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Cloning and Targeted Mutations of Gα7 and Gα8, Two Developmentally Regulated G Protein α-Subunit Genes in *Dictyostelium*

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GTP-binding protein (G protein)-mediated signal transduction pathways play essential roles during the aggregation and differentiation process of *Dictyostelium*. In addition to the five known G protein α-subunit genes, we recently identified three novel α-subunit genes, Gα6, Gα7, and Gα8, using the polymerase chain reaction technique. We present here a more complete analysis of Gα7 and Gα8. The cDNAs of these two genes were cloned, and their complete nucleotide sequences were determined. Sequence analyses indicate that Gα8 possesses some unusual features. It lacks the "TCATDT" motif, a sequence of amino acids highly conserved among Gα subunits, and has an additional 50 amino acids at its C-terminus consisting of long stretches of asparagine. Moreover, Gα8 is unusually resistant to protease digestion, which may indicate a slow GTP hydrolysis rate. The possible functions of these α-subunits were assessed by generating mutants lacking Gα7 or Gα8 by gene targeting through homologous recombination and by overexpressing Gα7 or Gα8 protein. Overexpression of Gα7 resulted in abnormal morphogenesis starting at the slug stage, whereas analysis of the other strains failed to reveal any obvious growth or developmental defects under either normal or stressful conditions. The implications of these results are discussed.

INTRODUCTION

Signal transduction via seven transmembrane domain receptors is an extremely widespread phenomenon that is involved in multiple processes such as vision, olfaction, cardiac oscillations, and cellular differentiation (Gilman, 1987; Bourne *et al.*, 1990; Dohlman *et al.*, 1991; Birnbaumer, 1992). When excited, these receptors activate heterotrimeric GTP-binding proteins (G proteins), catalyzing the exchange of GTP for GDP on the α-subunit and the dissociation of the α from the βγ-subunits. The activated subunits then interact with a variety of effectors, such as adenylyl cyclase, phospholipase, and ion channels, to elicit numerous physiological responses. In mammals, ~20 G protein α-subunits, four β-subunits, and six γ-subunits have been identified (Simon *et al.*, 1991; Birnbaumer, 1992). These subunits can presumably combine with each other to form a large variety of heterotrimers that interact with various effectors. In addition, >100 different G protein-coupled receptors and many effectors have been found. Being expressed in different tissues and/or different developmental stages, these receptors, G proteins, and effectors can form complicated networks in transmitting environmental signals.

The G protein signal transduction strategy appeared early in the evolution of eucaryotic cells and has been shown to serve important functions in *Dictyostelium discoideum*, a simple developing eucaryotic organism. The life cycle of *Dictyostelium* consists of distinct growth and developmental phases. In the developmental phase, triggered by starvation, ~10^5 individual amoebae aggregate to form a multicellular structure that undergoes further morphogenesis and eventually culminates to form a fruiting body. The cell-cell signaling processes

involved utilize extracellular cAMP that binds to cell surface receptors and, in turn, triggers numerous responses (Devreotes, 1989; Firtel, 1991). Genes encoding four highly related surface cAMP receptors have been identified (Klein *et al.*, 1988; Saxe *et al.*, 1991a,b). Some of these receptors have been shown to be needed for normal development (Sun *et al.*, 1990; Sun and Devreotes, 1991; Saxe *et al.*, 1993). All four contain the seven putative transmembrane domains characteristic of G protein–linked receptors.

By using oligonucleotides based on the sequences of the conserved GTP-binding domains of G protein α-subunits to screen a cDNA library or to perform polymerase chain reactions (PCR), genes encoding eight *Dictyostelium* G protein α-subunits and one β-subunit have been identified (Pupillo *et al.*, 1989; Hadwiger *et al.*, 1991; Wu and Devreotes, 1991; Cubitt *et al.*, 1992; Lilly *et al.*, 1993; Wu *et al.*, 1993; Pupillo and Devreotes, unpublished data). Northern blot analyses indicate that each of these genes has a distinct pattern of expression during development. The comparison of the preliminary sequence among the eight Gα-subunits, which presumably form heterotrimers with the unique β-subunit and an as yet unidentified γ-subunit, indicates that they do not seem to fall into any subgroups, nor can they be classified into any of the Gs, Gi, Gq, and G12 subtypes in mammals.

Gene targeting experiments indicate that Gα4 is required for the multicellular structure formation (Hadwiger and Firtel, 1992). Gα2, on the other hand, plays important roles during early aggregation. It has been shown that $g\alpha2^-$ cells do not aggregate and lack most of the cAMP-mediated responses such as the activation of adenylyl cyclase, guanylyl cyclase, PI-specific phospholipase C, and regulation of gene expression (Kesbeke *et al.*, 1988; Snaar-Jagalska and Van Haastert, 1988; Kumagai *et al.*, 1989, 1991; Okaichi *et al.*, 1992). It is not clear, however, what role the other Gα-subunits play and if they are functionally redundant.

We reported recently the isolation of PCR fragments of three Gα genes, Gα6, Gα7, and Gα8 from *Dictyostelium* (Wu and Devreotes, 1991; Cubitt *et al.*, 1992). Gα6 is expressed primarily in vegetative cells and very early development, whereas Gα7 and Gα8 are expressed maximally at the aggregation and mound stages, respectively. In this report, we describe the cloning of Gα7 and Gα8 cDNAs, their complete nucleotide sequences, and construction of gene disruption mutations. Furthermore, we have generated a specific antiserum against Gα8 and found that Gα8 is unusually resistant to trypsin digestion, which may indicate a slow rate of GTP hydrolysis. In addition, we show that overexpression of Gα7 results in abnormal morphological phenotypes distinct from those resulting from overexpression of other *Dictyostelium* Gα subunits.

MATERIALS AND METHODS

Cell Culture and Development

Cells were grown in HL5 media at 22°C as described (Watts and Ashworth, 1970). JH10, a thymidine auxotrophic mutant (Mann and Firtel, 1991; Hadwiger and Firtel, 1992), and DH1, a uracil auxotrophic mutant (Caterina *et al.*, 1994) cells were supplemented with 100 μg/ml thymidine and 20 μg/ml uracil, respectively. For development, cells were harvested from mid- to late-log phase, washed in development buffer (DB) consisting of 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$, and 0.2 mM $CaCl_2$ and plated on DB plates containing 1.5% agar at 5×10^7 cells per 10-cm petri dish. The plates were incubated at room temperature (22°C), and the morphology was examined. For stress condition tests, DB agar was supplemented with 100 mM NaCl, 0.2 mM cAMP, or 10× PB (1× PB is 5 mM Na_2HPO_4 and 5 mM KH_2PO_4 as in DB). Neutral red staining was performed essentially as described by Sternfeld and David (1981). Briefly, 10^8/ml cell suspensions were stained with 500 μg/ml neutral red for 1 min, washed twice, and resuspended in DB before plating for development. Stained cells showed normal development.

