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Mutations in the *Dictyostelium* heterotrimeric G protein α subunit Gα5 alter the kinetics of tip morphogenesis

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SUMMARY

Tip morphogenesis during the *Dictyostelium* developmental life cycle is a process by which prestalk cells sort to form the anterior region of the multicellular organism. We show that the temporal regulation of this morphological process is dependent on the copy number of the *Dictyostelium Gα5* gene. Tip formation is delayed in aggregates of *gα5* null mutant cells and accelerated in aggregates overexpressing the *Gα5* gene compared to tip formation in wild-type cells. The onset of cell-type-specific gene expression associated with mound formation and tip morphogenesis is also temporally altered in *Gα5* mutants. Tip morphogenesis in chimeric organisms of *Gα5* mutants and wild-type cells is dependent on the copy number of the *Gα5* gene, indicating that Gα5 function plays an integral role in the intercellular signaling of this stage of development. The *Gα5* gene encodes a Gα subunit that has 51% identity to the *Dictyostelium* Gα4 subunit. Like the *Gα4* gene, the *Gα5* gene is expressed in a subset of cells distributed throughout the multicellular organism, with a distribution that is similar to the anterior-like cell population. Amino acid substitutions in the Gα5 subunit analogous to substitutions altering guanine nucleotide binding and hydrolysis in other Gα subunits had no apparent effect on the rate of tip formation when a single copy of the mutant gene was used to replace the wild-type gene. Overexpression of these mutant *Gα5* genes by increased gene dosage resulted in cell death, suggesting that high levels of the altered subunits have detrimental effects during vegetative growth.

Key words: *Dictyostelium*, G protein, tip morphogenesis, temporal regulation, *Gα5*, mound formation

INTRODUCTION

Nutrient-deprived *Dictyostelium* cells aggregate to form a multicellular mound (typically 10^5 cells) that differentiates to form a migratory slug and then eventually a fruiting body (a mass of spores on top of a stalk) (Loomis, 1982). One of the earliest morphogenic processes of this developmental life cycle is the migration of cells to the top of the multicellular mound to form a tip, which represents the anterior end of the organism (Williams et al., 1989; Traynor et al., 1992; Esch and Firtel, 1991; Early et al., 1993). As the tip forms, the entire aggregate elongates upward to form a finger and then falls to the substratum as a migrating slug or pseudoplasmodium. The cells in the anterior region of the developing organism comprise multiple prestalk cell populations that give rise to the stalk structure and upper cup regions of the fruiting body (Early et al., 1993; Jermyn and Williams, 1991), whereas the majority of cells located in the central and posterior regions of the organism are prespore cells (precursors to the spore mass). In addition to the prestalk and prespore cells, the multicellular organism has anterior-like cells (ALCs) that exist scattered throughout the organism and these cells become localized as part of the lower cup and basal disk of the fruiting body (Williams et al., 1993). ALCs share many characteristics with prestalk cells such as the retention of the dye neutral red and expression of certain classes of developmentally regulated genes. ALCs can be recruited to form new prestalk cells in aggregates that have had their anterior regions surgically removed (Sternfeld and David, 1982; Sternfeld, 1992). In some large aggregates, multiple tips can form and lead to a division of the aggregate into multiple organisms, indicating that tip morphogenesis is also important for controlling the size of the multicellular organism. Moreover, analysis of the function of the heterotrimeric G protein α subunit Gα4, which is preferentially expressed in ALCs during the multicellular stages, suggests that ALCs play a cell non-autonomous role in regulating morphogenesis and cell-type differentiation (Hadwiger and Firtel, 1992; Hadwiger et al., 1994).

The role of extracellular cAMP in directing tip morphogenesis was proposed from the initial observations of directed migration of prestalk cells in response to exogenously added extracellular cAMP (Matsukuma and Durston, 1979; Sternfeld and David, 1981). Consistent with these observations, multicellular aggregates overexpressing an extracellular phosphodi-

esterase do not form tips, presumably due to the hydrolysis of extracellular cAMP, and marked prestalk cells sort to the base of the aggregate when mounds are placed on agar containing high levels of cAMP (Traynor et al., 1992). In addition, cells in which the gene encoding the cell surface cAMP receptor (cAR) cAR2 has been disrupted are developmentally blocked at the stage of tip formation, implying that cAR2 and cAMP are integral components of the signaling mechanisms required for this morphogenetic process (Saxe et al., 1993). However, some *car2* null aggregates eventually overcome this block in development after several hours, suggesting that other cARs can facilitate tip formation. In addition to regulating tip formation, extracellular cAMP is the primary signal for aggregation during early stages of development and an important inductive signal for developmental gene expression in the multicellular mound (Devreotes, 1994; Schnitzler et al., 1994, 1995).

Intensive searches for components involved in G protein-mediated signal transduction pathways have resulted in the identification of eight Gα subunit genes in *Dictyostelium* (Devreotes, 1994; Pupillo et al., 1989; Hadwiger et al., 1991; Wu and Devreotes, 1991; Cubitt et al., 1992; Wu et al., 1994). One of these genes, *Gα5*, was identified in a PCR (polymerase chain reaction) screen using degenerate primers to conserved regions of known Gα subunits (Hadwiger et al., 1991). Sequence analysis of the *Gα5* PCR product indicated homology between the Gα5 subunit and other Gα subunits. The *Gα5* gene was found to be expressed at low levels during growth and early development but at a significantly higher level during multicellular development, similar to the temporal expression pattern of the *Dictyostelium Gα4* gene (Hadwiger and Firtel, 1992; Hadwiger et al., 1991; Wu and Devreotes, 1991). In this report, we show that during the multicellular stages of development the *Gα5* gene is preferentially expressed in cells with a spatial pattern similar to that of *Gα4*-expressing cells and ALCs, and we provide evidence that the Gα5 subunit plays a functional role in the temporal control of tip morphogenesis.

MATERIALS AND METHODS

Strains and medium

In this study, the following axenic haploid *Dictyostelium* strains were used: KAx-3 (wild-type), JH10 (*thy1::PYR5-6*) [described in (Hadwiger and Firtel, 1992)], JH257(*gα5::THY1*), JH258 ($G\alpha5^{HC}$, contains a high copy number of the *Gα5* expression vector pJH206), and JH262 (strain JH257 with a low copy of the pJH206 construct). All strains used in this study were isogenic to the wild-type strain KAx-3, except at the loci noted, and all strains were grown axenically in HL5 medium or on *K. aerogenes* unless otherwise noted. Clonal isolates were obtained by plating transformed cell lines in 96-well microtiter plates. Electroporation of DNA into *Dictyostelium* cells was performed as described previously (Dynes and Firtel, 1989).

