Temperature-sensitive Gβ mutants discriminate between G protein-dependent and -independent signaling mediated by serpentine receptors

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Deletion of the single gene for the *Dictyostelium* G protein β-subunit blocks development at an early stage. We have now isolated temperature-sensitive alleles of Gβ to investigate its role in later development. We show that Gβ is directly required for adenylyl cyclase A activation and for morphogenetic signaling during the entire developmental program. Gβ was also essential for induction of aggregative gene expression by cAMP pulses, a process that is mediated by serpentine cAMP receptors (cARs). However, Gβ was not required for cAR-mediated induction of prespore genes and repression of stalk genes, and neither was Gβ needed for induction of prestalk genes by the differentiation inducing factor (DIF). cAMP induction of prespore genes and repression of stalk genes is mediated by the protein kinase GSK-3. GSK-3 also determines cell-type specification in insects and vertebrates and is regulated by the wingless/wnt morphogens that are detected by serpentine fz receptors. The G protein-dependent and -independent modes of cAR-mediated signaling reported here may also exist for the wingless/wnt signaling pathways in higher organisms.
Keywords: adenylyl cyclase/cell fate specification/ heterotrimeric G protein/serpentine receptors/ temperature-sensitive alleles

Introduction

Serpentine receptors transduce extracellular signals to intracellular effectors by interacting with heterotrimeric G proteins. Receptor activation of G proteins requires both the α and βγ subunits and both Gα and Gβγ can directly regulate effectors (Birnbaumer, 1992). The roles of G proteins *in vivo* have been assessed in a variety of genetic systems. In *Saccharomyces cerevisiae*, deletion of the single gene for either the Gβ or Gγ subunits blocks the capacity of mating pheromone to activate gene expression (Whiteway *et al.*, 1988), while in *Caenorhabditis elegans*, deletion of the single Gβ gene arrests development at gastrulation (Zwaal *et al.*, 1996). In *Drosophila melanogaster* there are two identified Gβ subunits. Mutants

defective in an eye-specific Gβ subunit display severe defects in light responsiveness (Dolph *et al.*, 1994). In *Dictyostelium discoideum*, deletion of the single Gβ gene blocks entry into the developmental program and eliminates multiple chemoattractant-mediated responses (Wu *et al.*, 1995a). These studies have been useful for elucidating the earliest requirement for Gβ function in different organisms. However, the functions of Gβ in later development remained elusive, because progression through the program is often too drastically impaired by earlier loss of function of Gβ.

The *D.discoideum* life cycle consists of a vegetative stage where cells feed on bacteria, and a multicellular stage where cells aggregate and differentiate. Following starvation, cells start to secrete cAMP pulses and chemotax towards the cAMP source to form multicellular aggregates of up to 100 000 amoebae. Cells differentiate into prestalk and prespore cells, and the multicellular mounds undergo a number of morphological changes that lead to formation of freely migrating slugs and fruiting bodies. Extracellular cAMP also controls gene expression during the entire course of development. Before aggregation, cAMP pulses strongly enhance expression of aggregative genes, encoding components of the cAMP signaling system. After aggregation, cAMP induces entry into the spore differentiation pathway as well as synthesis of a secreted factor, DIF, which induces entry into the stalk differentiation pathway. All effects of extracellular cAMP on gene expression are mediated by serpentine cAMP receptors (see Firtel, 1995).

Heterotrimeric G proteins have important regulatory functions during *Dictyostelium* development. Nine Gα subunits (Gα1–Gα9), one Gβ and one Gγ subunit have been identified (Devreotes, 1994; N.Zhang and P.N. Devreotes, unpublished results; J.Brzostowski and A.R. Kimmel, unpublished results). The single Gβ and Gγ subunits are expressed throughout growth and development, while Gα subunits are transiently expressed at specific stages.

The functions of G protein subunits have been examined in deletion mutants. Gα1 null mutants are defective in adaptation of phospholipase C (Bominaar and Van Haastert, 1994). Gα2 mediates cAMP-induced activation of guanylyl cyclase and phospholipase C (Okaichi *et al.*, 1992; Bominaar and Van Haastert, 1994) and by release of Gβγ of adenylyl cyclase (Wu *et al.*, 1995a). Gα3 null mutants show an as yet uncharacterized defect in cAMP signal production (Brandon and Podgorski, 1997). Gα4 mediates activation of guanylyl cyclase and, via Gβγ, of adenylyl cyclase by folate, a chemoattractant secreted by bacteria (Hadwiger *et al.*, 1994). Null mutants for the other Gαs either do not exhibit significant morphogenetic defects, or have not yet been investigated.
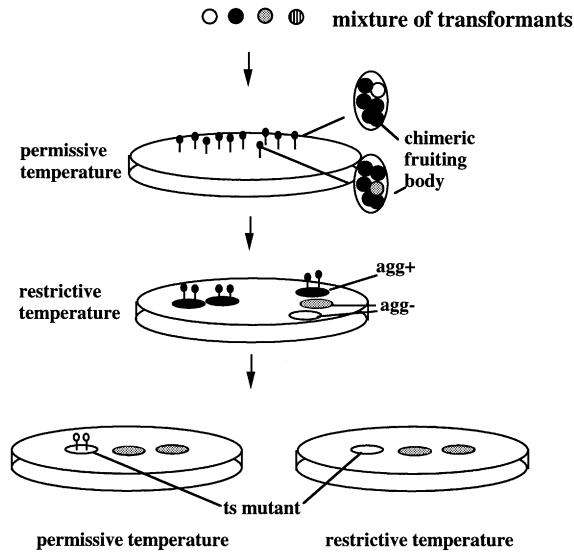
It seems apparent that the Gβ subunit must have

Fig. 1. Screening protocol for temperature-sensitive Gβ mutants. Approximately 10^4 transformants were amplified in shaking culture at 22°C. The mixture of ~10^8 cells expressing temperature-sensitive (empty circle), fully functional (black circle), conditional (light-shaded circle) or non-functional (striped circle) Gβ subunits was plated on non-nutrient agar for development at 22°C. Fruiting bodies had formed in 24 h, the spores were collected, and heated twice at 45°C for 30 min to kill non-spore cells. 2×10^4 spores were plated clonally on bacterial lawns at 27°C. Cells that formed plaques with an aggregation minus (agg⁻) phenotype at 27°C were picked and plated on replica bacterial plates incubated at 22°C and 27°C. The cells which formed fruiting bodies within plaques at 22°C and agg⁻ plaques at 27°C were isolated and grown in HL5 with G418.

important functions throughout development. Gβ null cells (gβ⁻) fail to enter development and remain completely deficient in chemoattractant-induced responses, since Gβ is required for all Gαs. To investigate the functions of Gβ, we isolated temperature-sensitive Gβ mutants, which allow G protein functions to be turned off at any stage of development. Two strains expressing the temperature-sensitive Gβ subunits were used to examine the role of G proteins in adenylyl cyclase activation, postaggregative morphogenesis and cell-type specification.