Cloning and Sequencing of Gα7 and Gα8

PCR fragments of Gα7 and Gα8 (Wu and Devreotes, 1991; Cubitt *et al.*, 1992) labeled by random priming were used for screening *Eco*RI-λgt10 and λZap (Schnitzler and Firtel, unpublished data) cDNA libraries (Stratagene, La Jolla, CA) representing the RNA of aggregation- and mound-stage cells of *Dictyostelium*, respectively. The inserts were subcloned to pBluescript (Stratagene) and sequenced using Sequenase (United States Biochemical, Cleveland, OH). Both strands were sequenced. To obtain the 3′-*Eco*RI genomic fragment of Gα8, the cDNA was used to screen an *Eco*RI-λZap genomic library constructed using the GIGAPACK II packaging extract (Stratagene).

Southern Blot, RNA Blot, and Other Recombinant DNA Techniques

Plasmids were constructed using standard cloning techniques (Maniatis *et al.*, 1982). Most constructs were made in pBluescript vector (Stratagene). Genomic DNA was isolated according to Nellen *et al.* (1987). Southern blot and RNA blot hybridization were carried out as described by Maniatis *et al.* (1982).

Generation of $g\alpha7^-$ and $g\alpha8^-$ Cells

The $g\alpha7^-$ and $g\alpha8^-$ cells were generated independently by two research groups at Johns Hopkins University (JHU) and University of California at San Diego (UCSD) using two sets of gene-disruption constructs and recipient cell lines. Details will be given here to describe each construct. In the RESULTS section, only the data from the Johns Hopkins group will be shown because the results obtained by the two groups are identical.

At JHU. For the Gα7 gene-disruption construct, plasmid pα7-thy (pLW72) was constructed by first subcloning the 1.3-kilobase (kb) *Eco*RI fragment of Gα7 cDNA into pBluescript, and then a 3-kb *Hin*dIII *thy1* fragment was inserted into the unique *Bcl* I site of the Gα7 gene. For the Gα8 gene-disruption constructs, the 5′-*Eco*RI Gα8 cDNA was fused to the 3′-*Eco*RI Gα8 genomic DNA to get a full length Gα8 gene (total 3.5 kb). The internal region of Gα8 (0.5-kb *Bcl* I-*Bal* I fragment) was replaced by either the 3-kb *thy1* fragment (pα8-thy or pLW75) or the 3.7-kb *ura* fragment (pα8-ura or pLW76). Plasmid pLW72, pLW75, or pLW76 (15–50 μg) was digested by enzymes in the polylinker region to excise the inserts, extracted by phenol-chloroform, precipitated by ethanol, and resuspended in 10 μl Tris-EDTA (pH 8). DNA was transformed into JH10 (pLW72 and pLW75) or DH1 (pLW76) cells by electroporation (Howard *et al.*, 1988) and selected in minimal medium (FM) medium (Franke and Kessin, 1977) in 96-well plates for clonal prototrophic transformants. Transformants grown in FM medium were further propagated in HL-5 medium, and genomic

DNA was isolated and subjected to Southern blot analyses. The *gα7*⁻ cells were designated as LW1 and *gα8*⁻ cells as LW3, but will be referred as *gα7*⁻ and *gα8*⁻ cells, respectively, in the subsequent text.

At UCSD. For the Gα7 gene-disruption construct, a PCR fragment corresponding to the region of Gα7 cDNA between TRYRE and KDILTR was inserted at the mid-*Bcl* I site with the *thy1* gene to produce plasmid pCG-gα7thy. For the Gα8 gene-disruption construct, two PCR fragments were generated that correspond to the regions between ESGKST and DVGGQR and between GPVHFR and WEEAKG, respectively. These two fragments were cloned into pSP72, and the *thy1* gene was inserted in between the two fragments to produce pCG-gα8thy. pCG-gα7thy and pCG-gα8thy was linearized and transformed into JH10 cells as described above. The *gα7*⁻ and *gα8*⁻ cells generated using these constructs were designated as CG1 and CG2 but will be referred as *gα7*⁻ and *gα8*⁻, respectively, in the subsequent text.

Generation of Antiserum Against Gα8 and Immunoblot Analysis

Two different rabbit antisera were generated against Gα8. For the first one, made at JHU, a synthetic peptide, identical to the carboxyl-terminal 14 amino acid residues of Gα8 with a cysteine added at the amino-terminus, was coupled to keyhole limpet hemocyanin by the method of Green *et al.* (1982), except 1.5% sodium dodecyl sulfate (SDS) was added to the coupling reaction. The second antiserum, made at UCSD, was generated against a GST-Gα8 fusion protein containing the C-terminal 113 amino acids of Gα8, which, by comparison to the other sequenced Gα genes, is unique to Gα8. The coupled peptide or the fusion protein was injected subcutaneously into a rabbit, and antisera were collected.

Total cellular proteins and ammonium sulfate–extracted membrane proteins (Theibert *et al.*, 1984) were analyzed by immunoblot according to the standard procedures using ^{125}I-labeled protein A (Towbin *et al.*, 1979), except for Figure 5B, where an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) was used as described by the manufacturer.

Trypsin Resistance of Gα8

Cells were developed for 4 h, washed, and resuspended in DB buffer without $CaCl_2$. The cells were lysed by filtration in the presence of 10 mM tris(hydroxymethyl)aminomethane pH 8.0, 1 mM $MgSO_4$ containing 80 μM GTPγS, 80 μM GDPβS, or neither. After incubating on ice for 30 min to permit nucleotide binding, membranes were pelleted and washed with the same buffer. The pellet was resuspended in 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM β-mercaptoethanol, and 0.64% lubrol PX (Berlot and Bourne, 1992) at 3×10^7 cell equivalents/ml (~6 mg proteins/ml). Solubilized proteins, separated from cellular debris by centrifugation at 12 000 rpm for 15 min at 4°C, were treated with 5 μg/ml L-1-tosylamide-2-phenylethylchloromethyl-trypsin for 5 min at 30°C. Soybean trypsin inhibitor (1 mg/ml) was then added to stop the digestion, and the products were subjected to immunoblot analyses.

Overexpression of Gα7 and Gα8 Subunits

Full length Gα7 or Gα8 cDNAs isolated from the λZap library were inserted into the *Spe* I and *Xho* I sites of pKZ-DIPj, a modified BS18 vector (Kumagai *et al.*, 1989) containing a polylinker (*Bgl* II/*Spe* I/*Kpn* I/*Xho* I) located in the 3′ untranslated region of actin 15 promotor and upstream of the SP70 (2H3) terminator (Zhou and Firtel, unpublished data). The vector also carries a gene fusion encoding resistance to G418 in *Dictyostelium*. The dominant active and negative mutations of Gα8 were made by site-directed mutagenesis using oligonucleotides. *Dictyostelium* cells were transformed by electroporation (Howard *et al.*, 1988), and stable, G418-resistant clones were selected. Transformants were screened for expression of Gα7 or Gα8 gene by quantitating at the transcript level (for Gα7) of the actin 15/Gα7 fusion gene by RNA blot hybridization and at the protein level (for Gα8) by immunoblot against anti-Gα8 antibody. Clones overexpressing Gα7 or Gα8 by ~15-fold compared to wild-type cells were selected.

