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Signal Transduction Pathways during *Dictyostelium* Development

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Mutations impairing the GTPase activity of G protein Gα subunits can result in activated Gα subunits that affect signal transduction and cellular responses and, in some cases, promote tumor formation. An analogous mutation in the *Dictyostelium* Gα4 subunit gene (Q200L substitution) was constructed and found to inhibit Gα4-mediated responses to folic acid, including the accumulation of cyclic nucleotides and chemotactic cell movement. The Gα4-Q200L subunit also severely inhibited responses to cAMP, including cyclic nucleotide accumulation, cAMP chemotaxis, and cellular aggregation. An analogous mutation in the Gα2 subunit (Q208L substitution), previously reported to inhibit cAMP responses (K. Okaichi *et al.*, 1992, *Mol. Biol. Cell* 3, 735–747), was also found to partially inhibit folic acid chemotaxis. Chemotactic responses to folic acid and cAMP and developmental aggregation were also inhibited by a mutant Gα5 subunit with the analogous alteration (Q199L substitution). All aggregation-defective Gα mutants were capable of multicellular development after a temporary incubation at 4°C and this development was found to be dependent on wild-type Gα4 function. This study indicates that mutant Gα subunits can inhibit signal transduction pathways mediated by other Gα subunits.

Key Words: **G protein; *Dictyostelium;* chemotaxis; development.**

INTRODUCTION

Heterotrimeric G proteins transduce signals from cell surface receptors to downstream signaling pathways, allowing eukaryotic cells to respond a wide variety of external stimuli (Bourne *et al.*, 1991; Hildebrandt, 1997; Neer, 1995; Simon *et al.*, 1991; Spiegel, 1997). Of the three subunits (α, β, and γ) forming these G proteins, the α subunit plays a key role in regulating the active and inactive states of this signal transduction component. Stimulated receptors activate G proteins by facilitating the loss of bound GDP from the Gα subunit and then the Gα subunit binds GTP and dissociates from the Gβγ dimer. The active state of the G protein is terminated by the hydrolysis of GTP to GDP, a process carried out by the intrinsic GTPase activity of the Gα subunit and enhanced by other Gα interacting components. Genetic and biochemical analyses have identified conserved regions of Gα subunits as being important to this GTPase activity and the inactivation of Gα subunit function (Kalinec *et al.*, 1992; Landis *et al.*, 1989; Lyons *et al.*, 1995; Spiegel, 1997; Vallar *et al.*, 1987). Many aberrant signaling phenotypes have been associated with specific mutations within these conserved regions, particularly with substitutions of leucine for the glutamine residue in the G3 region of the Gα subunit. This mutation in some mammalian Gα genes can result in altered cell growth control and tumor formation (Hermouet *et al.*, 1991; Kalinec *et al.*, 1992; Landis *et al.*, 1989; Lyons *et al.*, 1995). In some thyroid and pituitary tumors, elevated levels of cAMP suggest that the mutant Gαs subunit is constitutively activating its downstream effector, adenylyl cyclase (Landis *et al.*, 1989; Lyons *et al.*, 1995; Suarez *et al.*, 1991; Yoshimoto *et al.*, 1993). Constitutively active Gαi2 or Gαz subunits have both been reported to cause cell transformation in fibroblasts and

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the analogous mutation in the Gαq subunit can produce tumor formation (Kalinec *et al.*, 1992; Pace *et al.*, 1991; Wong *et al.*, 1995).

While G protein function has been examined in a wide variety of organisms, the role of these proteins in developmental processes has been intensively studied in *Dictyostelium discoideum*, a soil ameoba with a relatively simple developmental life cycle (Devreotes, 1994; Firtel, 1995; Firtel *et al.*, 1989; Parent and Devreotes, 1996). *Dictyostelium* grow vegetatively as solitary amoebae but upon nutrient deprivation the cells aggregate (~10^5 cells/aggregate) to form a multicellular mound (Loomis, 1982). The aggregate can undergo a series of morphological stages before development culminates with a fruiting body structure, consisting of a mass of spores on top of a stalk. Several G protein-mediated signal transduction pathways have been shown to be important for the multicellular developmental life cycle as indicated by genetic analyses (Brandon and Podgorski, 1997; Brandon *et al.*, 1997; Dharmawardhane *et al.*, 1994; Hadwiger *et al.*, 1994, 1996; Hadwiger and Srinivasan, 1999; Kumagai *et al.*, 1991, 1989; Lilly *et al.*, 1993; Wu *et al.*, 1994, 1995). At least four Gα subunits and one Gβ subunit are involved with some aspect of multicellular development (Brandon *et al.*, 1997; Hadwiger *et al.*, 1994, 1996; Kumagai *et al.*, 1991; Lilly *et al.*, 1993).