DNA constructs

Genomic DNA libraries used to isolate the *Gα5* gene were constructed by digesting genomic DNA with *Xho*I/*Bcl*I or *Bcl*I only and then ligating size-specific fragments to the vector pJH81 digested with *Sal*I/*Bcl*I or *Bcl*I only, respectively. pJH81 was created by inserting an *Xba*I/*Bcl*I linker (5′-CTAGAGGGTGATCACCCT-3′) into the *Xba*I site of the vector pT7T318U vector (Pharmacia) to create a novel *Bcl*I site between duplicated *Xba*I sites. *Gα5* genomic fragments [*Xho*I/*Bcl*I (2.1 kb) and *Bcl*I (1.0 kb)] were joined together in pJH81 to create the plasmid pJH198 that contains a contiguous 3.1 kb *Gα5* genomic insert. The *Gα5* expression vector pJH206 was created from pJH198 by the insertion of a 2.2 kb *Eco*RI fragment carrying a *pAct6::Neo* gene fusion (described in (Hadwiger and Firtel, 1992)) at the *Eco*RI site of the polylinker to confer resistance to the drug G418. The pJH206 vector was linearized at the remaining *Xba*I site in the polylinker region before electroporating into *Dictyostelium* cells to increase the probability of random integration.

To create a disrupted *gα5* allele, an *Eco*RV/*Xba*I fragment of the *Gα5* gene from pJH198 was inserted into the same sites of the pBluescriptSK+ vector (Stratagene). A 1.6 kb region containing primarily the *Gα5* open reading frame was PCR amplified from this construct using the oligonucleotide JH129 (5′-GTTTGAATTCTTCCAACTC-TACC-3′, complementary to a sequence 80 bases downstream of the *Gα5* open reading frame and also containing an *Eco*RI site) and an M13 universal primer (United States Biochemical) was used to generate an 1.6 kb fragment by PCR. This fragment was digested with *Eco*RI and *Sal*I and then inserted into the same sites of pT7T318U to create the plasmid pJH213. The *Gα5* open reading frame of this plasmid was disrupted by inserting a 3.0 kb *Pst*I/*Xba*I fragment containing the *THY1* gene into the *Pst*I/*Spe*I sites of the *Gα5* sequence. The resulting plasmid, pJH214, was digested with *Eco*RV and *Eco*RI and then electroporated into the strain JH10 to create a *gα5* gene disruption. Genomic DNA blots probed with the *Gα5* genomic insert of pJH213 revealed 7 and 0.9 kb *Bcl*I fragments and 5.5 and 1.6 kb *Cla*I fragments for *gα5* null cells, compared to the 4 and 0.9 kb *Bcl*I fragments and the 6 kb *Cla*I fragment for wild-type cells. These results are consistent with homologous recombination at the *Gα5* locus.

The *pGα5::lacZ* gene fusion vector was created by ligating a *Bam*HI linker (12 mer) to the *Eco*RV site of the *Gα5* genomic fragment. Then a 1 kb *Sph*I/*Bam*HI fragment containing the *Gα5* upstream sequence was inserted into the *Sph*I/*Bam*HI sites of pJH146 as described (Hadwiger and Firtel, 1992), creating an coding region fusion of the *Gα5* gene and the *Escherichia coli lacZ* gene. The 2.2 kb *Eco*RI fragment containing the *pAct6::Neo* gene fusion was inserted at the *Eco*RI site to create the plasmid pJH210.

Gα5 point mutations were created by site-directed in vitro mutagenesis as described (Kunkel, 1985). Oligonucleotides 5′-CTTCTTTCTGATCTTAGTCCACCAACATC-3′ ($G\alpha5^{Q198L}$) or 5′-CTGATCTTTGTCCAGTAACATCTAACATTC-3′ ($G\alpha5^{G196T}$) were used as primers to mutagenize a 0.3 kb *Spe*I/*Bcl*I fragment of the *Gα5* gene. The mutagenized *Gα5* fragments were used to replace wild-type fragments in pJH230 which consisted of the *Xho*I/*Hin*cII *Gα5* genomic fragment in the *Sal*I/*Xba*I sites of pT7T318U. The 1.0 kb *Hin*dIII/*Eco*RV fragment of these resulting plasmids was deleted to remove the *Gα5* promoter and initial coding region. A 3.2 kb *Bam*HI fragment containing the *Dictyostelium THY1* gene (Dynes and Firtel, 1989) was then inserted into the *Bam*HI site of these vectors to create the vectors pJH250 ($G\alpha5^{Q198L}$) and pJH252 ($G\alpha5^{G196T}$). These vectors and a wild-type control vector pJH255 (identical to pJH250 and pJH252 except for point mutations) were linearized at the *Spe*I site within the *Gα5* coding region and electroporated into the strain JH10. Transformants with a single copy of these vectors integrated precisely into the *Spe*I site of the *Gα5* locus were identified by genomic DNA blot analysis. Approximately 40% of the transformants contained the correct integration event. For high-copy integration of *Gα5* point mutant alleles, the *Bam*HI/*Spe*I fragments of pJH250 and pJH252 were used to replace the *Spe*I/*Bam*HI fragment of pJH206 to create the vectors pKN1 and pKN2, respectively, which were electroporated into KAx-3 cells.

Analysis of morphology and β-galactosidase activity

Analyses of morphology and β-galactosidase activity during multicellular development were performed on cells grown to mid-log phase

($1\text{-}2\times10^6$ cells/ml) in shaking cultures of HL5 medium. Cells were washed free of medium in 12 mM sodium/potassium phosphate buffer (pH 6.1), resuspended in phosphate buffer at 2×10^7 cells and then spread directly on non-nutrient plates for development. For β-galactosidase staining, cells were developed on nitrocellulose filters resting on top of non-nutrient agar plates and then stained for β-galactosidase activity as described previously (Haberstroh and Firtel, 1990).

Assays of the β-galactosidase activity of developing aggregates were performed by subjecting cells to a freeze/thaw cycle and then incubating extracts with ONPG (o-nitro phenyl-β-D-galactoside) as described (Dingermann et al., 1989).

DNA and RNA blots

Dictyostelium DNA blots were performed as described (Sambrook et al., 1989) and RNA blots were done as described previously (Mann and Firtel, 1987). DNA probes were generated by random primer probe synthesis using a Prime-a-Gene kit (Promega) or a Genius kit (Boehringer Mannheim) according to the manufacturers' instructions.

The GenBank/EMBL accession number for the *Gα5* DNA sequence is #U20806.

RESULTS

Cloning and structure of the *Gα5* gene

Using a previously isolated *Gα5* PCR-generated fragment (Hadwiger et al., 1991) as a hybridization probe, the *Gα5* gene was isolated as two fragments (2.1 kb *Xho*I/*Bcl*I and 1.0 kb *Bcl*I fragments) from genomic DNA libraries (see Materials and Methods). These fragments were combined to make a contiguous 3.1 kb genomic segment that contained the entire *Gα5* open reading frame, 1.1 kb of upstream sequence and 0.7 kb of downstream sequence. Sequence analysis revealed this open reading frame encodes 345 amino acids distributed on three exons divided by two small introns (Fig. 1). The predicted primary sequence of the Gα5 subunit is more related to the *Dictyostelium* Gα4 subunit (~51% identical) than any other Gα subunit from *Dictyostelium* or other organisms. In addition to the identity at the amino acid sequence level, the *Gα5* and *Gα4* genes both have introns located in analogous positions with respect to the open reading frames (Fig. 2). Both the Gα5 and the Gα4 subunits are more closely related to the members of the Gαi class (Simon et al., 1991) than to any other class of mammalian subunits (data not shown).