RESULTS

Cloning and Nucleotide Sequences of Gα7 and Gα8

The PCR fragments corresponding to the region between the conserved sequences GAGESK and DVGGQR of Gα7 and Gα8 (Wu and Devreotes, 1991; Cubitt *et al.*, 1992) were used as probes to screen cDNA libraries prepared from mRNAs at the aggregation and mound stages of *Dictyostelium* development. Among $\sim 3 \times 10^5$ phage screened, ~30 and 200 positive clones were obtained for Gα7 and Gα8, respectively. Four (Gα7) or eight (Gα8) positive clones were further purified and shown to comprise full length Gα7 and Gα8 cDNAs by restriction analyses and sequencing.

The complete nucleotide sequence of Gα7 and its deduced amino acid sequence are shown in Figure 1. The first methionine codon at nucleotide position 55 in the large open reading frame (ORF) marks the translation start site. This ATG is preceded by an AT-rich sequence, which is typical for a *Dictyostelium* start codon. The stop codon TAA is at position 1225. The predicted ORF of 1170 nucleotides encodes a protein of 390 amino acids with a calculated molecular mass of 43.9 kDa.

Figure 2 shows the nucleotide and deduced amino acid sequence of Gα8. Gα8 gene was cloned as two *Eco*RI fragments with the internal *Eco*RI site at nucleotide 1031. Its putative ATG start codon, also preceded by a AT-rich sequence, is at position 133 and the first in-frame stop codon is at 1342. The ORF of 1209 nucleotides encodes a 403 amino acid protein with a calculated molecular mass of 46.3 kDa. The amino acid sequence of the carboxyl-terminal region of Gα8, after the internal *Eco*RI site, possesses some very unusual features. It is ~50 residues longer than most of the other Gα proteins (see Figure 3) and contains a long stretch of asparagine residues (14) interrupted by two serines (underlined in Figure 2). Most of these asparagines are encoded by AAT codons (13 out of 14).

Several lines of evidence indicate that these unusual sequence features of Gα8 are not a cloning artifact. First, the sequence was found in several independent cDNA clones from two independently made libraries. Second, when either the 3′- or the 5′-*Eco*RI fragment was used as a probe to hybridize RNA isolated from different stages of development, identical results were obtained. Third, genomic Southern blot analysis indicates that the 3′-*Eco*RI fragment is linked to the rest of the Gα8 gene. Fourth, the 3′-*Eco*RI genomic fragment was sequenced and found to be identical to the cDNA in that region.

Sequence Comparison

The predicted amino acid sequences of Gα1, Gα2, Gα4, Gα5, Gα6, Gα7, and Gα8 are aligned in Figure 3. Visual

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   1  AAAAATAAAATAAAATAAAATAAATACTAGAATAGTAATAATAATTAAAAGAAAATGAGTAGCACTACAACAAATACAACAACAGCAACA   90
                                                              M  S  S  T  T  T  N  T  T  T  A  T

  91  CCAGCAATTCAAGTGAATGGTAATCAATCATCATCACCACAATCACCATCATCATCAACATCAACTTTATCACCACCAATGTCACCATCA  180
      P  A  I  Q  V  N  G  N  Q  S  S  S  P  Q  S  P  S  S  S  T  S  T  L  S  P  P  M  S  P  S

 181  CTTTTAACTCGTTATAGAGAGCAAAAAGCAGTCAATAAACAAATTGAAAAACAATTAAAAGAAGAGAAAAAGATAATGGATAGTGAATTA  270
      L  L  T  R  Y  R  E  Q  K  A  V  N  K  Q  I  E  K  Q  L  K  E  E  K  K  I  M  D  S  E  L

 271  AAATTATTATTACTTGGTACTGGTGATTCTGGTAAATCAACAGTCGTTAAACAAATGAAAATCTTACATCTTGAAGGTTACTCTCAAGAG  360
      K  L  L  L  L  G  T  G  D  S  G  K  S  T  V  V  K  Q  M  K  I  L  H  L  E  G  Y  S  Q  E

 361  GAAAGAATTAATCAAAGACAATTTGTTTATAGAAATATCATTGAAATTGCTTATTCAATCATTCGTGGTTGTGGTGTTTTAAATTTAACA  450
      E  R  I  N  Q  R  Q  F  V  Y  R  N  I  I  E  I  A  Y  S  I  I  R  G  C  G  V  L  N  L  T

 451  ATTCCATCACAATTTGATTCAATTTGTTCATCTATTGAAGAAATTTATGAAACTAAAAATTATACAAATTTAGATAAAAATGTATTAAAA  540
      I  P  S  Q  F  D  S  I  C  S  S  I  E  E  I  Y  E  T  K  N  Y  T  N  L  D  K  N  V  L  K

 541  GGAGTTGCAGAACTTTCTAAAAATGAATCATTCATTAATGCTGCTAATAATAGTGGTTCAAATTTCCAATTACATTCATCTTCACAATAC  630
      G  V  A  E  L  S  K  N  E  S  F  I  N  A  A  N  N  S  G  S  N  F  Q  L  H  S  S  S  Q  Y

 631  TTTTTAGATGACATTGCAAGATTTTCAGAGGAAGATTATATACCAACAGATCAAGATATTTTATATACTAGAGTTGCATCAACATCTGTT  720
      F  L  D  D  I  A  R  F  S  E  E  D  Y  I  P  T  D  Q  D  I  L  Y  T  R  V  A  S  T  S  V

 721  AGCGAAACTAGATTTTCAGTTAGGGGTATTAAATTTAGAATGATTGATGTAGCAGGTCAAAGAGGACATAGAGATAAATGGATTCATCAT  810
      S  E  T  R  F  S  V  R  G  I  K  F  R  M  I  D  V  A  G  Q  R  G  H  R  D  K  W  I  H  H

 811  TTTAGTGAAGTTACAGCAATATTATTTGTAATTTCATTATGTGAATATGATCAAGTTTTGGAAGAAGATGGTAAAACTAATCGTATGATT  900
      F  S  E  V  T  A  I  L  F  V  I  S  L  C  E  Y  D  Q  V  L  E  E  D  G  K  T  N  R  M  I

 901  GAATCAATTAAAGTATTTGGTGATATAATTAATCAACGTTGGTTCAAAGATATTCCAATCATTCTTTTCTTAAATAAACGTGATTTATTC  990
      E  S  I  K  V  F  G  D  I  I  N  Q  R  W  F  K  D  I  P  I  I  L  F  L  N  K  R  D  L  F

 991  GCAGAGAAAATTAAAAAGACTGGTATTTCAATTTGTTTCCCTGATTACACTGGTCCATCCGATGATTACGAACAATCTTTGGTCTTTTTG 1080
      A  E  K  I  K  K  T  G  I  S  I  C  F  P  D  Y  T  G  P  S  D  D  Y  E  Q  S  L  V  F  L

1081  AAAAAGAAAATTTTATCTGCAAATAAAACTTCAAAAGCAGTTTATACAAATGTTACAACCGCAACTGATACAACTAATATTGGTCATGTT 1170
      K  K  K  I  L  S  A  N  K  T  S  K  A  V  Y  T  N  V  T  T  A  T  D  T  T  N  I  G  H  V

1171  TTTGAAGCTGTAAAAGATATTCTTACTCGTCAAACTATGGAGGAAGGAGGTATCTAAAAGAATGAAATAATTTTTTTTTTTTTTTATTTT 1260
      F  E  A  V  K  D  I  L  T  R  Q  T  M  E  E  G  G  I

1261  GTTTTTCTTCCATTAAAACGCGTTCAAATATAAATAATAATTAAAAA 1308
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Figure 1. Nucleotide and deduced amino acid sequences of Gα7. The GenSeq accession number is L33847.

comparison and computer analyses indicate that the *Dictyostelium* Gα subunits all share 30–50% identity to each other and to mammalian G protein α-subunits. Based on amino acid sequences, it is not possible to divide them into subgroups.