While *Dictyostelium* development is relatively simple compared to higher eukaryotes, the roles of the individual *Dictyostelium* Gα subunits appear to be quite distinct with respect to developmental morphology and cellular differentiation as indicated by the phenotypes of gene disruption or overexpression mutants (Brandon *et al.*, 1997; Hadwiger *et al.*, 1994, 1996; Kumagai *et al.*, 1989, 1991; Wu *et al.*, 1994). The Gα2 subunit (*gpaB* gene) is required for cellular responses to the extracellular cAMP signal that directs the aggregation process during development (Kumagai *et al.*, 1989, 1991). These responses include the accumulation of cGMP and cAMP, inositol triphosphate (IP_3) production, and chemotactic movement to cAMP. The Gα3 subunit (*gpaC* gene) is also required for aggregation but only in absence of exogenous cAMP, suggesting that Gα3 subunit is not directly involved with responses to extracellular cAMP (Brandon and Podgorski, 1997; Brandon *et al.*, 1997). Finally, the Gα4 and Gα5 subunits (*gpaD* and *gpaE* genes, respectively) play important roles in the development of spores and the anterior prestalk cells, respectively, consistent with the increased expression of these subunits upon aggregate formation (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999; Hadwiger *et al.*, 1991, 1996). The Gα4 subunit is required for proper multicellular morphogenesis, spore production, and chemotactic responsiveness to folic acid (Hadwiger and Firtel, 1992; Hadwiger *et al.*, 1994; Hadwiger and Srinivasan, 1999). Overexpression of the Gα4 subunit inhibits prestalk cells development, as does exogenous folic acid, and the overexpressed Gα4 subunit also promotes cell-autonomous localization of cells to the prespore region of development (Hadwiger and Srinivasan, 1999). Conversely, the Gα5 subunit regulates formation of the anterior prestalk cell region and inhibits folic acid chemotaxis indicating that it plays an antagonistic role to that of the Gα4 subunit during development (Hadwiger *et al.*, 1996). The Gα2 and Gα4 subunits are specifically required for chemotaxis, since the loss of the Gα2 subunit only affects cAMP chemotaxis and the loss of the Gα4 subunit only affects folic acid chemotaxis (Hadwiger *et al.*, 1994). Similarly, the loss of other Gα subunits does not appear to affect these chemotactic responses implying that Gα subunits are pathway specific (Brandon *et al.*, 1997; Hadwiger *et al.*, 1996; Kumagai *et al.*, 1991; Wu *et al.*, 1994).

In addition to gene disruption and overexpression mutants, *Dictyostelium* Gα subunit function has been characterized by the analyses of activated Gα subunits containing a leucine for glutamine residue substitution within the conserved G3 region (Dharmawardhane *et al.*, 1994; Gundersen, 1997; Hadwiger *et al.*, 1996; Okaichi *et al.*, 1992). The expression of a Gα1 subunit with this alteration in wild-type cells produces large aggregates even though Gα1 function does not appear to be required for multicellular development (Dharmawardhane *et al.*, 1994). Expression of a Gα2 subunit with this same alteration in wild-type cells inhibits Gα2-mediated responses to cAMP, including the accumulation of cGMP, cAMP, and IP_3 and the chemotactic movement of cells to cAMP (Okaichi *et al.*, 1992). The analogous alteration in the Gα5 subunit appears to be detrimental to vegetative growth when overexpressed as a result of increased gene dosage even though the loss or overexpression of the wild-type Gα5 subunit does not noticeably alter vegetative growth (Hadwiger *et al.*, 1996). In this study we examined the phenotypes associated with an analogous alteration in the Gα4 subunit and determined that this mutant subunit interferes with responses to both folic acid and cAMP stimulation. We also found analogous alterations in the Gα5 or Gα2 subunits to inhibit folic acid and cAMP responses.

METHODS

Strains and media. All of the strains used in this study were isogenic to the axenic strain KAx3 except at the loci noted. The *gpaD-1* cells (previously designated as *gα4* null cells—strain JH142) contain a *gpaD* locus disrupted with the *thyA* gene (previously designated as the THY1 gene) (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999). The *gpaD-HC* cells (previously designated as $G\alpha4^{HC}$ cells—strain JH384) are KAx-3 cells with a high-copy number of the wild-type Gα4 expression vector pJH154 as previously described (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999). Electroporation of DNA into *Dictyostelium* cells was performed as previously described (Dynes and Firtel, 1989) and multiple *Dictyostelium* transformants were isolated and characterized from each electroporation unless otherwise noted. Vector copy number was verified by genomic DNA blots and the expression of Gα subunit genes was determined by RNA blot analysis. DNA blots were performed as previously described (Sambrook *et al.*, 1989). RNA blots were performed as previously described (Mann and Firtel, 1991). DNA probes were generated by random primer probe synthesis (Feinberg and Vogelstein, 1984). All strains were

grown axenically in HL5 medium or on lawns of *Klebsiella aerogenes* unless otherwise noted.

Development and chemotaxis assays. Cells were grown in shaking cultures to mid-log phase (~2×10^6 cells/ml), washed in phosphate buffer (12 mM NaH_2PO_4, adjusted to pH 6.1 with KOH), and suspended in phosphate buffer at 10^7 or 10^8 cells/ml before spotting onto nonnutrient plates (phosphate buffer, 15% agar) for development or chemotaxis assays. Chemotaxis assays were conducted by spotting 1 μl of cell suspension onto a nonnutrient plate and then immediately spotting 1 μl of chemoattractant solution (1 mM folic acid or 100 μM cAMP) approximately 2 mm away from the cell suspension droplet, as previously described by Hadwiger and Srinivasan (1999). In some cases, cells were starved on nonnutrient plate for 3–6 h, harvested in phosphate buffer, and then spotted onto fresh nonnutrient plates for chemotaxis assays. Development of cells for RNA isolation was conducted by spotting phosphate-buffer-washed cells (10^8 cells/ml) onto Whatmann 50 filters saturated in phosphate buffer. For temperature shift development, cells plated on nonnutrient plates were incubated for 24 h at 4°C before returning them to 22°C.

Treatment of starved cells with exogenous cAMP was conducted as previously described by Schnitzler *et al.* (1995). Briefly, cells suspensions in phosphate buffer (1×10^7 cells/ml) were shaken at 125 rpm and cAMP was added to the concentration of 100 μM after 6 h of starvation and then after every subsequent 2-h interval up to 12 h. Cells were then plated on nonnutrient agar plates for development.

Cells were labeled with the fluorescent dye CMFDA (Celltracker Green, Molecular Probes) as described by Hadwiger and Srinivasan (1999). Briefly, cells were grown to mid-log phase, washed in phosphate buffer, and resuspended to a density of 2×10^7 cells/ml. Cells were incubated in 1 mM CMFDA for 30 min in the dark. Chimeric organisms were created by mixing the labeled cells with unlabeled wild-type cells in a ratio of 1:10. The cell mixture was washed twice in phosphate buffer and then spotted on nonnutrient plates at a density of 2×10^7 cells/ml for development.