Gα5 mutant strains show temporally aberrant development

To gain insight into the function of the Gα5 subunit, a *gα5* null mutant was created by gene disruption. The *Dictyostelium THY1* gene was inserted into the *Gα5* open reading frame at the *Pst*I and *Spe*I sites of a PCR-generated fragment containing the majority of the *Gα5* open reading frame (see Materials and Methods). This construct was electroporated into the thymidine auxotrophic strain JH10 and then prototrophic transformants were selected and screened for disruption of the

```
                                30                                  60
tttaaaaataaattgtgcataaaatcacacagactcacacaaacacacatacatacacacacatatatag
                     90                         120
tcacttaataaattaatagacattaaaaagATGGGTTGTATATTAACAATTGAAGCAAAGAAATCAAGAG
                                  M  G  C  I  L  T  I  E  A  K  K  S  R
       150                        180                                 210
ATATCGACTATCAATTAAGAAAAGAAGAAGGAAGTAAAAATGAAACCAAATTACTTTTATTGGGACCTGG
D  I  D  Y  Q  L  R  K  E  E  G  S  K  N  E  T  K  L  L  L  L  G  P  G
                               240                         270
TGAATCAGGTAAATCAACTATATTTAAACAAATGAAGATTATTCAAGACGATGGTGGTTTCTCAATAGAC
  E  S  G  K  S  T  I  F  K  Q  M  K  I  I  Q  D  D  G  G  F  S  I  D
                     300                         330
GAAAGATTAGAATACAGATATATTATTTATGGTAATTGCATAAGCCAAATGAAAGTTTTGGTAACTGCTG
 E  R  L  E  Y  R  Y  I  I  Y  G  N  C  I  S  Q  M  K  V  L  V  T  A
       360                        390                                 420
CTATCTCTCAAGATTTAAAACCAAATAATCCTGATAATGAGgtaagatatatatatatattaataatc
           A  I  S  Q  D  L  K  P  N  N  P  D  N  E
                               450                         480
attctcttcattcattatcatacactattcatcaatttttctttctttttttttttaaaaattattatc
                     510                         540
ttttttttttttttttttttttttttcttcgttgtaaacactttcatcacctttttttttatttttttt
       570                        600                                 630
atttattttcgccgcactgaagaaaatatgacgtgtttttctcaaactttttttccgctcaaaaaaaaaa
                               660                         690
aaaaaaaaaataatcaaaaaaaaaaaaaaaaaaaatcaaaaaaatcaaaaaaatcaaaaaaataattcga
                     720                         750
ttttatttttttctatttttatttaaatgattaaatgattaatttttatataatttttattaatataat
       780                        810                                 840
tatagACTAGATTTGAAAAGTTTTCAAAGATTTCACCAGGTGGCAACTCTTGGACATTAGAGATTGCAGA
      T  R  F  E  K  F  S  K  I  S  P  G  G  N  S  W  T  L  E  I  A  E>
                               870                         900
AGATATTAAACAATTATGGAGCGATGATAGTATACAAAATATTTATAGAATGAAAGATAAATTTTATCAA
  D  I  K  Q  L  W  S  D  D  S  I  Q  N  I  Y  R  M  K  D  K  F  Y  Q>
                               930                         960
TTAAATGATTCTGCAGCATAgtatgtatactagtattattagtagttgaagaagtagtagtaatagttaa
 L  N  D  S  A  A  Y
       990                             1020                          1050
ttttttttttttaagataactaattaatttttttttttttttttttaaaattatagCTTTTTGATA
                                                                F  F  D
                               1080                          1110
ATATTGGTAGATTTGCAAATGAAAATTATGTACCAACACAAGATGATGTATTAAGATCAAGAGTACGTAC
N  I  G  R  F  A  N  E  N  Y  V  P  T  Q  D  D  V  L  R  S  R  V  R  T
            1140                          1170          *     *
AACTGGTATTCAAGAAGCACACTTTAAATTCATTAATATAGAATTTAGAATGTTAGATGTTGGTGGACAA
  T  G  I  Q  E  A  H  F  K  F  I  N  I  E  F  R  M  L  D  V  G  G  Q
       1200                          1230                          1260
AGATCAGAAAGAAGAAAATGGATTCATTGTTTTGATTCAGTTACAGCAGTTATATTTTGCGTTGCTTTGA
 R  S  E  R  R  K  W  I  H  C  F  D  S  V  T  A  V  I  F  C  V  A  L
                               1290                          1320
GCGAATATGATCAAACATTACGTGAAGAAGAATCTCAAAATAGAATGAAGGAATCGTTAATGTTATTCGA
S  E  Y  D  Q  T  L  R  E  E  E  S  Q  N  R  M  K  E  S  L  M  L  F  D
                     1350                          1380
TGAAATTGTTAATAGTCATTGGTTTAGAAATACAGCATTCATTATATTTTTTAATAAAGTCGATCTTTTC
  E  I  V  N  S  H  W  F  R  N  T  A  F  I  I  F  F  N  K  V  D  L  F
       1410                          1440                          1470
CGTGAGAAAATCGCAAAGATTGATTTAGGTGATTATTTCCCCGCTTATACTGGTGGTTTATCATTTGATA
 R  E  K  I  A  K  I  D  L  G  D  Y  F  P  A  Y  T  G  G  L  S  F  D
                               1500                          1530
ACTCTACTCAATTTATAAAGAAAATGTTTTTAGACCTTTCAACTGGAAATCAAAGAATCTTTGCTCATTT
N  S  T  Q  F  I  K  K  M  F  L  D  L  S  T  G  N  Q  R  I  F  A  H  F
                     1560                          1590
CACTTGTGCAATTGATACTGCAAATATTCAATTTGTATTTCATGCTGTTAGAGAAACTTTATTAAAAAAT
  T  C  A  I  D  T  A  N  I  Q  F  V  F  H  A  V  R  E  T  L  L  K  N
       1620                          1650                          1680
ATCTTTAATACAATCATAAATTATTAAatctttttttttttttttttttttttttgaaataaaagaaaaa
 I  F  N  T  I  I  N  Y  *
aaaaaaaaaaagtaataataataat
```

Fig. 1. Genomic nucleotide sequence and deduced amino acid sequence of the *Gα5* gene. Lower-case sequence represents nontranslated regions. The termination codon is indicated by an asterisk (*). GenBank/EMBL accession number for the *Gα5* DNA sequence is #U20806.

```
Gα5: MG..CILTIEAKKSRDIDYQRKEEGSK..NETKLLLLGPGESGKSTIFKQMKIIQDDGGFSIDERLEYRYIIYGNCISQM
Gα4: -RFK-FGSE-TEQ-SK--KSIETDRR-LRKDV----------------------E---Y-VE-L----AFV-S------
                        i                                                       i
Gα5: KVLVTAAISQDLKPNNPDNETRFEKFSKISPGGNSWTLEIAEDIKQLWSDDSIQNIYRMKDKFYQLNDSAAYFFDNIGRF
Gα4: EA-L--SAKLNIELEVENK..QRAANVLRRTI--EPW-LL-A---H--E-KG-KET-AQ---HF------------D-Y
                                        * *
Gα5: ANENYVPTQDDVLRSRVRTTGIQEAHFKFINIEFRMLDVGGQRSERRKWIHCFDSVTAVIFCVALSEYDQTLREEESQNR
Gα4: MR-DF--NEQ----C---------SE-T-DK-RLKIV-------Q---------C------VA-M-D---V---D--V--

Gα5: MKESLMLFDEIVNSHWFRNTAFIIFFNKVDLFREKIAKIDLGDYFPAYTGGLSFDNSTQFIKKMFLDLSTGNQRIFAHFT
Gα4: TR---A--K----CDY-KE-PIVL-L--K---K--LKRVP-QSC-SD---PNKYKDAAM--QSQY-AQGPSPRT-YT-A-

Gα5: CAIDTANIQFVFHAVRETLLKNIFNTIINY
Gα4: --V--E--K---R---Q-I-SQALEHF
```

