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Amino Acid Substitutions in the *Dictyostelium* Gα Subunit Gα2 Produce Dominant Negative Phenotypes and Inhibit the Activation of Adenylyl Cyclase, Guanylyl Cyclase, and Phospholipase C

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Previous studies have demonstrated that the *Dictyostelium* Gα subunit Gα2 is essential for the cAMP-activation of adenylyl cyclase and guanylyl cyclase and that *g*α2 null mutants do not aggregate. In this manuscript, we extend the analysis of the function of Gα2 in regulating downstream effectors by examining the in vivo developmental and physiological phenotypes of both wild-type and *g*α2 null cells carrying a series of mutant Gα2 subunits expressed from the cloned Gα2 promoter. Our results show that wild-type cells expressing Gα2 subunits carrying mutations G40V and Q208L in the highly conserved GAGESG (residues 38–43) and GGQRS (residues 206–210) domains, which are expected to reduce the intrinsic GTPase activity, are blocked in multicellular development. Analysis of downstream effector pathways essential for mediating aggregation indicates that cAMP-mediated activation of guanylyl cyclase and phosphatidylinositol-phospholipase C (PI-PLC) is almost completely inhibited and that there is a substantial reduction of cAMP-mediated activation of adenylyl cyclase. Moreover, neither mutant Gα2 subunit can complement *g*α2 null mutants. Expression of Gα2(G43V) and Gα2(G207V) have little or no effect on the effector pathways and can partially complement *g*α2 null cells. Our results suggest a model in which the dominant negative phenotypes resulting from the expression of Gα2(G40V) and Gα2(Q208L) are due to a constitutive adaptation of the effectors through a Gα2-mediated pathway. Analysis of PI-PLC in *g*α2 null mutants and in cell lines expressing mutant Gα2 proteins also strongly suggests that Gα2 is the Gα subunit that directly activates PI-PLC during aggregation. Moreover, overexpression of wild-type Gα2 results in the ability to precociously activate guanylyl cyclase by cAMP in vegetative cells, suggesting that Gα2 may be rate limiting in the developmental regulation of guanylyl cyclase activation. In agreement with previous results, the activation of adenylyl cyclase, while requiring Gα2 function in vivo, does not appear to be directly carried out by the Gα2 subunit. Our data are consistent with adenylyl cyclase being directly activated by either another Gα subunit or by βγ subunits released on activation of the G protein containing Gα2.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* grows vegetatively as single-celled amoebas in the presence of a food source. On starvation, cells initiate a multicellular developmental program, which commences several hours after starvation with the chemotactic aggregation of ~10^5 individual cells in response to pulsatile waves of cyclic AMP (cAMP). cAMP mediates this process by binding to G protein-coupled cell surface receptors, thereby activating a set of coordinately con-

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trolled intracellular pathways that activate adenylyl cyclase (AC), guanylyl cyclase (GC), phosphatidylinositol-phospholipase C (PI-PLC), and Ca^{2+} uptake (Loomis, 1982; Europe-Finner and Newell, 1987a,b; Gerisch, 1987; Janssens and van Haastert, 1987; Devreotes, 1989; Firtel *et al.*, 1989; van Haastert *et al.*, 1989; Firtel, 1991; Kimmel and Firtel, 1991; Milne and Coukell, 1991). These pathways control chemotaxis, signal relay, and the induction of aggregation-stage gene expression. Increases in Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and cGMP can be detected within 3 s of cAMP receptor occupation and peak by ~10 s, although kinetic differences do exist between strains (Europe-Finner *et al.*, 1988). These pathways adapt shortly after receptor occupation and second messenger levels fall rapidly. Activation of AC initiates after a short lag and increases for ~1.5 min, after which this response also adapts. Application of continuous levels of cAMP results in inhibition of all of these pathways due to adaptation and/or downregulation of the receptors and second messenger pathways (Dinauer *et al.*, 1980; Kesbeke and van Haastert, 1985). Activation of AC and GC have been shown to regulate signal relay between cells and chemotactic movement, respectively (see above references), but are not thought to be essential for cAMP-pulse induction of gene expression (Kimmel, 1987; Mann and Firtel, 1987, 1989; Mann *et al.*, 1988). Candidates for the regulation of aggregation-stage genes include Ca^{2+} and 1,2-diacylglycerol, which have been implicated for induction of prestalk and prespore gene expression (Blumberg *et al.*, 1988; Ginsburg and Kimmel, 1989). However, there are, as yet, no experiments that directly demonstrate that these messengers mediate gene expression during aggregation.

The Gα protein Gα2 and the cAMP receptor cAR1 are both developmentally regulated and are preferentially expressed during aggregation (Klein *et al.*, 1988; Kumagai *et al.*, 1989; Pupillo *et al.*, 1989). *Frigid*A strains, which contain mutations within the Gα2 gene and do not express functional Gα2 protein, and *g*α2 null strains, created via disruption of the *G*α2 gene using homologous recombination, do not aggregate (Coukell *et al.*, 1983; Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989, 1991). Furthermore, *g*α2 null cells have been shown to lack cAMP-mediated activation of AC, GC, and expression of pulse-induced genes (Kesbeke *et al.*, 1988; Mann *et al.*, 1988; Kumagai *et al.*, 1989, 1991) but retain receptor-activated Ca^{2+} uptake in vivo (Milne and Coukell, 1991). Somewhat surprisingly, however, relatively high concentrations of GTPγS can activate adenylyl cyclase in membranes from *Frigid*A cells (Kesbeke *et al.*, 1988). This suggests that, although the *G*α2 gene product is essential in vivo for coupling cell surface receptors to adenylyl cyclase, a Gα subunit other than Gα2 may directly activate AC or AC may be regulated directly by $\beta\gamma$ subunits or via another mechanism. Similarly, strains that do not express the cAMP receptor cAR1 (due to either antisense mutagenesis or gene disruption) do not aggregate and lack cAMP-mediated activation of AC (Sun *et al.*, 1990; Sun and Devreotes, 1991), suggesting that cAR1 may be the cAMP receptor regulating this effector. Moreover, biochemical studies indicate that cAR1 and the G protein containing the Gα2 subunit are directly coupled in vivo (Kumagai *et al.*, 1991). The results of these studies therefore suggest that Gα2, coupled to cAR1, plays a central role in regulating the signal transduction processes involved in controlling chemotaxis, cAMP relay, and the induction of gene expression in response to pulses of cAMP during aggregation.

The α subunits of G proteins bind guanine nucleotides and are capable of exchanging and hydrolyzing these nucleotides on activation by an occupied receptor (Gilman, 1987; Lochrie and Simon, 1988; Johnson and Dhanasekaran, 1989; Bourne *et al.*, 1990, 1991; Simon *et al.*, 1991). It is now widely accepted that exchange of GDP for GTP on the Gα subunit, catalyzed by occupied receptors, results in a conformational change in subunit structure G protein activation (Bourne *et al.*, 1990, 1991). In many cases, this leads to the release of the G$\beta\gamma$ complex and the interaction of the Gα subunit and/or G$\beta\gamma$ with its downstream effector. In the case of stimulatory α subunits, activation of the downstream effector continues until the intrinsic GTPase activity hydrolyzes the GTP to GDP, leading to the reassociation with the $\beta\gamma$ subunits. In the case of the *ras* G protein, mutations of glycine at residue 12 (e.g., G12 → V12 [G12V]) and of the glutamine at residue 61 (Q61L) in the GTP-binding and GTPase domains lead to a reduction in the intrinsic GTPase activity and result in *ras* remaining in its active (GTP bound) conformation (Bourne *et al.*, 1991). Recently, it has been demonstrated that in some cases, homologous mutations in Gαs conserved domains lead to the constitutive activation of adenylyl cyclase in mammalian cells (Landis *et al.*, 1989; Master *et al.*, 1989; Woon *et al.*, 1989). This results from a reduction of the intrinsic GTPase activity, with the Gαs(G49V) and Gαs(Q227L) having a 3- to 4-fold and ~100-fold reduction in GTPase activity, respectively (Graziano and Gilman, 1989). Although these mutations have been shown to lead to dominant activating phenotypes in the case of Gαs, other mutations within the same highly conserved GTP binding/GTPase domains have been shown to act as hypomorphic mutations in that cells expressing these mutations show a reduced in vivo activation of adenylyl cyclase. One such mutation, Gαs(G226A), is impaired in the ability of assuming the necessary conformational change to release $\beta\gamma$ subunits but has a normal GTPase activity and is capable of activating adenylyl cyclase in vitro (Lee *et al.*, 1992).