Results

Screen for temperature-sensitive mutations in the Gβ subunit

Cells of a gβ⁻ parental strain were transformed with a library of randomly mutagenized Gβ cDNAs and ~10^4 neo^R transformants were collected. As shown in Figure 1, each transformant will have a Gβ protein, that is either fully functional, temperature-sensitive, conditional or non-functional. First, we incubated the transformants collectively on DB agar at the permissive temperature (22°C). The cells expressing functional or conditional Gβ subunits will develop into chimeric fruiting bodies, while the cells expressing non-functional Gβ subunits will not participate. Secondly, the spores from the chimeric fruiting bodies were collected, plated clonally on bacterial lawns and incubated at the restrictive temperature (27°C). Cells expressing functional Gβ subunits will form plaques with fruiting bodies (agg⁺), while those cells expressing a temperature-sensitive (ts) or otherwise conditional Gβ subunit will form smooth aggregation minus (agg⁻)

plaques. We selected ~100 independent transformants from this second plating, which made agg⁻ plaques at 27°C. Thirdly, to distinguish the transformants expressing ts versus other conditional mutations, we replicated them individually on bacterial lawns and incubated them at both 22°C and 27°C. Those mutants which for various reasons can respond to, but not propagate, cAMP signals will be agg⁻ at both temperatures. We selected ~20 independent transformants which formed agg⁺ plaques at 22°C and agg⁻ plaques at 27°C. To prove that these phenotypes are plasmid-dependent, we rescued plasmids from four candidates and then retransformed them into parental gβ⁻ cells. All of the new transformants formed agg⁺ plaques at 22°C and agg⁻ plaques at 27°C. These results confirmed that the developmental defects were dependent on the plasmids.

For further studies, we chose two of the new transformants, designated as *gpbA1* and *gpbA2*, which formed fruiting bodies at 22°C. Figure 2A shows the development of wild-type, gβ⁻, *gpbA1* and *gpbA2* cells at the permissive (22°C) and restrictive (27°C) temperatures. Wild-type cells formed fruiting bodies at both temperatures, while the gβ⁻ cells always failed to aggregate and remained as a monolayer. At 22°C, *gpbA1* and *gpbA2* cells formed fruiting bodies indistinguishable from those of wild-type, while at 27°C, *gpbA1* and *gpbA2* completely failed to enter the developmental program. In wild-type cells, the Gβ protein level is constant at all stages of growth and development (Lilly *et al.*, 1993). Figure 2B shows that in both wild-type, *gpbA1* and *gpbA2* cells, the levels of Gβ protein were constant during development, and remained unchanged for 6 h after a shift to 27°C. We therefore assume that the Gβ subunits in the *gpbA1* and *gpbA2* cells show conformational instability at the restrictive temperature.

The *gpbA1* and *gpbA2* mutants allowed us to determine whether Gβ is required for postaggregative development. We incubated *gpbA1* or *gpbA2* cells on non-nutrient agar at 22°C until they formed aggregates (Figure 3a), tipped mounds (Figure 3d) or slugs (Figure 3g), and then shifted them to 27°C. *gpbA2* aggregates formed abnormal mounds after 2.5 h at 27°C (Figure 3b), which showed no further progress for 26 h (Figure 3c). *gpbA2* cells shifted at the tipped mound stage, formed a first finger after 2.5 h at 27°C (Figure 3e), but did not develop further (Figure 3f). *gpbA1* cells shifted at the slug stage showed abnormal slug morphology after 2.5 h (Figure 3h), which became progressively more aberrant after 26 h (Figure 3i). These results suggested a continuous requirement for G protein-mediated signaling for proper tip-, slug- and fruiting body formation.

To determine whether the developmental block was reversible, we developed *gpbA1* cells to tipped mounds at 22°C (Figure 3j), and then shifted them to 27°C for 26 h until development completely arrested (Figure 3k). We shifted cells back to 22°C and after another 22 h, fruiting bodies were observed (Figure 3l), demonstrating that the developmental block could be reversed. However, it should be noted that not all cells participated in fruiting body formation, so the reversal was not complete.

Adenylyl cyclase A activation in Gβ ts mutants

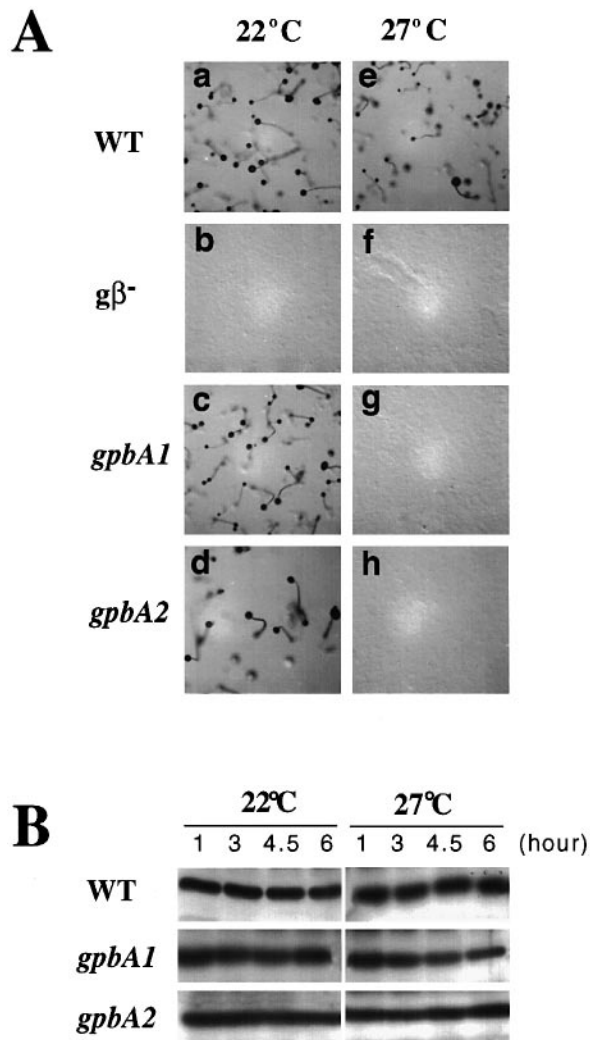To demonstrate that G protein function was lost when *gpbA1* and *gpbA2* cells were shifted to the restrictive

A

B

Fig. 2. Phenotype and Gβ protein levels of cells expressing ts Gβ mutations. (**A**) Developmental phenotype. Wild-type (a and e), gβ⁻ (b and f), *gpbA1* (c and g) and *gpbA2* (d and h) cells were harvested from shaking cultures, plated on DB agar, incubated at either 22°C (a–d) or 27°C (e–h) for 72 h and photographed. (**B**) Immunoblot analysis of Gβ proteins. Wild-type (WT), *gpbA1* and *gpbA2* cells were incubated in DB at either 22°C or 27°C and stimulated with pulses of 50 nM cAMP at 6-min intervals. Samples were taken after the indicated periods of incubation. Total protein of 10^6 cells was size-fractionated by SDS–PAGE and Western blots were probed with Gβ antibodies.

