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A G-protein β-subunit is essential for *Dictyostelium* development

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Recent studies have demonstrated that G-protein-linked signal transduction pathways play a significant role in the developmental program of the simple eukaryotic organism *Dictyostelium*. We have reported previously the isolation of a G-protein β-subunit and present here a more complete analysis of this gene. Low-stringency Southern blots and RFLP mapping studies suggest that the β-subunit is a unique gene found on linkage group II. Its deduced amino acid sequence of 347 residues is ~60% identical to those of the human, *Drosophila*, and *Caenorhabditis elegans* β-subunits. The carboxy-terminal 300 residues are about 70% identical; the amino-terminal 50 residues are quite divergent, containing only 10 identities. At all stages of growth and development, a single 1.9-kb β-subunit mRNA is present at a high level, and a specific antibody detects a single 37-kD protein. We propose that G-protein heterotrimers are formed when this β-subunit couples with each of the eight distinct G-protein α-subunits that are transiently expressed during development. Targeted disruption of the β-subunit gene had no effect on the viability of haploid cells, but resulted in the inability of cells to aggregate.

[*Key Words:* Signal transduction; chemotaxis; gene targeting; heterotrimeric G-proteins]

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Signal transduction via seven transmembrane receptors is an extremely widespread phenomenon in higher mammals, mediating the action of numerous hormones and neurotransmitters (Dohlman et al. 1987). When excited, these receptors activate heterotrimeric G-proteins, catalyzing the exchange of GTP for GDP on the α-subunit and dissociation of the α- from the βγ-subunits (Gilman 1987). In mammals, there are almost 20 G-protein α-subunits, divided into four classes represented by α_s, α_i, α_q, and α_{12} (Simon et al. 1991). There are four β- and six γ-subunits, which presumably can combine with the α-subunits to form a huge variety of heterotrimers (Birnbaumer 1992). Effectors of G-proteins include adenylyl cyclase, phosphodiesterase, phospholipases, and ion channels (Hepler and Gilman 1992).

The G-protein-linked signal transduction strategy appeared early in the evolution of eukaryotic cells. Pheromone receptors of the seven transmembrane type, coupled to heterotrimeric G-proteins, mediate the mating response in *Saccharomyces cerevisiae* (Herskowitz and Marsh 1987). A variety of G-protein α-subunits, some of which fall into the mammalian classes, have also been found in *Caenorhabditis elegans* and *Drosophila* (Provost et al. 1988; Yoon et al. 1989; Quan and Forte 1990; Silva and Plasterk 1990); and G-protein-coupled signal transduction pathways, very similar to those in higher mammals, play a key role in the developmental program of *Dictyostelium* (Van Haastert et al. 1991).

The life-cycle of *Dictyostelium* consists of distinct growth and developmental phases (Devreotes 1982). Starvation initiates the developmental phase in which ~10^5 individual amoebae aggregate to form a multicellular structure. This process is organized by extracellular adenosine 3′,5′-monophosphate (cAMP) that is secreted by cells at aggregation centers. Surrounding cells respond by moving chemotactically toward the signaling cells and by relaying the signal to cells farther from the center. The signaling system continues to play a role as the resulting multicellular aggregate undergoes further morphogenesis forming a mound, then a slug. Cells in the anterior or posterior of these structures differentiate into prestalk or prespore cells, which eventually form the stalk and spore mass of a fruiting body. Many of these cell–cell signaling processes occur via G-protein-linked signal transduction pathways (Snaar-Jagalska and Van Haastert 1988).

The key components of these pathways are highly analogous to their mammalian counterparts. There are four surface cAMP receptors (cARs), which comprise a family of highly related seven transmembrane domain proteins (Klein et al. 1988; Saxe et al. 1991; Johnson et al. 1993), and eight G-protein α-subunits, which share 35–50% identity with the mammalian α-subunits (Pupillo et al. 1989; Hadwiger et al. 1991; Wu and Devreotes 1991). The known effectors include an adenylyl cyclase

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that is homologous to the mammalian enzymes (Pitt et al. 1992) and a ligand-stimulated phospholipase C (PLC) that is homologous to the mammalian PLC-δ (Drayer and Van Haastert 1992). Most of these components are expressed transiently at different times in development.

Targeted gene disruptions by homologous recombination have shown that a surface cAMP receptor (cAR1), a G-protein α-subunit (Gα2), and an adenylyl cyclase (ACA) play essential roles in early development (Kumagai et al 1991; Sun and Devreotes 1991; Pitt et al. 1992). The loss of each of these genes results in an aggregation-deficient phenotype. *Gα2*$^-$ cells, designated *Frigid A*, cannot sense extracellular signals and fail to carry out chemotaxis or differentiate under any conditions (Kesbeke et al. 1988; Kumagai et al. 1991). In this respect, the *Dictyostelium* system resembles many mammalian systems where the activated α-subunit has been shown to interact with a variety of effectors to mediate the physiological action of the hormone or neurotransmitter (Stryer and Bourne 1986). Recently, the βγ-subunits have also come into the limelight as interacting with specific surface receptors and directly activating certain effectors (Birnbaumer 1992). For example, ion channels and certain adenylyl cyclase subtypes are activated by βγ (Logothetis et al. 1987; Tang and Gilman 1991). These observations suggest avenues for crosstalk between signal transduction pathways and the formation of interacting networks (Ross 1992).