Cyclic nucleotide assays. The accumulation of cGMP and cAMP in response to extracellular folic acid or cAMP was conducted as previously described by De Wit and Rinke de Wit (1986). Cells were starved on nonnutrient plates for 3 or 6 h before harvesting and stimulating with folic acid or cAMP, respectively. 2′-Deoxy cAMP was substituted for cAMP in assays measuring cAMP accumulation. Stimulated cells were collected and assayed for the accumulation of cGMP or cAMP using radioimmunoassays (Amersham). Concentrations of cGMP and cAMP were normalized with respect to the protein concentration of stimulated cell extracts. All assays were repeated multiple times.

Construction of expression vectors and mutagenesis. The Gα4-Q200L subunit expression vector was created from the wild-type Gα4 subunit expression vector pJH56 that contains the complete wild-type Gα4 subunit gene (*gpaD*) (Hadwiger and Firtel, 1992). Site-directed mutagenesis of an 0.8-kb *Cla*I/*Sca*I segment of the Gα4 subunit gene with the oligonucleotide 5′ CTTCTTTGAGATCTTAGACCACCGACATCTAC 3′ was accomplished by using the mutagenesis procedure described by Kunkel (1985). This mutated segment was sequenced by Sequenase (Amersham) dideoxy chain termination sequencing and then used to replace the wild-type segment in pJH56 to create the vector pJS4. A 2.2-kb *Eco*RI fragment containing the *pactF(act6)::Neo*^{r} gene fusion was inserted into the unique *Eco*RI site of pJS4 to create the Gα4-Q200L expression vector, pJS11, that confers resistance to the drug G418. The wild-type Gα4 subunit control vector, pJH154, is identical to pJS11 except for the mutated sequence (Hadwiger and Firtel, 1992). The insertion of a *Kpn*I/*Eco*RI Bsr gene segment (1.5 kb) from pUCBsrΔBam (Sutoh, 1993) into the same sites of pJS4 created a Gα4-Q200L expression vector, pJS10, that confers resistance to the drug Blasticidin S. The wild-type Gα4 subunit control vector, pJH287, that confers resistance to Blasticidin S is identical to pJS11 except for the mutated Gα4 sequence. Vectors expressing the Gα4-Q200L subunit from the *disA* (discoidin 1) promoter were constructed by inserting the *Kpn*I/*Sal*I fragment of the *disA* promoter of vector pVEII (Blusch *et al.*, 1992) into the same sites of pBluescriptSK to create vector pJS22. Then a *Sal*I/*Xba*I fragment containing the Gα4-Q200L subunit gene and the *pactF::Neo*^{r} gene was cloned into the same sites of pJS22 to give pJS23. A *Sal*I/*Eco*RI fragment of the wild-type Gα4 subunit gene was inserted into the same sites of pJS23 to create pJS32, a vector expressing the wild-type Gα4 subunit from the *disA* promoter. The construction of the Gα5-Q199L (*gpaE-2*) expression vector, pKN1, has been previously described (Hadwiger *et al.*, 1996). The Gα2-Q208L expression vector used in this study expresses the Gα2-Q208L subunit from the *actO* (*actin15*) promoter and the construction of this vector has been previously described (Gundersen, 1997).

RESULTS

Aggregation Defects Associated with the Gα4-Q200L Subunit

To characterize the effects of an activated Gα4 subunit on chemotactic movement and development, site-directed mutagenesis was used to create a *gpaD* allele (*gpaD-2*) encoding a Gα4 subunit containing the Q200L substitution within the G3 region of the Gα4 subunit. A Gα4-Q200L expression vector was constructed with a Blasticidin S-resistance selectable marker gene, capable of conferring resistance to the drug when the vector is present in a single copy. Transformation of wild-type cells with this Gα4-Q200L vector produced approximately one-half the number of viable transformants as did side by side transformations with a wild-type Gα4 subunit control vector containing the Blasticidin S-resistance gene. The majority of these Gα4-Q200L vector transformants failed to aggregate when plated on nonnutrient plates for development (Fig. 1). On bacterial lawns, cells expressing the Gα4-Q200L subunit consumed bacteria to form plaques (clearings on the lawn) but the starved cells within the central regions of the plaques did not form aggregates like cells without the Gα4-Q200L subunit (data not shown). Cells expressing the Gα4-Q200L subunit were only capable of forming loose mounds at the plaque perimeters where cells accumulate at high densities. These loose mounds typically did not proceed any further in development. The Gα4-Q200L expression vector was also transformed into *gpaD-1* cells, that do not produce the wild-type Gα4 subunit, and *gpaD-HC* cells, that overexpress the wild-type Gα4 subunit due to increased gene dosage. In both strains, the majority of transformants were defective in the aggregation phase of development (Fig. 1).

The defective aggregation phenotype was also observed in wild-type, *gpaD-1*, and *gpaD-HC* cells transformed with the Gα4-Q200L vector containing the G418-resistance gene

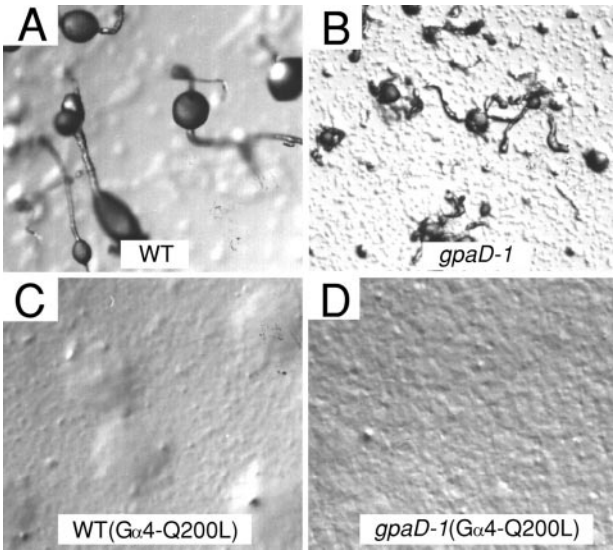


FIG. 1. Developmental phenotypes of wild-type and *gpaD-1* cells expressing the Gα4-Q200L subunit. Cells were grown in shaking cultures and developed on nonnutrient plates as described under Methods. Developing wild-type cells (A, fruiting bodies), *gpaD-1* cells (B, extended tip morphology), wild-type cells expressing the Gα4-Q200L subunit (C, no aggregates), and *gpaD-1* cells expressing the Gα4-Q200L subunit (D, no aggregates) were photographed 25 h after nutrient depletion.