Fig. 3. Multicellular morphogenesis of *gpbA1* and *gpbA2* mutants. *gpbA1* and *gpbA2* cells were incubated on DB agar at 22°C until they had reached the late aggregation (**a**), tipped mound (**d**) and slug stage (**g**) and plates were then placed at 27°C and photographed after 2.5 h (**b**, **e** and **h**) and 26 h (**c**, **f** and **i**). *gpbA1* cells were developed to tipped mounds 22°C (**j**), shifted to 27°C for 26 h (**k**), and back to 22°C for 22 h (**l**). *gpbA2* cells were photographed in panels (a–f) and *gpbA1* cells in panels (g–l).

Table I. Activation of adenylyl cyclase A (ACA) in wild-type (WT) cells and ts mutants

Cell line	Unstimulated ACA activity[a]		Stimulated ACA activity[a]	
	22°C	27°C	22°C	27°C
WT	11	23 (26)	89	86 (91)
gpbA1	9	13 (20)	46	17 (22)
gpbA2	12	13 (24)	63	22 (34)

[a]Activity expressed in pmol/min/mg.
Wild-type, *gpbA1* and *gpbA2* cells were stimulated for 5 h at 22°C with cAMP pulses to induce optimal expression of cAMP signaling components. Cells were subsequently pre-incubated at 22°C or 27°C for 2 h and lysed in the absence (unstimulated activity) or presence (stimulated activity) of GTPγS. Adenylyl cyclase activity in the lysates of wild-type, *gpbA1* and *gpbA2* cells was measured at 22°C as described in Materials and methods. Data in parentheses represent an adenylyl cyclase assay performed at 27°C instead of 22°C. All data represent the means of an assay performed in duplicate. An independent experiment was done and yielded similar results.

temperature, we assayed GTPγS activation of adenylyl cyclase A (ACA). In gβ⁻ cells, cAR- and G protein-mediated activation of ACA is completely absent (Wu *et al.*, 1995a). However, since the gβ⁻ cells are blocked at an early stage of development, it might be argued that this defect is not directly due to the absence of Gβ, but to reduced expression of components other than Gβ, that are essential for ACA activation. Analysis of *gpbA1* and *gpbA2* ruled out this possibility and allowed us to assess how quickly G protein function was lost.

We first stimulated *gpbA1* and *gpbA2* cells during 5 h at 22°C with cAMP pulses to allow them to express all aggregative genes, and then shifted them to 27°C to 'turn-off' the functions of the Gβ subunit. We measured activity in lysates stimulated with GTPγS to bypass the receptor and directly assess the G protein. When *gpbA2* cells were
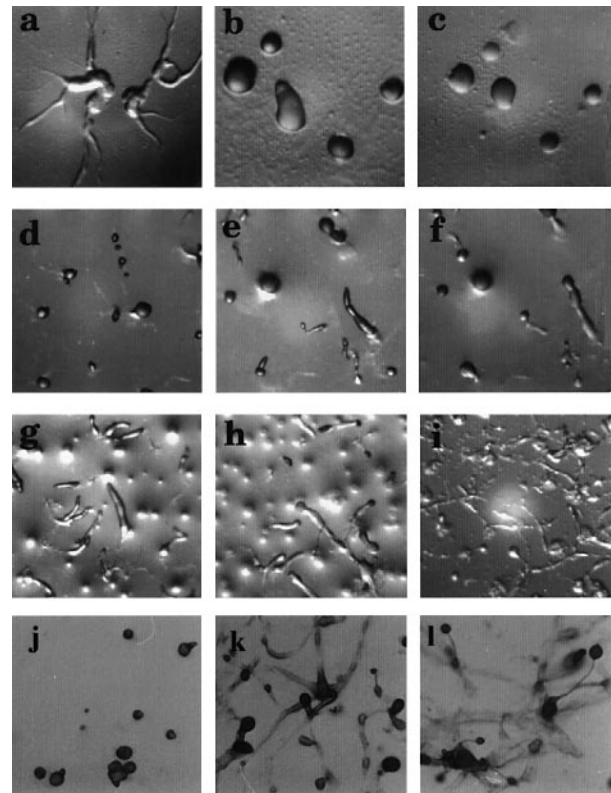
incubated at 27°C for 1 h, GTPγS-stimulated ACA activity was reduced 3-fold when compared with cells incubated at 22°C (data not shown). We then extended incubation at 27°C to 2 h. Table I shows that in cells incubated at 22°C, GTPγS induced a 5.1- or 5.3-fold stimulation of ACA activity in *gpbA1* and *gpbA2* lysates, respectively. However, in cells incubated at 27°C, GTPγS stimulation was reduced to ~1.3- and ~1.7-fold in the *gpbA1* and

gpbA2 mutants, respectively. Under the same conditions, GTPγS significantly stimulated ACA activity in wild-type cells incubated at both 22°C and 27°C. These results indicate that a functional Gβ subunit is directly required for GTPγS activation of ACA.

Induction of aggregative gene expression in Gβ ts mutants

The expression levels of several genes that encode components required for aggregation such as cAR1, Gα2, ACA, phosphodiesterase (PDE) and the cell adhesion glycoprotein csA/gp80, increase dramatically upon starvation. Cell density sensing factors, that are secreted upon starvation, induce sufficient levels of PDE, cAR1, Gα2 and ACA to initiate oscillatory cAMP secretion. cAMP pulses then enhance further transcription of the *cAR1*, *Gα2* and *PDE* genes and induce transcription of the *gp80* gene (Firtel, 1995).

We first examined gp80 and cAR1 protein levels in wild-type cells and gβ⁻, *gpbA1* and *gpbA2* mutants, that were stimulated with cAMP pulses at 22°C and 27°C. In wild-type cells, gp80 (Figure 4A) and cAR1 (Figure 4B) proteins were induced at both 22°C and 27°C, while in gβ⁻ cells, the level of gp80 and cAR1 protein was extremely low at both temperatures. In the *gpbA1* and *gpbA2* mutants, gp80 and cAR1 proteins were induced to the same levels as in wild-type cells at 22°C, but expression was strongly reduced at 27°C. We also measured basal and cAMP-stimulated levels of the *gp80*, *cAR1* and *PDE* mRNAs in *gpbA1* and *gpbA2* cells (Figure 4C). There was a low level of *cAR1* and a moderate level of *PDE* mRNA accumulation in the absence of cAMP stimuli at both 22°C and 27°C. Transcription of both *cAR1* and *gp80* was at least 10-fold stimulated by cAMP pulses at 22°C, but not at all at 27°C. Transcription of PDE was stimulated only ~2-fold by cAMP pulses; this stimulation seemed to be reduced at 27°C. These data indicated that the Gβ subunit is required for stimulation of aggregative gene expression by cAMP pulses, but most likely not for basal induction by cell density sensing factors.

