gain functional interactions with different mammalian hormone receptors or yeast mating type receptors. In one case, a chimeric $G\alpha_q$ subunit with a carboxyl terminus resembling that of $G\alpha_i$ subunits was shown to mediate chemotaxis to signals that stimulate $G\alpha_i$ -specific receptors (Neptune and Bourne, 1997). In this study, *Dictyostelium* $G\alpha$ subunit function and specificity were examined using the chimeric $G\alpha$ subunits, $G\alpha_{2/4}$ and $G\alpha_{5/4}$, that contain a carboxyl terminus specific to the $G\alpha_4$ subunit so as to allow the chimeric subunits to interact with $G\alpha_4$ -specific receptors. These chimeric $G\alpha$ subunits, along with wild-type $G\alpha$ subunits, were tested for the ability to compensate for the absence of the $G\alpha_4$ subunit with respect to developmental morphogenesis, chemotaxis, and cGMP accumulation. The $G\alpha$ subunits were also analyzed for the ability to rescue the loss of $G\alpha$ specific function in $ga2^-$ and $ga5^-$ cells. The results of this study support critical roles for $G\alpha$ subunits in chemotaxis and development in addition to the release of the $G\beta\gamma$ dimers from specific receptors.

Materials and methods

Strains and Media

Analysis of $G\alpha$ subunit function was conducted in the *Dictyostelium* strain KAx-3 and $G\alpha$ subunit gene disruption mutants derived from this strain. The creation of $ga4^-$, $ga2^-$, and $ga5^-$ strains was previously described (Hadwiger and Firtel, 1992; Hadwiger et al., 1996; Kumagai et al., 1991). *Dictyostelium* were grown in axenic medium HL5 or on bacterial lawns (Watts and Ashworth, 1970). DNA vectors were electroporated into *Dictyostelium* as previously described except the healing solution treatment omitted (Dynes and Firtel, 1989). Transformed cells were selected and maintained in medium containing 4–10 $\mu\text{g/ml}$ of the drug G418 but cells were grown in the absence of the drug at least 18 hours prior to analysis. Folate solutions were prepared by neutralizing folic acid with NaHCO_3 .

Recombinant DNA Constructs and Nucleic Acid Detection

Chimeric $G\alpha$ subunit genes were created by PCR amplification of cloned $G\alpha$ subunit genomic or cDNA sequences using oligonucleotides with *Hind*III (upstream) or *Xba*I (downstream) restriction sites. The upstream oligonucleotide used in the amplification of $G\alpha_2$ and $G\alpha_{2/4}$ subunits was (gccggcaagCTTAAAAAATGGGTATTTGTGCATCATCAATGGAAGGAG) and the downstream oligonucleotides were (cggcgctctagaTTAAGAATATAAACCAGCTTTCATAACACATTG) and (cggcgctctagattagaagtgttctaatgcttgagatAAAATAATATCTTTTACAGCTTCAAAAACG), respectively (lower case nucleotides differ from those in the template DNA). The upstream oligonucleotide used in the amplification of the $G\alpha_5$ and $G\alpha_{5/4}$ subunits was (gccggcaageTTAAAAAGATGGGTTGTATATTAACAATTGAAGCAAAGAAATC) and the downstream oligonucleotides were (cggcgctctagaTTAATAATTTATGATTGTATTAAGATATTTTTTAATAAAG) and (cggcgctctagattagaagtgttctaatgcttgagaTAATAAAGTTTCTCTAACAGCATGAAATAC), respectively. The upstream and downstream oligonucleotides used in the amplification of the $G\alpha_4$ subunit were (gccggcaagcttAAAAAATGAGATTCAAGTGTGGATCAGAAGAAACTG) and (cggcgctctagaTTAGAAGTGTCTAAAtGCTTGAGATAAAATTGTTTGTCTAAC), respectively. All of the downstream oligonucleotides coding for the $G\alpha_4$ subunit carboxyl terminus had a silent mutation to remove a *Hind*III site to facilitate the cloning of the PCR products. Each amplified $G\alpha$ subunit gene was sequenced to screen out any PCR-related mutations and then inserted into the *Dictyostelium* expression vector pDXA-GFP2 (*Ddp2*-based plasmid), replacing the *Hind*III/*Xba*I fragment that contained the *GFP2* reading frame

downstream of the *act15* promoter (Levi et al., 2000). These vectors integrate into the genome unless the cell also contains the pREP vector. All *pact15::Ga* gene constructs were also transferred as *Sall/XbaI* fragments into the *Ddp1*-based pTX-GFP vector replacing the *pact15::GFP2* gene (Levi et al., 2000). Each *Ga* subunit expression vector was electroporated into *Dictyostelium* and multiple transformants were examined for folate chemotaxis, growth on bacterial lawns, and multicellular development. The *Ddp2*-based *Ga* subunit expression vectors were also co-electroporated with the pREP vector allowing the *Ddp2*-based vectors to be maintained as extrachromosomal plasmids. Total RNA was isolated from *Dictyostelium* and then RNA blots and hybridizations were performed as described (Hadwiger et al., 1996).

Development and chemotaxis assays

Dictyostelium were grown to mid-log phase (approximately 2×10^6 cells/ml), washed twice in phosphate buffer (12 mM NaH_2PO_4 adjusted to pH 6.1 with KOH), and suspended in phosphate buffer before spotting on nonnutrient plates (phosphate buffer, 1.5% agar) for development or chemotaxis as described (Hadwiger et al., 1996). Chemotaxis assays were performed by spotting droplets of cell suspensions (10^7 to 10^8 cell/ml) on non-nutrient plates followed by the spotting of 1 μl droplets of folate solutions (10^{-2} – 10^{-4} M) approximately 2–3 mm away from the cell droplet. Cell movement was monitored with a dissecting microscope and a digital camera. Cell density and folate concentrations were varied experiments but all strains were treated identically for each experiment.

Cyclic GMP assays

Dictyostelium were grown to mid-log phase (approximately 2×10^6 cells/ml), washed twice in phosphate buffer and suspended in phosphate at 5×10^7 cells/ml. Cell suspensions were bubbled with air for 10 minutes prior to treatment with 100 μM folate. At times indicated cellular responses were terminated by the addition of perchloric acid and then the samples were neutralized with ammonium sulfate as described (Hadwiger et al., 1994). The concentration of cGMP in each sample was determined using a radioimmunoassay kit (Amersham).

Results

Creation and expression of chimeric and wild-type *Ga* subunits

The distinct chemotactic and developmental phenotypes associated with the loss of either *Ga2*, *Ga4*, or *Ga5* subunits in *Dictyostelium* suggest these subunits and/or their respective coupled-receptors provide signaling specificity in chemotactic and developmental pathways but the extent of *Ga* subunit contribution to the specificity of these pathways beyond receptor coupling has not previously been determined. Sequence comparison between the *Dictyostelium* *Ga2*, *Ga4* and *Ga5* subunits indicates a slightly greater overall sequence similarity between the *Ga4* and *Ga5* subunits and this similarity is also indicated by a phylogenetic analysis of *Dictyostelium* *Ga* subunits (Fig. 1A and B) (Chisholm et al., 2006; Eichinger et al., 2005). However, the *Ga4* and *Ga2* subunits share some identity between residues that are distinct from those found at the same positions in the *Ga5* subunit, including a cluster of residues near the amino terminus. All three subunits share considerable identity with each other in regions highly conserved among all *Ga* subunits (Simon et al., 1991). Each of these *Dictyostelium* *Ga* subunits differs from each other in the last several residues of the carboxyl terminus consistent with the coupling of these subunits to different receptors. All of the *Dictyostelium* *Ga* subunits are expected to interact with the same $\text{G}\beta\gamma$ dimer because only single genes for these subunits are found in BLAST searches of the sequenced *Dictyostelium* genome (Eichinger et al., 2005).