To better understand the function of Gα2 in the activation and adaption of effector pathways during aggregation, we employed a molecular genetic approach to understand the mechanisms of effector pathway regulation during aggregation. To do this, we have examined the effect of mutations in the highly conserved GAGES and GGQRS domains within the *G*α2 gene on

cAMP-mediated activation of second messenger systems in *Dictyostelium*, when expressed in wild-type and *gα2* null strains. In addition, we have assessed the influence of these changes on the morphogenesis and developmental kinetics of the second messenger systems. We show that Gα2 mutations homologous to those known to reduce the intrinsic GTPase activity in other Gα subunits produce dominant negative phenotypes, including a reduced activation of AC, GC, and PI-PLC, when expressed in wild-type cells. Overexpression of Gα2 in a wild-type background results in a decrease in AC activity, whereas it results in an increased and developmentally precocious activation of GC in response to cAMP. We also present results indicating that Gα2 directly activates PI-PLC.

MATERIALS AND METHODS

Construction of Expression Vector Cassettes

The Gα2 coding region was amplified by polymerase chain reaction (PCR) using a 5′ oligonucleotide that contained a *Hin*dIII and an *Spe* I restriction site followed by six A residues and the AUG initiation codon. The 3′ oligonucleotide encompassed the UAA termination codon followed by an *Spe* I restriction site. The 5′ *Spe* I site allows the Gα2 coding cassette to be coupled to a variety of promoters within the 5′ untranslated region and, therefore, to be translated from the Gα2 AUG initiation codon. The amplified product was cloned into Bluescript SKII (Stratagene, La Jolla, CA) and sequenced to ensure that the PCR amplification did not result in any nucleotide changes.

The Gα2 promoter cloning has been reported previously (Kumagai *et al.*, 1991). To make a promoter cassette that can be readily coupled to the various Gα2 constructs, as well as constructs such as Gα2:Gα1 chimeras, the restriction fragment containing the promoter and part of the coding region was digested with *Exo* III, starting from the restriction site in the coding region. Time points were taken and an *Spe* I linker was added to deletions of the appropriate length. A deletion in which the linker was 10 nucleotides 5′ to the AUG initiation codon was used for the promoter cassette. The promoter cassette was combined with the Gα2 coding region, the 2-H3 terminator from the *Dictyostelium SP70* gene (Crowley *et al.*, 1985; Haberstroh and Firtel, unpublished data). This construct also contained the origin of replication and the gene for the *trans*-acting factor required for origin function from pDeΔ1 (a clone from the endogenous plasmid Ddp2 [Leiting and Noegel, 1988; Leiting *et al.*, 1990]) cloned into the plasmid backbone pAT153L (Haberstroh and Firtel, 1990). This allows a high frequency of transformation into *Dictyostelium* cells with a uniform level of expression and copy number in individual cells within transformed populations. The separate promoter, coding region, terminator, and Ddp2-derived cassettes allow the rapid construction of various expression vectors with different promoter or coding sequences.

Mutagenesis

Mutations within the coding regions were made by oligonucleotide-directed mutagenesis, using a mutagenesis kit from Amersham (Arlington Heights, IL) and single-strand DNA template. The template was made by cloning the Gα2 coding cassette into M13 and isolating single-strand DNA from purified phage particles (Sambrook *et al.*, 1989). After mutagenesis, the clones were sequenced to confirm changes in nucleotide sequences. Each of the mutant Gα2 coding regions was used to construct the appropriate expression vector using the promoter, terminator, and pDeΔ1 cassettes.

Development

For analysis of morphological phenotypes or for in vivo assays of cAMP activation of AC, GC, and PI-PLC, cells were developed on Na^+/K^+ phosphate-buffered (pH 6.2) agar or filters (Millipore, Bedford, MA) as previously described (Firtel and Chapman, 1990; Kumagai *et al.*, 1991).

Assays

Assays for in vivo cAMP activation of AC, GC, and PI-PLC were done as previously described (Kesbeke *et al.*, 1986; van Haastert, 1989; Firtel and Chapman, 1990). For the activation of AC, cells were stimulated with 2′dcAMP, which does not interfere with the cAMP assay (van Haastert, 1983). To ensure that assays could be compared, all strains were assayed with wild-type parental strains at the same time. In most experiments, the Gα2 expression strains were also assayed at the same time. Each strain was assayed at least three times and duplicate time points were taken in each experiment.

Western Blots

Western blots were performed as previously described (Kumagai *et al.*, 1989, 1991) using an anti-Gα2 peptide antibody (Kumagai *et al.*, 1989; Gundersen and Devreotes, 1990).

Transformation of Dictyostelium Cell Lines

Dictyostelium cells were transformed by electroporation and stable transformants selected using G418 (Howard *et al.*, 1988).

Dictyostelium Strains

The wild-type axenic strain used was KAx-3. A *gα2* null strain, JH104, was created by a gene disruption via homologous recombination and has been described previously (Kumagai *et al.*, 1991). A *gα2* null strain was made in strain JH010, a thymidine-requiring strain that is isogenic to KAx-3, by homologous recombination using the same *Gα2/Thy1* construct used to create JH104 (Kumagai *et al.*, 1991; Mann and Firtel, 1991; Hadwiger and Firtel, unpublished data).

c-AMP Receptor Levels

Receptor levels were determined by quantitating total cell surface cAMP binding using the $(NH_4)_2SO_4$ method (Devreotes *et al.*, 1987).

Cloning of a Mutant Gα2 Allele

The Gα2 alleles from *Frigid*A strain HC112 and the parental strain HC6 (Coukell *et al.*, 1983) were isolated by PCR amplification using primers from the 5′ and 3′ ends of the coding regions. These were cloned and sequenced using standard techniques.