When compared to other Gα subunits, both Gα7 and Gα8 possess some interesting and unusual features. The N-terminal region of Gα7 before the GTP-binding domain (···GAGESG···) is ~40 amino acids longer than that of other Gα subunits. Gα8, as discussed above, has an additional 50 amino acid stretch at its C-terminus.

Most G protein α-subunits possess several highly conserved regions that are important for their intrinsic functions. These sequences are thought to be directly involved in interaction with the guanine nucleotide (Simon *et al.*, 1991). When these sequences were compared among the *Dictyostelium* Gα subunits, it is noted that region C is completely conserved among all of them except Gα7 that has a conservative substitution of an Ala for a Gly. Region G is also highly homologous among these proteins with only Gα8 having a conservative substitution of Val for Phe, and in Gα5 Tyr is replaced by Leu. The other two regions, A and I, are relatively more divergent. Gα4 has a sequence divergence in region A (···GPGES··· instead of ···GAGES···), which is the region thought to be involved in the release of βγ subunits and in GTP hydrolysis (Simon *et al.*, 1991). Analysis of Gα7 sequence reveals that Gα7 has even more substitutions in this region (···GTGDS··· instead of ···GAGES···). Finally, region I of Gα8 is markedly different from that of other Gα subunits. This TCATDT motif near the C-terminus of other Gα subunits is totally missing in Gα8. At the corresponding position, Gα8 has IAARYK.

Identification and Developmental Expression of Gα8

An antiserum against Gα8 was generated using a synthetic peptide corresponding to the deduced carboxyl-terminal 14 amino acid residues of Gα8 (see Figure 2).

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   1 TTTTTTTCCTCTTCAATACACTCACACACACCAGTTATAATATTAATAGTAATATAATTAAATAACAGTACAAACATTAATAATAATTAT  90

  91 AAAAAAAAAAAAACCAATAATAAATATAGTTACGGAAATAAAATGGGTTGCTATCAATCACGTGTTCAAGTAGAAGATAAAACAAACGAA 180
                                                 M  G  C  Y  Q  S  R  V  Q  V  E  D  K  T  N  E

 181 CAATTAGATAAATATCTTGTACAAGCCGGTAAAGAGGGTTTGTTAGATTTTAGAATATTATTGTTGGGTGCTGGTGAAAGTGGTAAATCC 270
      Q  L  D  K  Y  L  V  Q  A  G  K  E  G  L  L  D  F  R  I  L  L  L  G  A  G  E  S  G  K  S

 271 ACTGTTGTAAAACAATTAAAATCAATATATAAAATTCAAGTCGATGATGATGAATTACATAGTTATGCTGTAAATATTCATAAAAATACA 360
      T  V  V  K  Q  L  K  S  I  Y  K  I  Q  V  D  D  D  E  L  H  S  Y  A  V  N  I  H  K  N  T

 361 GTACTTTGTATGCAAGTTTTATTAGAAGCTGGTGAAACTTTAGGTATTGAATTAACTGACCCCGAAACAAAGAAAAGAGCAATTAATGTT 450
      V  L  C  M  Q  V  L  L  E  A  G  E  T  L  G  I  E  L  T  D  P  E  T  K  K  R  A  I  N  V

 451 AAATCATTCCAATTTGAACCAGAAGTTAAACAAATGCCAGTTAGTATAGGTTTAGATATTGAAGAATTATGGAGGGATGAAGATATTCAA 540
      K  S  F  Q  F  E  P  E  V  K  Q  M  P  V  S  I  G  L  D  I  E  E  L  W  R  D  E  D  I  Q

 541 AAGATTTGGGATAGAAGATCAGAATATTGGTTTTTAGATGCAACACCATATTATTTCGAAAATATTCAAAGATTTTTAGATGATGATTTC 630
      K  I  W  D  R  R  S  E  Y  W  F  L  D  A  T  P  Y  Y  F  E  N  I  Q  R  F  L  D  D  D  F

 631 GTACCAACTGAAGAAGATTGTATTATGACACGTGTTAGAACTACAGGTATCTCTGTAACTGAATTTGATGAAGGTCCAGTTCATTTCCGT 720
      V  P  T  E  E  D  C  I  M  T  R  V  R  T  T  G  I  S  V  T  E  F  D  E  G  P  V  H  F  R

 721 GTTGTTGATGTTGGTGGTCAAAGAAATGAAAGAAAGAAATGGATTCATTGTTTTGATGATGTTAAAGCATTATTATTTGTTGTAAATTTA 810
      V  V  D  V  G  G  Q  R  N  E  R  K  K  W  I  H  C  F  D  D  V  K  A  L  L  F  V  V  N  L

 811 GCTGGTTATGATCAAGTTATGTTTGAAGATCCTTCACAAAATAGAATGCAAGAATCATTAACATTATTTGGACAAATTTGTAATAATCCA 900
      A  G  Y  D  Q  V  M  F  E  D  P  S  Q  N  R  M  Q  E  S  L  T  L  F  G  Q  I  C  N  N  P

 901 ATATTTTCAGAGACCCCAACATTTTTGGTATTAAATAAAAAAGATTTATTTGAACAAATGATTCAAAAAACTGATCTTAGTAAATGTTTC 990
      I  F  S  E  T  P  T  F  L  V  L  N  K  K  D  L  F  E  Q  M  I  Q  K  T  D  L  S  K  C  F

 991 CCAGACTATAAGGGTGGTTCAGATGTAAAGACAGCATTGGAATTCATTCAAATGAAATATCAACAAAAGATTCAAGAATCAAATAAACCT 1080
      P  D  Y  K  G  G  S  D  V  K  T  A  L  E  F  I  Q  M  K  Y  Q  Q  K  I  Q  E  S  N  K  P

1081 TTACATACATTCCATATTGCTGCTCGTTATAAAAAAGATATTAAATACACATGGGAAGAAGCAAAAGGTATACTTTTAGAAGAAAATAAA 1170
      L  H  T  F  H  I  A  A  R  Y  K  K  D  I  K  Y  T  W  E  E  A  K  G  I  L  L  E  E  N  K

1171 AAGGTTTTAATGAAAGCAACAAAAGATTTAAAGAAATCTTCAAAACAATCAAGTAAATCATCTTTAGGTAATAGTACTCAAAATAATAGT 1260
      K  V  L  M  K  A  T  K  D  L  K  K  S  S  K  Q  S  S  K  S  S  L  G  N  S  T  Q  N  N  S

1261 AATAATAACAATAATAATAATAATAGTAATAATAATAATGGCCAAACTACAATTGATGGTGCAACCGCCAAAATTAATTCTTAAAAAAAA 1350
      N  N  N  N  N  N  N  N  N  S  N  N  N  N  N  G  Q  T  T  I  D  G  A  T  A  K  I  N  S

1351 AAAAAAACATCAAAAAAAAAAATC 1375
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Figure 2. Nucleotide and deduced amino acid sequences of Gα8. ___, reiterated amino acids; ═, peptide sequence used to generate one of the Gα8-specific antisera described. The GenSeq accession number is L33848.