To determine if regions other than the carboxyl terminus of *Dictyostelium* *Ga* subunits provide functional specificity, the carboxyl terminus of the *Ga2* and *Ga5* subunits were replaced with

the corresponding sequence specific to the $G\alpha 4$ subunit (Fig. 1C). In these chimeric $G\alpha$ genes, only the sequence coding for the last 12 residues of the $G\alpha 2$ subunit and the last 8 residues of the $G\alpha 5$ subunit were replaced with the corresponding $G\alpha 4$ sequence because the adjacent sequences coded for conserved residues (Brown et al., 2000). To eliminate distinctions in $G\alpha$ subunit expression, each chimeric subunit gene, as well as each wild-type subunit gene, was inserted into a *Dictyostelium Ddp1*-based expression vector downstream of the *act15* promoter. Others have shown gene expression from this vector to be relatively homogeneous among individual cells within a clonal population of cells (Levi et al., 2000). RNA blot analysis indicated the expression level of the $G\alpha$ subunit transcripts was abundant for all constructs in this *Ddp1*-based expression vector (Fig. 2). Expression of these $G\alpha$ subunit genes from integrating *Ddp2*-based vectors gave similar phenotypes but with more variability in the strength of the phenotype from clone to clone suggesting that differences in gene copy number might be the basis for such variability (data not shown except where noted).

Analysis of developmental morphology

Expression of $G\alpha$ subunit genes from a heterologous promoter (e.g., *act15*) rather than $G\alpha$ -specific promoters could potentially alter the spatial and temporal distributions of $G\alpha$ subunits during development and possibly affect the ability of the $G\alpha$ expression vectors to rescue phenotypes in $G\alpha$ subunit mutants. Therefore, $G\alpha$ subunit expression vectors were introduced into strains with $G\alpha$ subunit gene knockout strains ($ga2^-$, $ga4^-$, or $ga5^-$ mutants) and then examined for the ability to complement $G\alpha$ subunit-specific developmental phenotypes. Only the $G\alpha 4$ subunit expression vector complemented $ga4^-$ cells restoring wild-type morphological development (Fig. 3). The $G\alpha 2/4$, $G\alpha 2$, $G\alpha 5/4$, or $G\alpha 5$ subunit expression vectors failed to complement or noticeably change the terminal $ga4^-$ morphology of arrested aggregates with extended tip structures. The $G\alpha 2$ but not the $G\alpha 2/4$ subunit expression vector was capable of restoring aggregate formation and fruiting body development in $ga2^-$ cells (Fig. 4). The formation of cell aggregates was slightly delayed but subsequent morphological development was similar to wild-type aggregates. Consistent with this result, $ga2^-$ cells carrying the $G\alpha 2$ but not the $G\alpha 2/4$ subunit expression vector were capable of chemotactic movement to cAMP (data not shown). The $G\alpha 5$ and $G\alpha 5/4$ subunit expression vectors were both capable of rescuing the large aggregate phenotype of $ga5^-$ cells and producing a precocious tip development phenotype associated with the over-expression of $G\alpha 5$ function (Fig. 5). The $G\alpha 5$ and $G\alpha 5/4$ subunit expression vectors also produced similar small aggregate and precocious tip development phenotypes in $ga4^-$ and wild-type cells (data not shown). The ability of $G\alpha 5/4$ subunit to provide phenotypes similar to the $G\alpha 5$ subunit suggested that $G\alpha 5/4$ subunit might be activated by receptors that recognize the $G\alpha 4$ -specific carboxyl terminus.

Chemotaxis to folate

To examine $G\alpha$ subunit specificity in cellular responses, $ga4^-$ and wild-type cells with or without $G\alpha$ subunit expression vectors were assayed for chemotaxis to folate. Chemotaxis assays were conducted on a nonnutrient agar surface where droplets of cells and folate solution are placed in close proximity of each other resulting in the chemotactic migration of cells toward the source of folate (Fig. 6A). The chemotaxis index (provided as a percentage) in this assay was defined as the number of cells migrating toward the source of folate divided the total number of cell migrating away from the original cell droplet location. Using this definition, chemotactic cells are expected to have a chemotaxis index significantly greater than 50% whereas cells without chemoattractant-directed movement are expected to have a chemotaxis index of near 50%. Only $ga4^-$ cells carrying the $G\alpha 4$ or $G\alpha 2/4$ subunit expression vectors displayed a chemotaxis index substantially larger than 50% suggesting that the other $G\alpha$ subunit vectors were not capable of rescuing chemotactic movement (Fig. 6B). The distance traveled by cells expressing the $G\alpha 2/4$ subunit was noticeably less than that exhibited by cells expressing the $G\alpha 4$ subunit. This reduced migration distance of cells expressing the $G\alpha 2/4$ subunit could

possibly be due to a decrease in cell speed across the substratum and/or an increase in cell turning. To examine these possibilities, cell movement during chemotaxis assays was monitored by time-lapsed photography and paths of individual cells were mapped over a 30 min period (Fig 6C). Cells were plated at very low densities and only cells nearest to the source of folate were monitored. Only a few cells were examined in each strain but the migration distance of cells expressing the $G\alpha 4$ subunit appeared to be much greater at each interval (5 minutes) than that observed for all other strains, regardless of the direction of movement. Cells expressing the $G\alpha 2/4$ subunit migrated distances similar to cells without a $G\alpha$ subunit expression vector and cells expressing the $G\alpha 5/4$ subunit traveled the shortest distances each interval. In general, the paths observed for each strain appeared relatively similar with respect to changes in cell direction suggesting that cell speed is likely to account for much of the differences in cell migration distance. The maximum distance traveled by each strain toward the folate source was also examined by measuring the distance from the original cell droplet perimeter to the leading edge of cells after a specific length of time. The distance traveled by $ga4^-$ cells with the $G\alpha 4$ subunit was substantially greater than that traveled by the other strains consistent with the $G\alpha 4$ subunit providing a greater migratory speed than the other $G\alpha$ subunits (Fig. 7A). The distance traveled by cells expressing the $G\alpha 5/4$ or $G\alpha 5$ subunit was less than that observed for $ga4^-$ cells without a $G\alpha$ subunit expression vector suggesting these subunits actually inhibit cell migration. This inhibition of cell migration was also observed for wild-type cells with either the $G\alpha 5/4$ or $G\alpha 5$ subunit expression vector (Fig. 7B). The $G\alpha 2/4$ or $G\alpha 2$ subunit expression vectors only had marginal effects on the distance traveled by wild-type cells.

Some *Dictyostelium* mutants, such as $ga4^-$ or $g\beta^-$ cells, do not chemotax to folate and they also form small plaques with slow expansion rates when grown on bacterial lawns (Hadwiger et al., 1994; Wu et al., 1995). These mutants also have less accumulation of cells on the plaque perimeter, probably reflecting a reduced capacity to migrate toward the bacterial food source surrounding the plaque. Several clones of wild-type cells expressing the $G\alpha 5/4$ subunit from the integrating *Ddp2*-based vector displayed a reduction in the accumulated cells on the plaque perimeter and slightly retarded plaque expansion rate as compared to wild-type cells without a $G\alpha$ subunit expression vector (Fig. 8A). These phenotypes were not observed for wild-type cells carrying any of the other $G\alpha$ subunit expression vectors, including the $G\alpha 2/4$ subunit expression vector. The impact of the $G\alpha 5/4$ subunit on cell accumulation at the plaque perimeter is consistent with the ability of this $G\alpha$ subunit to inhibit chemotactic responses to folate. However, cells expressing the $G\alpha 5/4$ subunit were capable of aggregating and completing fruiting body development (Fig. 8B) with viable spores whereas aggregates of $ga4^-$ cells terminate development with aberrant morphology and a severe reduction in viable spores. The slow plaque growth rate, reduced cell accumulation on the perimeter, and aberrant developmental morphology of $ga4^-$ cells on bacterial lawns was not altered by the expression of the $G\alpha 5/4$ or $G\alpha 2/4$ subunit suggesting neither subunit can provide sufficient $G\alpha 4$ subunit function to correct these phenotypes (Fig. 8A).