Although disruption of Gα2 results in a strong phenotype in early development, a few receptor and/or G-protein-mediated responses persist in these mutants. Disruption of most of the other known α-subunits also fails to cause a defect in these responses (Milne and Devreotes 1993; Wu et al. 1993). These data, together with the observations that βγ-subunits may play an active role in some systems, prompted us to turn our attention to the β-subunit. We have reported previously the isolation of a β-subunit cDNA from *Dictyostelium* (Pupillo et al. 1988), and here we provide a complete analysis of this gene and its protein product. Furthermore, we find that disruption of the β-subunit is not lethal but results in a failure to aggregate, a phenotype similar to that of the *gα2*$^-$ cells.

Results

Isolation and sequence analysis of the G-protein β-subunit gene

To isolate a G-protein β-subunit cDNA from *Dictyostelium*, we designed redundant oligonucleotides that were based on conserved regions among the human β1- and β2-subunits and the *Drosophila* β-subunit. The positions of the oligonucleotides, roughly equidistant along the length of the coding sequence, are illustrated in Figure 1. We initially screened with oligonucleotide 0-1 and then rescreened with oligonucleotides 0-2, 0-3 and 0-4 to eliminate false positives and isolate full-length clones. Approximately 1.2×10^4 phage from a λgt11 cDNA library, prepared from mRNA isolated at 2–4hr of development, were screened. Approximately 48 positive clones were detected, and 14 were selected for further study. Restriction mapping of multiple independent phage revealed an apparently common internal *Eco*RI site. The 5′ and 3′ *Eco*RI fragments of the inserts, as well as a full-length insert from a partial digest, were subcloned into a Bluescript vector and sequenced. Three independent 5′ fragments and two independent 3′ fragments each overlapped. The nucleotide sequences were identical in the overlapping regions and with that surrounding the *Eco*RI site in the full-length insert.

The single deduced amino acid sequence, illustrated in Figure 1, encodes a polypeptide of 347 amino acid residues with a calculated molecular mass of 38,579. Figure 2, in which we have arranged the sequences according to the internal repeat structure described previously (Fong et al. 1986), compares the *Dictyostelium* β to human β-2 (Fong et al. 1987; Gao et al. 1987), *C. elegans* β (van der Voorn et al. 1990), and *Drosophila* β (Yarfitz et al. 1988). Overall, the sequences are ~60% identical. The carboxy-terminal 300 residues of the *Dictyostelium* β-subunit are ~70% identical, or ~90% homologous allowing for conservative replacements. An abrupt switch in the extent of homology appears within the amino-terminal 50 amino acid residues, which contain only 10 identities and seven additional residues. A similar tendency for divergence near the amino-terminal region has been noted among the previously sequenced mammalian and lower eukaryotic β-subunits; the *Dictyostelium* sequence provides the most striking example. The sharp transition may represent the separation of two functionally distinct domains in the molecule.

Amplification of genomic DNA with primers corresponding to the ends of the β-subunit coding region, which contains 1041 bp, produced a band of ~1.8 kb, indicating the presence of ~800 bp of intron(s) within the coding region. The *Eco*RI fragments of the genomic PCR product were subcloned and sequenced to localize the introns. Three introns were identified; the positions and partial sequences are illustrated in Figure 1. The most 5′ is ~160 bp; the middle intron is ~500 bp, and the 3′-most is ~100 bp.

Figure 3 shows a restriction map of the β-subunit locus and illustrates one of the Southern blot analyses from which the restriction map was derived. A single intense band was detected with a variety of enzymes at very low-stringency hybridization conditions. Under slightly more stringent conditions (i.e., 37°C wash), we readily detected cross-hybridization among the four cAMP receptor subtypes, which are ~60% identical. This suggests the existence of a unique β-subunit gene and no other closely related β-subunit subtypes. The 4.5-kb 5′ *Eco*RI fragment overlapping with the β-subunit coding sequence was found to be polymorphic in *Dictyostelium* strains (Table 1). This observation allowed a restriction fragment length polymorphism (RFLP) analysis and assignment of the β-subunit to linkage group II. The details of the analysis are provided in Table 1.

Time course of expression of the β-subunit gene

Figure 4 illustrates the time course of expression of the

Figure 1. Nucleotide and deduced amino acid sequence of the *Dictyostelium* G-protein β-subunit. The nucleotides are numbered relative to the ATG. Coding sequence is represented by uppercase letters, whereas lowercase letters show the splice junctions of the introns. Oligonucleotides used in screening are represented by stippled bars above the nucleotide sequence.

β-subunit gene. On RNA blots, a single 1.9-kb band is detected at a constant level at all stages of growth and development, indicating that expression of the β-subunit gene is not significantly regulated during the life cycle of the organism. The high frequency of independent β-subunit positive phage in the λgt11 library (1 : 250) suggests that the β-subunit mRNA is abundant. A peptide corresponding to the deduced amino-terminal 15 amino acid residues of the β-subunit protein was synthesized, coupled to keyhole limpet hemocyanin (KLH), and injected into a rabbit. After two subsequent boosts, a strong response was seen on immunoblots. As shown in Figure 4, a band at ~37 kD, roughly the molecular mass calculated from the cDNA sequence, was detected in membranes. Preimmune serum failed to stain these bands, and preincubation of the antiserum with the KLH-conjugated or free peptide reduced (by 90%) the staining intensity (data not shown). An approximately constant amount of the 37-kD band was present at all stages of growth and development, again indicating that the level of β-subunit is not developmentally regulated.