As shown in Figure 4, a band of 48 kDa was detected on immunoblots of proteins isolated from growth or various stages of development using this antibody. This molecular mass is roughly the same as that calculated from the deduced amino acid sequences (46.3 kDa). The same results were obtained when using an antiserum raised against a GST-Gα8 fusion protein containing the C-terminal 113 amino acids of Gα8.

The expression of Gα8 mRNA is highly regulated during development (Wu and Devreotes, 1991; Cubitt *et al.*, 1992). It is expressed at very low levels in growth stage and is greatly induced upon starvation. It reaches a maximum level during aggregation and then declines. Similarly, the level of Gα8 protein in vegetative cells is very low and is enhanced greatly after the initiation of development. In contrast to the mRNA expression, however, Gα8 protein remains at a constant level throughout development. Similar observations have been made for Gα2. After its initial appearance at the onset of development, the Gα2 protein level remains high even though the level of its major transcript decreases (Gunderson, personal communication).

Trypsin Resistance of Gα8

Because of the unusual amino acid sequences of Gα8 at the C-terminus, we wanted to determine if this feature has any correlation with the intrinsic activity of G proteins such as a GTP-induced conformational change. To do so, we used the trypsin resistance technique. It has been reported that binding of GTP analogues, such as GTPγS, to certain Gα-subunits prevents trypsin from cleaving the polypeptide chains at a conserved arginine residue, corresponding to R231 in Gαs (Fung and Nash,

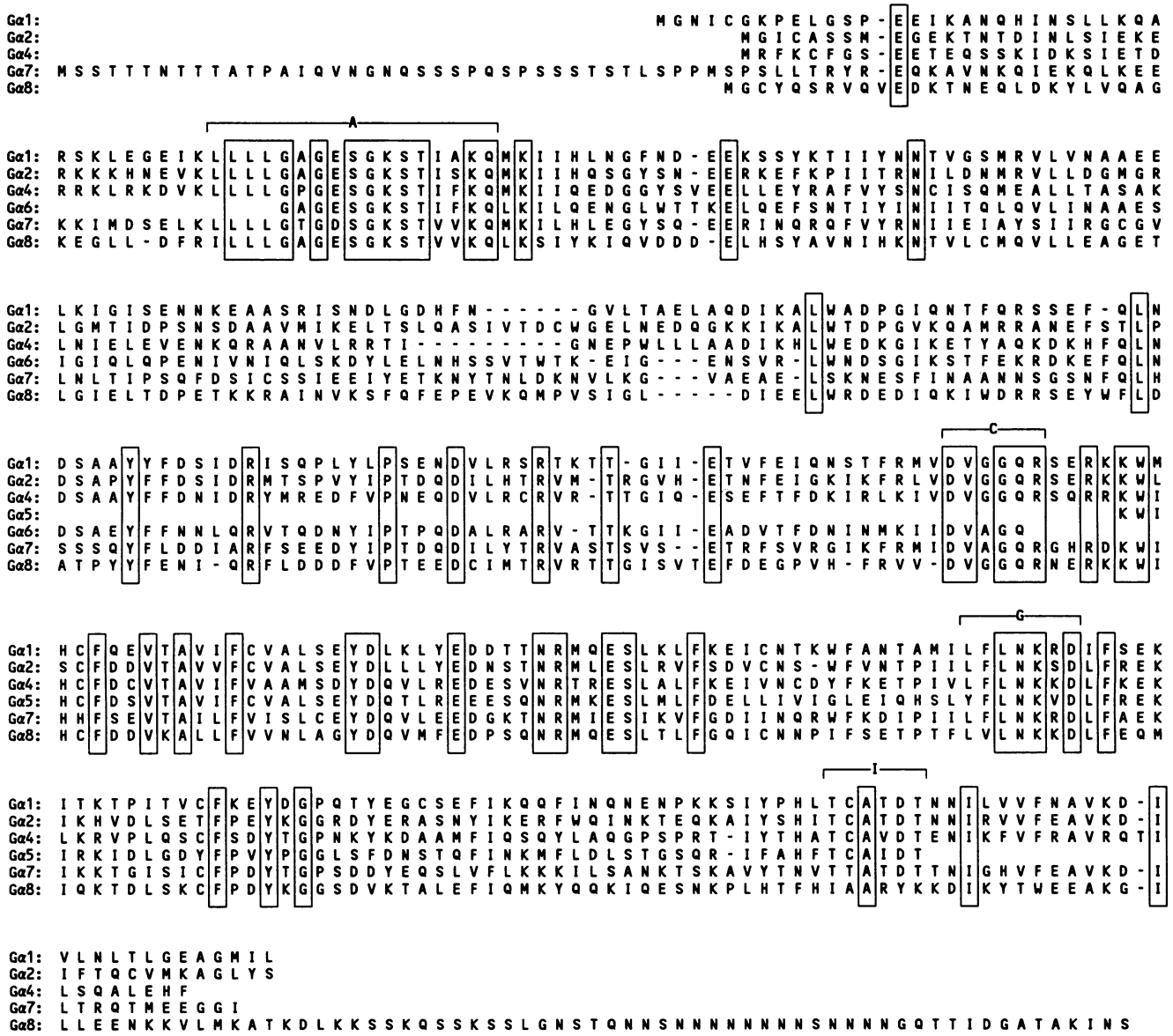

Figure 3. Amino acid sequence comparison of *Dictyostelium* Gα subunits. The sequences of Gα1 and Gα2 (Pupillo *et al.*, 1989), Gα4 and Gα5 (Hadwiger *et al.*, 1991), and Gα6 (Wu and Devreotes, 1991) are shown aligned with Gα7 and Gα8. The box indicates the amino acid residues that are identical among all the Gα subunits sequenced so far. -, gaps introduced to align the conserved sequences. Capital letters A, C, G, and I indicate the highly conserved regions. The complete sequences of Gα5 and Gα6 have not been determined. Gα3 sequence is not shown and is cloned by Pupillo and Devreotes (unpublished observations).

1983; Hurley *et al.*, 1984; Miller *et al.*, 1988). This effect is interpreted to reflect an altered and activated conformation. We have noted a similar GTPγS-induced protection of Gα1 and a Gα2/Gα1 chimera (Chen and Devreotes, personal communication) in *Dictyostelium*. The presence of GTPγS in the reaction protects these proteins from being completely degraded, resulting in a band ~2 kDa shorter than the undigested protein. In the presence of GDPβS, they are completely lost.