cGMP accumulation

Folate stimulates a rapid accumulation of cGMP in *Dictyostelium* and this response is dependent on $G\alpha 4$ subunit function (Fig. 9). Expression of the $G\alpha 2/4$ but not the $G\alpha 2$ subunit partly restores this response in $ga4^-$ cells but the rise in cGMP concentration was much less than that observed for wild-type cells. While lower in amplitude, the accumulation of cGMP in $ga4^-$ cells expressing the $G\alpha 2/4$ subunit occurred with kinetics similar to those observed for wild-type cells. An increase in cGMP concentration was not observed for $ga4^-$ cells with the $G\alpha 5/4$ or $G\alpha 5$ subunit expression vector, suggesting the $G\alpha 5/4$ subunit is not sufficient to rescue the stimulation of cGMP accumulation. However, cells with either of these $G\alpha$ subunits

displayed a higher basal level of cGMP than did cells without any of the $G\alpha$ subunit expression vectors.

Discussion

The phenotypic analysis of developing $G\alpha$ mutant and wild-type cells containing the chimeric $G\alpha 2/4$ or $G\alpha 5/4$ subunits suggests that the functional specificity of these subunits impacts downstream interactions and not just the release of the $G\beta\gamma$ dimer from a specific receptor, consistent with the studies of similar chimeric $G\alpha$ subunits in other organisms (Conklin et al., 1993; Conklin et al., 1996). The $G\alpha 5$ over-expression phenotypes (i.e., small aggregates with precocious tip formation and folate chemotaxis inhibition) observed in $ga5^-$ and wild-type cells expressing the $G\alpha 5/4$ subunit support this idea. The ability of the $G\alpha 5/4$ subunit to produce these developmental phenotypes could potentially result from the activation of $G\alpha 5$ -specific function of this subunit by $G\alpha 4$ -specific receptors. This possibility cannot be confirmed without evidence of direct subunit activation by $G\alpha 4$ -specific receptors. However, the strong similarities in temporal and spatial parameters of signal transduction pathways mediated by the $G\alpha 5$ and $G\alpha 4$ subunits allow for the possibility that the endogenous signals activating these pathways might be available to the same subset of cells during the various stages of development (Hadwiger and Firtel, 1992; Hadwiger et al., 1991; Hadwiger et al., 1996). Alternatively, the developmental phenotypes might result from the $G\alpha 5/4$ subunit acting as a dominant-negative component in $G\alpha 4$ -mediated signaling pathway. However, the $G\alpha 5/4$ only inhibits chemotaxis and reduces aggregate size but does not alter subsequent morphological development suggesting the $G\alpha 5/4$ subunit only affects some $G\alpha 4$ -specific functions. The inability of the $G\alpha 2/4$ subunit to rescue aggregation of $ga2^-$ cells is likely due to the lack of coupling to cAMP receptors because these cell were incapable of chemotaxis to cAMP. Neither the $G\alpha 2/4$ or $G\alpha 5/4$ subunit could rescue developmental $G\alpha 4$ function in $ga4^-$ cells but this observation agrees with chimeric $G\alpha$ subunit function not being defined solely by the coupled receptor. The failure of $G\alpha 2/4$ and $G\alpha 5/4$ subunits to rescue development in $ga4^-$ cells cannot be explained by insufficient expression from a heterologous promoter because the same expression vectors provided sufficient wild-type $G\alpha$ subunits (i.e., $G\alpha 4$, $G\alpha 2$, and $G\alpha 5$) to restore wild-type development to the respective $G\alpha$ subunit mutants.

The similar chemotactic indexes of $ga4^-$ cells expressing the $G\alpha 2/4$ or $G\alpha 4$ subunit suggest the $G\alpha 2/4$ subunit can partially restore directional movement in folate gradients but the extent of the migration is significantly compromised. The very limited folate-stimulated cGMP response in cells expressing the $G\alpha 2/4$ subunit is also consistent with this compromised chemotactic response. Given that the $G\alpha 4$ and $G\alpha 2$ subunits mediate chemotaxis for different cell fates (foraging versus aggregation, respectively) the failure of the $G\alpha 2/4$ subunit to fully rescue folate chemotaxis might be attributed to inefficient coupling of the $G\alpha 2/4$ subunit with the folate receptors or to $G\alpha 2/4$ interactions with downstream signaling components that potentially desensitize responses to folate. The desensitization of folate responses by $G\alpha 2$ function is consistent with the reduction in folate chemotaxis as the $G\alpha 2$ subunit mediates chemotactic responses to cAMP at the onset of aggregation (De Wit and De Wit, 1986). Mechanisms of heterologous desensitization in *Dictyostelium* are poorly understood but $G\alpha$ subunits could potentially play important roles in this process.

The ability of the $G\alpha 5/4$ subunit to inhibit wild-type cell migration to folate is probably not due to an interference in the coupling of the $G\alpha 4$ subunit to folate receptors because the inhibition of migration also occurs in $ga4^-$ cells. Consistent with this idea, activating mutations that impair GTPase activity and receptor association in $G\alpha$ subunits produce $G\alpha 5$ subunits ($G\alpha 5^*$ or $G\alpha 5/4^*$) that are very effective in inhibiting chemotaxis to folate and also cAMP (Watson and Hadwiger, unpublished results, (Srinivasan et al., 1999)). Analogous activating mutations in the $G\alpha 4$ or $G\alpha 2$ subunit also inhibit folate and cAMP chemotaxis suggesting the

constitutive activation of G α subunits adversely affects chemotactic signaling. These observations suggest that the inhibition of migration is dependent on downstream G α 5/4 subunit function rather than specific receptor associations. The unexpectedly high basal level of cGMP in *ga4*⁻ cells expressing the G α 5/4 or G α 5 subunit could possibly inhibit chemotaxis by altering myosin assembly through the cGMP binding protein GpbC (Bosgraaf et al., 2002). However, this possible mechanism of inhibition has not been verified.

The phenotypes associated with cells expressing the G α 2/4 or G α 5/4 subunit implies that *Dictyostelium* G α subunit specificity plays an important role in regulating developmental morphogenesis, chemotaxis, and cGMP accumulation in response to folate. If G α subunits were only important for the release and capture of G $\beta\gamma$ dimers from specific receptors then the G α 4, G α 2/4, and G α 5/4 subunits would be expected to give similar phenotypes but this is clearly not the case suggesting G α subunits interact with other signaling components. The distinct functions(s) of each *Dictyostelium* G α subunits could result from different interactions with RGS (regulators of G protein signaling) proteins or other downstream signaling components, allowing for pathway specific responses and heterologous desensitization. The involvement of G α subunits in competing cellular processes, such as foraging versus aggregation or prespore versus prestalk cell development, make these proteins good candidates for mediators of heterologous desensitization.