when transformants were selected at low drug concentrations (<5 μg/ml G418) to allow for survival of low-copy-number transformants (data not shown). At higher G418 concentrations, most drug-resistant colonies (usually less than 50 cells per colony) survived only 5–10 days after the transformation. Some of the Gα4-Q200L transformants were capable of continued proliferation and normal multicellular development but these transformants typically contained rearranged Gα4-Q200L vectors as indicated by genomic DNA blot analysis (data not shown). *Dictyostelium* cells transformed with a high-copy number of the control vector, expressing the wild-type Gα4 subunit, displayed no obvious defects in vegetative growth but exhibited an altered developmental morphology as described in earlier reports (Hadwiger and Firtel, 1992; Hadwiger and Srinivasan, 1999). The lack of viable transformants with a high-copy number of intact Gα4-Q200L expression vector was an observation similar to that observed for transformations with the activated Gα5-Q199L subunit (designated as Gα5-Q198L in previous report) expression vector (Hadwiger *et al.*, 1996). Defects in aggregation were also observed when the Gα4-Q200L subunit was expressed from the folic acid-repressible *disA* (*discoidin 1*) promoter in wild-type or *gpaD-1* cells (data not shown). Attempts to repress Gα4-Q200L expression from the *disA* promoter with 1 mM exogenous folic acid did not rescue the ability to aggregate, suggesting that the promoter was not completely repressed (data not shown). Expression of the wild-type Gα4 subunit from the *disA* promoter did not affect the developmental phenotype of wild-type cells but did rescue folic acid chemotaxis and early developmental morphology in *gpaD-1* cells, indicating that the expression from the *disA* and *gpaD* promoters overlap during early development. All subsequent analyses of Gα4-Q200L mutants were conducted using the Blasticin S-resistance low-copy vectors.

The Gα4-Q200L Subunit Inhibits Chemotactic Movement to Folic Acid and cAMP

Cells expressing the Gα4-Q200L subunit were examined for folic acid chemotaxis because wild-type Gα4 function has been previously shown to be required for this response (Table 1). Wild-type cells expressing Gα4-Q200L subunit were partially inhibited in their chemotactic movement to folic acid at 3 h poststarvation but this inhibition was primarily with respect to the quantity of chemotactic cells rather than the distance traveled by the cells. The inhibition of chemotaxis was absent by 6 h poststarvation (data not shown). The expression of the Gα4-Q200L subunit did not rescue the folic acid chemotaxis defect of *gpaD-1* cells, indicating that the Gα4-Q200L expression vector does not complement the *gpaD-l* allele. The Gα4-Q200L subunit was capable of inhibiting folic acid chemotaxis in *gpaD-HC* cells suggesting that the increased expression of the wild-type Gα4 subunit does not abolish the inhibitory effects of the Gα4-Q200L subunit (data not shown).

The defective aggregation phenotype of cells expressing the Gα4-Q200L subunit was similar to that observed for cells with altered Gα2 function, suggesting that the Gα4-Q200L subunit might also inhibit responses to extracellular cAMP during aggregation. Therefore, the ability of Gα4-

TABLE 1
Chemotactic Responses to Folic Acid and cAMP

Strain	Folic acid	cAMP
Wild-type (*gpaD*+)	+++	+++
gpaD-1	—	++
Wild-type (*gpaD*+)/Gα4-Q200L	+	—
gpaD-1/Gα4-Q200L	—	—
Wild-type (*gpaD*+)/Gα2-Q208L	+	—
Wild-type (*gpaD*+)/Gα5-Q199L	—	—

Note. Folic acid and cAMP chemotaxis assay of wild-type (*gpaD*+) and *gpaD-1* cells expressing the Gα4-Q200L, Gα2-Q208L, or Gα5-Q199L subunit. Cells were grown and prestarved on nonnutrient agar plates as described under Methods. Cells were harvested after 3 and 6 h of starvation and plated for chemotaxis assays to folic acid and cAMP, respectively. Droplets of cells were examined 2.5 h after cells and chemoattractants were plated. Relative chemotactic responsiveness is represented by number of plus symbols. No chemotactic responsiveness is represented by the dash.

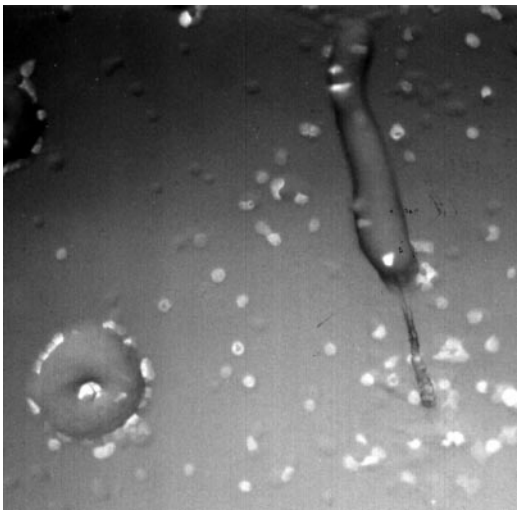


FIG. 2. Localization of wild-type cells expressing the Gα4-Q200L subunit when developed with wild-type cells. Gα4-Q200L subunit-expressing cells and wild-type cells were prepared for development as described under Methods. The Gα4-Q200L subunit-expressing cells were labeled with the fluorescent dye CMFDA, mixed with a 10-fold excess of unlabeled wild-type cells, and then spotted onto nonnutrient plates for development. Photographs were taken after 18 h of development. The anterior of the slug is located at the top.