Figure 5 shows the results of trypsin digestion of Gα8. In general, Gα8 is more resistant to trypsin digestion than Gα1 and Gα2. It is always protected in the presence or absence of GTPγS and often in the presence of GDPβS (Figure 5A). In some experiments, however, cleavage of Gα8 could be observed when an excess of GDPβS was added (Figure 5B). The 32- and 11-kDa bands detected in those samples not treated with trypsin or in the presence of a trypsin inhibitor are probably because of endogenous protease activity. The samples that are not exposed to any exogenous nucleotides or incubated with GTPγS are still protected from the proteolytic digestion. These results indicate that Gα8 is rel-

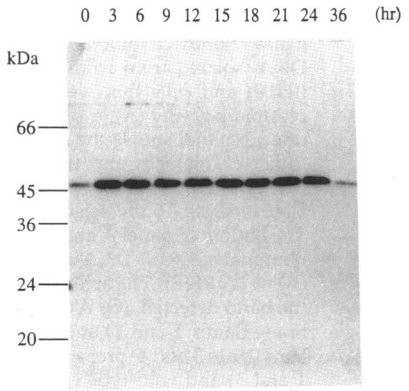

Figure 4. Time course of Gα8 protein expression. Membrane protein samples were prepared from wild-type AX3 cells at vegetative stage (0 h) and at the indicated times of development. These samples were run on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot analyses using anti-Gα8 peptide antibody. The molecular masses of protein standards are shown in this and the subsequent figures. During this development, aggregates were observed at 5 h, mounds were formed at 10–12 h, slugs were seen at 15 h, and ~25% of mounds developed to fruiting bodies at 24 h. By 36 h, most have developed into fruiting bodies.

atively resistant to trypsin digestion. It is possible that this Gα subunit has an unusual conformation that is protected from proteases. Alternatively, it may have a slow rate of GTP hydrolysis and thus the predominant form of the protein is GTP-bound, which prevents the protein from being completely degraded by trypsin.

Gene Targeting of Gα7 and Gα8

To investigate the functions of Gα7 and Gα8, null mutants of Gα7 and Gα8 were constructed. To disrupt Gα7, a linear fragment of Gα7 cDNA with a *Thy1* gene inserted at its midpoint was transformed into thymidine auxotroph JH10 cells (Mann and Firtel, 1991; Hadwiger and Firtel, 1992). Genomic DNA from the transformants was subjected to Southern blot hybridization. As shown in Figure 6A, parental wild-type genomic DNA digested by *Hin*dIII gave rise to a 2.9-kb fragment by hybridizing to the 5′ Gα7 DNA. In contrast, the same probe detected a 1.3-kb band in some transformants, which is the size expected from the desired double cross-over event. Other restriction analyses confirmed these results. These cells were designated $g\alpha7^-$.

The Gα8 gene was disrupted in two different recipient cell lines, JH10 and DH1. The plasmid constructs contained a 3.5-kb Gα8 fragment that was digested by *Bcl* I and *Bal* I to replace the 0.5-kb coding sequence with either a *Thy1* or *Ura* selectable marker. The plasmids were linearized and transformed into JH10 or DH1 (uracil auxotroph) cells, and transformants were selected. Genomic blot indicates that the parental wild-type cells yield a 3.5-kb *Eco*RI fragment when using Gα8 5′-region as a probe. In some transformants receiving the *Thy1* construct, however, this band was replaced by a 8.8-kb band (Figure 6B), indicating that the expected double cross-over event had occurred. Similar results were obtained with the *Ura* construct. To confirm these results, a probe corresponding to the 0.5-kb Gα8 sequence that should have been deleted was hybridized to the same blot. The wild-type DNA gave rise to two *Eco*RI fragments with a size of 3.5 and 2.5 kb as expected, and no hybridizing bands were observed for the transformants. In addition, immunoblot analyses of either membrane or total cellular proteins prepared from these cells detected no discernible proteins during growth or development (Figure 6C). The high molecular weight protein band detected in total protein samples from either wild-type or mutant cells probably result from a nonspecific cross-reaction to the Gα8 antibody. These combined results indicate that the Gα8 gene is disrupted in these cells, and we designate them $g\alpha8^-$.

Several independent $g\alpha7^-$ and $g\alpha8^-$ cell lines have been examined for their growth and developmental phenotypes in both JHU and UCSD. In comparison to several random integrants or the parental cell lines, these null cells showed no obvious defects in their growth rate and the timing or the morphological features of the development.

To assess whether $g\alpha7^-$ and $g\alpha8^-$ cells exhibit any defects that can only be detected under stressful con-

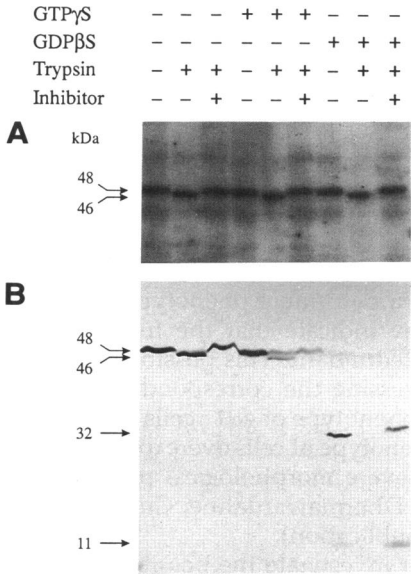

Figure 5. Trypsin protection assay of Gα8. Membrane proteins were solubilized as described in MATERIALS AND METHODS and subjected to trypsin digestion in the presence of GTPγS, GDPβS, or neither. To some samples, inhibitor (soybean trypsin inhibitor) was added before adding trypsin. Proteins were electrophoresed on SDS-PAGE, and immunoblot was performed using anti-Gα8 peptide antibody. (A) Same as described in MATERIALS AND METHODS. (B) Fresh GDPβS (80 μM) was added before adding trypsin and incubated at 30°C.

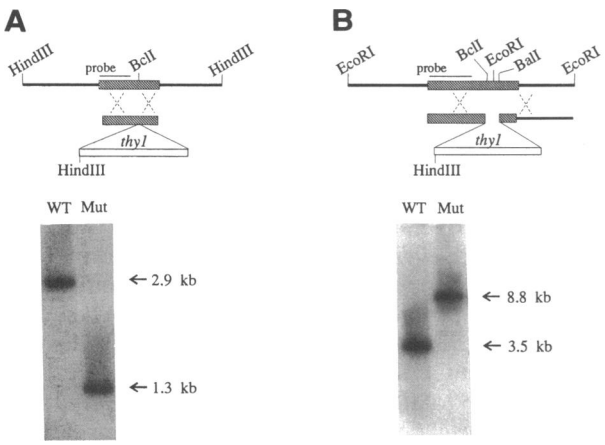

Figure 6. Disruption of the Gα7 and Gα8 genes. Southern blot analyses of genomic DNA isolated from wild-type parental cells (JH10) and cells that were transformed by plasmid pα7-thy (A) or pα8-thy (B). The top of each panel shows the restriction maps of the relevant locus and the gene disruption strategies employed. ▩, the coding region of Gα7 or Gα8; □, the selectable *thy1* marker. The bottom of panel A and B shows Southern blot analysis with DNA digested by *Hin*dIII (A) or *Eco*RI (B). The arrows show the size of the band detected. (C) Western blot of membrane (lanes 1 and 2) and total cellular proteins (lanes 3 and 4) prepared from $g\alpha8^-$ (lanes 1 and 3) and wild-type cells (lanes 2 and 4) developed for 4 h. The anti-Gα8 peptide antibody was used.

ditions that might occur in a natural environment, cells were developed on DB agar containing higher concentration of salts (100 mM NaCl or 100 mM PO_4^{3-}), high concentration of cAMP (0.2 mM), or at high (26°C) or low (6°C) temperatures. Under any of these conditions, the development was inhibited similarly for both mutant and wild-type cells.