The contrasting phenotypes produced by the G α 4, G α 2/4, and G α 5/4 subunits suggests that G α subunit structure outside of the carboxyl terminus significantly contributes to the specificity of G α function in *Dictyostelium*. The chimeric G α 5/4 subunit was more inhibitory to folate responses than the G α 2/4 subunit but this observation is somewhat surprising given that the G α 4 subunit primary sequence shares more identity overall with the G α 5/4 (52%) than the G α 2/4 (45%) subunit. However, some small regions of the G α 4 subunit have more identity with the G α 2 subunit than to the G α 5 subunit including a region (residues 12–32) near the amino. This particular region of the G α 4 subunit has 38% and 14% identity with the corresponding regions of the G α 2 and G α 5 subunit, respectively, and within this region only the G α 5 subunit contains a putative MAP kinase-docking site (KKSRDIDYQL) composed of a group of two or more basic residues separated by 2–8 residues from a hydrophobic-X-hydrophobic residue sequence (Bardwell et al., 2001). A similar MAP kinase-docking site (KRANDVIEQSLQL) has been previously identified in the analogous region of the yeast Gpa1 G α subunit (Metodieiev et al., 2002). The MAP kinase-docking site of the yeast Gpa1 protein is required for interactions with the Fus3 MAP kinase and for the desensitization to mating factor stimulation (Metodieiev et al., 2002). The *Dictyostelium* MAP kinase Erk2 is activated in G α 4- and G α 2-mediated signaling pathways and therefore the Erk2 protein might represent a target for the putative G α 5 subunit MAP kinase docking site (Maeda and Firtel, 1997; Segall et al., 1995). Requirement of this docking site for G α 5-mediated developmental phenotypes has been recently determined (Raisley and Hadwiger, unpublished data).

The expression of chimeric G α subunits in mammalian cell lines, yeast and now in *Dictyostelium* has allowed for the analysis of G α subunit specificity by coupling receptors in these cells to activate new or altered responses (Brown et al., 2000; Conklin et al., 1993; Conklin et al., 1996). This manipulation of signaling has resulted in the development of heterologous signaling systems useful for the analysis of orphan receptors and the screening of compounds that interact with specific G protein-coupled receptors (Coward et al., 1999; Howard et al., 2001; Stables et al., 1997). The development of similar technologies should also be possible in *Dictyostelium* because G α subunits play important roles in defining downstream signaling responses.

Acknowledgements

The author thanks B. Raisley for technical assistance. The research was supported by an NIH grant (R15 GM073698-01) and a grant from the Center for Sensors and Sensor Technology at Oklahoma State University awarded to J.A.H.

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Abbreviations

PCR	polymerase chain reaction
MAP kinase	mitogen-activated protein kinase
SDS	sodium dodecyl sulfate
GTPγS	guanosine 5' [γ -thio]triphosphate

cGMP cyclic guanosine monophosphate

cAMP cyclic adenosine monophosphate

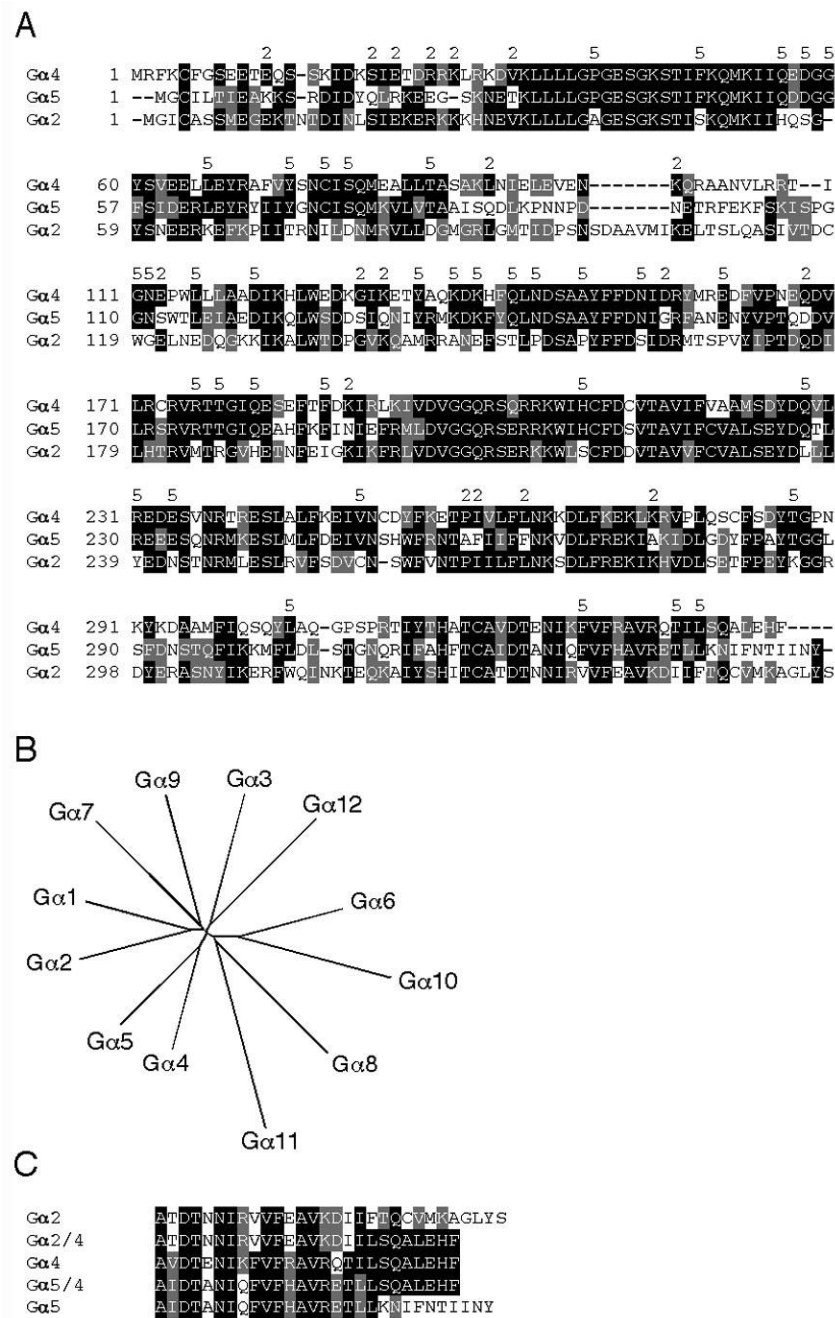


Fig. 1. Comparison of *Dictyostelium* $G\alpha$ subunits. (A) Alignment of *Dictyostelium* $G\alpha 4$, $G\alpha 5$ and $G\alpha 2$ subunit amino acid sequences using ClustalW program and formatted using BOXSHADE program to highlight identical (black background) and similar residues (gray background). The number (2) above the aligned sequences indicates residues identical between the $G\alpha 4$ and $G\alpha 2$ but not $G\alpha 5$ subunit. The number (5) above the aligned sequences indicates identity between the $G\alpha 4$ and $G\alpha 5$ but not $G\alpha 2$ subunit. (B) Unrooted phylogenetic tree of *Dictyostelium* $G\alpha$ subunits generated at <http://clustalw.genome.ad.jp/> by the neighbor-joining method from a ClustalW alignment. Sequence information was obtained using dictyBase at

<http://www.dictpbase.org/>. (C) Alignment of wild-type and chimeric G α subunit carboxyl terminal amino acid sequences formatted using the BOXSHADE program as described above.