Q200L-expressing cells to chemotax to cAMP was examined after 6 h of starvation when cells typically initiate the aggregation process. Wild-type cells expressing the Gα4-Q200L vector were completely inhibited in the ability to chemotax to cAMP in comparison to wild-type cells without the Gα4-Q200L subunit (Table 1). Even after 12 h of starvation, cells expressing the Gα4-Q200L subunit were still unable to exhibit cAMP chemotaxis, indicating that the Gα4-Q200L subunit does not merely delay chemotactic responsiveness to cAMP (data not shown). Cells expressing the Gα4-Q200L subunit were transformed with a high-copy-number wild-type Gα2 expression vector but none of the transformants were capable of aggregation, suggesting that the increased Gα2 subunit expression does not suppress the inhibitory effects of the Gα4-Q200L subunit (data not shown). Wild-type cells expressing the Gα4-Q200L subunit were also labeled with the fluorescent dye CMFDA and monitored for the ability to develop with wild-type cells. While some labeled cells were found in the chimeric aggregate, most of these cells were subsequently excluded as the aggregate differentiated into a migratory slug (Fig. 2).

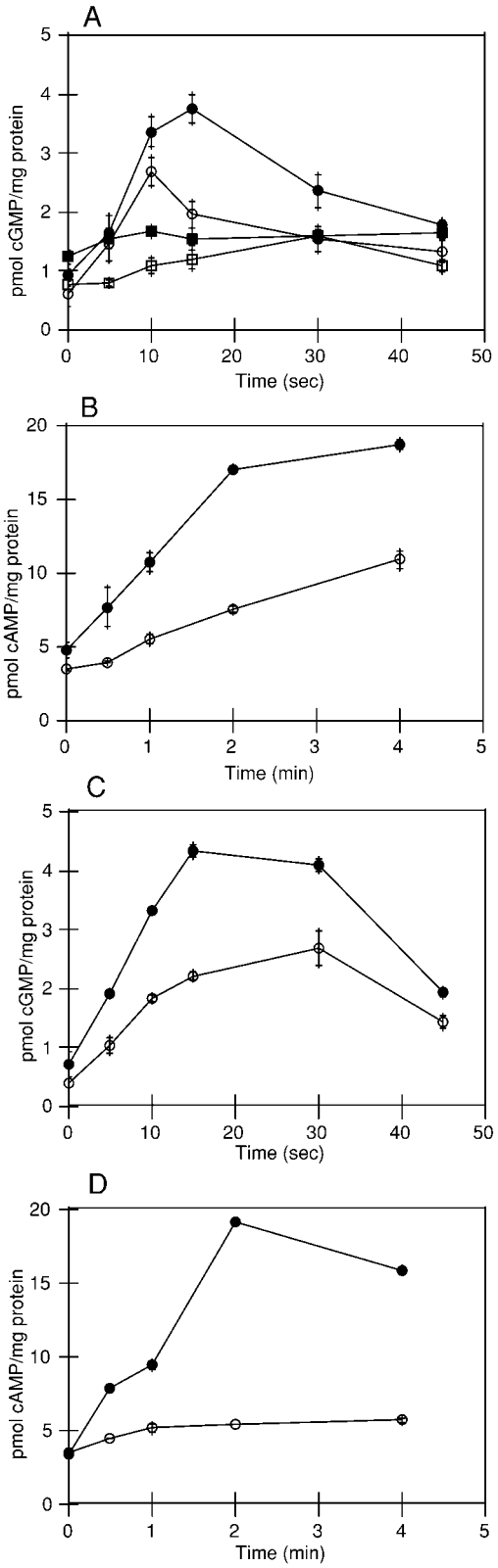
The Gα4-Q200L Subunit Inhibits Cyclic Nucleotide Accumulation in Response to Folic Acid and cAMP

The effects of the Gα4-Q200L subunit on folic acid-stimulated cyclic nucleotide accumulation were also examined since these responses are dependent on Gα4 function. Wild-type cells expressing the Gα4-Q200L subunit displayed a reduced accumulation of cGMP and cAMP in response to folic acid compared to wild-type cells without the vector, indicating that the Gα4-Q200L subunit inhibits both responses (Fig. 3). Expression of the Gα4-Q200L subunit did not rescue cGMP accumulation in *gpaD-1* cells, consistent with the inability of the mutant subunit to complement the loss of wild-type Gα4 subunit function in these cells. Wild-type cells expressing the Gα4-Q200L subunit were also tested for the ability to accumulate cGMP in response to extracellular cAMP stimulation since this response is important for chemotactic movement during aggregation. Wild-type cells expressing the Gα4-Q200L subunit displayed a reduced accumulation of cGMP in comparison to that observed for wild-type cells without the mutant subunit. The Gα4-Q200L subunit also inhibited the ability of wild-type cells to accumulate cAMP after stimulation with 2′-deoxy cAMP, an agonist of cAMP receptors. The ability of the Gα4-Q200L subunit to inhibit both cGMP and cAMP accumulation is consistent the defective aggregation phenotype associated with the subunit.

The reduced responsiveness to extracellular cAMP could potentially result from an absence of signal transduction components, such as cAMP receptors or the Gα2 subunit. Therefore, cells expressing the Gα4-Q200L subunit were examined for the expression of the *carA* (cAR1) and *gpaB* (Gα2) genes after nutrient deprivation. RNA blot analysis indicated both the *carA* and *gpaB* genes were expressed at levels similar to those found in wild-type cells without the Gα4-Q200L subunit up to at least 6 h of starvation (data not shown). At 15 h of development, the levels of these transcripts in Gα4-Q200L mutants were much lower than wild-type levels but this reduction in gene expression might be due to differences in developmental morphology. Cell-type-specific genes such as *cotC* (prespore cell specific) and *ecmA* (prestalk cell specific) were not expressed in the Gα4-Q200L mutants presumably because cells were unable to aggregate (data not shown).