Vital dyes have been used as cell type markers to study cell sorting during pattern formation (Sternfeld and David, 1981). To determine if Gα7 or Gα8 might affect the multicellular structure formation, $g\alpha7^-$ and $g\alpha8^-$ cells were stained with neutral red, a dye that specifically stains the prestalk and anterior-like cells in the slugs. Slugs of each cell line were stained darkly in the anteriors and lightly in the posteriors, a pattern indistinguishable from that of wild-type cells. These results suggest that deletion of either Gα7 or Gα8 does not significantly affect the pattern formation or the proportion of prestalk to prespore cells.

Overexpression of Gα7 and Gα8 Subunits

The lack of a detectable phenotype in $g\alpha7^-$ and $g\alpha8^-$ mutants may indicate that the function of these Gα subunits is redundant. This possibility might be tested by overexpressing the corresponding protein. For example, the phenotype of $g\alpha1^-$ cells is very subtle. However, the phenotype of cells overexpressing Gα1 resulted in a more severe morphological phenotype (Kumagai *et al.*, 1989; Dharmawardahne, Cubitt, and Firtel, submitted for publication).

To further investigate the possible functions of Gα7 and Gα8, we constructed cell lines that overexpress Gα7 or Gα8 protein. To do so, the Gα7 or Gα8 full length cDNA was cloned into an expression vector in which the inserted gene was driven by a promotor that expresses at high levels in all cell types during *Dictyostelium* development (Mann and Firtel, 1993). Wild-type *Dictyostelium* cells were transformed with each construct, and clonal isolates overexpressing Gα7 mRNA (determined by RNA blots) or Gα8 protein (determined by immunoblots) by 15- to 20-fold were selected. At least three independent transformants were analyzed for each construct for the morphology and timing of development.

The transformants overexpressing Gα8 protein do not show any defects when compared to wild-type cells. In addition, overexpression of Gα8-containing amino acid substitutions predicted to result in dominant active or negative mutations also did not yield any morphological phenotype. The transformants overexpressing Gα7 protein show normal developmental phenotype through aggregation and the formation of the first finger. However, as can be seen in Figure 7, starting at the slug stage, the morphogenesis becomes abnormal. Continued morphogenesis results in the formation of an abnormal fruiting body. Two independent transformations were carried out, many different transformants were examined, and the same results were obtained.

DISCUSSION

Dictyostelium has at least eight G protein α-subunits. They all share ~35–50% identity to each other and to their mammalian counterparts. Based on amino acid or nucleotide sequence, no two of the subunits fall into a distinct class whether or not the highly conserved regions are included in the analysis. Furthermore, it is not possible based on sequences to assign any of the *Dictyostelium* Gα subunits to one of Gαs, Gαi, Gαq, or Gα12 subtypes of higher eucaryotes. The complete nucleotide sequences of Gα7 and Gα8 cDNA reported in this study underscore these points.

Among the eight Gα subunits in *Dictyostelium*, Gα8 sequence has the most unusual features. It lacks the well conserved TCATDT or I region and possesses an extended C-terminus that includes long stretches of asparagine. Such triplet nucleotide repeats have been observed for several cAMP receptors (Johnson *et al.*, 1993), adenylyl cyclase genes (Pitt *et al.*, 1992), and the catalytic