Fig. 2. Alignment of the *Dictyostelium* Gα5 and Gα4 subunits. Dashes (-) represent identical amino acid residues. Periods (.) represent gaps created to enhance the alignment. Locations of *Gα5* and *Gα4* introns indicated by (i). Glycine (G) at position 196 and glutamine (Q) at position 198 are denoted by asterisks (*).

Gα5 locus by genomic DNA blot analysis. All *gα5* null mutants, as determined by disruption of the *Gα5* locus, showed a 3 hour delay in the formation of tip structures on multicellular mounds (Fig. 4). All subsequent developmental stages were also delayed by ~3 hours (data not shown). In addition, the *gα5* null mutants formed aggregates, slugs and spore masses that were generally larger than those of wild-type cells. The larger slugs of the *gα5* null mutants also had a greater tendency to lose cells from the posterior region compared to wild-type slugs (data not shown). These developmental phenotypes were rescued by the integration of the vector pJH206 (Fig. 3) into the genome at a single or low copy (~2-10 copies) number at loci other than *Gα5*, indicating that the 3.1 kb genomic fragment contains all the necessary sequences for complementing the *gα5* null mutation. However, the integration of many copies (>15) of this *Gα5* vector into wild-type or *gα5* null mutant cells resulted in the overexpression of the *Gα5* gene and aberrant temporal regulation of morphogenesis. Developing aggregates of $G\alpha5^{HC}$ (*Gα5* gene at high copy number) cells had precocious tip formation ~3 hours earlier than wild-type aggregates (Fig. 4) and this morphogenic process was initiated even before the aggregation stage was completed. All subsequent stages of the $G\alpha5^{HC}$ development were also ~3 hours precocious compared to wild-type development. In contrast to *gα5* null cells, $G\alpha5^{HC}$ cells formed aggregates, slugs and spore masses that tended to be much smaller than the corresponding structures of developing wild-type cells.

To determine whether the aberrant temporal regulation of tip morphogenesis in *Gα5* mutant aggregates could be affected by intercellular signaling, *Gα5* mutants and wild-type cells were mixed to create chimeric organisms. Aggregates containing equal portions of *gα5* null mutant and wild-type cells formed tips at the rate expected for wild-type cells, indicating that cells expressing the *Gα5* gene exert a dominant role in the rate of tip formation (Fig. 5). Cells overexpressing the *Gα5* gene also exerted a dominant effect on the chimeric organism with respect to tip formation, as aggregates containing mixtures of $g\alpha5/G\alpha5^{HC}$ or wild-type/$G\alpha5^{HC}$ cells formed tips at the rate expected for aggregates of only $G\alpha5^{HC}$ cells, indicating that Gα5 functions cell non-autonomously in regulating tip formation (Fig. 5).

The timing of late gene expression is altered in *Gα5* mutant strains

The correlation between Gα5 function and the temporal regulation of tip morphogenesis was also investigated with respect to developmental gene expression. RNA was isolated from developing *Gα5* mutants and wild-type cells at various times after starvation and the level of expression of the prespore gene *SP60* and two distinct prestalk genes, *ecmA* and *ecmB*, was

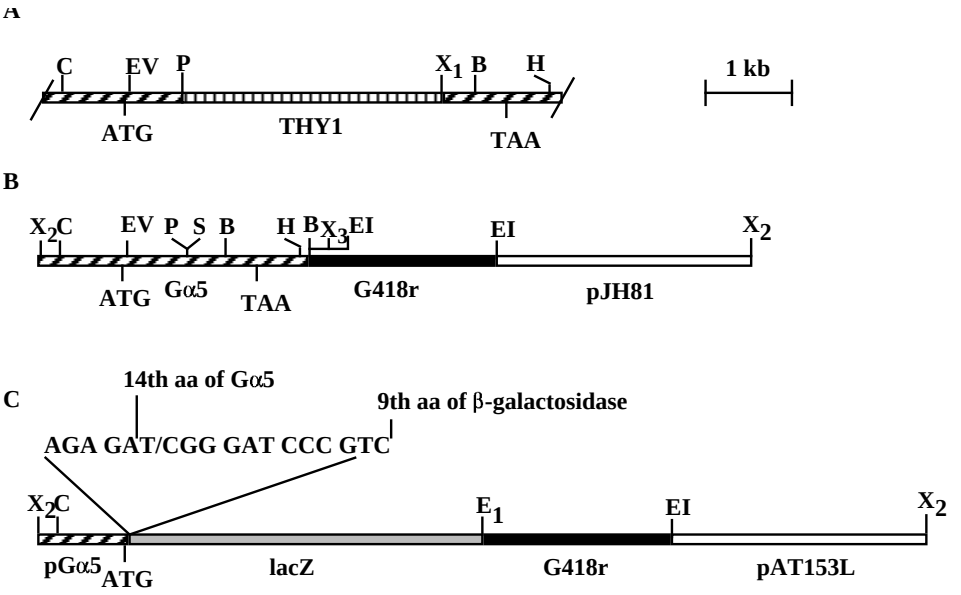

Fig. 3. *Gα5* gene constructs. (A) Map of *gα5::THY1* gene disruption. (B) Map of *Gα5* expression vector pJH206. Plasmids pKN1 and pKN2 are identical to pJH206 except for specific mutations within the *Gα5* coding region. (C) Map of *pGα5::lacZ* gene fusion vector pJH210. A detailed description of the constructs is presented in Materials and Methods. *Gα5* genomic sequences are represented by solid segments. The initiation and termination codons are represented by ATG and TAA respectively. G418r represents the *pACT6::Neo*^{r} gene fusion that confers resistance to the drug G418. Restriction enzymes are shown: *Bcl*I (B), *Cla*I (C), *Eco*RI (EI), *Eco*RV (EV), *Hinc*II (H), *Pst*I (P), *Spe*I (S), *Xba*I/*Spe*I junction (X_1), *Xho*I/*Sal*I junction (X_2), *Xba*I (X_3).