Prestalk and prespore gene expression in the Gβ ts mutants

After aggregates have formed, cAMP continues to be essential for regulation of prespore and prestalk gene expression. The prespore genes *psA* and *CotB* are induced by cAMP and repressed by DIF. The prestalk gene *ecmA* is induced by DIF and cAMP in synergy. The stalk gene *ecmB* is also induced by DIF, but this induction is inhibited by cAMP (see Firtel, 1995). We determined whether either cAMP- or DIF-induced gene regulation requires the Gβ subunit.

The prespore genes *psA* and *cotB* and the prestalk gene *ecmA* are optimally inducible in cells that have developed to the loose aggregate stage. Wild-type cells and the *gpbA1* and *gpbA2* mutants were developed on agar at 22°C until loose aggregates had formed, and were then incubated for 8 h in suspension at 22°C or 27°C with cAMP and/or DIF. As shown in Figure 5A and B, the expression of the prespore genes *psA* and *cotB* in wild-type and ts mutant cells was induced by cAMP and inhibited by DIF. The prestalk gene *ecmA* showed moderate levels of expression in the presence of DIF alone and
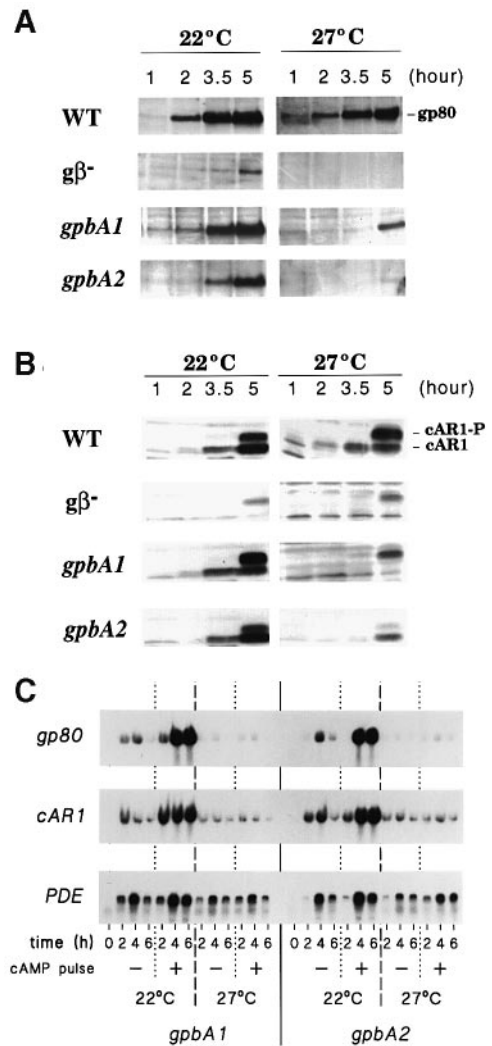

Fig. 4. Induction of aggregative gene expression by cAMP pulses. (**A** and **B**) gp80 and cAR1 protein levels in wild-type and mutant cells. Wild-type, gβ⁻, *gpbA1* and *gpbA2* cells were stimulated with 50 nM cAMP pulses at 6-min intervals for 5 h at either 22°C or 27°C. Samples were taken after the indicated incubation periods and subjected to SDS–PAGE and immunoblotting with gp80- and cAR1-specific antibodies. (**C**) Induction of *gp80*, *cAR1* and *PDE* mRNA in ts mutants. *gpbA1* and *gpbA2* cells were incubated at 22°C or 27°C with or without 30 nM cAMP pulses at 6-min intervals. mRNA was isolated after 0, 2, 4 and 6 h of incubation and Northern blots were probed with ^{32}P-labeled *cAR1*, *gp80* and *PDE* cDNA probes.

optimal expression in the presence of both cAMP and DIF. These patterns were essentially the same at 22°C and 27°C. Prespore gene expression requires at least 4 h of incubation with cAMP, which leaves sufficient time for loss of Gβ function at 27°C, but *ecmA* induction by DIF occurs within 1 h (Williams *et al.*, 1987). Figure 5C shows that some *ecmA* induction by DIF and cAMP was indeed evident in *gpbA2* cells after 1 h, but mRNA levels increased progressively up to 5 h of incubation at both 22°C and 27°C, well after the inactivation of Gβ. Absolute induction levels were somewhat lower at 27°C, but this was also the case in wild-type cells (data not shown) and could be due to enhanced degradation of DIF at the higher temperature. In general, the results indicate that Gβ does not mediate regulation of prespore or prestalk genes by cAMP or DIF.
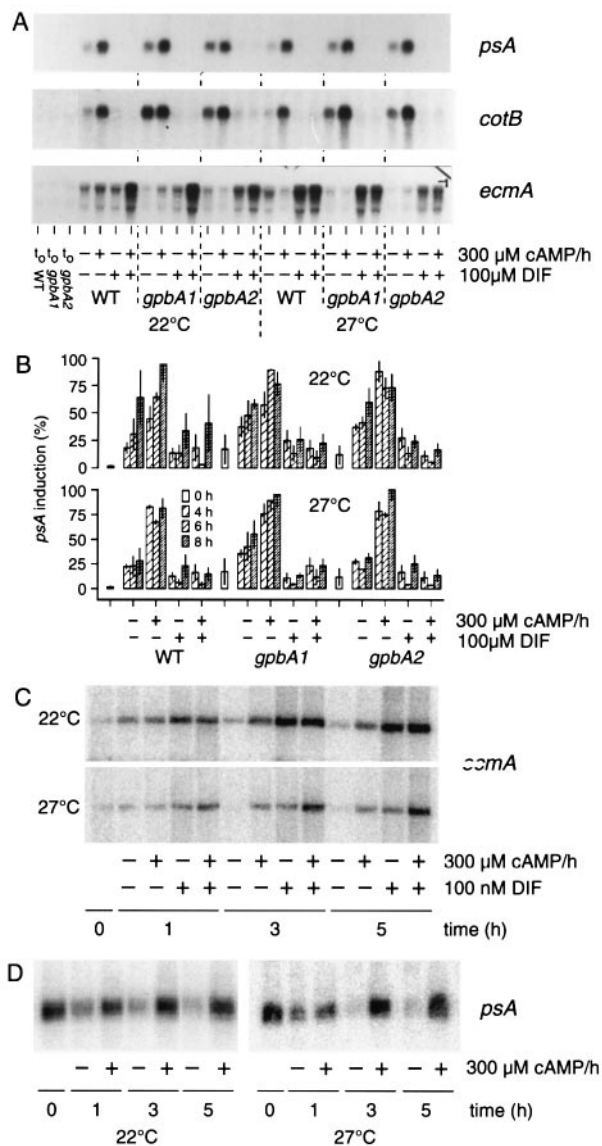
Fig. 6. Regulation of *ecmB* gene expression. Wild-type, *gpbA1* and *gpbA2* cells were first incubated for 16 h with 5 mM cAMP in stalk salts. Aggregates were dissociated into single cells, which were thoroughly washed and resuspended in stalk salts. Cells were incubated in Petri dishes at either 22°C or 27°C and challenged with 100 nM DIF and 1 μM Sp-cAMPS. RNA was isolated after 8 h and probed to ^{32}P-labeled *ecmB* cDNA. The experiment was repeated twice with similar results for *gpbA1* and *gpbA2* and equal levels of *ecmB* mRNA at 22°C and 27°C for wild-type cells.