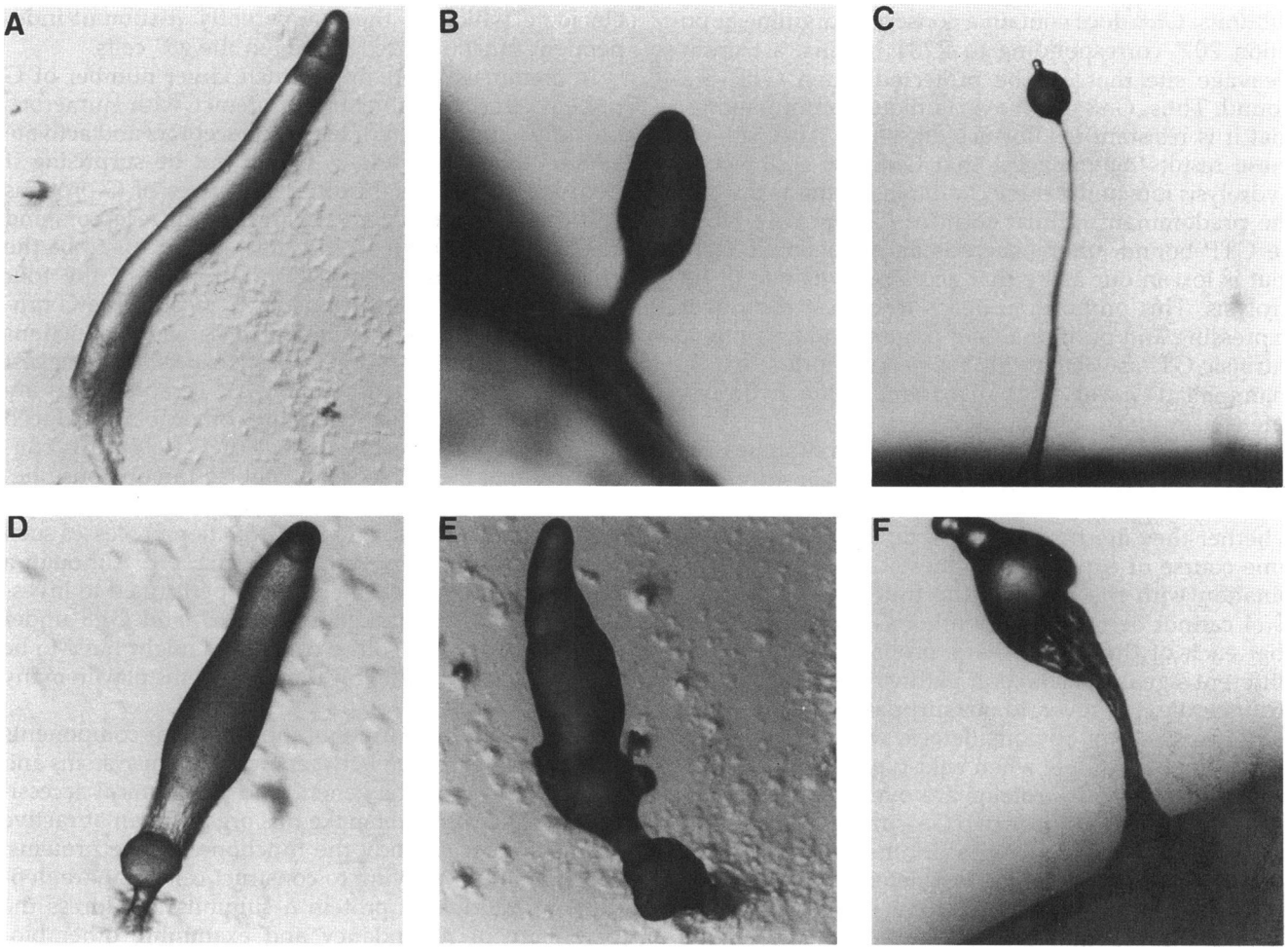

Figure 7. Developmental morphology of wild-type cells and cells overexpressing Gα7. Cells were plated on Na/K PO_4-buffered DB agar. A, B, and C show wild-type cells during the migrating slug stage, mid-culmination (21 h), and mature fruiting body (26 h), respectively. D, E, and F show the Gα7-overexpressor at the same times during development.

subunit of cAMP-dependent protein kinase (Burki *et al.*, 1991; Mann and Firtel, 1991) in *Dictyostelium*. Repeated amino acids are also present in a number of transcription factors, including developmentally regulated ones such as homeodomain proteins (Ross *et al.*, 1993). In addition, recent investigations reveal that expansion of trinucleotide repeats are found in genes related to the diseases of fragile X syndrome, spinal and bulbar muscular atrophy, myotonic dystrophy, and Huntington's disease (Ross *et al.*, 1993). To our knowledge, Gα8 is the first G protein α-subunit identified among all the systems possessing repeated single amino acids.

It has been proposed that the C-terminal region of the G protein is involved in receptor interactions (Simon *et al.*, 1991). This suggestion is supported by the observation that modification of Gα subunit at the C-terminus by pertussis toxin blocks its interaction with receptor. Antibodies that bind the C-terminus or peptides with sequences corresponding to the C-terminal regions, including the TCATDT sequence, also disrupt Gα subunit/receptor interaction (Deretic and Hamm, 1987; Sullivan *et al.*, 1987; Masters *et al.*, 1988). Moreover, Conklin *et al.* (1993) showed recently that the substitution of three amino acids at the C-terminus switches receptor specificity of Gqα to that of Giα (Conklin *et al.*, 1993; Conklin and Bourne, 1993), suggesting that the amino acid sequences at the C-terminus play an important role in the determination of the abilities of individual G proteins to discriminate among specific subsets of receptors. The unusual structure of Gα8 at the C-terminal region suggests that Gα8 may interact with a structurally different receptor or more than one receptors, and thus represent a very distinct class within the G protein superfamily.

In addition to its unusual sequence features, Gα8 is highly resistant to trypsin digestion even in the absence of GTPγS or the presence of GDPβS. As most other Gα

subunits, Gα8 does contain a conserved arginine at position 207, corresponding to R231 in Gαs, a trypsin-cleavage site that can be protected when GTPγS is bound. Thus, Gα8 may have a unique conformation so that it is resistant to protease digestion. Alternatively, these results may suggest that Gα8 has a slow GTP hydrolysis rate under these conditions. If this is the case, the predominant cellular form of Gα8 protein would be GTP-bound, unless there is an endogenous factor that is lost in our assay that accelerates its rate of hydrolysis. This proposition needs to be tested by overexpressing and purifying Gα8 protein and assaying its intrinsic GTPase activity. It has been reported that the mammalian Gαz also has an extremely slow hydrolysis rate (Casey *et al.*, 1990).

The presence of at least eight G protein subtypes during development is intriguing. It is unclear why there is such a diversity of G proteins in the slime mold and whether they are functionally redundant. The distinct time course of expression of these G proteins in combination with the fact that the functions of Gα2 and Gα4 cannot be replaced by other G proteins suggests that each of these proteins is probably involved in a different signal transduction pathway and thus plays a distinct role. However, to our surprise, neither Gα7 nor Gα8 displays any obvious defects when disrupted or, in the case of the Gα8, when wild-type, dominant active or negative mutant proteins are overexpressed. These results suggest that these two Gα-subunits either 1) are functionally redundant with another Gα subunit, 2) have subtle effects that are not detected in the null or overexpressor cells, or 3) function in a pathway that has not been identified and is not essential for multicellular development as followed in the laboratory. The absence of phenotypes using the putatively dominant active and negative mutations of Gα8 tends to favor the latter two possibilities for Gα8. Overexpression of Gα7 results in a distinct morphological phenotype during the multicellular stages that is different from that caused by overexpression of other *Dictyostelium* Gα subunits. Therefore, this result favors the possibility that Gα7 is redundant with another Gα subunit, but further experiments are needed to test this hypothesis and to elucidate the possible role of Gα7 during the late stage of development.

Both *gα7*$^-$ and *gα8*$^-$ cells have normal cAMP-stimulated Ca^{2+} influx (Milne and Devreotes, 1993), receptor phosphorylation, and loss of ligand binding (Caterina, and Devreotes, personal communication), suggesting that neither of the two Gα subunits mediates these Gα2-independent responses. In fact, none of these responses is affected by disruption of seven of the eight α-subunits (Gα6 has not been disrupted) (Kumagai *et al.*, 1991; Hadwiger and Firtel, 1992; Milne and Devreotes, 1993). It is possible that these processes are not mediated through G proteins (Caterina *et al.*, 1994). The recent construction of the *gβ*$^-$ cells will make it possible to elucidate which of these apparently α-subunit-independent functions are retained in the *gβ*$^-$ cells.

In mammals, there are a much larger number of G proteins that can potentially interact with numerous identified and as yet unidentified receptors and activate various effectors. Thus, it would not be surprising if *Dictyostelium* had eight different classes of G proteins and each of them interact with its own receptors and effectors. It has been shown that Gα2 seems to be the one that transmits signals from any one of the four cAMP receptors and thus mediates most of the G protein–dependent, cAMP-stimulated signal transductions (Kumagai *et al.*, 1989, 1991). Recent experiments suggest that Gα4 might interact with folate receptors that are involved in folate chemotaxis and other folate-induced responses (Hadwiger, Lee, and Firtel, 1994). It is conceivable that the Gα subunits that lack an obvious phenotype when disrupted, including Gα7 and Gα8, couple to other unidentified signal transduction processes such as light-sensing, temperature-sensing, etc. Although a few preliminary experiments were performed to investigate the possible functions of Gα7 and Gα8 under stressful conditions, other conditions might have to be tested to elucidate the role that G proteins play in many other processes.

The striking similarities of the molecular components of signal transduction between mammalian systems and *Dictyostelium* and the genetic and biochemical accessibility of *Dictyostelium* make this organism an attractive model system to study the function of these proteins. We are currently trying to construct cells that are deficient in multiple G protein α-subunits to address the possibility of redundancy and examining other biochemical and physiological properties of *gα7*$^-$ and *gα8*$^-$ cells to elucidate the pathways in which Gα7 and Gα8 may be involved.

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