RESULTS

Developmental Consequences of Expression of Gα2 Mutations in Wild-Type Cells

To examine the role of Gα2 in cAMP-mediated signal transduction pathways, a series of amino acid substitutions in the Gα2 coding region was made by site-directed mutagenesis (see MATERIALS AND METHODS). These include G40V (G at position 40 changed to V [homologous to G12V in *ras* and G43V in Gαs]), Q208L (equivalent to Q61L in *ras* and Q227L in Gαs), G43V, and G207V, all of which are within the conserved GTP binding/GTPase domains. All lie in the highly conserved GAGESG (positions 38–43) and GGQRS (positions 206–210) domains (see INTRODUCTION). By analogy to *ras* and Gαs, one might expect that Gα2(G40V) and Gα2(Q208L) would have a reduced

intrinsic GTPase activity that would result in the in vivo stimulation of Gα2-mediated effectors (see INTRODUCTION), whereas Gα2(G207V) might be expected to produce a Gα subunit with reduced in vivo ability to activate downstream effectors in response to receptor activation (see INTRODUCTION; Lee *et al.*, 1992). Constructs were made by PCR amplification of the Gα2 coding region using 5′ and 3′ primers containing appropriate restriction sites for construction of expression vectors (see MATERIALS AND METHODS). The wild-type and mutant Gα2-coding constructs were then inserted into an expression vector in which Gα2 is expressed from the cloned Gα2 promoter on a *Dictyostelium* extrachromosomal shuttle vector (Figure 1). These were then transformed into the wild-type axenic cell line KAx-3 and the *g*α2 null mutant strain JH104 (Kumagai *et al.*, 1991), enabling both dominant and recessive phenotypes to be examined (see MATERIALS AND METHODS for details). Extrachromosomal vectors were used to ensure that expression was not affected by potential differences in the chromosomal site of integration in different clones and that cells within the population have a uniform copy number of the vector (Ahern *et al.*, 1988; Leiting and Noegel, 1988; Leiting *et al.*, 1990).

As we have shown previously, Gα2 expression from the cloned Gα2 promoter in an extrachromosomal vector results in a level of Gα2 that is ~10- to 15-fold higher than that of endogenous Gα2, with a temporal pattern of expression similar to that seen with the endogenous gene in axenic cell lines (see Kumagai *et al.*, 1991; data not shown). In these strains, Gα2 protein is expressed in vegetative cells at moderate levels, with expression increasing severalfold by the time of aggregation. Expression from this promoter then decreases and expression from a more proximal promoter is activated (Carrel and Firtel, unpublished data). This later expression is spatially localized to the anterior tip region of the culminating fruiting body. Analysis of the levels of expression of wild-type and each of the mutant Gα2 proteins in the transformed strains was consistent with earlier results (Kumagai *et al.*, 1991).

The developmental consequences of expressing the mutated Gα2 proteins in wild-type KAx-3 cells were determined visually by following developmental morphology when cells were plated on nonnutrient agar (see MATERIALS AND METHODS). In wild-type KAx-3 cells, aggregation streams started to become apparent at ~6 h of development, mounds were formed by ~10 h, and culmination was initiated ~18 h after starvation, leading to the formation of mature fruiting bodies by ~24 h (see Figure 2 for photographs of strains at various times after starvation). Cells transformed with the wild-type Gα2, Gα2(G43V), and Gα2(G207V) constructs

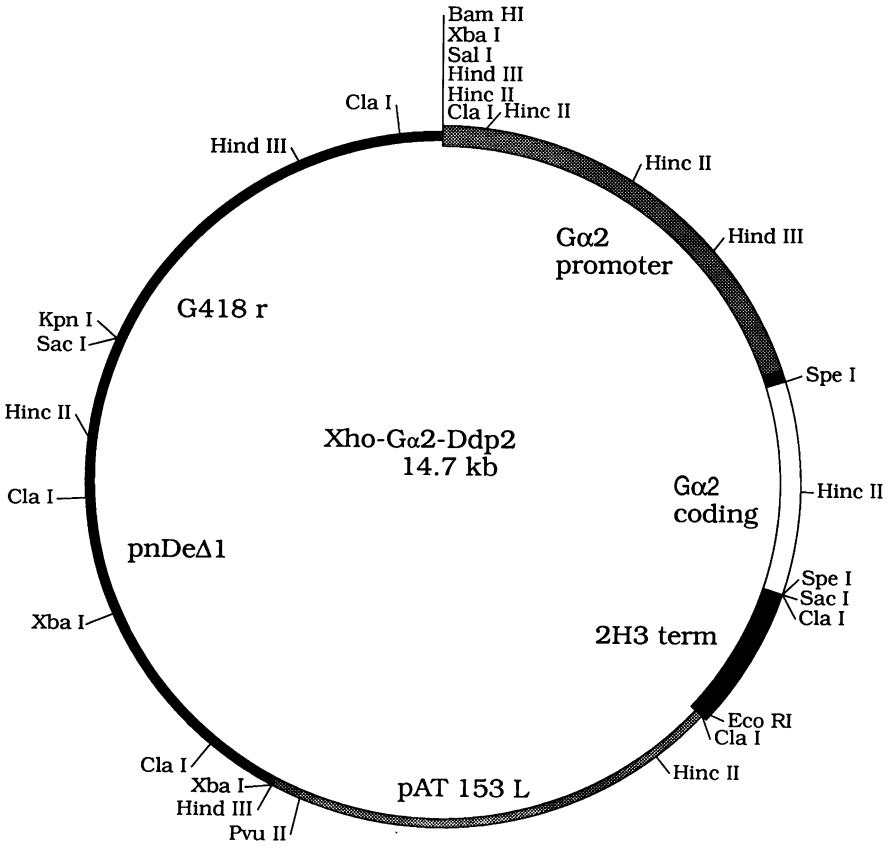

Figure 1. Vector construct used for expression studies. See MATERIALS AND METHODS for details of construction.

displayed no distinguishable differences in the timing of the initiation of aggregation. Multicellular development, as determined by the time to form migrating slugs, was unaffected in cells transformed with the wild-type Gα2 and only slightly delayed in Gα2(G43V) and Gα2(G207V) transformed cells. For all three, the sizes of the aggregates were slightly smaller than those of KAx-3 cells (see Figure 2, plates I and II). Development in all three strains was indistinguishable from wild-type and all formed mature fruiting bodies between 24 and 26 h (see Figure 2, plate III).

In contrast, the expression of either Gα2(G40V) or Gα2(Q208L) resulted in a substantial delay of development. Cells expressing Gα2(G40V) initiated aggregation only after 10 h, developed small aggregates by 15 h, and slugs at ~20 h after starvation (see Figure 2, plates I and II). Fruiting bodies approximately one-fourth the size of wild-type fruiting bodies were formed at ~30 h (see Figure 2, plates III and IV). These results suggest that progression through culmination occurred with kinetics similar to those of wild-type cells and that most of the delay occurred at the aggregation and earlier multicellular stages. Cells expressing Gα2(Q208L) displayed an even stronger suppression of developmental morphogenesis. The appearance of aggregates was delayed until ~20 h, with only 20–40% of the cells forming small mounds and the rest remaining as a lawn of cells. This result was seen with transformed whole populations and with clonal isolates (result not shown). The fruiting bodies that were produced were very small and their appearance was delayed until ~36 h. Thus, cells transformed with Gα2(Q208L) exhibit inhibition and delays in both aggregation and culmination, suggesting that this mutation affects signal transduction mediated by Gα2 in both the early and late stages of development. In contrast, the Gα2(G40V) mutation affected early signal transduction events more severely than later development.

Effect of Gα2 Mutants on the Activation of AC and GC

To characterize the biochemical basis of the delayed developmental phenotypes, we examined the ability of cells starved for 6 h to activate both GC and AC in response to cAMP. Figure 3 shows the kinetics of cAMP activation of GC in KAx-3 wild-type cells and cells transformed with the different Gα2 expression constructs. Cells expressing wild-type Gα2 display kinetics similar to those of control untransformed cells, but with a reproducible elevation in the level of activation as determined by the maximum level of cGMP production. Expression of the Gα2(G43V) or Gα2(G207V) mutants resulted in a small reduction in the levels of cGMP. In contrast, expression of Gα2(G40V) or Gα2(Q208L), mutations that would be expected to have an activating phenotype on effector pathways (see INTRODUCTION), resulted in an almost complete inhibition of GC activation, with Gα2(Q208L) having a stronger dominant negative phenotype. This observation is unexpected, considering the effect of homologous mutations in Gαs on AC in mammalian cells and suggests that the dominant negative phenotype seen in our studies may be due to a constitutive down-regulation or adaptation of this pathway (see DISCUSSION).