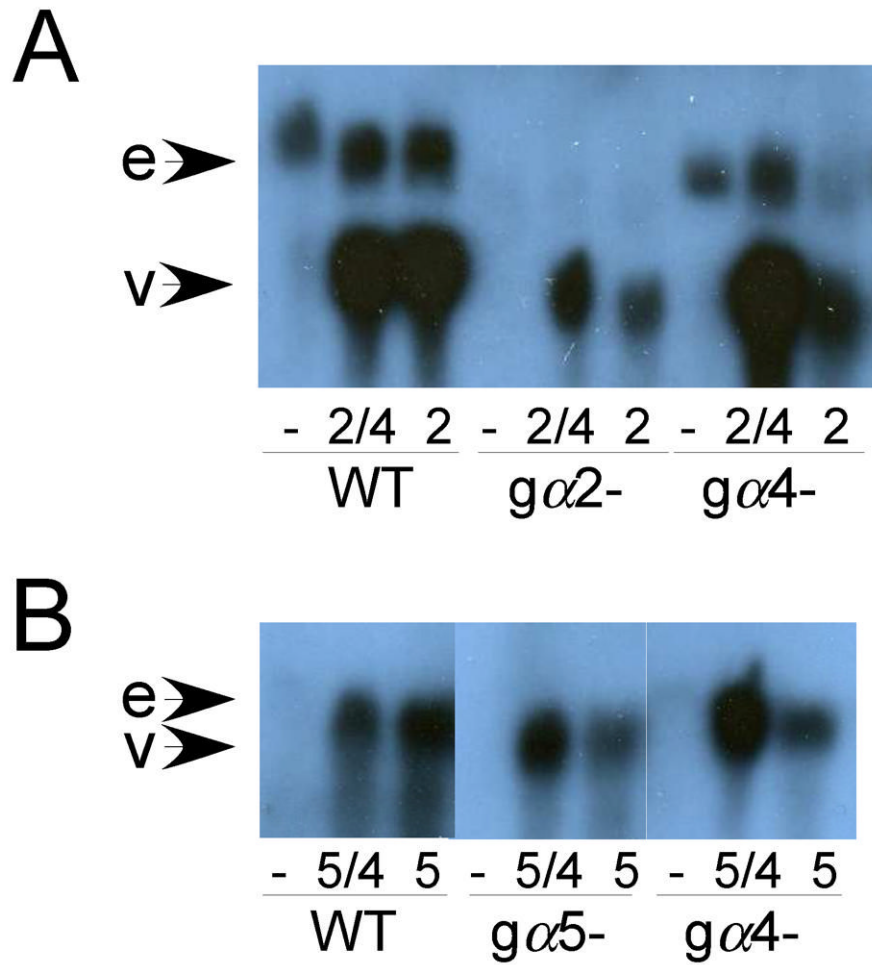


Fig. 2. Expression of $G\alpha$ subunit transcripts in wild-type and $G\alpha$ subunit mutants. Total RNA was isolated from cells in late log phase of growth and subjected to RNA blot analysis using $Ga2^-$ or $Ga5^-$ specific probes as describe in Materials and methods. Each lane was loaded with 4 μ g of total RNA. All lanes from each panel were run on same gel and then blotted and hybridized together but the scanned image of the autoradiograph was spliced to bring relevant lanes together. (A) Endogenous $Ga2$ (e) and vector-expressed $Ga2/4$ or $Ga2$ transcripts (v) in wild-type, $ga2^-$, and $ga4^-$ cells with no vector (-), the $Ga2/4$ vector (2/4), or the $Ga2$ vector (2). (B) Endogenous $Ga5$ (e) and vector-expressed $Ga5/4$ or $Ga5$ transcripts (v) in wild-type and $ga4^-$ cells with the $Ga5/4$ vector (5/4), $Ga5$ vector (5), or no vector (-). Variability in the level of vector-expressed transcripts is likely due to gel loading inconsistencies but in each case the level of vector-expressed transcripts is higher than the level of endogenous gene transcripts. Endogenous $Ga2$ and $Ga5$ transcripts were more visible upon longer exposures.

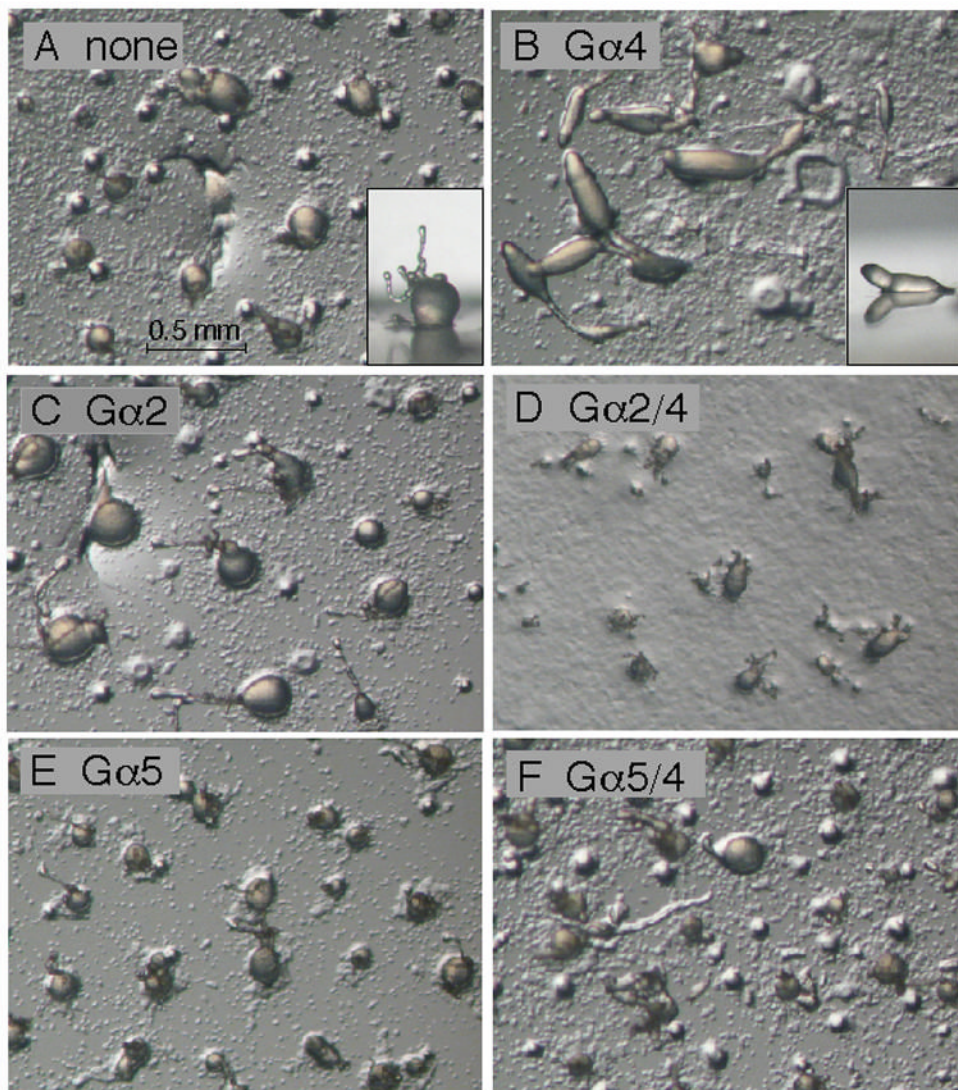


Fig. 3. Development of *ga4⁻* cells with or without $G\alpha$ subunit expression vectors. Cells were grown in axenic medium and washed in phosphate buffer and then cell suspensions (2×10^8 cells/ml) were plated for development as described in the Materials and methods section. Panel (A) aggregates of *ga4⁻* cells with no $G\alpha$ subunit expression vector. Insert displays a side view of a typical aggregate with the round mound and extended tip morphology. Panel (B) aggregates of *ga4⁻* cells with $G\alpha 4$ subunit expression vector in the early stages of culmination. Insert displays a side view of a typical aggregate displaying wild-type slug morphology. Panel (C) *ga4⁻* cells with $G\alpha 2$ subunit expression vector. Panel (D) *ga4⁻* cells with $G\alpha 2/4$ subunit expression vector. Panel (E): *ga4⁻* cells with $G\alpha 5$ subunit expression vector. Panel (F): *ga4⁻* cells with $G\alpha 5/4$ subunit expression vector. Developing cell aggregates were photographed 23 hrs after initial plating using a dissecting microscope (20X magnification).