1983). We investigated whether maintenance of prespore gene expression required the Gβ subunit. Wild-type and *gpbA2* cells were developed to the tight mound stage, when the prespore gene *psA* is already expressed. Cells were then incubated at either 22°C or 27°C in the presence and absence of cAMP. Figure 5D shows that in wild-type and *gpbA2* cells, cAMP stabilized *psA* mRNA at both 22°C and 27°C, suggesting that cAR-mediated mRNA stabilization is also independent of the Gβ subunit.

To study regulation of the prestalk gene *ecmB*, cells were prestimulated in monolayers with 5 mM cAMP during 16 h at 22°C (Berks and Kay, 1988). cAMP was removed and cells were incubated at 22°C and 27°C with 100 nM DIF and 1 μM Sp-cAMPS. Sp-cAMPS mimics the nanomolar cAMP levels, which are most effective to repress *ecmB* induction (Soede *et al.*, 1996). Figure 6 shows that DIF induced *ecmB* expression in both wild-type cells and the ts mutants, while Sp-cAMPS almost completely inhibited this induction. These effects were essentially the same at 22°C and 27°C, and indicated that neither DIF induction nor Sp-cAMPS repression of *ecmB* required the Gβ subunit.

Determination of mutations in the ts Gβ subunits

We sequenced the Gβ genes on the plasmids rescued from the *gpbA1* and *gpbA2* cells, and found that each contained multiple mutations. Figure 7A shows the amino acid alignment of bovine $G_t\beta$, human Gβ2, mouse Gβ4 and *D.discoideum* Gβ, and the amino acid substitutions in *gpbA1* and *gpbA2* mutants. The *gpbA1* mutant contained three amino acid substitutions: M48T, I52T and E310G, and the *gpbA2* contained seven, namely R13G, N74S, S79P, H149R, V178A, V194R and F293L. Figure 7B illustrates the positions of these mutations superimposed on a ribbon structure of the bovine $G_t\beta\gamma$ dimer.

Although it is not our purpose here to investigate structure, we separated some of the mutations. Using a convenient restriction site in the Gβ gene, we replaced a portion of the coding regions of *gpbA1* and *gpbA2* (from aa 187–346, or from aa 1–186) with the corresponding coding regions of the wild-type gene. These swaps yielded the mutant Gβ genes *gpbA1*a (M48T and I52T), *gpbA1*b (E310G), *gpbA2*a (R13G, N74S, S79P, H149R and V179A) and *gpbA2*b (V194R and F293L). We transformed the gβ− cells with plasmids of *gpbA1*a, *gpbA1*b, *gpbA2*a

Fig. 5. Induction and maintenance of prestalk and prespore gene expression. (**A**) Prespore and *ecmA* mRNA accumulation. Wild-type, *gpbA1* and *gpbA2* cells were developed to loose aggregates, dissociated and resuspended in DB. Cells were shaken at 150 r.p.m. at either 22°C or 27°C and challenged with 100 nM DIF added once at the onset of the incubation, 300 μM cAMP added at 60-min intervals, or a combination of the two stimuli. After 0, 4, 6 and 8 h of incubation, RNA was isolated and probed to ^{32}P-labeled *psA*, *cotB* or *ecmA* cDNA. Only the results at t = 0 and t = 6 h are shown in this panel. (**B**) Quantitation of the time-course of *psA* induction. *psA* RNA bands from three independent experiments were quantitated by densitometry. All optical density values were calculated as percentage of induction at t = 8 h in *gpbA2* cells incubated in the presence of 300 μM cAMP at 27°C. The data are presented as mean ± SEM. (**C**) Time-course of *ecmA* induction. *gpbA2* cells, developed to loose aggregates, were incubated with cAMP and DIF in shaken suspension at 22°C and 27°C. RNA was isolated at the indicated time points and probed to *ecmA* cDNA. (**D**) Maintenance of prespore gene expression. Wild-type and *gpbA2* mutants were developed at 22°C until tight mounds had formed. Mounds were dissociated and cells were incubated in suspension at 22°C or 27°C with 300 μM cAMP for the indicated time periods. RNA was isolated and probed to ^{32}P-labeled *psA* cDNA.

Prespore differentiation requires the continued presence of cAMP, since transcription ceases and prespore mRNAs become destabilized in its absence (Mangiarotti *et al.*,
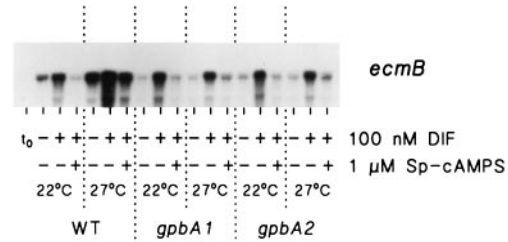
A

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Bovine      .....MSELDQLRQEAEQLKNQIRDARKACADATLSQITNNIDPVGR 42  IQ..MRTRRTLRGHLAKIYAMHWGTDSRLLVSASQDGKLIIWD  83
Human   2   .....MSELEQLRQEAEQLRNQIRDARKACGDSTLTQITAGLDPVGR 42  IQ..MRTRRTLRGHLAKIYAMHWGTDSRLLVSASQDGKLIIWD  83
Mouse   4   .....MSELEQLRQEAEQLRNQIQDARKACNDATLVQITSNMDSVGR 42  IQ..MRTRRTLRGHLAKIYAMHWGYDSRLLVSASQDGKLIIWD  83
Dicty       MSSDISEKIQQARRDAESMKEQIRANRDVMNDTTLKTFTRDLPGLPK 47  MEGKIKVRRNLKGHLAKIYAMHWAEDNVHLVSASQDGKLLVWD  90
Mutations                     *                                    *  *                   *      *
gpbA1       ..............................................      ..........................................
gpbA2       .....................G........................      ...............................S....P.....

Bovine      SYTTNKVHAIPLRSSWVMTCAYAPSGNYVACGGLDNICSIYN 125  LKTREGNVRVSRELAGHTGYLSCCRFLDDNQIVTSSGDTTCALWD 170
Human   2   SYTTNKVHAIPLRSSWVMTCAYAPSGNFVACGGLDNICSIYS 125  LKTREGNVRVSRELPGHTGYLSCCRFLDDNQIITSSGDTTCALWD 170
Mouse   4   SYTTNKMHAIPLRSSWVMTCAYAPSGNYVACGGLDNICSIYN 125  LKTREGDVRVSRELAGHTGYLSCCRFLDDGQIITSSGDTTCALWD 170
Dicty       GLTTNKVHAIPLRSSWVMTCAYSPTANFVACGGLDNICSIYN 132  LRSREQPIRVCRELNSHTGYLSCCRFLNDRQIVTSSGDMTCILWD 177
Mutations                                                                      *
gpbA1       .........................................      .............................................
gpbA2       .........................................      ...............................R.............