The ability of cells expressing mutant Gα2 subunits to activate AC was also assessed at 6 h after starvation. Cells transformed with either Gα2(G40V) or Gα2(Q208L) displayed marked reductions in cAMP accumulation (see Figure 4). In general, however, the quantitative effects of expressing these mutant Gα2 subunits on cAMP accumulation were not as severe as the effects on cGMP accumulation. Somewhat unexpectedly, expression of wild-type Gα2 resulted in a reduction in cAMP accumulation compared to wild-type cells, even though the same Gα2-transformed cells exhibited enhanced stimulation of cGMP production under these conditions. Gα2(G43V) had no observable effect on AC activation, whereas Gα2(G207V) showed a level of inhibition similar to that seen in Gα2-transformed cells.

To better understand the parameters affecting the developmental appearance of these two signaling pathways, the levels of activation of GC and AC were examined at various times in early development. As shown in Figure 5, wild-type cells exhibit a very low level of cAMP-mediated activation of GC in vegetative cells but display increasing levels of stimulation after 2, 4, and 6 h of starvation, as previously described (Kesbeke *et al.*, 1986). Expression of the wild-type Gα2 increases cAMP-mediated GC stimulation at 0, 2, and 4 h of development to a level similar to that seen in 6-h control cells. These results suggest that Gα2 may be the rate-limiting factor in cAMP receptor-mediated activation of cGMP during vegetative growth and 2–4 h developing cells. Expression of Gα2(Q208L) results in the total suppression of GC activation at all times in development, whereas cells expressing Gα2(G40V) showed a small activation in vegetative cells and no activation at the other developmental time points.

Previous results have shown that the cAMP activation of AC is also developmentally regulated, with low activation in growing and early developing cells and a high level of activation during aggregation (Kesbeke *et al.*, 1986). Transformation of wild-type cells with wild-type Gα2 had no observable effect on the kinetics of AC activation in vegetative and 2-h cells, suggesting that other components of the pathway that activates AC are rate limiting in mediating that developmental response (results not shown).

Effect of Gα2 Mutations on the Activation of PLC

In 6-h starved aggregation-competent KAx3 cells, cAMP stimulation has been demonstrated to transiently activate PI-PLC, leading to small increases in inositol 1,4,5-

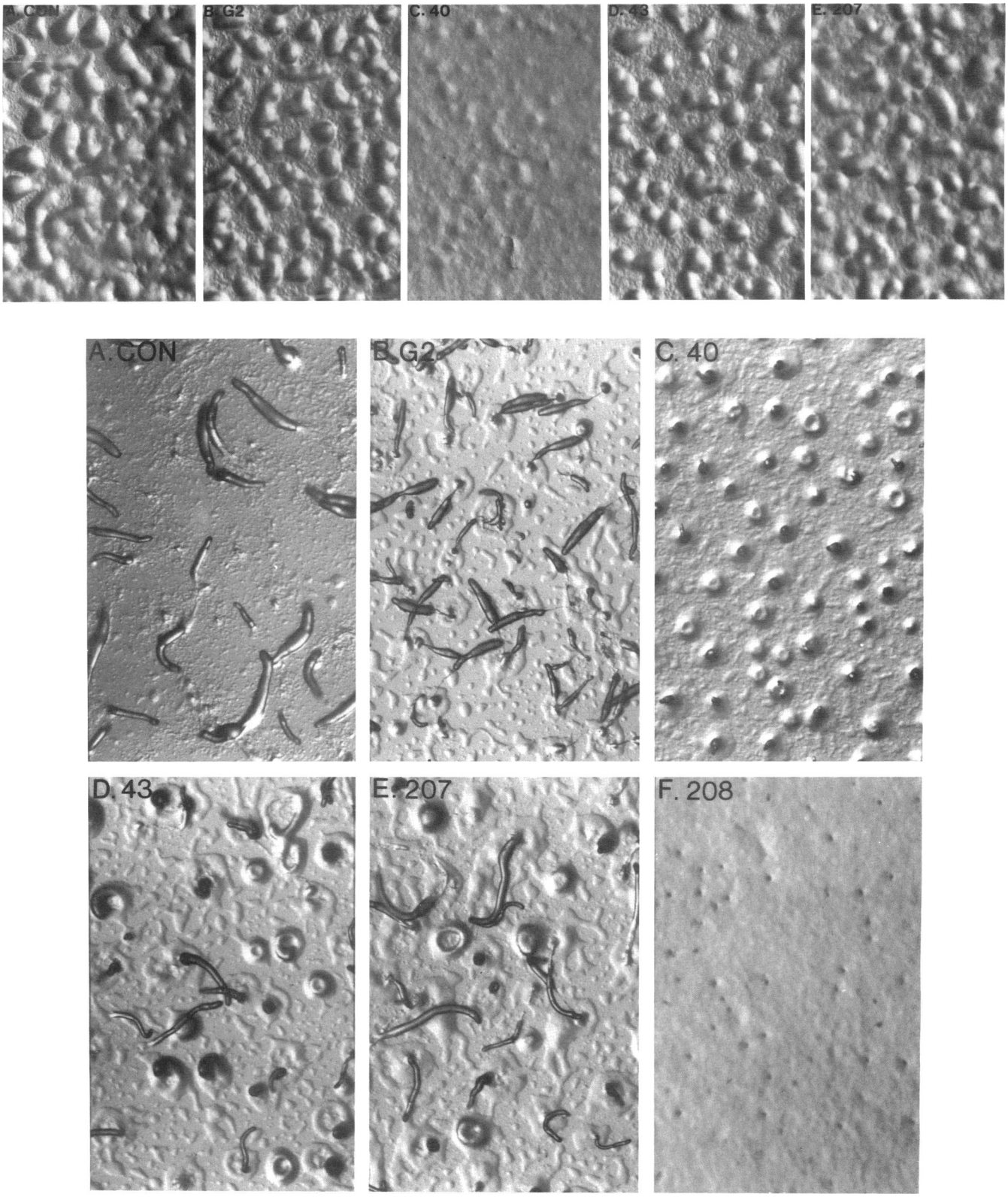

Figure 2. Photographs of development in wild-type KAx-3 cells and strains overexpressing wild-type and mutant Gα2 proteins. Cells were plated on nonnutrient agar and incubated at 20°C. Photographs on Plates I–III were taken from above. Plate I, cells at 10 h. (A) KAx-3; (B) Gα2 wild-type; (C) Gα2(G40V); (D) Gα2(G43V); (E) Gα2(G207V). Plate II, cells at 15 h. (A) KAx-3; (B) Gα2 wild-type; (C) Gα2(G40V); (D) Gα2(G43V); (E) Gα2(G207V); (F) Gα2(Q208L). Plate III, cells at 24 or 30 h. (A) KAx-3; (B) Gα2 wild-type; (C) Gα2(G40V); (D) Gα2(G43V); (E) Gα2(Q208L). Plate IV, side view of fruiting bodies taken at 30 h. (A) KAx-3; (B) Gα2(G40V).