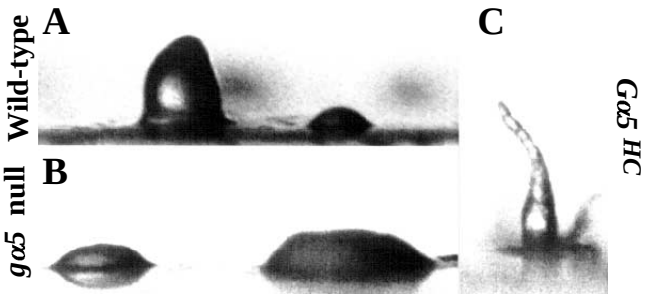

Fig. 4. Developmental morphology of (A) wild-type, (B) *gα5* and (C) *Gα5*^{HC} cells at 12 hours after starvation. Mutant and wild-type cells were grown and plated for development as described in Materials and Methods.

analyzed by RNA blot analysis. Using RNA isolated every 6 hours during development, it was clear that the induction of all three genes was delayed in the *gα5* null strain as compared to that in the wild-type strain (Fig. 6A). The same blots showed that expression of the two prestalk markers was induced earlier relative to starvation in *Gα5*^{HC} cells, but these blots could not distinguish differences in the kinetics of expression of the prespore marker between the overexpressors and wild-type cells. To examine the kinetics more accurately, the temporal regulation of cell-type-specific gene expression was examined using RNA isolated from developing cells at 1-hour intervals starting at 9 hours of development. Prespore- and prestalk-specific gene expression was delayed in *gα5* null mutants but was precocious in *Gα5*^{HC} mutants in comparison to the temporal pattern of gene expression in wild-type cells (Fig. 6B). These differences in temporal gene regulation correlated with differences in the temporal regulation of tip morphogenesis. In all three strains, the genes were induced at approxi-

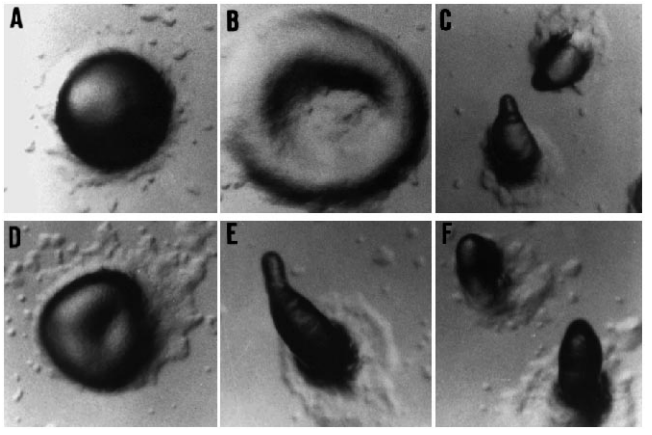

Fig. 5. Developmental morphology of *gα5*, wild-type and *Gα5*^{HC} chimeras at 12 hours after starvation. Mutant and wild-type cells were grown and plated for development as described in Materials and Methods. Cells were developed as a homogeneous population or mixed with an equal number of cells of another strain. Photographs were taken from above (birds-eye view) at the same magnification. (A) Wild-type cells alone, (B) *gα5* null cells alone, (C) *Gα5*^{HC} cells alone, (D) wild-type cells mixed with *gα5* null cells, (E) wild-type cells mixed *Gα5*^{HC} cells, (F) *gα5* null cells mixed with *Gα5*^{HC} cells.

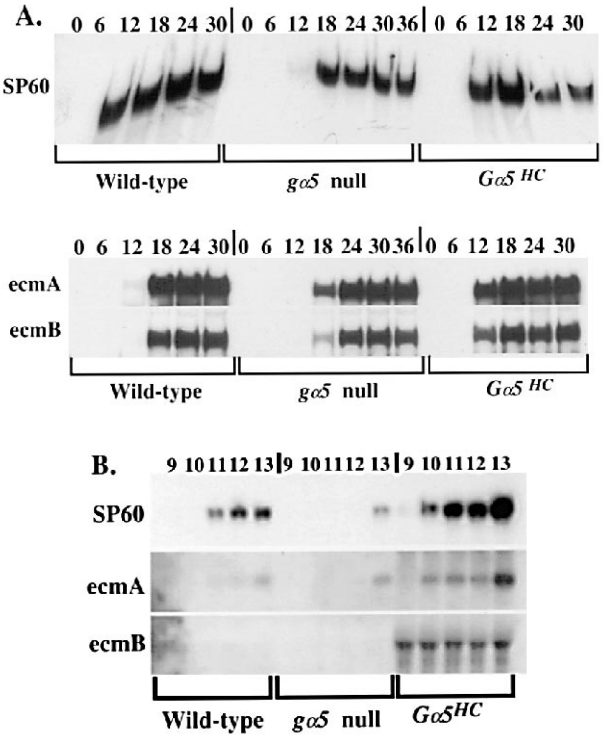

Fig. 6. Developmental expression of *SP60* (prespore), *ecmA* (prestalk) and *ecmB* (prestalk) genes in wild-type, *gα5* null and *Gα5*^{HC} cells. Total RNA was isolated from cells at times indicated, size-fractionated on denaturing gels (4 μg of RNA loaded in each lane) and blotted onto nylon membranes. Blots were hybridized with *SP60*, *ecmA* or *ecmB* probes. (A) RNA isolated every 6 hours. '0' represents vegetative cells. (B) RNA isolated every hour from 9 to 15 hours of development. Dark areas in the *ecmB* blot of wild-type RNA at 9 hours are not bands. For both time courses, at 12 hours, wild-type cells are tight aggregates, *gα5* null cells are loose aggregates and *Gα5*^{HC} cells are tipped aggregates forming fingers.

mately the same morphological stage and the maximal level of expression of each of the genes was the same.

To further examine the possible intercellular signaling-dependent Gα5 function in controlling the timing of cell-type-specific gene expression, we examined the ability of wild-type and *Gα5*^{HC} cells to accelerate prespore and prestalk gene expression specifically in *gα5* null cells when these cells are developed as chimeras. This was achieved by using *gα5* null cells transformed with either a prespore-specific (*SP60::lacZ* gene fusion) or prestalk-specific (*ecmA::lacZ* gene fusion) reporter gene. As shown in Fig. 7, *SP60::lacZ* expression in *gα5* null mutant cells was consistently accelerated in the presence of wild-type or *Gα5*^{HC} cells in chimeric aggregates (Fig. 7). However, the difference in the level of induction between the *gα5*/wild-type and *gα5*/*Gα5*^{HC} chimeras was variable, indicating that parameters other than the level of Gα5 function are important for the levels of induction. Prestalk-specific gene expression (*ecmA::lacZ* gene fusion) in *gα5* null cells was also precociously induced by the presence of wild-type or *Gα5*^{HC} cells and the level of this induction was consistently greater in *gα5*/*Gα5*^{HC} chimeras as compared to *gα5*/wild-type chimeras, suggesting