Rescue of Multicellular Development in Gα4-Q200L Mutants Requires Gα4 Function

Previous reports by others indicate that developmental defects associated with altered Gα2 subunit function are alleviated by exogenous cAMP treatments or by temporary incubation at lower temperatures (Okaichi *et al.*, 1992; Schnitzler *et al.*, 1995). Therefore, cells expressing the Gα4-Q200L subunit were starved and treated with exogenous cAMP in shaking cultures and then transferred to nonnutrient plates for development. During the cAMP treatment, wild-type cells expressing the Gα4-Q200L subunit formed small aggregates capable of differentiating into fruiting bodies on agar plates (Fig. 4). However, the majority of treated cells did not participate in multicellular development, implying that cAMP treatment does not efficiently rescue multicellular development. In contrast, *gpaD-1* cells expressing the Gα4-Q200L subunit formed aggregates dur-



ing the cAMP treatment but none of the aggregates proceeded further in development, indicating a requirement for wild-type Gα4 function for bypassing the aggregation defect associated with the Gα4-Q200L subunit. Under these same conditions, *gpaD-1* cells without the Gα4-Q200L subunit were capable of aggregation and subsequent development, indicating that the development of these cells is not inhibited by the cAMP treatment.

The ability of Gα4-Q200L subunit expressing cells to undergo multicellular development after incubation at lower temperatures was also tested by incubating nutrient-deprived cells at 4°C on nonnutrient plates for 24 h and then returning them to 22°C for development. After the cold temperature incubation, wild-type cells expressing the Gα4-Q200L subunit formed small aggregates and then fruiting bodies, indicating a rescue of multicellular development under these conditions (Fig. 5). However, *gpaD-1* cells expressing the Gα4-Q200L subunit failed to produce aggregates under these conditions, consistent with the requirement of wild-type Gα4 function for bypassing the inhibitory effects of the Gα4-Q200L subunit on multicellular development. Without the Gα4-Q200L subunit, *gpaD-1* cells are capable of aggregation and subsequent development after incubation at the lower temperature, indicating that the development of these cells is not inhibited by the temperature shift. Strains expressing the Gα4-Q200L subunit were also tested for development when plated at different densities (Table 2). Wild-type cells expressing the Gα4-Q200L subunit only formed aggregates after the cold-temperature shift when cells were plated from solutions containing greater than 5×10^7 cells/ml. Wild-type and *gpaD-1* cells without the Gα4-Q200L subunit were capable of forming aggregates after the cold-temperature shift when plated from solutions of 1×10^7 cells/ml but these aggregates were much smaller than aggregates obtained in higher cell density or without the temperature shift. Expression of the Gα4-Q200L subunit in *gpaD-1* cells resulted in aggregation-deficient phenotypes at all cell densities tested.

FIG. 3. Cyclic nucleotide accumulation of Gα4-Q200L mutants in response to extracellular folic acid or cAMP stimulation. Wild-type cells (closed circles), *gpaD-1* cells (closed squares), wild-type cells expressing the Gα4-Q200L subunit (open circles), and *gpaD-1* cells expressing the Gα4-Q200L subunit (open squares) were grown and prestarved on nonnutrient agar plates. Cells were prestarved 3 h prior to folic acid stimulation and 6 h prior to cAMP stimulation as described under Methods. The accumulation of cGMP (A) and cAMP (B) were measured at the times indicated after stimulation with 30 μM folic acid. The accumulation of cGMP (C) and cAMP (D) was measured at the times indicated after stimulation with 100 nM cAMP or 5 μM 2′-deoxy cAMP, respectively. The cGMP and cAMP concentrations were normalized with respect to the protein concentration of the stimulated cell suspension. Data represent the mean value of duplicate assays from one of several independent experiments that produced similar results. Error bars represent the deviation from the mean value.

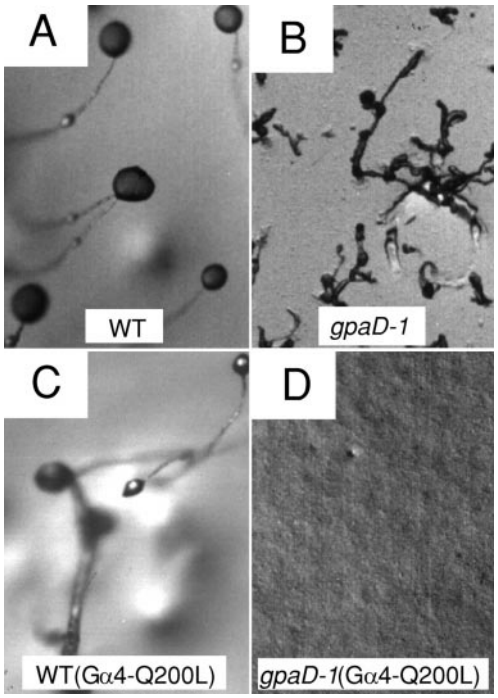


FIG. 4. Developmental phenotypes of wild-type and *gpaD-1* cells expressing the Gα4-Q200L subunit after treatment with cAMP. Cells were grown in shaking culture and then washed and suspended at 1×10^7 cells/ml in phosphate buffer. Cells were then shaken at 125 rpm for 6 h with additions of exogenous cAMP (100 μM concentration) every 2 h and then plated at 5×10^7 cells/ml on nonnutrient plates. The cAMP-treated wild-type cells (A, fruiting bodies), *gpaD-1* cells (B, extended tip morphology), wild-type cells expressing the Gα4-Q200L subunit (C, fruiting bodies), and *gpaD-1* cells expressing the Gα4-Q200L subunit (D, no aggregates) were photographed 24 h after plating on nonnutrient agar plates. (A) 15× magnification; (B–D) 75× magnification.