triphosphate (1,4,5-IP_3), with levels rising ~20–40% within 7–10 s after stimulation and then rapidly falling to basal levels (van Haastert *et al.*, 1989; results not shown). To study the possible effects of Gα2 mutants on PI-PLC activation, we examined the basal levels of 1,4,5-IP_3 and the developmental kinetics of the activation of PI-PLC during development in the cell lines expressing various mutant Gα2 subunits. As seen in Figure 6, cAMP stimulation of vegetative cells results in a reproducible 2- to 2.5-fold rise in 1,4,5-IP_3 levels extending over ~20 s, with the level of 1,4,5-IP_3 remaining constant for at least an additional 40 s. At 4 and 6 h of development, a cAMP-mediated response was observable but tended to be of substantially lower and variable magnitude compared with the response seen in vegetative cells, consistent with the level of activation seen by others in aggregation-competent cells (Europe-Finner and Newell, 1987a; van Haastert *et al.*, 1989). Because of the low level of activation and the variability of the response displayed at 6 h of development, we confined our analysis to vegetative cells. Vegetative cells transformed with the wild-type Gα2 construct show kinetics of activation of PI-PLC similar to control cells. In contrast, cells transformed with either a Gα2(G40V) or a Gα2(Q208L) vector show no observable cAMP-mediated stimulation (results not shown). We have also examined a *g*α2 null strain that is isogenic to KAx-3, except for the Gα2 locus and the auxotrophic marker used as the selectable marker for the homologous recombination (see MATERIALS AND METHODS). This strain showed no cAMP-mediated activation of PI-PLC (results not shown) in either vegetative cells or in 6-h starved cells, suggesting that Gα2 may be involved directly in mediating this response (see DISCUSSION).

Previous studies in this lab have demonstrated that Ca^{2+}-activated inositol-lipid-specific PLC activity decreases from vegetative cell levels during the first 6 h of development. To determine if this was reflected by decreases in 1,4,5-IP_3 levels, these levels were measured in vegetative and 2-, 4-, and 6-h cells. In wild-type cells, there is a progressive decrease in the basal (unstimulated) level of 1,4,5-IP_3 through 6 h of development (see Figure 7), consistent with the developmental re-

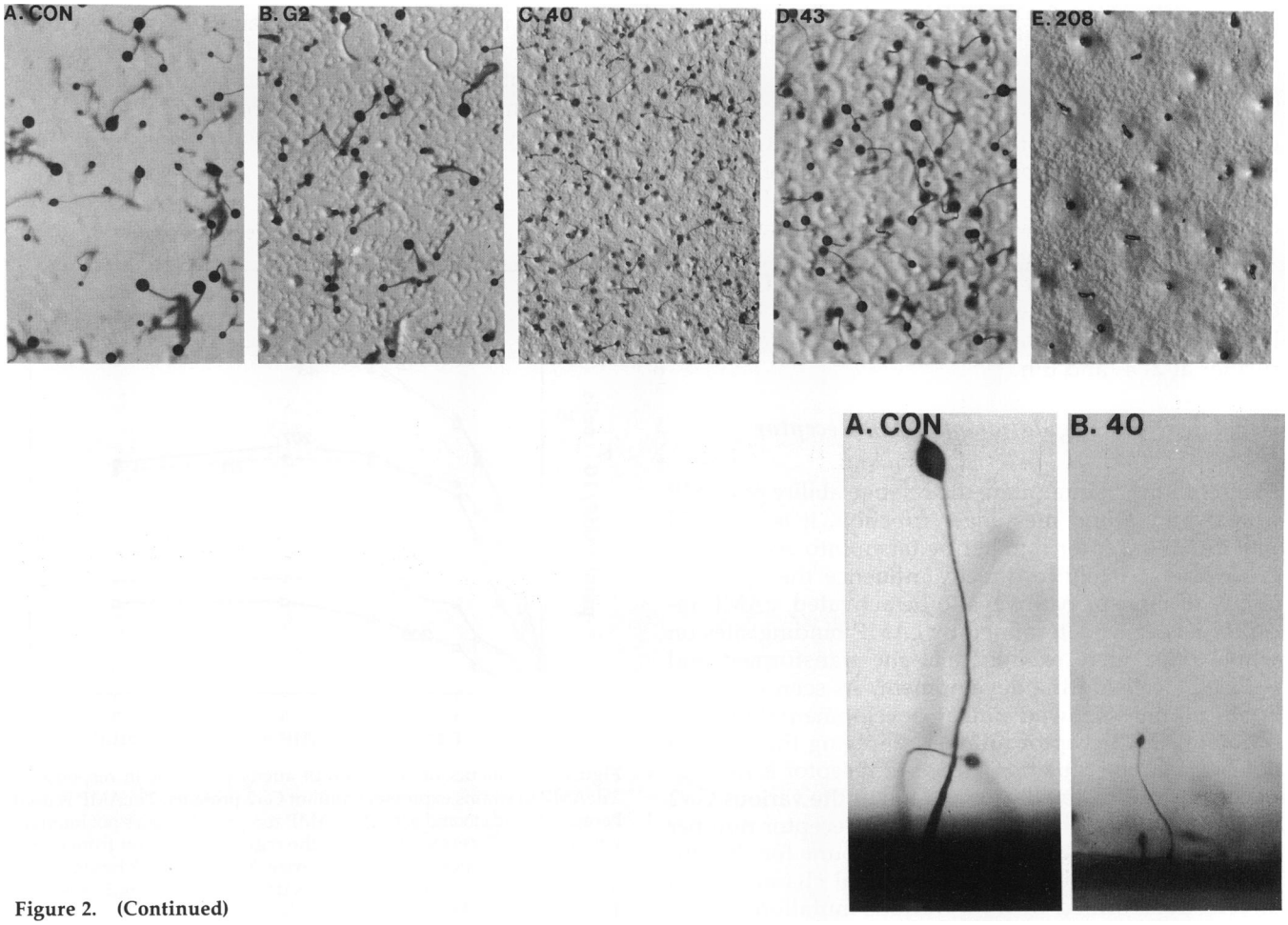

Figure 2. (Continued)

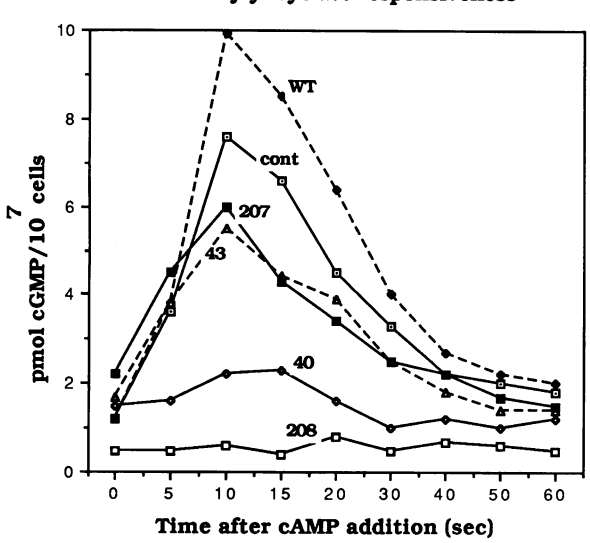

Figure 3. Kinetics of activation of guanylyl cyclase in response to cAMP in strains expressing mutant Gα2 proteins. Wild-type KAx-3 cells were transformed with extrachromosomal vectors to express wild-type Gα2 and Gα2 carrying specific amino acid substitutions. Stable transformants were isolated by selection in G418 and grown axenically. Cells were washed and plated for development. Aggregation competent cells were harvested, washed, and activated with cAMP as described in MATERIALS AND METHODS. Samples were taken at the times indicated and levels of cGMP were assayed. See MATERIALS AND METHODS for details and references.

duction in the levels of Ca^{2+}-activated PI-PLC activity measured in vitro (Cubitt and Firtel, 1992). Cells transformed with wild-type Gα2 have high 1,4,5-IP_3 levels during growth but show a more rapid decrease in basal 1,4,5-IP_3 levels through development than occurs in wild-type cells. Cells expressing either Gα2(G40V) or Gα2(Q208L) cells show substantially reduced 1,4,5-IP_3 levels during vegetative growth, with levels decreasing further at 2, 4, and 6 h.