Attempt to alter β-subunit expression levels by antisense mutagenesis and overexpression

A 5′ *Eco*RI fragment of the β-subunit cDNA, containing 6 bp 5′ of the ATG, was ligated in both the sense and antisense directions into an actin-15-based expression vector (B18) that drives expression constitutively. As shown in Figure 5A a very large amount of β-subunit mRNA was produced from both the sense and antisense constructs. The level of endogenous mRNA and the total amount of protein, however, were not altered (Fig. 5A,B). To test whether additional sequences (either the entire coding sequence or 5′- and 3′-untranslated regions) are necessary for successful antisense mutagenesis, the full-

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1    MSSDISEKIQQARRDAESMKEQIRANRDVMNDTTLKTFTRDLPGLPKMEGKIKVRRN
2         MSELEQLRQEAEQLRNQIRDARKACGDSTLTQITAGLDPVGRIQ--MRTRRT
3         MSELDQLRQEAEQLKSQIREARKSANDTTLATVASNLEPIGRIQ--MRTRRT
4         MNELDSLRQEAESLRNAIRDARKACGDSSLLQATTSLDPVGRIQ--MRTRRT
             o  o● o●●      ●●  ●    ● ●      ●            ●●

1         LKGHLAKIYAMH---WAEDNVHLVSASQDGKLLVWDGLTTNKVHA
2         LRGHLAKIYAMH---WGTDSRLLVSASQDGKLIIWDSYTTNKVHA
3         LRGHLAKIYAMH---WASDSRNLVSASQDGKLIVWDSYTTNKVHA
4         LRGHLAKIYAMH---WGNDSRLLVSASQDGKLIIWDSYTTNKVHA
          ●o●●●●●●●●●●●   ●o ●   ●●●●●●●●●●oo●● o●●●●●●●

1         IPLRSSWVMTCA---YSPTANFVACGGLDNICSIYNLRSREQPIRVCRE
2         IPLRSSWVMTCA---YAPSGNFVACGGLDNICSIYSLKTREGNVRVSRE
3         IPLRSSWVMTCA---YAPSGSFVACGGLDNICSIYSLKTREGNVRVSRE
4         IPLRSSWVMTCA---YAPSGNYVACGGLDNMCSIYSLKTREGNVRVSRE
          ●●●●●●●●●●●   ● ●o  o●●●●●●●●o●●●● ●ooo●  o●● ●●

1         LNSHTGYLSCCR----FLNDRQIVTSSGDMTCILWDVENGTKITE
2         LPGHTGYLSCCR----FLDDNQIITSSGDTTCALWDIETGQQTVG
3         LPGHTGYLSCCR----FLDDNQIITSSGDMTCALWDIETGQQCTA
4         LPGHGGYLSCCR----FLDDNQIITSSGDTSCGLWDIETGQQVVS
          ●  ● ●●●●●●●    ●● ● ●●o●●●●●  ● ●●● ● ●

1         FSDHNGDVMSVS---VSPDKNYFISGACDATAKLWDLRSGKCVQT
2         FAGHSGDVMSLS---LAPDGRTFVSGACDASIKLWDVRDSMCRQT
3         FTGHTGDVMSLS---LSPDFRTFISGACDASAKLWDIRDGMCKQT
4         FLGHSGDVMALS---LAPDGRTFVSGACDASIKLWDVRESVCRQT
          ●  ● ●●●●        ●●   ● ●●●●●●  ●●●● ●   ● ●●

1         FTGHEADINAVQ---YFPNGLSFGTGSDDASCRLFDIRADRELM
2         FIGHESDINAVA---FFPNGYAFTTGSDDATCRLFDLRADQELL
3         FPGHESDINAVA---FFPSGNAFATGSDDATCRLFDIRADQELA
4         FIGHESDINAVT---FFPNGQAFTTGSDDATCRLFDLRADQELL
          ● ●●● ●●●●●    ●●● ●   ● ●●●●●●●●●●●●●●●● ●●●

1         QYTHDNILCGITSVGFSFSGRFLFAGYDDFTCNVWDTLKGERVLS
2         MYSHDNIICGITSVAFSRSGRLLLAGYDDFNCNIWDAMKGDRAGV
3         MYSHDNIICGITSVAFSKSGRLLFAGYDDFNCNVWDSMRQERAGS
4         MYSHDNIICGITSVAFSRSGRLFFAGYDDFNCNIWDTMKADRSGI
           ● ●●●●●●●●●●●● ●● ●●●●●●●●●●●● ●● ●● ●●   ●