Bovine      IETGQQTTTFTGHTGDVMSLSLAPDTRLFVSGACDASAKLWD 212  VREGMCRQTFTGHESDINAICFFPNGNAFATGSDDATCRLFD 254
Human   2   IETGQQTVGFAGHSGDVMSLSLAPDGRTFVSGACDASIKLWD 212  VRDSMCRQTFIGHESDINAVAFFPNGYAFTTGSDDATCRLFD 254
Mouse   4   IETGQQTTTFTGHSGDVMSLSLSPDLKTFVSGACDASSKLWD 212  IRDGMCRQSFTGHISDINAVSFFPSGYAFATGSDDATCRLFD 254
Dicty       VENGTKITEFSDHNGDVMSVSVSPDKNYFISGACDATAKLWD 219  LRSGKCVQTFTGHEADINAVQYFPNGLSFGTGSDDASCRLFD 261
Mutations   *                           *                      *
gpbA1       .........................................      .........................................
gpbA2       A..................R.....................      .........................................

Bovine      LRADQELMTYSHDNIICGITSVSFSKSGRLLLAGYDDFNCNVWD 298  ALKADRAGVLAGHDNRVSCLGVTDDGMAVATGSWDSFLKIWN 340
Human   2   LRADQELLMYSHDNIICGITSVAFSRSGRLLLAGYDDFNCNIWD 298  AMKGDRAGVLAGHDNRVSCLGVTDDGMAVATGSWDSFLKIWN 340
Mouse   4   LRADQELLLYSHDNIICGITSVAFSKSGRLLLAGYDDFNCSVWD 298  ALKGGRSGVLAGHDNRVSCLGVTDDGMAVATGSWDSFLRIWN 340
Dicty       IRADRELMQYTHDNILCGITSVGFSFSGRFLFAGYDDFTCNVWD 305  TLKGERVLSLTGHGNRVSCLGVPTDGMALCTGSWDSLLKIWA 347
Mutations                             *                           *
gpbA1       ............................................      ..........G...............................
gpbA2       .........................................L..      ..........................................
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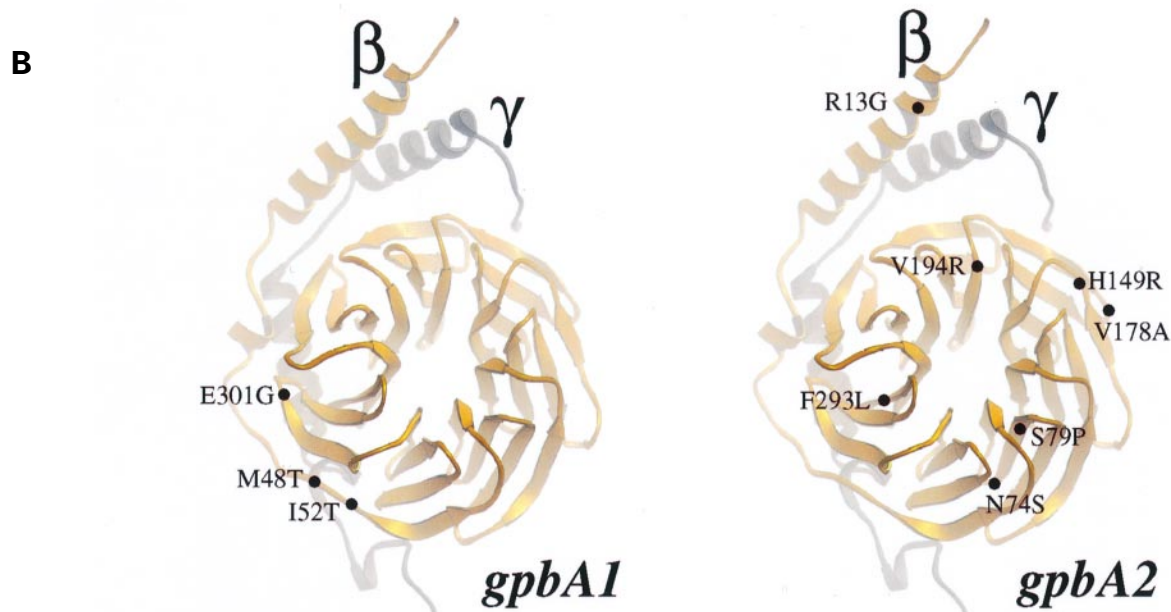
B

Fig. 7. Sequence identification of ts alleles in Gβ mutants. (**A**) Amino acid sequence alignment of four Gβ subunits and the substitutions in ts Gβ subunits. (**B**) Ribbon diagram showing the positions of the substitutions in ts Gβ subunits.

and *gpbA2*b, and examined the development of these transformants at 22°C and 27°C. At 22°C, all of these transformants made agg⁺ plaques on bacterial lawns, suggesting that they expressed a functional Gβ protein at the permissive temperature. At 27°C, *gpbA1*a and *gpbA2*a formed agg⁻ plaques, while *gpbA1*b and *gpbA2*b formed plaques with small aggregates. When plated on DB agar, cells of *gpbA1*a and *gpbA2*a remained as a monolayer at 27°C and formed fruiting bodies at 22°C, while cells of *gpbA1*b and *gpbA2*b formed aggregates at 27°C. These observations suggested that *gpbA1*a with M48T and I52T mutations, and *gpbA2*a with the R13G, N74S, S79P, H149R, V178A mutations resulted in temperature-

sensitive Gβ subunits, while *gpbA1*b with E310G, and *gpbA2*b with V194R and F293L substitutions showed only weak temperature sensitivity.

The crystal structure of a $G_t\beta\gamma$ heterodimer showed that Gβ forms extensive, mainly hydrophobic, interactions with Gγ (Sondek *et al.*, 1996). For *gpbA1*a, the substitutions M48T and I52T are located at a loop that links the N-terminal helix to the first β sheet. Interestingly, the residue corresponding to I52 has been implicated in the formation of a specific hydrophobic interaction between Gβ and Gγ (Sondek *et al.*, 1996), the substitutions in *gpbA1*a may weaken or disrupt the hydrophobic interaction, and therefore possibly destabilize the Gβγ complex.

For *gpbA2*a, substitution of N74S changed the diverged N in the Gβ of *D.discoideum* back to the conserved S of other Gβ subunits, and mutation of V179A occurred at the residue which is V, I or L on the other Gβ subunits, while mutations of R13G, S79P and H149R represent more significant substitutions occurring at highly conserved residues in the identified Gβ subunits of all organisms. Thus, it is likely that the temperature-sensitive character of *gpbA2* is due to the R13G, S79P or H149R mutations. None of the changes in *gpbA2* is in positions that have been implicated in interaction with γ or α subunits and functions of βγ.