Analogous Mutations in Other Gα Subunit Genes Result in Phenotypes Similar to Those of Gα4-Q200L Mutants

The ability of the Gα4-Q200L subunit to inhibit chemotactic responses to extracellular cAMP resembles the phenotype previously reported for cells expressing an analogous alteration in the Gα2 subunit, suggesting that mutant Gα4, Gα2, and related Gα subunits might affect common signaling mechanisms (Okaichi *et al.*, 1992). Wild-type cells were transformed with a Gα2-Q208L or Gα5-Q199L subunit expression vector conferring resistance to the drug G418. In previous studies, cells transformed with Gα5-Q199L subunit expression vectors displayed no defects in chemotaxis but this phenotype might have been overlooked if the selection for vector copy number had been too stringent (Hadwiger *et al.*, 1996). Many of transformants containing either the Gα2-Q208L or the Gα5-Q199L subunit expression vector were partially or completely inhibited in aggregation (Fig. 6). However, these transformants were capable of multicellular development after incubation at 4°C similar to the phenotype observed for wild-type cells expressing the Gα4-Q200L subunit. However, *gpaD-1* cells expressing these the Gα2-Q208L subunit were not able to undergo multicellular development, indicating the requirement of wild-type Gα4 function for multicellular development under these conditions. Transformations of *gpaD-1* cells with the Gα5-Q199L expression vector produced very few viable transformants in comparison to transformations with the Gα2-Q208L or Gα4-Q200L expression vectors suggesting that these cells are more sensitive to the adverse effects of the Gα5-Q199L subunit.

Cells expressing the Gα2-Q208L or Gα5-Q199L subunit were defective in cAMP and folic acid chemotaxis (Table 1). Similar to the Gα4-Q200L subunit, the Gα2-Q208L or

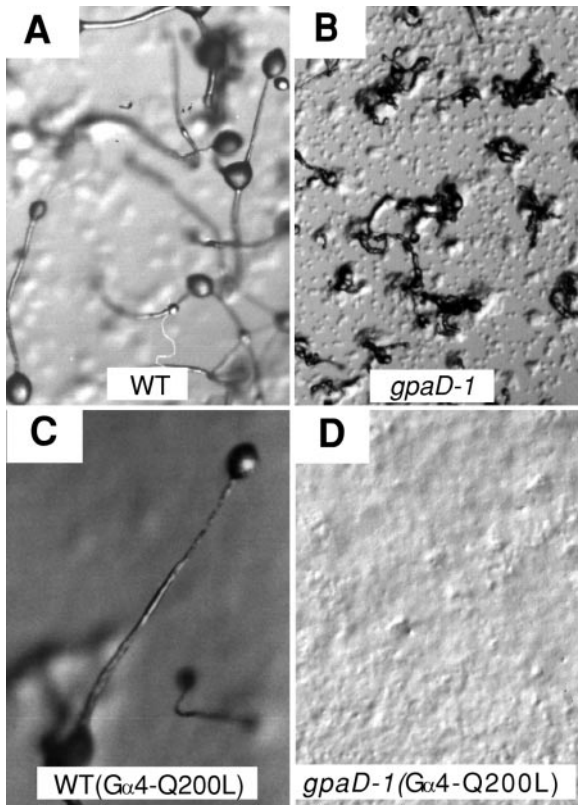


FIG. 5. Developmental phenotypes of wild-type and *gpaD-1* cells expressing the Gα4-Q200L subunit after cold-temperature shift. Cells were grown in shaking culture and plated for development on nonnutrient agar plates from solutions of 1×10^8 cells/ml in phosphate buffer. The plates were incubated at 4°C for 24 h and then returned to 22°C. Wild-type cells (A, fruiting bodies), *gpaD-1* cells (B, extended tip morphology), wild-type cells expressing the Gα4-Q200L subunit (C, small fruiting bodies), and *gpaD-1* cells expressing the Gα4-Q200L subunit (D, no aggregates) were photographed 24 h after plates were returned to 22°C. (A) 15× magnification; (B–D) 75× magnification.

TABLE 2
Development of Gα4-Q200L-Expressing Cells at Different Cell Densities

Strain	Cold-temperature shift				No cold-temperature shift			
	1×10^8	5×10^7	1×10^7	5×10^6	1×10^8	5×10^7	1×10^7	5×10^6
Wild-type (*gpaD+*)	+	+	+*	−	+	+	+	+
gpaD-1	+	+	+*	−	+	+	+	+
wild-type (*gpaD+*)/Gα4-Q200L	+*	−	−	−	−	−	−	−
gpaD-1/Gα4-Q200L	−	−	−	−	−	−	−	−

Note. Multicellular development of wild-type and *gpaD-1* cells expressing the Gα4-G200L subunit after cold-temperature shift. Cells were grown in shaking culture and plated for development on nonnutrient agar plates from solutions of 1×10^8, 5×10^7, 1×10^7, or 5×10^6 cells/ml in phosphate buffer. The plates were incubated at 4°C for 24 h and then returned to 22°C for the cold-temperature shift. Mound formation or the absence of mound formation are designated by the plus and minus symbols, respectively. Small mound size is designated by an asterisk.

Gα5-Q199L subunit inhibited cAMP chemotaxis completely but only inhibited folic acid chemotaxis during the first few hours of starvation. The Gα5-Q199L subunit inhibition of folic acid chemotaxis was notably more severe than the inhibition observed by the Gα2-Q208L or Gα4-Q200L subunits. The inhibition of cAMP chemotaxis by the Gα2-Q208L subunit confirmed earlier reports by others (Okaichi *et al.*, 1992).