1         LTGHGNRVSCLGV---PTDGMALCTGSWDSLLKIWA
2         LAGHDNRVSCLGV---TDDGMAVATGSWDSFLKIWN
3         LAGHDNRVSCLGV---TEDGMAVCTGSWDSFLKIWN
4         LAGHDNRVSCLGV---TDNGMAVATGSWDSFLRVWN
          ● ●● ●●●●●●●●       ●●●● ●●●●●●●●●●●
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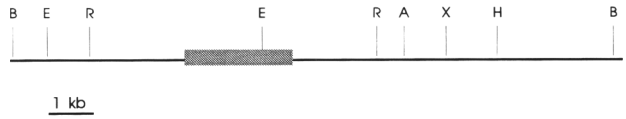
Figure 2. Homology of the *Dictyostelium* β-subunit to that of other species. The *Dictyostelium* (1), human β-2 (2), *C. elegans* (3), and *Drosophila* (4) β-subunits are aligned according to the repeated structure described by Fong (Fong et al. 1986). (●)Positions at which all four sequences are identical; (○) conservative replacements.

length cDNA fragment, obtained from a partial *Eco*RI digest, was inserted in the antisense direction into the expression vector. Again, an immunoblot of membranes revealed no alteration in expression level of the β-subunit protein (Fig. 5B). These results suggest that the β-subunit mRNA is sequestered from the antisense RNA. Consistent with the failure to alter expression levels, no discernible phenotype was displayed by the transformants. Surprisingly, similar overproduction of the full-length sense mRNA (not shown) also did not substantially increase the level of β-subunit protein (Fig. 5B). These observations suggest that the β-subunit expression level is tightly regulated at a post-transcriptional level.

Targeted disruption of the β-subunit gene

To study the function of the G-protein β-subunit, we decided to create a β-subunit null cell line by gene targeting. A thymidine auxotrophic mutant, JH10, which can be complemented by transformation of the *thy1* gene (Dynes and Firtel 1989), was used as a recipient cell line. A linear Gβ cDNA fragment, interrupted at the internal *Eco*RI site by the *thy1* gene, was transformed into JH10 cells, and clonal transformants were selected in minimal medium. The genomic DNA from various transformants was subjected to Southern blot analyses. As shown in Figure 6A, hybridization of a fragment located 5′ to the β-subunit coding sequence to wild-type genomic DNA digested by *Eco*RI and *Hin*dIII yielded a 4-kb band. In contrast, similar analyses of genomic DNA isolated from three of the transformants (one is shown) gave rise to a 7-kb band, which is the size predicted to result from a double crossover event. Twelve of the transformants examined retained an unaltered β-subunit gene structure and were probably random integrants. One of these cell lines and one of the $g\beta^-$ cell lines were selected for subsequent experiments.

To verify that $g\beta^-$ cells do not produce β-subunit protein, an immunoblot was performed on membrane proteins from $g\beta^-$ and control cells (Fig. 6B). Consistent with Figure 4, in the control cells the β-subunit was detected as a relatively abundant protein in growing and developing cells. In contrast, the β-subunit antibody rec-



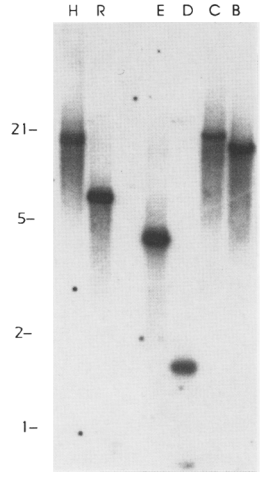


Figure 3. Restriction map and Southern analysis of the β-subunit locus. Genomic DNA isolated from strain AX-3 was digested with various restriction enzymes, both individually and in pairs, and probed with either the 5′ or 3′ *Eco*RI fragment of the cDNA to determine the restriction map. An example of one of the blots, probed with the 5′ *Eco*RI fragment at low stringency as described in Materials and methods, is shown below. (B) *Bgl*II; (E) *Eco*RI; (R) *Eco*RV; (A) *Acc*I; (X) *Xba*I; (H) *Hin*dIII.

Table 1. *Linkage group analysis*

Strain	*Eco*RI restriction fragment (kb)	Linkage group I	II	III	IV	VI	VII
HU1628	3.5	*cycA1*	*acrA1823*	*bsgA5*	*whiC351*	*manA2*	*couA351*
AX3K	4.5	*cycA*$^+$	*acrA*$^+$	*bsgA*$^+$	*whiC*$^+$	*manA*$^+$	*couA*$^+$
HUD238	4.5	+	+	*bsgA5*	+	*manA2*	*couA351*
HUD239	4.5	*cycA1*	+	*bsgA5*	+	*manA2*	+
HUD240	4.5	+	+	+	*whiC351*	*manA2*	+
HUD244	4.5	*cycA1*	+	*bsgA5*	*whiC351*	+	+
HUD246	3.5	+	*acrA1823*	+	+	+	*couA351*
HUD247	3.5	+	*acrA1823*	+	*whiC351*	+	+
HUD248	3.5	*cycA1*	*acrA1823*	*bsgA5*	*whiC351*	*manA2*	*couA351*
HUD249	3.5	+	*acrA1823*	*bsgA5*	*whiC351*	*manA2*	+
HUD257	3.5	*cycA1*	*acrA1823*	*bsgA5*	+	+	*couA351*
HUD259	4.5	*cycA1*	+	+	*whiC351*	+	*couA351*
HUD260	3.5	+	*acrA1823*	*bsgA5*	*whiC351*	+	*couA351*
HUD261	4.5	*cycA1*	+	+	+	*manA2*	*couA351*

Analysis of RFLPs in segregants of marked parental strains allows assignment to a linkage group within the genome.

ognized no proteins isolated from *gβ*$^-$ cells under the same conditions. Similar results were obtained when total SDS-solubilized proteins were analyzed (data not shown). Although the antibody is directed against the amino-terminal 15 amino acids of the protein and would be expected to detect truncated fragments of the protein, if present, no smaller fragments of the β-subunit protein were observed.