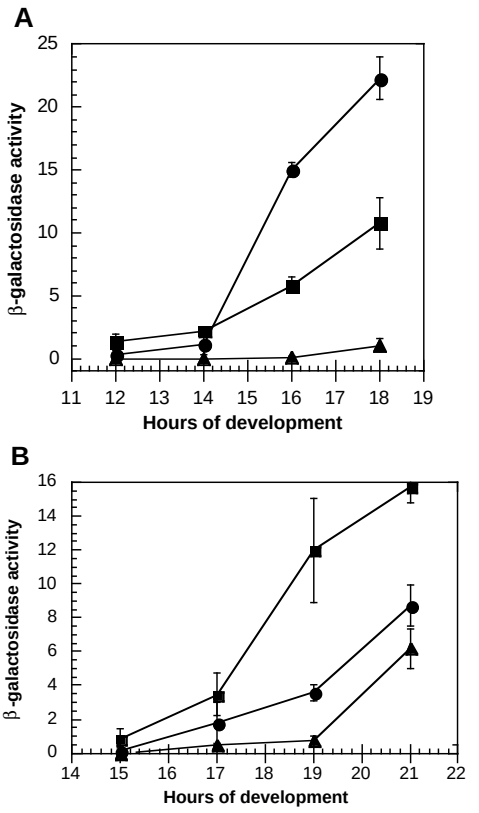

Fig. 7. Expression of cell-type-specific gene fusions in *gα5* null mutant cells developed in the presence of *gα5* null, wild-type and $G\alpha5^{HC}$ cells. *gα5* null cells carrying a *pSP60::lacZ* (A) or *pecmA::lacZ* (B) gene fusion were mixed at a ratio of 1:4 with *gα5* null cells (triangles), wild-type (circles), or $G\alpha5^{HC}$ cells (squares) and developed as chimeras. At the times indicated the chimeras were assayed for β-galactosidase activity as described Materials and Methods. The values represent the mean of activities measured from two sets of chimeric organisms developed simultanenously and the error bars represent the standard deviation of these activities. β-galactosidase activities are given as pmoles of substrate hydrolyzed/(μg protein/minute) at 22°C.

that prestalk gene expression is dependent on the level of Gα5 function in accompanying cells (Fig. 7). Cytological staining of the *gα5* null cells carrying the prespore- or prestalk-specific *lac*Z reporter gene fusions showed a spatial pattern of localization of prespore and prestalk cells that was similar to that of wild-type cells (Fig. 8A,D). However, we notice that when *gα5* cells expressing *SP60::lac*Z were mixed with wild-type cells, there was a greater concentration of the *gα5* null cells towards the anterior of the prespore region (Fig. 8B). In contrast, when *gα5* cells expressing *ecmA::lac*Z were mixed with $G\alpha5^{HC}$ cells, the region of greatest staining (prestalk A/O cells) appeared to be more extended (Fig. 8F) compared to when to mixing of *gα5* cells expressing *ecmA::lac*Z with *gα5* null cells (Fig. 8C) or wild-type cells (Fig. 8E). While these effects were not dramatic, they suggested that while Gα5 function was not essential in a cell-autonomous manner for cell-type sorting, it may have some affect on the spatial pattern. Gα5 function could potentially be important for the rate of cell sorting.

Gα5 gene is preferentially expressed in a pattern similar to the distribution of ALCs during the multicellular stages

Our previous report revealed that the *Gα5* gene is expressed at very low levels during vegetative growth and early development, but the expression increases significantly during multicellular development with a temporal pattern similar to that of the *Gα4* gene (Hadwiger et al., 1991). To determine the spatial pattern of *Gα5* gene expression during the multicellular stages, a segment of the *Gα5* gene that included the presumptive promoter (determined by the complementation analysis described earlier) and amino-terminal coding region was fused in-frame with the *E. coli lacZ* gene (Fig. 3), transformed into wild-type and *gα5* null cells, and individual clones were isolated. Wild-type or *gα5* null cells carrying the *pGα5::lacZ* construct expressed β-galactosidase activity in a small subset of cells distributed over the entire organism at all stages of multicellular development (Fig. 9). Although the cells expressing the *pGα5::lacZ* construct were found in all areas of the multicellular organism, there appeared to be a greater abundancy of these cells near the posterior end of slugs. Staining was also observed in the stalk, basal disc and cup regions of the fruiting bodies. This spatial pattern of expression is very similar to the pattern described for *Gα4* gene expression (Hadwiger and Firtel, 1992; Hadwiger et al., 1994) and the pattern of ALCs (Jermyn and Williams, 1991; Sternfeld and David, 1982), and is distinct from that of cells expressing prespore- or prestalk-specific genes (Williams et al., 1989; Haberstroh and Firtel, 1990).

Analysis of mutant Gα5 subunits

Mutations that inhibit GTPase activity in G proteins or Ras proteins often result in increased stimulation of downstream effectors and, in some cases, oncogenic transformation (Lyons et al., 1990; Landis et al., 1989; Kalinec et al., 1992; Bourne et al., 1991). A mutation commonly associated with these phenotypic properties is the substitution of a glutamine residue in the highly conserved G3 region (DVGGQR, using nomenclature described in Bourne et al., 1991) with a leucine residue. The analogous Q→L substitution mutation was created in the *Gα5* gene by site-directed mutagenesis to give the allele $G\alpha5^{Q198L}$ (Fig. 2). A plasmid containing this mutant *Gα5* allele and also a G418-resistance marker gene (see plasmid pKN1; Fig. 3) was transformed into wild-type cells to examine possible phenotypes of the $G\alpha5^{Q198L}$ allele. Transformants selected in growth medium containing 10 μg/ml G418 formed colonies but then gradually died, whereas transformants selected with lower concentrations of G418 (2-5 μg/ml) remained viable, suggesting that a high copy number of the plasmid was lethal. As a control, side-by-side transformations using the plasmid pJH206 (a plasmid identical to pKN1 except for the $G\alpha5^{Q198L}$ mutation) resulted in several hundred high-copy-number transformants that remained viable in medium containing 15 μg/ml G418 and possessed the precocious developmental phenotypes described above. Genomic DNA blots of the viable $G\alpha5^{Q198L}$ transformants indicated a very low copy number of the $G\alpha5^{Q198L}$ mutant plasmid (approximately 1-5 copies using the endogenous wild-type *Gα5* gene as a reference), consistent with the lower resistance to G418 medium (data not shown). None of these low-copy-number $G\alpha5^{Q198L}$ transformants displayed altered developmental characteristics. The $G\alpha5^{Q198L}$ allele was also transformed into

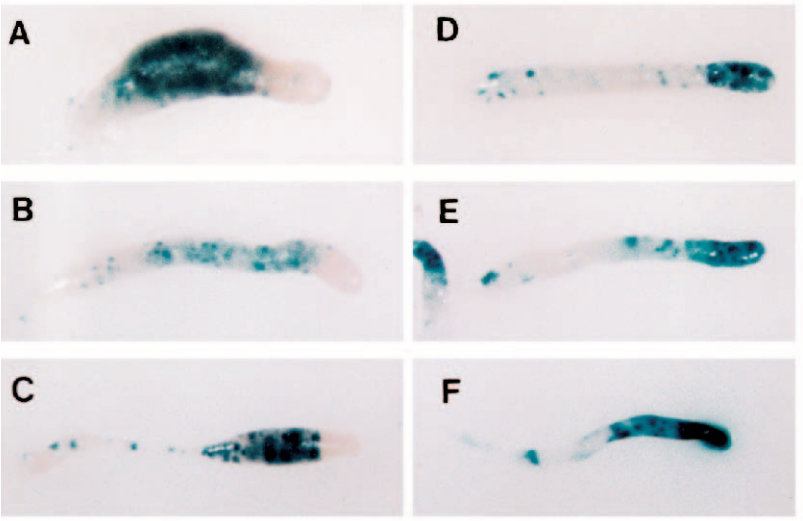

Fig. 8. Cytological staining of *gα5* null mutant cells developed in the presence of wild-type and $G\alpha5^{HC}$ cells. *gα5* null cells carrying a *pSP60::lacZ* (A-C) or *pecmA::lacZ* (D-F) gene fusion were mixed at a ratio of 1:4 with *gα5* null cells (A,D), wild-type (B,E), or $G\alpha5^{HC}$ cells (C,F) and developed as chimeras. Chimeras were fixed and histochemically stained as described in Materials and Methods.