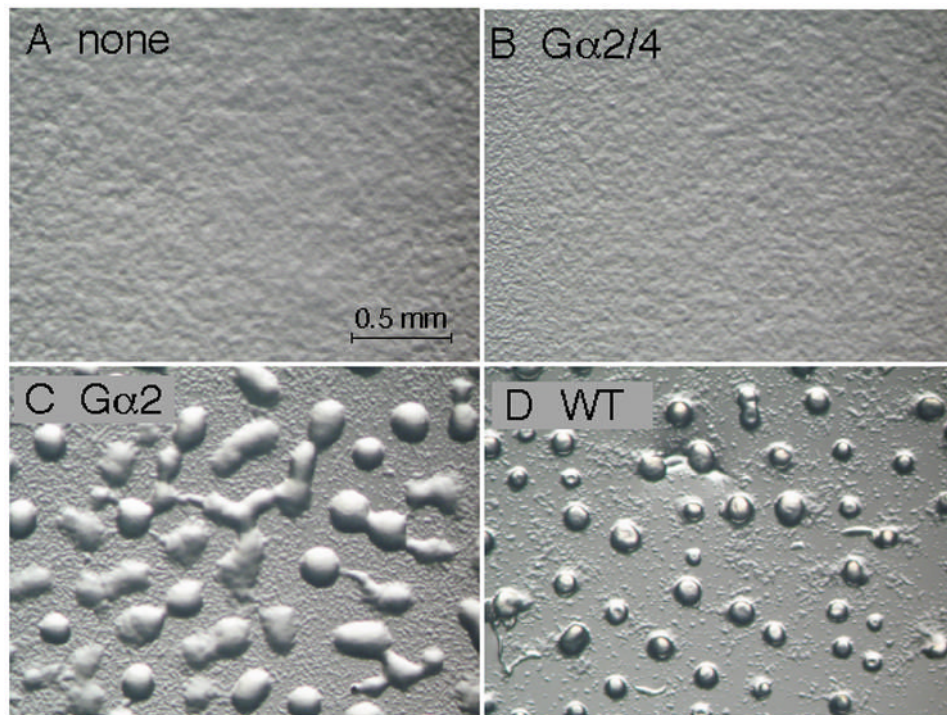


Fig. 4. Development of $ga2^-$ cells with or without $G\alpha$ subunit expression vectors. Cells were grown in axenic medium and washed in phosphate buffer and then cell suspensions (2×10^8 cells/ml) were plated for development as described in the Materials and methods section. Panel (A) $ga2^-$ cells with no $G\alpha$ subunit expression vector. Panel (B) $ga2^-$ cells with $G\alpha2/4$ subunit expression vector. Panel (C) aggregates of $ga2^-$ cells with $G\alpha2$ subunit expression vector. Panel (D) aggregates of wild-type cells with no $G\alpha$ subunit expression vector. Developing cells were photographed 12 hrs after initial plating using a dissecting microscope (20X magnification).

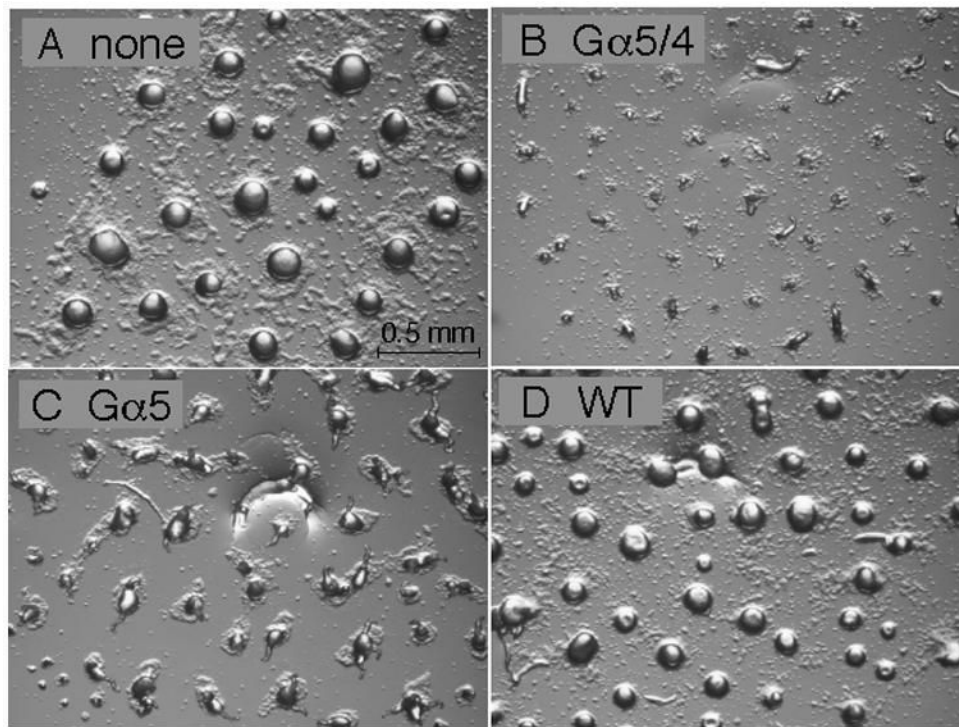


Fig. 5. Development of *ga5⁻* cells with or without *Gα* subunit expression vectors. Cells were grown in axenic medium and washed in phosphate buffer and then cell suspensions (2×10^8 cells/ml) were plated for development as described in the Materials and methods section. Panel (A) aggregates of *ga5⁻* cells with no *Gα* subunit expression vector. Panel (B) aggregates of *ga5⁻* cells with *Gα5/4* subunit expression vector. Panel (C) aggregates of *ga5⁻* cells with *Gα5* subunit expression vector. Panel (D) aggregates of wild-type cells with no *Gα* subunit expression vector. Developing cell aggregates were photographed 12 hrs after initial plating using a dissecting microscope (20X magnification).