The developmental morphology of the *gβ*$^-$ cells was examined by plating them on non-nutrient agar plates. Although the control cells began to aggregate within a few hours, the *gβ*$^-$ cells failed to enter the developmental program and remained as monolayer cells for several days (Fig. 6C). The cell lines were also examined on nutrient agar plates carrying a lawn of *Klebsiella aerogenes*. The control cells cleared the lawn, then promptly aggregated and formed fruiting bodies; the *gβ*$^-$ cells cleared the lawn and remained as a monolayer for several weeks. Several independent control and *gβ*$^-$ clones were examined in this assay, and they all showed consistent phenotypes, indicating that the defects are caused by the absence of the β-subunit protein.

Discussion

The *Dictyostelium* G-protein β-subunit shows a remarkable homology to the β-subunits found in humans, *Drosophila*, and *C. elegans* (Fong et al. 1987; Gao et al. 1987; Yarfitz et al. 1988; van der Voorn et al. 1990). Two common features of these homologous sequences have been noted previously, but the addition of the *Dictyostelium* sequence to the comparison markedly underscores these points. First, the extent of sequence conservation is localized (van der Voorn et al. 1990). The highly divergent amino terminus shows only 20% identity among the β-subunits, but the identity increases to 70% when the amino-terminal 50 amino acid residues are not considered. This sharp transition in extent of identity may represent a separation of two functionally distinct domains. We propose that the amino-terminal region confers specificity and predict that heterologous chime-

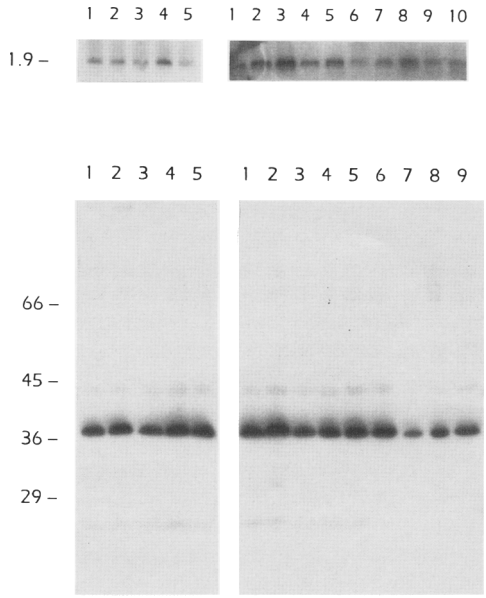


Figure 4. Northern and immunoblot analysis of the β-subunit. (*Top*) The *left* panel shows RNA prepared from AX-3 cells developed at 2 × 10^7/ml in shaking culture. Lanes *1–5* show samples (5 μg/lane) prepared after 0, 1.5, 3, 4.5, and 6 hr. The *right* panel shows cells developed on agar surfaces. Lanes *1–10* show samples prepared after 0, 3, 6, 9, 12, 15, 18, 21, 24, and 36 hr of development. A single 1.9-kb mRNA is detected at all stages. (*Bottom*) Membranes were prepared from cells developing in shaking culture (*left* panel) or on agar surfaces (*right* panel). Lanes *1–5* at *left* correspond to 0, 2, 4, 6, and 8 hr. Lanes *1–9* at *right* correspond to 0, 0.5, 2, 4, 6, 8, 13, 18, and 24 hr. The β-subunit appears as a constitutively expressed protein of 37 kD.

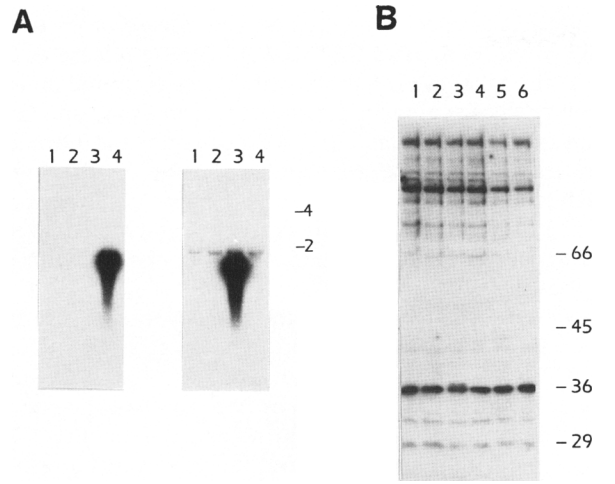


Figure 5. Attempts to alter the expression levels of the β-subunit. (*A*) RNA prepared from wild-type cells (lane *1*), from vector control-transformed cells (lane *2*), from cells transformed with the 5′ *Eco*RI fragment in the sense orientation (lane *3*), or from cells transformed with the 5′ *Eco*RI fragment in the antisense orientation (lane *4*) was probed with an RNA corresponding to the coding strand of the message (*left*) or to the noncoding strand (*right*). The positions of the RNA bands (2 and 4kb) are indicated for reference. (*B*) Membranes were prepared from cells transformed with the expression vector pB18 containing the following inserts: (Lanes *1* and *4*) No insert; (lane *2*) the 5′ *Eco*RI fragment in the sense orientation; (lane *3*) the 5′ fragment in the antisense orientation; (lane *5*) a full-length cDNA in the sense orientation;(lane *6*) the full-length cDNA in the antisense orientation.