Developmental Regulation of cAMP Receptor Number

In attempting to quantitate the relative ability of cAMP to activate second messenger function, it is essential that cAMP receptor number be taken into account because this is likely to directly influence the apparent ability of effector pathways to be activated. cAMP receptor levels, as determined by cAMP binding sites on whole cells, were examined in the transformed and wild-type cells during development. As seen in Figure 8, all cell lines showed similar developmental kinetics of total cAMP receptor number, implying that normal developmental regulation of cAMP receptor is not significantly affected by the expression of the various Gα2 mutant proteins. Thus, differences in receptor number that are observed are unlikely to account for the observed biochemical and developmental characteristics of cells transformed with the various mutations.

Effect of Gα2 Mutations in gα2 Null Cells

We previously described the construction of a *gα2* null strain (JH104) produced by homologous recombination of the *Thy1* marker into the *Gα2* locus in the thymidine auxotroph HPS400 (Kumagai *et al.*, 1991). These cells do not aggregate (aggregation-minus) and do not display cAMP-mediated stimulation of either AC or GC (Kumagai *et al.*, 1991). *gα2* null cells (JH104) transformed with the wild-type Gα2 vector show normal development (Kumagai *et al.*, 1991; results not shown) and exhibit cAMP-mediated activation of AC and GC at similar or slightly higher levels compared with the parental strain HPS400 (Kumagai *et al.*, 1991; results not shown). Expression in JH104 cells of the putative activating mutations Gα2(G40V) and Gα2(Q208L) does not restore the ability of the cells to aggregate or their responsiveness to cAMP when the cells are plated and developed at 20°C. Previously, a series of alleles of the *FrigidA* locus were identified as being aggregation-minus due to the inability to respond to cAMP. These strains have been shown to lack cAMP-activation of AC and GC and to be mutant in the gene encoding Gα2 (Coukell *et al.*, 1983; Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989, 1991). Although several alleles contained either a deletion in the Gα2 gene or did not express Gα2 mRNA (Kumagai *et al.*, 1989), *FrigidA* strain HC112 was shown to have normal Gα2 mRNA levels and no major rearrangements within the Gα2 gene. The Gα2 coding regions from HC112 and the parental strain HC6 were isolated by PCR, cloned, and sequenced. The DNA from

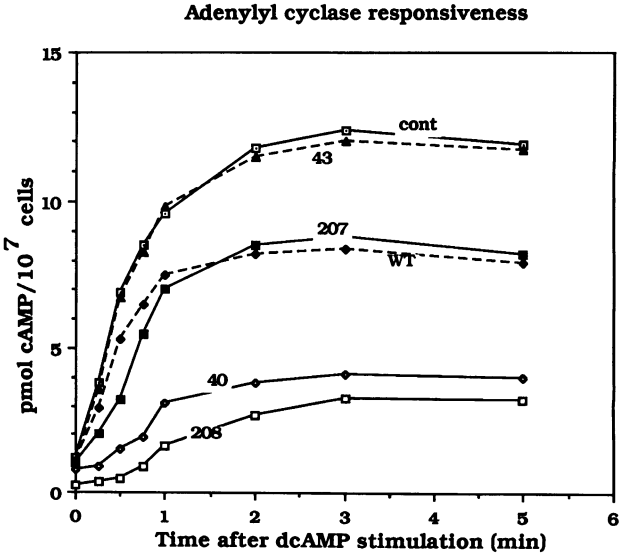

Figure 4. Kinetics of activation of adenylyl cyclase in response to 2'dcAMP in strains expressing mutant Gα2 proteins. 2'dcAMP is used because it binds to and activates cAMP receptors but does not interfere with the cAMP assay, which uses the regulatory subunit from mammalian cAMP-dependent protein kinase A as the cAMP binding protein. Experiments were performed as described in Figure 3. See MATERIALS AND METHODS for details and references.

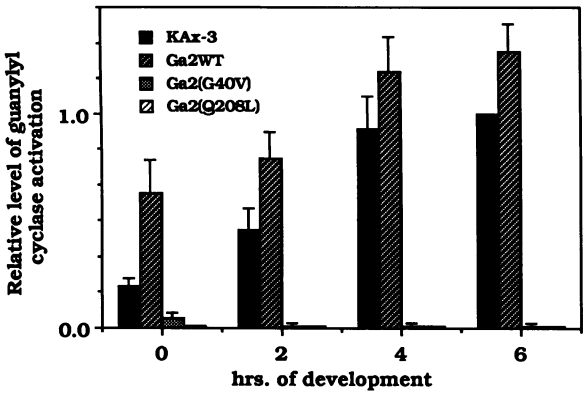

Figure 5. Relative level of guanylyl cyclase responsiveness during early development for various strains. Cells were plated on nonnutrient agar containing buffered salts, incubated at 20°C, and harvested at the times indicated. Experiments were performed as described in Figure 3. The maximum levels of cGMP produced at 10 s after cAMP activation are compared. "0 h" cells represent harvested, washed, log-phase vegetatively growing cells.

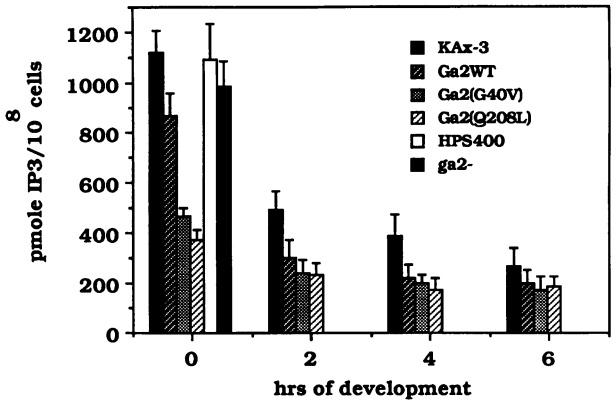

Figure 7. Relative levels of 1,4,5-IP_3 in various strains during early development. Experiments were performed as described in Figures 5 and 6. Expression of Gα2 wild-type and mutant subunits was carried out in KAx-3 wild-type cells as described above. HPS400 is the parental strain for the *g*α2 null mutant used (strain JH104, see Kumagai *et al.*, 1991).

HC112 showed a G → A change at nucleotide 189, resulting in a G40 to D40 missense mutation (data not shown). Sequence analysis indicated that the mutation should also ablate an *Hph* I restriction site. This was confirmed by restriction digestion of the two strains followed by Southern blot analysis, indicating that the Gα2 gene in HC112 contained a G40 mutation. Western blot analysis was done using anti-peptide antibodies that were either specific for Gα2 (an NH_2-terminal peptide) or were against a Gα-common region (GAGESGK) (Kumagai *et al.*, 1989; Gundersen and Devreotes, 1990). The Gα2 anti-peptide antibody showed a normal level of Gα2 protein in the HC112 strain compared with the control parental, but no detectable protein was seen using the Gα-common antibody against the GAGESGK region (results not shown; Gundersen and Devreotes, unpublished data). This antibody showed a normal level of Gα2 in the control cells. These results confirm that HC112 contains a mutation in the GAGESGK region, consistent with the PCR results, and confirms the results showing that the Gα2(G40V) construct is not capable of complementing the *g*α2 null phenotype.