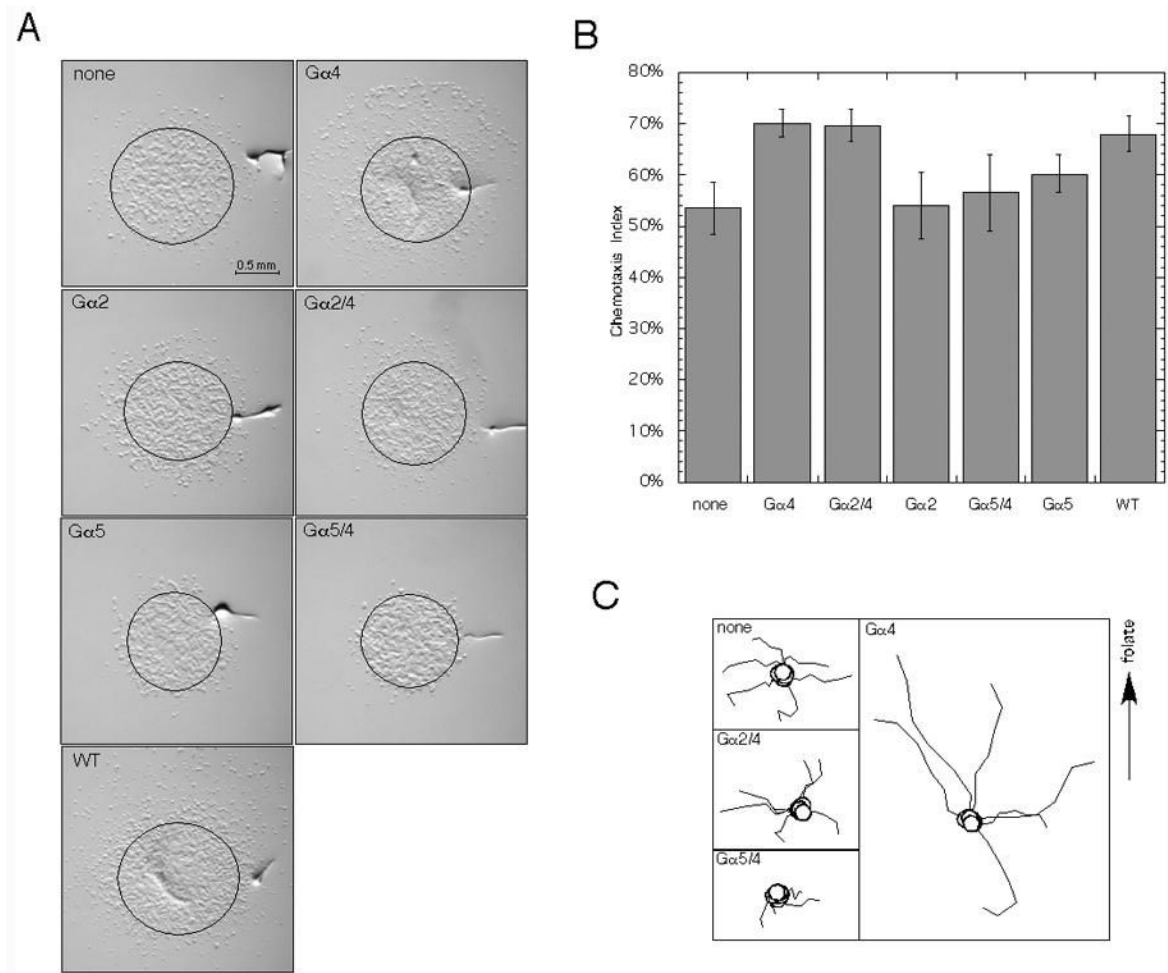


Fig. 6. Chemotaxis of *ga4⁻* cells with or without G α subunit expression vectors. Cells were grown in axenic medium, washed in phosphate buffer, and plated for chemotaxis as described in the Materials and methods section. Droplets of cell suspensions (2×10^7 cells/ml) were spotted onto nonnutrient plates followed by the spotting of one microliter droplets of 1 mM folate solution approximately 2–3 mm away from the cell droplet. (A) Micrographs of chemotaxis assays. In all panels the source of folate is above (upper side) cell droplet. Deformations in agar were created by a needle and used as a reference points to align images captured at different times during the assay. Cell distribution was photographed 3 hrs later using a dissecting microscope (20X magnification) and indirect light. Original cell droplet perimeter is indicated by drawn circle. Developing cell aggregates were photographed 23 hrs after initial plating using a dissecting microscope (20X magnification). Data is representative of one of several assays with similar results. (B) Chemotaxis index of *ga4⁻* cells with or without G α subunit expression vectors. Cell suspensions (1×10^7 cells/ml) were plated for chemotaxis assays and cell distribution was photographed after 3 hrs. Chemotaxis index was determined as the number cells outside the original drop perimeter on the side facing the source of folate divided by the total number of cells outside the original droplet perimeter. Each index was determined as the mean of six separate droplets. Data is representative of one of three different experiments with similar results and the vertical error bars represent the standard deviation. (C) Migration maps of *ga4⁻* cells with or without G α subunit expression vectors. Cell suspension (5×10^6 cells/ml) were plated for chemotaxis assays as described above and then 2.5 hours after the initial

plating time-lapse photography was used to follow the movement of cells over a 30 minute period. Images corresponding to 5 min intervals were used to trace the movement of individual cells. Only cells with minimal or no cell-cell contact were chosen for creating maps. Multiple paths representing different cells of the each strain are shown in each panel and the initial point of each path is designated with an open circle. All initial points were positioned at a central location in each panel with the orientation of the folate source at the top of each panel. Data is representative of one of two experiments with similar results. In all panels and graph, $ga4^-$ cells without $G\alpha$ subunit expression vector (*none*), $ga4^-$ cells with the $G\alpha2/4$ subunit ($G\alpha2/4$), $G\alpha2$ subunit ($G\alpha2$), $G\alpha5/4$ subunit ($G\alpha5/4$), or $G\alpha5$ subunit ($G\alpha5$) expression vectors, and wild-type cells with no $G\alpha$ subunit expression vector

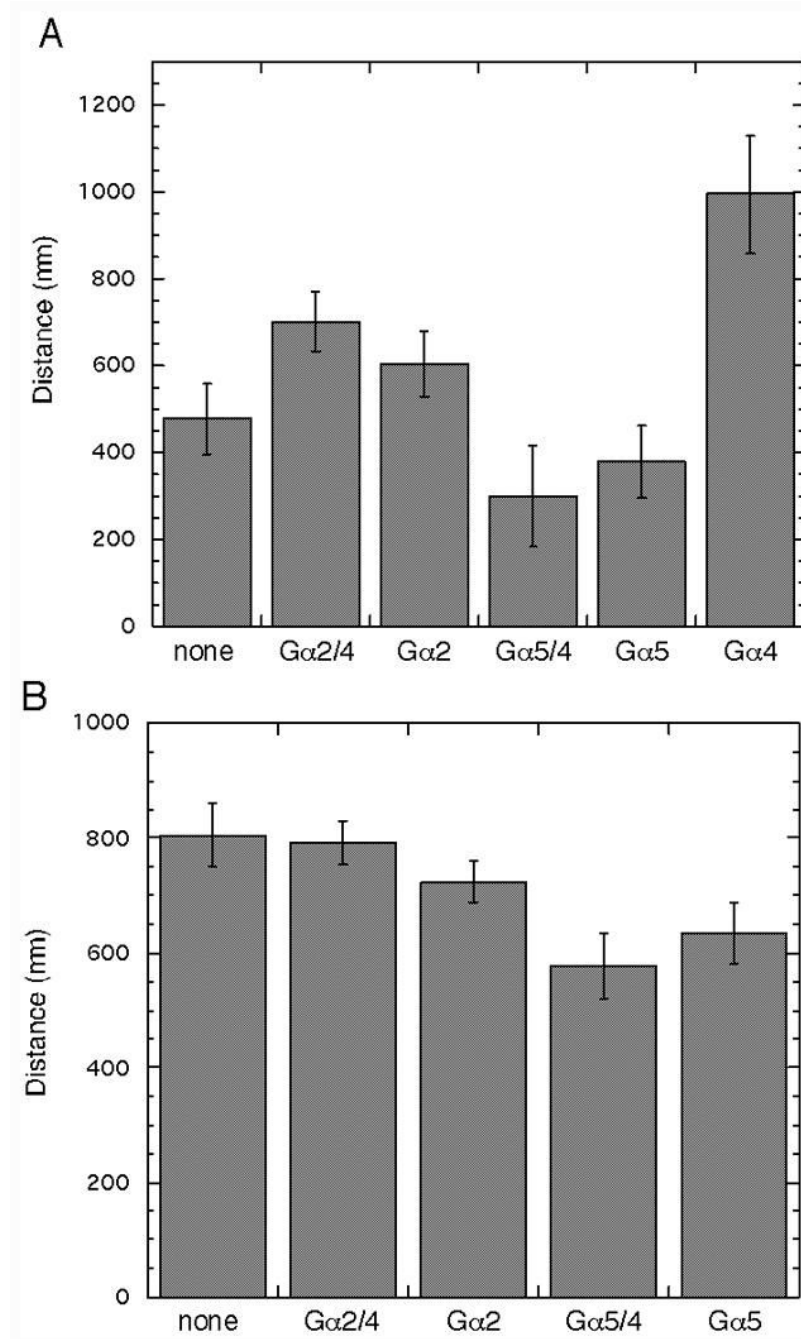


Fig. 7. Chemotaxis distance of *ga4⁻* or wild-type cells with or without Ga subunit expression vectors. Chemotaxis assays were conducted as described in Figure 6 and the maximum distance from the original cell droplet perimeter to the leading edge of migrating cells in the direction of the folate source was recorded. (A) Chemotaxis distance traveled by *ga4⁻* cells without a Ga subunit expression vector (*none*) and cells with the Gα2/4 subunit (*Gα2/4*), Gα2 subunit (*Gα2*), Gα5/4 subunit (*Gα5/4*), Gα5 subunit (*Gα5*), or Gα4 subunit (*Gα4*) expression vectors after 3.5 hrs. (B) Chemotaxis distance traveled by wild-type cells without a Ga subunit expression vector (*none*) and cells with the Gα2/4 subunit (*Gα2/4*), Gα2 subunit (*Gα2*), Gα5/4 subunit (*Gα5/4*), or Gα5 subunit (*Gα5*) expression vectors after 3 hrs. Each value is the mean

of six independent cell droplets. Error represents the standard deviation. Data is representative of one of at least two assays with similar results.