ras between other β-subunits and that of *Dictyostelium* will function most efficiently in the system from which the amino-terminal region is derived. Second, the β-subunits appear to be comprised of internally repeated homologous segments (Fong et al. 1986). The presence of this entire repeated structural motif in the *Dictyostelium* sequence suggests that it arose early in evolution. Although the coding sequence of the *Dictyostelium* β-subunit is derived from four exons, the introns do not subdivide the sequence on the basis of the repeat structure or the abrupt transition in extent of conservation.

The *Dictyostelium* β-subunit gene appears to be a unique gene expressed at a high level in growth and development. Low-stringency Southern blot analyses indicate that no additional β-subunit subtypes, within ~50% identity, are present. A single band is detected on RNA blots of samples taken at all stages, supporting the speculation that there is a single β-subunit gene. Constitutive expression of the β-subunit is also evident in immunoblots. The β-subunit appears to be expressed at a relatively high level as evidenced by the large number of positive clones found in the cDNA library and strong signals on both RNA blots and immunoblots. We propose that this β-subunit participates in the formation of heterotrimers with each of the eight G-protein α-subunits that have been described previously (Pupillo et al. 1989; Hadwiger et al. 1991; Wu and Devreotes 1991).

It will be interesting to investigate the regulation of β-subunit expression. The untranscribed portions of the β-subunit mRNA consists of 800–900 bp, slightly larger

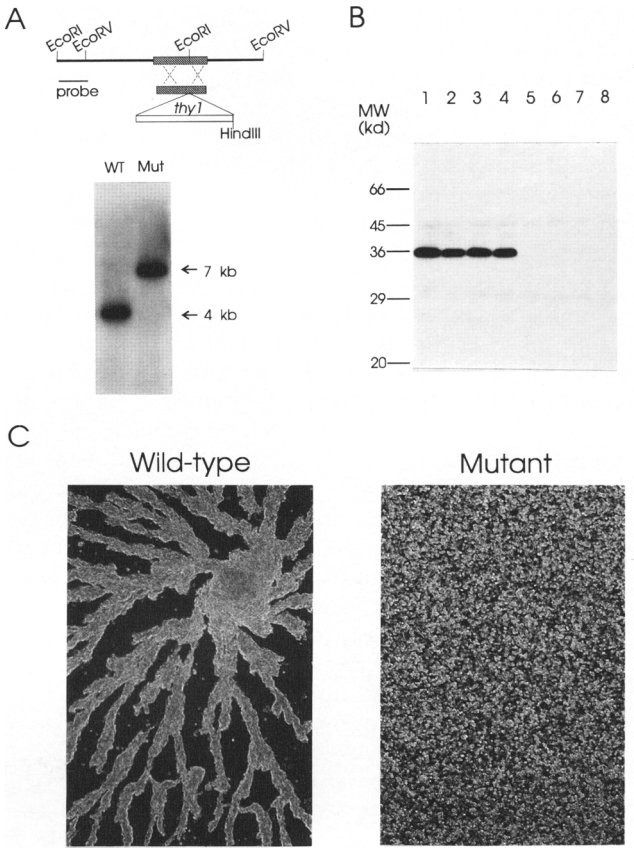


Figure 6. Disruption of the Gβ locus in *Dictyostelium* results in the inability to enter the developmental program. (*A*) Southern blot analysis of genomic DNA isolated from wild-type parental cells (JH10, a thymidine auxotrophic mutant) and cells that were transformed with a linear fragment from plasmid pPL6. (*Top*) Restriction map of the relative β-locus and the fragment of pPL6. Note that the internal *Eco*RI site from the Gβ locus was lost as a result of the insertion of the *thy1* marker. The shaded box represents the coding region of Gβ; the open box depicts the *thy1* gene, which can complement the JH10 thymidine auxotroph. (*Bottom*) A Southern blot analyses probed by the indicated *Eco*RI–*Eco*RV fragment labeled by random priming. DNA was digested by *Eco*RI and *Hin*dIII. The arrows show the size of the band detected. (*B*) Western blot of proteins prepared from wild-type and $g\beta^-$ cells in vegetative or different developmental stages. Cells were either grown in HL-5 medium or developed in DB with pulses of 50 nM cAMP at 6-min intervals for various times. Membrane proteins were extracted by ammonium sulfate, run on a 10% SDS-PAGE, and subjected to immunoblot by a Gβ peptide antibody. (MW) Molecular mass markers are shown at *left*. (Lanes *1–4*) Proteins prepared from wild-type cells; (lanes *5–8*) proteins prepared from $g\beta^-$ cells. The length of the development is: 0 hr (lanes *1* and *5*); 4 hr (lanes *2* and *6*); 6 hr (lanes *3* and *7*); 8 hr (lanes *4* and *8*). (*C*) Developmental phenotype of $g\beta^-$ cells. Wild-type and $g\beta^-$ cells were plated on starvation plates, and photographs were taken at 7 hr.