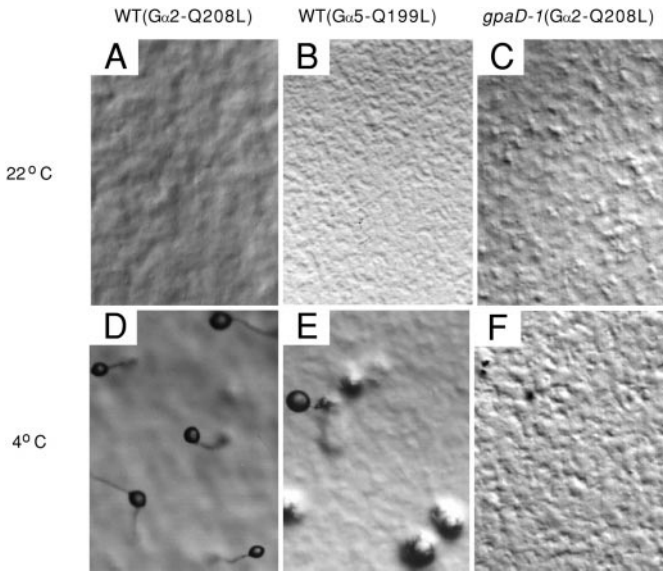


FIG. 6. Developmental phenotypes of wild-type and *gpaD-1* cells expressing the Gα2-Q208L or Gα5-Q199L subunits. Cells were grown in shaking culture and plated for development on nonnutrient agar plates as described in Figure 5. Wild-type cells expressing the Gα2-Q208L subunit (A and D), wild-type cells expressing the Gα5-Q199L subunit (B and E), and *gpaD-1* cells expressing the Gα2-Q208L subunit (C and F) were incubated continuously at 22°C for 24 h (A–C) or incubated at 4°C for 24 h and then at 22°C for an additional 24 h (D–F). Cell morphology was photographed after the incubations at 22°C.

DISCUSSION

The inability of the Gα4-Q200L subunit to rescue folic acid-stimulated cAMP and cGMP accumulation and chemotactic movement indicates that the altered subunit does not provide wild-type Gα4 function. Furthermore, the reduced cGMP and cAMP accumulation and chemotactic movement in response to folic acid suggests that the Gα4-Q200L subunit inhibits the Gα4-mediated signal transduction pathway. The inhibition of cAMP-stimulated chemotaxis and cyclic nucleotide accumulation argues that the Gα4-Q200L subunit is capable of interacting with multiple signal transduction pathways during development. The Gα4-Q200L subunit might also interfere with responses during vegetative growth as suggested by the inability to obtain viable transformants with a high-copy number of the Gα4-Q200L subunit expression vector. The ability of other activated Gα subunits to inhibit both cAMP and folic acid chemotaxis provides further support for multiple signal transduction pathways being inhibited by a single mutant Gα subunit and indicates that this inhibition is not limited to a specific Gα subunit. The mutant subunits could potentially interact with a common signaling component (e.g., the Gβ subunit, guanylyl cyclase, or adenylyl cyclase) shared by multiple pathways or interact with pathway-specific components capable of binding a variety of mutant Gα subunits. Studies of Gα subunits in other systems suggest that activated Gα subunits are defective in GTPase activity, preventing the inactivation of the Gα subunit. Therefore, the altered Gα subunits are predicted to interact with downstream effectors, including GTPase-activating proteins (GAPs) or regulators of G protein signaling (RGS) with proteins, rather than proteins associated with the inactive Gα subunit state (e.g., receptors or Gβγ dimers). RGS proteins are thought to regulate signal transduction by providing GAP activity to activated Gα subunits but this activity is often used as a mechanism to down regulate signal transduction (Dohlman *et al.*, 1998; Hepler *et al.*, 1997; Watson *et al.*, 1996). Sequence compari-

sons have identified many potential RGS genes in a wide variety of eukaryotic organisms, including *Dictyostelium* (D. Hereld, personal communication).

Extracellular cAMP signals are typically generated and detected in pulses during the aggregation stage of development (Loomis, 1982). Continuous or high concentrations of cAMP can prevent cells from adapting and regaining chemotactic sensitivity. In contrast, continuous or high concentrations of folic acid do not appear to effect chemotactic responsiveness. Therefore, differences in signal adaptation might explain the distinction in pathway sensitivity to inhibitory Gα subunits. Differences in sensitivity might also be related to the period of development in which these responses occur. A general block in the procession of development might have a greater impact on cAMP responses because cells become responsive to cAMP after approximately 6 h of starvation whereas they can be responsive to folic acid during vegetative growth and early starvation. There is no indication that the inhibitory mechanism involves a general loss of signal transduction component expression since at least some of the components, cAR1 and Gα2, are expressed in Gα4-Q200L mutants.

The ability of activated Gα mutants to undergo multicellular development after cAMP treatment or a cold-temperature shift indicates that the developmental block by the mutant Gα subunits can be bypassed. The formation of cell–cell contacts by plating cells at high densities or allowing aggregates to form in shaking culture might circumvent the standard aggregation process mediated by the wild-type Gα2 subunit. Previous analysis of the aggregation deficiency associated with the Gα2-Q208L subunit has led others to suggest that cold-temperature shifts allow the Gα2-Q208L subunit to provide Gα2 subunit-mediated aggregation (Okaichi *et al.*, 1992). However, cells lacking Gα2 function, due to an insertional mutation at the *gpaB* locus, can also form small aggregates under these conditions without the Gα2-Q208L subunit, negating the need for Gα2 function in development (Natarajan and Hadwiger, unpublished data). The wild-type Gα4 subunit is required for the multicellular development of activated Gα mutants and the basis of the requirement might be related to the role of this subunit in positioning cells within multicellular aggregates, as previously reported for wild-type cells (Hadwiger and Srinivasan, 1999).

The ability of mutant *Dictyostelium* Gα subunits to interfere with multiple G protein-mediated signaling pathways supports the presence of G protein pathway cross-talk as a mechanism for coordinating cellular responses to developmental signals. While this study focused on mutant subunits, other studies with overexpressed Gα subunits also indicate the presence inhibitory cross-talk mechanisms between G protein signal transduction pathways (Hadwiger and Srinivasan, 1999). Similar pathway interactions might also occur in other organisms where the structural identity between Gα subunits is even greater. Therefore, phenotypes associated with activated Gα subunits in mammals, such as alterations in cell growth characteristics and tumor formation, might potentially result from the inhibition of G protein signal transduction pathways rather than the overstimulation of a single pathway.

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