Discussion

We designed a screening procedure based on the essential role of the Gβ subunit in early *Dictyostelium* development and isolated the temperature-sensitive Gβ mutants *gpbA1* and *gpbA2*. Both mutant Gβ subunits function at the permissive temperature, but could be 'turned off' by shifting the cells to restrictive temperature. The mutants completed development and formed normal fruiting bodies at 22°C, but failed to aggregate at 27°C. We used these conditional mutants to investigate the roles of the Gβ in cell–cell signaling and gene regulation during the entire course of development.

The Gβ subunit directly mediates ACA activation
At the aggregation stage, cAR1-mediated activation of ACA reaches its highest level. Activation of ACA through βγ-subunits is a complex pathway, requiring at least three additional components, CRAC, Aimless and Pianissimo, that are all developmentally regulated (Insall *et al.*, 1994, 1996; Chen *et al.*, 1997). ts mutants, developed at the permissive temperature to induce all signaling components, showed an almost complete loss of GTPγS-induced ACA activation after 2 h at the restrictive temperature and reduced activation after 1 h. Since the Gβ protein is not actually degraded, this indicates that it requires ~2 h to become fully destabilized.

Induction of aggregative gene expression by cAMP pulses requires the Gβ subunit
Induction of aggregative genes, as *PDE*, *cAR1*, *Gα2* and *gp80* occurs in two steps. First, protein factors that are secreted upon starvation induce a basal level of transcription that allow cells to initiate oscillatory cAMP signaling. Second, periodic cAMP stimulation then triggers full expression of these genes (see Firtel, 1995). We found that, in the Gβ ts mutants, basal induction of expression of the *PDE* and *cAR1* genes occurred at both the permissive and restrictive temperatures. However, cAMP pulse-induced expression of the *cAR1* and *gp80* genes could be detected only at the permissive temperature. This is consistent with earlier data showing that pulse-induced gene expression does not occur in *fgdA* mutants that lack a functional Gα2 subunit (Mann *et al.*, 1988; Kumagai *et al.*, 1989). Previous studies show that induction of the PDE inhibitor, PDI, which is typically induced by starvation factors and repressed by cAMP does not require the Gβ subunit, whereas induction of PDE mRNA by 1 mM cAMP was also Gβ-independent (Wu *et al.*, 1995b). In our hands, induction of PDE mRNA by 30 nM cAMP

pulses (a physiologically relevant stimulus at this developmental stage) was so modest, that requirement of Gβ was difficult to determine. In general, it seems evident that Gβ does not mediate basal induction of aggregative genes, but is an essential component of the signaling pathway that mediates cAMP pulse-induced transcription.

The Gβ subunit is required for postaggregative morphogenesis, but not for postaggregative gene induction
The development of ts mutants plated on agar can be blocked at any stage of development up to fruiting body formation by transferring the plates to the restrictive temperature. These developmental arrests are reversible and indicate that the Gβ subunit is required for both aggregation and formation of multicellular structures such as mounds, tips, slugs and fruiting bodies. Morphogenesis is the result of intercellular signals that control processes as cell movement, cell adhesion and differentiation of prespore and prestalk cells.

We analyzed in detail whether Gβ is required for induction of prestalk and prespore gene expression. Transcription of prespore genes is induced by persistent stimulation with micromolar cAMP, and maintenance of expression as well as stabilization of prespore mRNAs depends on the continued presence of cAMP. Micromolar cAMP synergizes with the stalk-inducing factor DIF to induce the early prestalk gene *ecmA*, while nanomolar cAMP antagonizes DIF-induced expression of the late prestalk gene *ecmB* (Berks and Kay, 1988, 1990; Soede *et al.*, 1996). The ts mutants showed all responses mentioned above at both the permissive and restrictive temperatures, which indicates that Gβ is neither directly involved in cAMP induction of prespore gene transcription and stabilization of prespore mRNAs, nor in DIF-induction of *ecmA* and *ecmB*, nor in cAMP repression of *ecmB*.

These results contrast rather strongly with the universal requirement of Gβ for morphogenesis in all stages of development. Gene regulation is only one of the factors that govern morphological changes. A fair body of evidence indicates that after aggregation oscillatory cAMP signaling persists to coordinate morphogenetic movement (Siegert and Weijer, 1992). The morphogenetic defects of the Gβ mutants are most likely a consequence of the essential role of Gβ in ACA activation and in chemoattractant-induced changes in the cytoskeleton, that govern directed cell movement.

Serpentine receptors do not always function by coupling to heterotrimeric G proteins
The lack of Gβ requirement is not completely surprising for responses induced by the highly lipophilic morphogen DIF, which is unlikely to activate G protein-coupled receptors. However, it is quite remarkable that none of the cAR-mediated postaggregative gene regulation events appears to require G proteins. One trivial explanation could be that the genetic lesions in the ts mutants only affect coupling to target proteins that mediate preaggregative gene induction and ACA activation. We consider this unlikely since *gpbA1* and *gpbA2* harbor entirely different sets of mutations. Both sets of mutations block the two early responses, which are each mediated by a different signaling pathway (Pitt *et al.*, 1993). One of the

mutations in *gpbA1* is at a critical region for interaction with Gγ and may be expected to affect all functions of Gβ. The presence of a second Gβ is also not very likely, considering the high level of conservation (60% amino acid identity) between Gβs of different organisms and the absence of additional bands hybridizing to Gβ DNA in low-stringency Southern blots (Lilly *et al.*, 1993). In addition, GTPγS modulation of cAR1 affinity is entirely lost in Gβ− cells, indicating that all functional coupling between cAR1 and G proteins has disappeared (Wu *et al.*, 1995a).

Earlier evidence for G protein-independent gene regulation was provided by studies showing that another class of cAMP-inducible non-cell type-specific genes as *LagC*, *rasD* and *CP2*, could be prematurely induced by cAMP in gβ− cells that overexpressed the transcription factor GBF (Schnitzler *et al.*, 1995). Our current data also agree with earlier observations that null mutants for the Gα5, Gα7 and Gα8 subunits, that are all expressed after aggregation, show normal developmental gene expression, although this may have been due to functional redundancy of individual Gα subunits. Gα4 null cells do show a defect in prespore differentiation *in vivo*, but this defect can be overcome by stimulation with cAMP *in vitro*, indicating that Gα4 does not mediate cAMP signal transduction (Hadwiger and Firtel, 1992; Hadwiger *et al.*, 1994).