than most previously characterized *Dictyostelium* genes. Whether these regions provide regulatory control, or serve some other function, is not yet known. Although it was possible to significantly overexpress a variety of both sense and antisense transcripts of the β-subunit, little alteration of the protein levels resulted. These observations suggest the existence of efficient post-transcriptional controls on β-subunit protein expression. In other systems, it has been found that β and γ cannot be overexpressed independently (Simonds et al. 1991). We have not yet identified a *Dictyostelium* γ-subunit. The overall similarity of the G-protein-linked signal transduction system to that in mammals, however, suggests there are likely to be one or several γ-subunits.

Because this β-subunit appears to be the only β-subunit in *Dictyostelium* and it is present during growth, we were concerned that its disruption might be lethal or cause major growth defects. The fact that several independent viable $g\beta^-$ cell lines were obtained indicates that this protein is not essential for growth. Preliminary examination shows that these cell lines divide and grow normally, suggesting that heterotrimeric G-proteins are not required for cell cycle and cell growth-related processes. We have noted that there is a tendency for the $g\beta^-$ cells to generate smaller plaques than wild-type cells on bacterial lawns, a phenotype noted previously for $g\alpha4^-$ cells (Hadwiger and Firtel 1992). We are currently exploring the basis for the smaller plaque size.

The β-subunit does play an essential role in development. The $g\beta^-$ cells fail to aggregate under a variety of conditions. In this sense, the $g\beta^-$ cells are similar to those lacking Gα2. $G\alpha2^-$ cells correspond to *Frigid A*, one of five complementation groups designated *Frigid* that fail to respond to cAMP (Coukell et al. 1983). Because Gα2 should require the β-subunit to couple to cAR1, the $g\beta^-$ cells should be at least as defective as the *Frigid A* cells. Therefore, we anticipate that the $g\beta^-$ cell lines will belong to the *Frigid* class of mutants. Two of these groups, A and D, map to linkage group II and thus it is possible that *Frigid D* is the β-subunit.

Although cells lacking Gα2 fail to differentiate, several receptor- and/or G-protein-mediated functions have been shown to be retained in the $g\alpha2^-$ cell lines. For instance, although these cells do not sense cAMP, they are able to carry out chemotaxis to other attractants such as folic acid, yeast, and bacteria extracts (Kesbeke et al. 1988). cAMP receptor-mediated Ca^{2+} influxes and ligand-induced receptor phosphorylation also occur normally in the $g\alpha2^-$ cell lines (Milne and Coukell 1991; Pupillo et al. 1992; Milne and Devreotes 1993). None of these responses are affected by disruption of seven of the eight α-subunits (Gα6 has not been disrupted) (Kumagai et al. 1991; Hadwiger and Firtel 1992; L. Wu and P.N. Devreotes; M. Pupillo and P.N. Devreotes, both in prep.; R. Firtel pers. comm.). Furthermore, whereas cAMP-mediated increases in cAMP in vivo require Gα2, in vitro GTPγS activation of adenylyl cyclase is uninhibited in all of the $g\alpha^-$ cell lines (Pupillo et al. 1992 and unpubl.). This latter result could be explained if βγ stimulated the enzyme (Okaichi et al. 1992; Pupillo et al. 1992). In vivo, excitation of cAR1 would cause the release of βγ from Gα2, whereas in vitro GTPγS would cause release of βγ from any other G-protein. βγ stimulates mammalian adenylyl cyclase subtypes II and IV in vitro (Tang and Gilman 1991). Expression of the type II enzyme in COS cells renders it sensitive to stimulation by α-adrenergic ligands presumably via release of βγ from G_i (Federman et al. 1992). We are currently determining which of these apparently α-subunit-independent functions are retained in the $g\beta^-$ cells.

Excited seven transmembrane domain receptors interact with heterotrimeric G-proteins to facilitate GTP/GDP exchange and the dissociation of α- and βγ-subunits. The cycle is completed by hydrolysis of the bound GTP and reassociation of the α- and βγ-subunits. Because the β-subunit is essential for this cycle to occur, there can be no transmembrane signaling in its absence. If the β-subunit that we have identified and eliminated is unique, the $g\beta^-$ cells should be completely unable to receive environmental signals via G-protein coupled receptors and should provide a means for testing these hypotheses in an in vivo context.

Materials and methods

Oligonucleotide screening

Oligonucleotides, based on known conserved regions of G-protein β-subunits, were synthesized (0-1 and 0-4) or obtained (0-2 and 0-3) from M. Simon (California Institute of Technology, Pasadena). The position of the oligonucleotides along the length of the β-subunit coding sequence is indicated in Figure 1. The sequences of these oligonucleotides were

```
          T   T   T   T   T   T
01  TACGACGACTTCAACTGCAA

          A   A
02  CAGATGAT(A/G)TT(A/G)TC(A/G)TG
          T   T

              G       G
03  GC(A/G)CA(A/C)GTCAT(A/C)ACCCA
              T       T

              T   T   T
04  AA(A/G)ATCTACGCCATGCA(T/C)TGG
              A       A
```

0-1 was 5′-end labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear) and used to screen a *Dictyostelium discoideum* λgt11 cDNA library at 50°C by the method of Wood et al. (1985). Putative positive clones were then probed on grids with 0-2, 0-3, and 0-4 to ensure that cDNAs were full-length β-subunit clones.