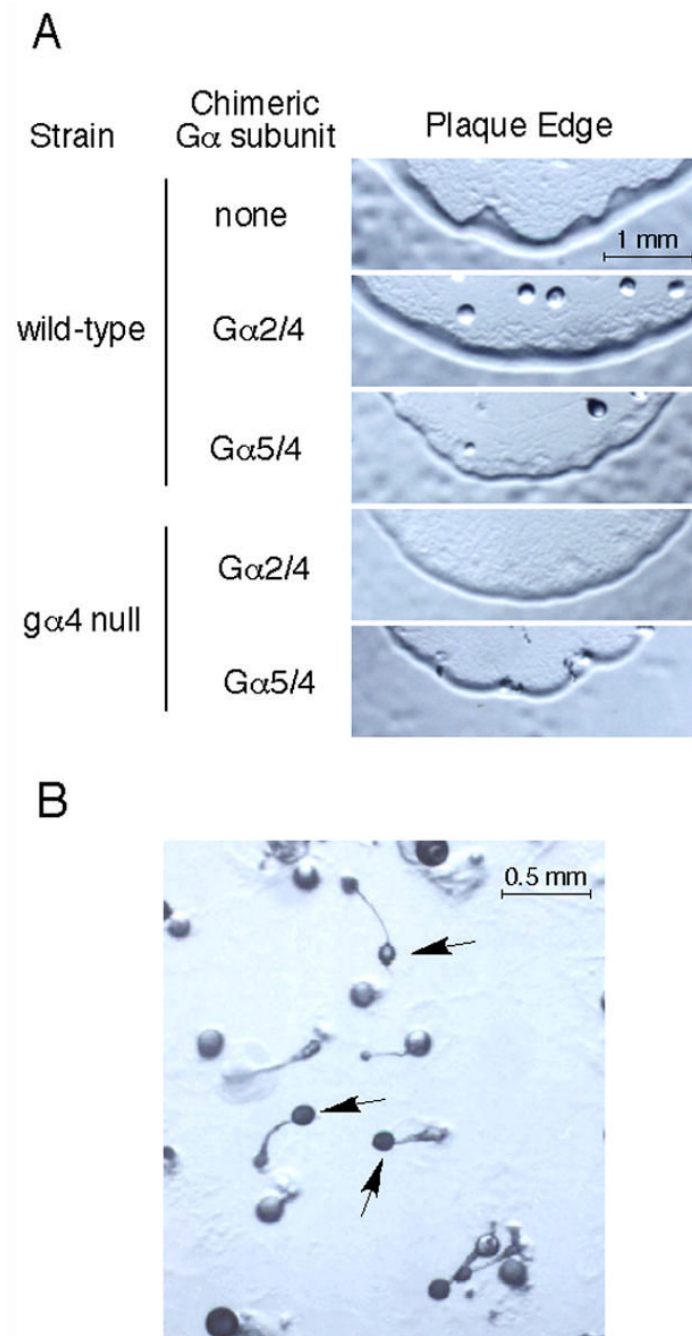


Fig. 8. Growth and development phenotypes on bacterial lawns. (A) Plaque edge phenotype of wild-type and $ga4^-$ cells with chimeric $G\alpha$ subunit vectors on bacterial lawns. Wild-type cells without $G\alpha$ subunit (none) expression vector or wild-type and $ga4^-$ cells with the $G\alpha 2/4$ subunit ($G\alpha 2/4$) or $G\alpha 5/4$ subunit ($G\alpha 5/4$) expression vector were placed on a bacterial lawns and allowed to form plaques. Plaque edges were photographed after 4 days using the same magnification (20X) on a dissecting microscope. (B) Wild-type cells with the $G\alpha 5/4$ subunit expression vector developing within a plaque on a bacterial lawn. Developing aggregates were photographed using a dissecting microscope (40X). Arrows indicate mature fruiting body formation.

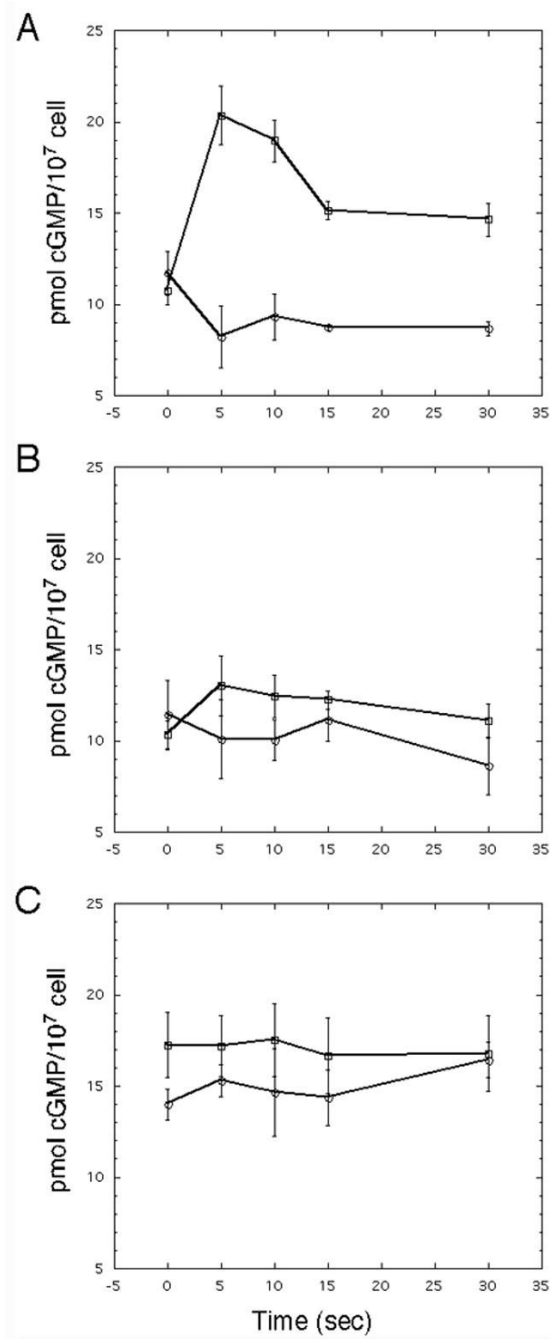


Fig. 9. Folate-stimulated cGMP accumulation in *ga4*⁻ cells with or without Ga expression vectors. Cells were grown, harvested, and stimulated with folate as described in the Materials and methods section. (A) cGMP accumulation in *ga4*⁻ cells with the Ga4 subunit (open squares) or without a Ga subunit (open circles) expression vector. (B) cGMP accumulation in *ga4*⁻ cells with the Ga2/4 subunit (open squares) or Ga2 subunit (open circles) expression vector. (C) cGMP accumulation in *ga4*⁻ cells with the Ga5/4 subunit (open squares) or Ga5 subunit (open circles) expression vector. Concentration of cGMP was normalized with respect to cell extract protein concentration and each cell extract was assayed in duplicate. The data represent the

average of three individual experiments and the vertical error bars represent deviation from the mean value.