One putative target for cAMP induction of prespore gene expression is GSK-3, a homolog of zeste white-3/shaggy in *Drosophila*. This protein also mediates cAMP repression of the *ecmB* gene and most likely acts downstream of a cAMP receptor (Harwood *et al.*, 1995; Ginsburg and Kimmel, 1997). In *Drosophila*, GSK-3 mediates effects of the secreted morphogen wingless, which has crucial functions during segmentation and wing development (Siegfried *et al.*, 1994). In vertebrates, the homologous wnt factors have equally important roles in many inductive events during embryogenesis, which are also mediated by GSK-3 (Perrimon, 1996). Wingless and wnt are detected by the frizzled (fz) family of serpentine receptors (Bhanot *et al.*, 1996). Fz receptors do not have the consensus sequence for interaction with heterotrimeric G proteins (Wang *et al.*, 1996), although recent data suggest a possible role for G proteins in wnt- and fz-activated Ca^{2+} signaling (Slusarski *et al.*, 1997). However, these data did not address a role for G proteins or Ca^{2+} in control of GSK-3 or cell fate decisions. Other studies indicate that fz receptors induce hyperphosphorylation and translocation of the cytoplasmic protein disheveled, which is an intermediate for GSK-3 regulation (Yanagawa *et al.*, 1995).

This study is the first demonstration that serpentine receptors, which are known to activate multiple G protein mediate responses, may control GSK-3-dependent cell fate decisions in a manner that is independent of heterotrimeric G proteins. This possibly involves a pathway common to the wingless/wnt signal transduction cascades.

Materials and methods

Cell growth and development

AX3 (wild-type) and LW6 (gβ−) cells were grown in HL5 axenic medium, which was supplemented with 10 μg/ml G418 (Sigma) for transformed cell lines. For development on solid substratum, cells were washed in development buffer (DB) (2 mM $MgCl_2$, 0.2 mM $CaCl_2$ in 10 mM Na/K phosphate pH 6.5) and incubated on non-nutrient agar (1.5% agar in DB) at the indicated temperature. For development in suspension, cells were harvested, washed twice in DB, then resuspended in DB and shaken at 120–180 r.p.m.

Screen for temperature-sensitive Gβ mutants

The Gβ cDNA was randomly mutagenized by error-prone PCR, and subcloned into the extrachromosomal expression vector pMC34, where the Gβ genes are under the control of the actin15 promoter. The gβ− cell line LW6 was transformed with the library of mutagenized Gβ genes and selected for 7 days on G418. Approximately 10^4 transformants were collected and amplified in shaking culture; ~10^8 cells were harvested, washed, plated on DB agar plates and incubated at 22°C until fruiting bodies had formed. The spores were collected, resuspended in 10% glycerol and heated at 45°C for two, 30-min periods to kill contaminating cells. 2×10^4 spores were then plated clonally on *Klebsiella aerogenes* bacterial lawns at 27°C for 8 days. One hundred plaques with agg− phenotype at 27°C were picked and plated on replica bacterial plates incubated at 22°C and 27°C. Twenty plaques, which were agg+ at 22°C and agg− at 27°C were picked, and clonal isolates were grown in HL5 with G418.

Plasmid recovery, construction and sequence analysis

Total DNA was prepared from 10^8 cells as described previously (Parent and Devreotes, 1995) and transformed into XL1-blue bacteria (Stratagene). The recovered plasmids were sequenced and then transformed into LW6 (gβ−) cells. To separate the mutated alleles in the *gpbA1* and *gpbA2* genes, the 1.05 kb *Bgl*II–*Not*I fragments of *gpbA1*, *gpbA2* and wild-type Gβ genes in the extrachromosomal expression vector (pMC34) were subcloned into *Bgl*II–*Not*I site of a modified Bluescript K/S plasmid in which a *Bgl*II linker was inserted into the *Eco*RV site. Using the unique *Eco*RI site located in the region encoding E186 and F187 in the Gβ gene, the *Bgl*II–*Eco*RI and *Eco*RI–*Not*I fragments of *gpbA1* and *gpbA2* genes were swapped with the corresponding fragments of wild-type Gβ gene. The *Bgl*II–*Not*I fragments of the resulting plasmids were then subcloned into the *Bgl*II–*Not*I site of the *Dictyostelium* extrachromosomal expression vector pMC34 and the resulting vectors that harbor Gβ mutations *gpbA1*a, *gpbA1*b, *gpbA2*a and *gpbA2*b were transformed into gβ− cells.

Immunoblotting

Samples of 10^6 cells were solubilized in sample buffer, subjected to electrophoresis on 10% low-bis or regular polyacrylamide gels, and blotted onto polyvinylidene difluoride membranes (Millipore). The membranes were probed with antibodies specific to Gβ, cAR1 or csA/gp80 as described previously (Klein *et al.*, 1988; Lilly *et al.*, 1993). Bands were visualized using an enhanced chemiluminescence kit (Amersham, UK).

Adenylyl cyclase assay

Cells were first stimulated in suspension with 50 nM cAMP pulses at 6-min intervals for 5 h at 22°C, and then shaken at 22°C or 27°C for 1 or 2 h. Cells were lysed in 2× lysis buffer (2 mM $MgSO_4$ in 20 mM Tris pH 8.0) in the presence and absence of 40 μM GTPγS and rapidly mixed with 1/10 volume of 10× reaction mix (100 mM Tris pH 8.0, 1 mM ATP, 100 mM DTT and [α-^{32}P]ATP). Reactions were terminated after 2 min of incubation at 22°C or 27°C, and assayed for ^{32}P-labeled cAMP as described previously (Pupillo *et al.*, 1992; Insall *et al.*, 1996).

Gene induction procedures

For induction of aggregative gene expression, cells were harvested at the late log phase of development, resuspended at 10^7 cells/ml in DB and stimulated with cAMP pulses at 6-min intervals. For induction of prespore and *ecmA* gene expression, cells were incubated on solid substratum until loose aggregates had formed (usually 16 h at 6°C and 1–2 h at 22°C), then resuspended at 5×10^6 cells/ml in DB and incubated for an additional 8 h with various stimuli as indicated in the figure legends. To test maintenance of prespore transcription and mRNA stability, cells were developed at 22°C on solid substratum until tight mounds had formed. Cells were dissociated, resuspended in DB at 5×10^6 cells/ml and shaken at 180 r.p.m. at either 22°C or 27°C. For induction of stalk gene expression, cells were resuspended in stalk salts (10 mM KCl, 2 mM NaCl, 1 mM $CaCl_2$ in 10 mM MES pH 6.2) to 5×10^6 cells/ml and incubated at 22°C in 10 ml Petri dishes. After 8 h, cAMP was added to a final concentration of 5 mM and incubation was continued for a further 16 h at 22°C (Berks and Kay, 1988). Cells had

then formed tight aggregates, which were dissociated by forcing them through a 21-gauge needle. Subsequently, cells were incubated in stalk salts at 5×10^6 cells/ml for 8 h with DIF (Affinity Research Products, UK) and Sp-cAMPS (Biolog, Germany) at 22°C or 27°C.

RNA isolation and analysis

Total cellular RNA was isolated from 2.5×10^7 cells as described by Nellen *et al.* (1987), size-fractionated on 1.5% agarose gels containing 2.2 M formaldehyde and transferred to Gene Screen membranes. Northern transfers were hybridized to [α-^{32}P]dATP-labeled DNA probes according to standard procedures, and exposed to X-ray films.

Acknowledgements

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