Recombinant DNA techniques

Plasmids were constructed by use of standard cloning techniques (Maniatis et al. 1982). Most constructs were made with Bluescript vector (Stratagene); the sense and antisense constructs utilized pB18 (Johnson et al. 1991).

Sequence was obtained by the dideoxynucleotide chain termination reaction by use of a kit from USBC.

A primer corresponding to the sense strand of the 5′ end (19

nucleotides) was synthesized as was one corresponding to the noncoding strand of the 3′ end (23 nucleotides). These primers were used in the PCR (Saiki 1990) on genomic DNA prepared from strain AX-3.

Southern blot hybridization was performed as described by Maniatis (Maniatis et al. 1982). For low-stringency Southern blots, the filters were hybridized overnight at room temperature in 50% formamide, 1% SDS, 1 M NaCl, and 10% dextran sulfate at 22°C. All washes were performed at room temperature as follows: 2× for 30 min in 2% SSC; 2× for 30 min in 2% SSC + 1% SDS; 2× for 15 min in 0.1% SSC. DNA probes were labeled by the random priming method (Feinberg and Vogelstein 1983) to a specific activity of $\sim 10^9$cpm/μg.

RNA was prepared at intervals during development by the guanidine–HCl method. Five micrograms of RNA per lane was sized on a denaturing gel and transferred to nitrocellulose for hybridization according to standard RNA blot procedures (Maniatis et al. 1982). Probes were prepared as described above for Southern blot analysis. Autoradiographs were exposed for 2–24 hr.

Expression in Dictyostelium

A 5′ *Eco*RI β-subunit cDNA fragment containing 6 bp of untranslated sequence was ligated into p18 in both the sense and antisense directions. The constructs were transformed by $CaPO_4$ precipitation (Knecht et al. 1986) into *Dictyostelium* and selected by use of G418 at 20 μg/ml.

Antiserum

A peptide corresponding to the amino-terminal 15 amino acid residues was synthesized with a cysteine at the amino terminus to enable cross-linking. This was coupled to KLH and used to immunize a rabbit (Green et al. 1982). High-titer antibody was obtained following the second boost.

Immunoblots

Immunoblots were done according to standard procedures with ^{125}I-labeled protein A as a second antibody (Towbin et al. 1979).

Cell culture and development

D. discoideum strain AX-3 cells were grown in HL-5 medium as described (Watts and Ashworth 1970). Development was initiated by washing the cells in development buffer (DB) and either plating them at 5×10^7/10-cm petri dish or shaking them at 2×10^7/ml in an Erlenmeyer flask (Devreotes et al. 1987).

Creation of a gβ$^-$ cell line

Plasmid pPL6 was constructed by insertion of the *Xmn*I cDNA fragment into the *Eco*RV site of pBluescript, and a 3-kb *Hin*dIII *thy1* fragment was filled in and subcloned into the filled-in *Eco*RI site of the Gβ gene. pPL6 (15–50 μg) was digested by *Bam*HI and *Xho*I in the polylinker region to drop the insert, extracted by phenol/chloroform, precipitated by ethanol, and resuspended in 10 μl of TE (pH 8). DNA was transformed into JH10 cells (a thymidine auxotrophic mutant, a gift from R.A. Firtel, University of California, San Diego) by electroporation (Howard et al. 1988) and selected in minimal medium (FM) (Franke and Kessin 1977) in 96-well plates for clonal thymidine protrophic transformants. Transformants grown in FM medium were propagated further in HL-5 medium, and genomic DNA was isolated (Nellen et al. 1987) and subjected to Southern blot analyses.

RFLP mapping

These experiments followed previously developed techniques (Welker et al. 1986; Welker 1988). *Eco*RI RFLPs were identified by screening Southern blots of nuclear DNA preparations from well-marked tester strains and from sets of wild isolates and sets of mutant laboratory stocks. A diploid strain bearing different alleles was generated by fusion of haploid tester strain HU1628 and laboratory strain AX3K, using the dominant *cob*354 cobalt resistance trait and the recessive *cou*A351 coumarin and temperature sensitivity trait of HU1628 to select the diploid. The genotypes of parasexually derived haploids produced from the diploid were determined by use of the markers for each of the six established linkage groups present in the HU1628 tester strain. The markers were group I, *cyc*A1, cycloheximide resistance; group II, *acr*A1823, methanol resistance; group III, *bsg*A5, inability to use *Bacillus subtilis* as a food source; group IV, *whi*C351, white sori; group VI, *man*A2, α-mannosidase-1 deficient; and group VII, *cou*A351, coumarin and temperature sensitivity. The presence of the Gβ allele derived from HU1628 or AX3K was determined in a subset of these haploid segregants and correlated with the presence or absence of the genetic markers from HU1628.

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Note added in proof

The nucleotide and amino acid sequence data for the G-protein β-subunit have been submitted to the EMBL/GenBank data libraries.

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