*g*α2 cells expressing Gα2(G43V), a mutation that has no dominant phenotype, show no activation of GC or AC, and the cells do not aggregate. Gα2(G207V) restores a level of GC activation that is ~18 ± 5% of that observed for wild-type Gα2 and a very low activation of AC (~15 ± 4%), but no aggregation is observed (results not shown).

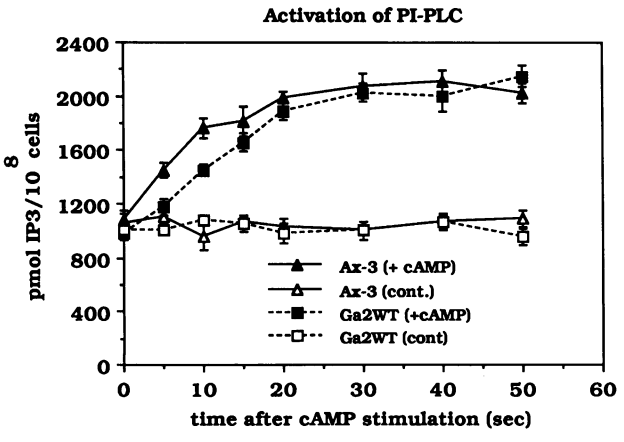

Figure 6. Kinetics of activation of PI-PLC in response to cAMP in strains expressing mutant Gα2 proteins in vegetative cells. Log-phase vegetative cells were harvested, washed, and activated with cAMP. Time points were taken and total 1,4,5-IP_3 was assayed. See MATERIALS AND METHODS for details and references.

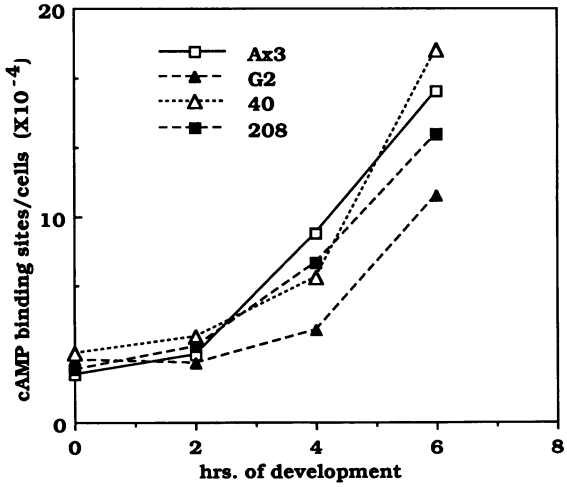

Figure 8. Developmental kinetics of cell surface cAMP binding sites in different strains. Cells were plated as described in Figure 5, harvested at the times indicated, and assayed for total cAMP binding sites. See MATERIALS AND METHODS for details and references.

When wild-type cells are plated at 5°C, they enter the developmental program; however, aggregation and subsequent multicellular development are not observed at this temperature. When these cells are incubated at 5°C for 16 h and then placed at 20°C, aggregation rapidly ensues after ~2 h followed by multicellular differentiation. As described above, when Gα2(G40V) is expressed in *g*α2 null cells plated at 20°C, the cells do not aggregate and there is no cAMP activation of AC or GC. However, when these cells are placed at 5°C for 16 h and then placed at 20°C for another 22 h, the JH104 Gα2(G40V)-expressing cells aggregate and approximately one-half develop further and produce small fruiting bodies. With Gα2(Q208L)-expressing cells, approximately one-half of the cells aggregate to form small mounds but do not proceed further.

DISCUSSION

In this study, we have used molecular genetic approaches to examine the in vivo effect of specific amino acid substitutions within the Gα2 coding region on the ability of Gα2 to activate AC, GC, and PI-PLC when expressed in Gα2 wild-type and *g*α2 null backgrounds. Two of the mutations that were examined, Gα2(G40V) and Gα2(Q208L), are homologous to mutations in *ras* and the Gα subunit Gαs that cause a reduction in the intrinsic GTPase activity, leading to constitutively active G protein (see INTRODUCTION). In *Dictyostelium*, however, these mutations resulted in a dominant negative phenotype in vivo. Wild-type KAx-3 cells expressing Gα2(G40V) or Gα2(Q208L) showed substantially delayed development and lacked cAMP-activation of GC and PI-PLC. Furthermore, the inhibition of aggregation in these strains is consistent with a proposed role of GC, and possibly PI-PLC, in regulating chemotaxis (see INTRODUCTION).

The developmental phenotypes of the two mutants Gα2(G40V) and Gα2(Q208L) were somewhat different. Expression of Gα2(G40V) in wild-type cells resulted in retarded early development but relatively normal kinetics during the multicellular stages. By comparison, the construct expressing Gα2(Q208L) resulted in the suppression of both early and late developmental processes. The lack of an observed phenotype late in development in Gα2(G40V) cells suggests that Gα2 may have a different function in late development than during aggregation or that the effect of Gα2(G40V) at this stage produces a phenotype that is too subtle to be seen. Because the overexpression of wild-type Gα2 does not inhibit GC and PI-PLC activation during aggregation, we expect the dominant negative phenotypes of Gα2(G40V) and Gα2(Q208L) are not due to unusually high levels of a Gα subunit that could compete for a possible limited amount of βγ subunits. Expression of Gα2(G43V) and Gα2(G207V) resulted in a small inhibition of GC activation when expressed in wild-type cells. Gα2(G43V) showed no effect on AC activation, whereas Gα2(G207V) resulted in a low level of inhibition similar to that seen with overexpression of wild-type Gα2. The relative strength of the two mutant Gα2 subunits in inhibiting signaling processes parallels the known relative effect on inhibiting the intrinsic GTPase activity (Graziano and Gilman, 1989). Expression of Gα2(G207V) partially complemented GC and AC activation in *g*α2 null cells, suggesting it can couple with cAMP receptors and stimulate downstream effectors with an apparent lower efficiency than wild-type Gα2. This could be due to a lower efficiency of activation or an impaired ability to undergo conformational changes required for the efficient release of βγ subunits, as has been shown for the homologous mutation in Gαs (Lee *et al.*, 1992).

Role of Gα2 in GC and PI-PLC Responses

Our analyses further define the role of Gα2 in the activation of GC and PI-PLC. Although previous reports showed Gα2 is essential for cAMP-activation of GC (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989, 1991), no analysis has been reported on the role of Gα2 in cAMP receptor activation of PI-PLC stimulation. cAMP-mediated activation of GC is low in vegetative cells and progressively increases at 2, 4, and 6 h of development. Transformants overexpressing Gα2 show a high level of cAMP-mediated GC activation in vegetative cells that is similar to that seen at 4 h of development in control cells, whereas cells starved for 6 h show a supermaximal response. The observation that the level of cAMP receptors is similar in control and Gα2-overexpressor lines suggests that during vegetative growth, and at later stages of development, the level of Gα2 may be rate limiting for the activation of GC activity. Our molecular genetic approaches, combined with earlier analysis showing that GC is not activated in *g*α2 cells, suggest that Gα either directly activates GC or is the Gα subunit that is upstream from GC in the signal transduction pathway. These experiments do not exclude the possibility that expression of Gα2 during vegetative growth induces the expression of other proteins required for GC activation. Therefore, although we think it is unlikely, it is possible that the effect of Gα2 on the precocious developmental activation of GC is indirect.