wild-type cells as a truncated gene on a plasmid linearized within the *Gα5* coding region so as to direct the integration of the plasmid into the *Gα5* locus (Fig. 10). This strategy resulted in approximately 40% of the transformants having a single copy of a full-length $G\alpha5^{Q198L}$ gene and a truncated *Gα5* gene (no promoter or amino-terminal coding region), as determined by DNA blot analysis. Transformants with this insertional configuration displayed no aberrant developmental phenotypes, suggesting that the $G\alpha5^{Q198L}$ allele was capable of complementing the loss of the wild-type *Gα5* gene.

In addition to the analysis of the $G\alpha5^{Q198L}$ allele, another *Gα5* mutant allele, $G\alpha5^{G196T}$, was created by site-directed mutagenesis and assessed for function. The G→T substitution mutation, also in the conserved G3 region of Gα subunits, is analogous to mutations resulting in dominant negative alleles of the mammalian *Gαs* gene (Osawa and Johnson, 1991) and the *Dictyostelium Gα2* gene (Okaichi et al., 1992). The $G\alpha5^{G196T}$ allele, like the $G\alpha5^{Q198L}$ allele, appeared to be lethal at high copy number, as stable transformants contained <10 copies of the plasmid carrying the $G\alpha5^{G196T}$ allele (see plasmid pKN2; Fig. 3). None of the viable transformants displayed aberrant tip formation or any other apparent developmental phenotypes. Using the strategy previously described for the single-copy insertion of the $G\alpha5^{Q198L}$ allele, a truncated copy of the $G\alpha5^{G196T}$ allele was inserted into the *Gα5* locus to test the ability of the $G\alpha5^{G196T}$ allele to complement for the loss of the wild-type *Gα5* gene. All transformants with a single copy of the $G\alpha5^{G196T}$ allele and no wild-type allele (determined by genomic DNA blots) displayed no aberrant phenotypes with respect to developmental morphology or kinetics. This ability to complement the wild-type allele suggests that the $G\alpha5^{G196T}$ allele can provide sufficient Gα5 function and may not behave as a dominant negative allele.

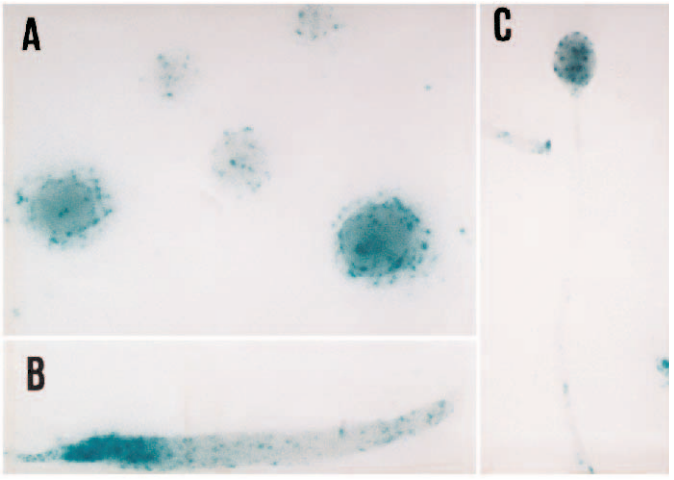

Fig. 9. Cytological staining of clonal wild-type cells expressing the *pGα5::lacZ* gene fusion vector pJH210. Development and staining procedures are described in Materials and Methods. (A) Early tipped-mound stage (13 hours after starvation), (B) migrating slug stage (16 hours of development), (C) fruiting body stage (24 hours of development).

DISCUSSION

The delayed tip morphogenesis and developmental gene expression of *gα5* null mutants suggest that the Gα5 subunit plays an important role in the temporal regulation of these developmental processes. This suggestion is further supported by the precocious tip morphogenesis and gene expression of the $G\alpha5^{HC}$ cells. The ability of wild-type or $G\alpha5^{HC}$ cells to accelerate the expression of prepsore- and prestalk-specific genes specifically in *gα5* null cells, as determined using *gα5* null cells expressing either *SP60::lacZ or ecmA::lacZ*, suggests that the Gα5-mediated signal transduction pathway is important for production rather than reception of an intercellular signal that regulates late developmental gene expression. The dependence of this signal on *Gα5* gene-dosage for the induction of prestalk- but not prespore-specific gene expression is consistent with the role of Gα5 function in regulating tip formation, as this process involves the accumulation of prestalk-specific cells on the top of the cellular aggregate. Whether this induction of prestalk-gene expression is due to increased cell differentiation and/or migration has not

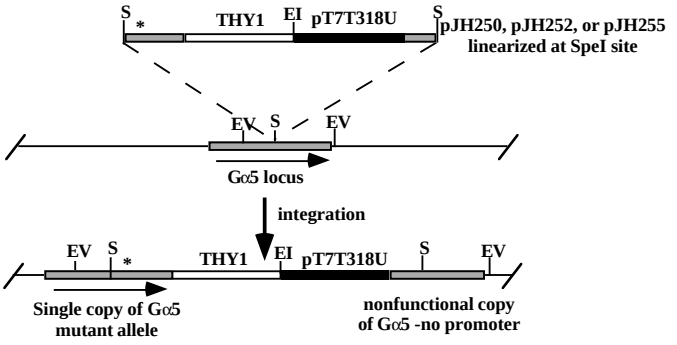

Fig. 10. Illustration of strategy used to introduce *Gα5* alleles at a single copy into the *Gα5* locus. Vectors containing *Gα5* alleles truncated by the deletion of promoter and amino terminal coding region (upstream of *Eco*RV restriction enzyme site) were linearized at a unique *Spe*I site within the *Gα5* open reading frame and electroporated into the strain JH10 (*thy1* null mutant). Tranformants with mutant or wild-type *Gα5* alleles inserted into the *Spe*I site of the *Gα5* locus were detected by genomic DNA blot analysis using the *Eco*RI/*Eco*RV fragment of pJH213 as a probe (see Materials and Methods). *Eco*RI/*Eco*RV digests of genomic DNA from insertional mutants produced 5.2 kb and 4.8 kb fragments compared to the 2.0 kb fragment of wild-type cells, consistent with a single insertion of the vector at the *Spe*I site of the *Gα5* locus. *Spe*I/*Eco*RV digests of the insertional mutant DNA produced 8.0 kb, 1.2 kb and 0.8 kb fragments compared to 1.2 kb and 0.8 kb fragments of wild-type DNA, indicating the *Spe*I sites were retained upon integration of the vectors. Restriction sites *Eco*RI (EI), *Eco*RV (EV) and *Spe*I (S) are shown. The region of the *Gα5* gene altered by mutagenesis is indicated by an asterisk (*).

been determined but previous studies have indicated that the expression of many cell-type-specific genes (e.g. *SP60, ecmA and ecmB*) can be initiated before cell sorting, suggesting that cell-type-specific gene expression does not require cell sorting (Esch and Firtel, 1991; Williams et al., 1989; Jermyn and Williams, 1991; Haberstroh and Firtel, 1990; Berks and Kay, 1990). However, the level of cell-type-specific gene expression might be dependent on the location of a cell within the aggregate. Whether the Gα5-dependent intercellular signal directly affects cell differentiation or migration, the cellular aggregate must have alternative but less effective mechanisms to carry out these processes and Gα5 appears to play an important role in these processes. Our results also showed that Gα5 dosage did not have a significant effect on the spatial patterning of the cells types within chimeric organisms.

The temporal induction and spatial expression pattern of the *Gα5* gene expression is very similar to that described for the related *Dictyostelium Gα4* gene (Hadwiger and Firtel, 1992; 1994; Hadwiger et al., 1991; Wu and Devreotes, 1991). Both genes are expressed at low levels before cellular aggregation and then at higher levels during the multicellular stages of development. The higher level of expression during multicellular stages suggests these subunits play important roles in the multicellular stage, which is further supported by the observation that both subunits affect intercellular signaling. However, the very low levels of expression during early development may also play important roles, as suggested by the requirement of the Gα4 subunit in preaggregated cells for responses to folic acid (Hadwiger et al., 1994). The spatial expression patterns of the *Gα4* and *Gα5* genes suggest neither gene is expressed in prespore or prestalk cells in the multicellular stages, but rather in a subpopulation of ALCs which can function as precursors or regulators of the two major cell types (Jermyn and Williams, 1991; Sternfeld, 1992; Hadwiger and Firtel, 1992; Hadwiger et al., 1994; Sternfeld and David, 1981; Abe et al., 1994). Whether the *Gα5* gene is expressed in the same subset of cells as the *Gα4* gene remains to be determined. For our analysis, we have used an upstream regulatory region that we believe contains the full upstream regulatory sequences (see results); however, we cannot exclude that additional sequences may result in additionals patterns of expression. While the expression of *Gα5* is highly enriched in ALCs in the multicellular stages, it is also expressed at earlier stages of development. It is therefore possible that the expression of *Gα5*, while low, during the earlier stages of development may be important in influencing the timing of tip formation. A possible role of Gα5 in earlier development and growth is consistent with the lethal effects that we observed when we tried to overexpress certain mutant Gα5 proteins. Moreover, since *Gα5* is expressed during these earlier stages, including vegetative growth, one might expect a low level of Gα5 protein to be present in most or all cells of the developing aggregate.

Although cAR2 and Gα5 are both required for the proper timing of tip formation, they probably function in different signal transduction pathways that independently facilitate tip morphogenesis. cAR2 expression has been proposed to be prestalk-specific (Saxe et al., 1993), whereas we have shown that Gα5 is expressed at low level during early development and induced to a higher level in the multicellular stages in which it is preferentially expressed in ALCs. Moreover, tip morphogenesis in *car2* null mutant aggregates is delayed significantly longer than *gα5* null mutant aggregates: *car2* cells overexpress prespore-specific genes, whereas *gα5* cells are delayed in prespore gene expression. The kinetics of tip morphogenesis in the *Gα5* mutant/wild-type chimeras indicate that Gα5 functions cell non-autonomously, presumably by affecting intercellular signaling during tip morphogenesis. Although cAMP appears to be the chemoattractant for prestalk cell migration during tip morphogenesis (Traynor et al., 1992; Matsukuma and Durston, 1979; Sternfeld and David, 1981; Saxe et al., 1993), *Gα5*-expressing cells are not likely to be the source of this cAMP, as these cells are poorly represented in the anterior region where cAMP levels are the highest. However, *Gα5*-expressing cells might modulate cAMP levels throughout the multicellular organism.

The mutations created in the G3 region of the Gα5 subunit, $G\alpha5^{Q198L}$ and $G\alpha5^{G196T}$, do not appear to affect tip morphogenesis, as suggested by the replacement of the wild-type allele with a single copy of the mutant alleles. However, the inability to obtain viable transformants carrying high copy numbers of these alleles suggests that the overproduction of these mutant proteins affects processes essential for vegetative growth. Whether these mutant *Gα5* subunits affect only the Gα5-mediated signal transduction pathway or other pathways remains to be determined. Although Gα5 function is not essential for vegetative growth and the expression of the *Gα5* gene is relatively low before multicellular development, the Gα5 signal transduction pathway might be involved in preaggregative functions. This is the case for the *Gα4* gene, which has a expression pattern similar to that of the *Gα5* gene

(Hadwiger et al., 1994). Although mutations analogous to the Q198L mutation have been analyzed in a variety of Gα subunits from different systems, mutations homologous to the G196T substitution in the Gα5 subunit have only been examined in the mammalian Gαs and *Dictyostelium* Gα2 subunits, for which the downstream effector pathways are understood and thus the effect of a mutant subunit could be evaluated (Osawa and Johnson, 1991; Okaichi et al., 1992). Although the G3 region of all known Gα subunits is highly conserved, it is not clear whether such mutations would function as dominant negative muations in all Gα subunits. Thus, it is not possible to evaluate the absence of a phenotype in strains in which the wild-type gene was replaced with the $G\alpha5^{G196T}$ allele.

Mutations affecting the temporal regulation of *Dictyostelium* development have been reported for genes other than the *Gα5* gene. These include rapidly developing (rde) mutants, *rdeA* and *rdeC*, which result in precocious formation of spore and stalk cells (Sonneborn et al., 1963; Kessin, 1977; Abe and Yanagisawa, 1983). In the case of *rdeC* mutants, spores and stalk cells mature soon after mound formation, resulting in disorganized mounds of stalk cells and spores. The defects of the *rdeC* mutants have been shown to result in a nonfunctional regulatory subunit of protein kinase A, suggesting that an unregulated catalytic subunit of protein kinase A (PKAcat) stimulates differentiation (Simon et al., 1992; Mann et al., 1992; Anjard et al., 1992). This implication is supported by the accelerated spore development of cells expressing the *PKAcat* gene from a prespore-specific promoter (Mann and Firtel, 1993; Mann et al., 1994). In contrast to *rdeC* mutants, *rdeA* mutants have accelerated aggregation and spores are formed at the base of the stalk rather than the top. The gene affected by the *rdeA* mutations remains to be determined. Both *rdeA* and *rdeC* mutants show precocious cellular differention (spore maturation) with respect to multicellular morphogenesis, whereas the coordination of differentiation and morphogenesis remains intact during the development of *Gα5* mutants. Thus, *Gα5* mutants appear to alter the time at which certain morphogenetic and gene expression pathways are induced but do not alter the actual morphogenesis.

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