We also have shown that activation of PI-PLC is dependent on Gα2, because no increase in 1,4,5-IP_3 can be measured in *g*α2 null cells. Because we can examine this activation in vegetative cells, we have reduced, but not eliminated, the possibility that PI-PLC activation requires other developmentally regulated genes. These results, therefore, are also support the model that PI-PLC is also directly activated by Gα2. Furthermore, we have demonstrated that IP_3 levels are developmentally regulated and decrease for the first 6 h of development from the level found in vegetative cells. This observation is consistent with our findings that Ca^{2+}-stimulated PI-PLC is high in vegetative cells and decreases through

early development (Cubitt and Firtel, 1992). Interestingly, the characteristics and responsiveness of PI-PLC activation also display developmental changes. In vegetative cells, cAMP stimulation leads to a 2- to 2.5-fold increase in 1,4,5-IP_3 levels over 20 s, at which point a steady-state level of 1,4,5-IP_3 is reached that does not decline for ≥40 s. This could be due to the adaptation of the PI-PLC in the absence of 1,4,5-IP_3 degradation or to reaching a steady state of continued phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and 1,4,5-IP_3 degradation. This is in stark contrast to the very transient and small response seen in aggregation-stage cells, which adapts in 7–10 s (van Haastert *et al.*, 1989). In vegetative cells, the basal level of 1,4,5-IP_3 is very high relative to the levels required for calcium release in other systems (van Haastert *et al.*, 1989). This high steady-state level of 1,4,5-IP_3 in vegetative cells is consistent with a high steady-state level of diacylglycerol at these stages (Cubitt and Firtel, unpublished data) and may indicate that this second messenger plays an important role in regulating vegetative cell growth or gene expression.

From studies in other systems, we expected that the Gα2(G40V) and Gα2(Q208L) mutations would lead to a constitutive activating phenotype. In fact, in *Dictyostelium*, wild-type cells expressing these Gα subunits lack receptor-mediated activation of GC and PI-PLC, implying that they exert a dominant negative phenotype. One possible explanation for this observation is that expression of the constitutively activating G proteins leads to the down-regulation and adaptation of these second messenger systems. In this model, these mutant Gα subunits produce a low level constitutive activation of GC and PI-PLC that result in a feedback adaptation of these pathways. In wild-type cells, both the GC and PI-PLC pathways adapt within 7–10 s during the aggregation stage. In support of the above model, Small *et al.* (1987) showed that in permeabilized cells, addition of GTPγS resulted in adaption of the ability of cAMP to activate GC and suggested that this adaptation is mediated via a G protein. A result that is consistent with our model is that 1,4,5-IP_3 levels are reduced in vegetative cells expressing Gα2(G40V) or Gα2(Q208L). We also observed that when JH104 *gα2* null cells expressing Gα2(G40V) are incubated at 5°C for 16 h and then placed at 20°C, the cells aggregate and form some fruiting bodies. JH104 cells expressing Gα2(Q208L) under the same conditions will form loose aggregates at a low frequency. These results suggest that one or more of the following is true: 1) cells expressing Gα2(G40V) or Gα2(Q208L) can either bypass Gα2 function when incubated at 5°C; 2) Gα2(G40V) and Gα2(Q208L) can function sufficiently at this temperature in vivo; and/or 3) the adaptation pathway does not function at 5°C. Previous results from van Haastert (1987) show that both AC and GC can be activated at 0°C, but the pathways do not adapt at this temperature. These observations are thus consistent with our hypothesis that the expression of "activated" Gα2 subunits leads to a constitutive adaption of downstream pathways. Although the mechanism of adaptation is unknown, both the receptor cAR1 and Gα2 have been demonstrated to be phosphorylated (Vaughan and Devreotes, 1988; Gunderson and Devreotes, 1990). The kinetics of these phosphorylations are too slow to account for the adaptation of GC and PI-PLC but are consistent with the adaptation of AC. We also note that the majority of Gα2 is not phosphorylated in Gα2(G40V)- and Gα2(Q208L)-expressing cells (results not shown).

One possible mechanism for the postulated adaptation of GC and PI-PLC is that the second messenger pathways may stimulate a kinase that specifically phosphorylates the effectors themselves, preventing activation by the Gα2 subunit. Depending on the kinetics of adaptation and deadaptation, the constitutively activating Gα subunits could result in the effectors being nonresponsive to exogenous stimulation. We also observed that overexpression of wild-type Gα2 resulted in a higher level of GC activation. It is possible that Gα2 may be rate limiting even at 6 h of development and would be consistent with our observations that Gα2 expression in vegetative cells results in a substantial increase in the ability of cAMP to stimulate GC activity in vivo. Overexpression of wild-type Gα2 would not be expected to produce the same phenotype as the Gα2(G40V) and Gα2(Q208L) mutations because the wild-type Gα subunit would be fully dependent on receptor stimulation to elicit a response and would be rapidly converted to the Gα2-GDP form, which would not activate downstream effectors.

Regulation of AC

In contrast to the effects observed on GC, overexpression of Gα2 did not result in a precocious ability to activate AC but decreased the maximum response in 6-h cells. The actual mechanism of AC activation in *Dictyostelium* is unknown and occurs after a delay compared with the immediate activation of GC and PI-PLC. Kesbeke *et al.* (1988) previously has shown that in *gα2* null cells, AC can be activated in vitro by 100 μM of GTPγS. This implies that either Gα2 is not the only Gα subunit required for activating AC or that AC is stimulated by released βγ subunits and/or indirectly through Ca^{2+}/calmodulin, as has been described for some mammalian AC (Tang and Gilman, 1991; Tang *et al.*, 1991; Federman *et al.*, 1992). The possibility that AC in *Dictyostelium* is activated via released βγ subunits from the G protein containing the Gα2 subunit is supported by the observation that overexpression of Gα2 inhibits AC activation. In this model, the observation that GTPγS can activate AC in vitro in *gα2* null cells can be explained by the release of βγ subunits from other G proteins expressed at the same time in development (e.g., Gα1, Gα6, Gα7, and Gα8) (Kumagai *et*

al., 1989; Pupillo *et al.*, 1989; Wu and Devreotes, 1992; Gaskins, Zhou, and Firtel, unpublished data). In $g\alpha 2$ null strains, cAMP occupation of cAR1 would not produce free $\beta\gamma$ subunits. Although it is possible that the inhibition of AC is caused by a Gα2-induced down-regulation of cAMP receptor or adaptation of other effector pathways, this seems unlikely given the enhanced levels of cGMP accumulation seen at the same time in these cells. Furthermore, the Gα2(G40V) and Gα2(Q208L) mutations, which strongly suppress the activation of GC and PI-PLC, only weakly inhibit AC. This is consistent with the model that Gα2 couples directly to GC and PI-PLC but only indirectly to AC, possibly via the release of $\beta\gamma$ subunits. In further support of our model, Gα2(G207V), which would be expected to associate with $\beta\gamma$ subunits but not efficiently release them upon receptor activation (se INTRODUCTION; Lee *et al.*, 1992), suppress AC activation to the same extend as overexpressing wild-type Gα2. Further characterization of the Gα subunits Gα7 and Gα8, which are expressed during aggregation, may shed light on the mechanisms by which these second messenger pathways are regulated during aggregation and the degree of cross talk possible between the pathways.

Our results on the possible mechanisms controlling signal transduction processes activated by cAMP and mediated through Gα2 are based on molecular genetic approaches, examining the in vivo phenotype resulting from the expression of mutant Gα2 protein subunits in wild-type and $g\alpha 2$ null backgrounds. Further analysis will require establishing the appropriate in vitro assay systems that can be used to directly examine the role Gα2 plays in the activation of these pathways and the pathways that lead to adaptation of these pathways.

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