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Multiple α subunits of guanine nucleotide-binding proteins in Dictyostelium

(signal transduction/receptor/chemotaxis)

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ABSTRACT Previous results have shown that chemotaxis and the expression of several classes of genes in Dictyostelium discoideum are regulated through a cell surface CAMP receptor interacting with guanine nucleotide-binding proteins (G proteins). We now describe cloning and sequencing of cDNAs encoding two Gα protein subunits from Dictyostelium. The derived amino acid sequences show that they are 45% identical to each other and to Gα protein subunits from mammals and yeast. Both cDNAs are complementary to multiple mRNAs that are differentially expressed during development. This evidence and analysis of mutants presented elsewhere suggest that they have distinct physiological functions.

When deprived of nutrients, Dictyostelium discoideum amoebae cease growth and initiate a developmental program. Within a few hours cells, guided by chemotaxis and intercellular signaling, spontaneously aggregate and form a multicellular organism. Cells in specific positions of the multicellular structure differentiate into the stalk and spore cells of a mature fruiting body. The signal molecule is extracellular CAMP that interacts with a cell surface receptor. Present evidence indicates that the CAMP receptor also regulates aggregation stage and cell-type-specific gene expression, cell patterning in the migrating pseudoplasmodium, and morphogenesis during culmination (1–4).

There appear to be two signal transduction pathways that may involve different kinetic classes of the cell surface receptor (5). The first is a "signaling" pathway in which CAMP, presumably binding to the rapidly dissociating class of receptors, results in the activation of adenylate cyclase with the subsequent synthesis and secretion of CAMP from the cell (6). Emitted CAMP can activate receptors on the same cell, creating a positive feedback loop, or on adjacent cells, relaying the signal. This response reversibly adapts and the cycle is repeated with a periodicity of 6–7 min (7, 8).

A second, or "chemotactic," pathway is proposed to be mediated by the slowly dissociating class of cell surface receptors. This pathway is believed to involve activation of phospholipase C with the production of inositol 1,4,5-trisphosphate and diacylglycerol (9). Additional responses triggered by extracellular CAMP, such as activation of guanylate cyclase and the transient production of cGMP, actin polymerization, and myosin phosphorylation, also appear to be associated with the chemotactic pathway (10).

Numerous biochemical studies indicate that both signal transduction pathways in Dictyostelium involve guanine nucleotide-binding proteins (G proteins). In addition, the primary structure of the CAMP receptor displays seven putative transmembrane domains, a structure identical to other G protein-linked receptors (11). We now report the complete cDNA sequences and deduced amino acid sequences of two distinct G protein α subunits. We also show that genes encoding these proteins are expressed at different times in development.

MATERIALS AND METHODS

Oligonucleotide Screening. Oligonucleotides were 5' end-labeled using T4 polynucleotide kinase (Promega or New England Biolabs) and [γ-32P]ATP (NEB or ICN). D. discoideum cDNA libraries were screened with two oligonucleotides at 50°C by the method of Wood et al. (12). The sequence of the first oligonucleotide was GGTGTTCAAGGGTAGTGAA. The sequence of the second oligonucleotide was CGTGTGAGATAGTGAAATGG.

Construction of Plasmids. cDNA inserts obtained from EcoRI digestion of phages were subcloned into the EcoRI site of pGEM2 or pSP73 (Promega) using standard techniques (13).

RNA Blots. For the development time course Gα1 and Gα2 expression, D. discoideum strain NC-4 cells were grown on SM (15 mM KH2PO4/5 mM K2HPO4/1% dextrose/1% yeast extract/1% agar) plates in association with Klebsiella aerogenes, washed, and plated for development. RNA was isolated from cells harvested at specific times in development, sized on denaturing gels, and analyzed by RNA blot hybridization as described (1).

RESULTS

Identification of Two Distinct α Subunits. cDNA clones for α subunits of G proteins were obtained by screening libraries prepared from 3.5-, 5-, and 11-hr stages of Dictyostelium development. Two oligonucleotide probes, corresponding to the highly conserved putative guanine nucleotide-binding (GGQSERKKW) and GTPase (GAGESGK) regions of the known mammalian α subunit sequences were designed. These probes included 80–90% of the possible codons by biasing for the highly (A+T)-rich codon usage of Dictyostelium (14). An initial collection of 25 cDNAs was obtained from screens of about 250,000 plaques. These separated into two classes defined by the relative intensity of cross-hybridization and the presence or absence of an internal EcoRI site (Fig. 1). Further restriction site analysis confirmed the existence of these two classes that were designated Gα1 (with an internal EcoRI site) and Gα2 (without an internal EcoRI site). The two Gα1 clones tested (RF1 and RF3) weakly cross-hybridized to the Gα2 class, whereas the Gα2 clone tested (FR1) did not cross-hybridize to the Gα1 class. Two distinct size classes were

Abbreviations: G protein, guanine nucleotide-binding protein; Gα, G protein of unknown function; Gβ, inhibitory G protein; Gγ, stimulatory G protein; Tr, transducin.

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The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M25060 for Gα1 and M25061 for Gα2).

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found repeatedly among the G1 clones. RF1, FR7, FR11, AD1, AD3, and AD4 are less than or equal to 1.2 kilobases (kb), whereas RF3, FR5, and FR5 are 2.4 kb.

The nucleotide sequence and the deduced amino acid sequence for G1 are shown in Fig. 2. Two clones, RF1 and AD4, were sequenced in both directions. The sequences

| RF1 | RF3-5’ RF3-5’ RF1 KB EcoRI |
|-----|-----------------|-----|-----|
| 1   | AAG TCT CAA AAA AAA AAA AAC ATC CAC AAC TAC TAC AAC AAC |
| 130 | ATG GGT ATT CTT GGT AAA CCA GAA TTA GTA GCA TCA CCA GAA GAT ATT AAA GCC CAA CAC ATT CAT ATT AAT GGT TGG TGG AAA GCA |
| 173 | MET Gly Asn Ile Gly Lys Pro Gly Leu Gly Ser Ile Lys Ala Asn Gin Gin His Ile Asn Ser Leu Leu Leu Leu|
| 217 | GAG CTT AAA TTA GAG GAT GTA ATT AAA GAG CAA GAT GAC TAT TAT AAA CCA GTA TAT GAG TAT GTA TAT GTA |
| 260 | Arg Ser Lys Leu Gly Gly Leu Lys Leu Leu Leu Gly Ala Gly Glu Ser Gly Ser Thr Ile Ala Ile Leu Gly Met Gly Le |
| 304 | GAT CAT TAT GAG TCT AAC GAT GAG GAG ACG TCA TAT AAA ACC ACC ATC ATC TAC AAT AAT ACA GGT GGT GTC GGT TTG |
| 347 | Gln His Leu Asn Gly Phe Asn Asp Gly Leu Lys Ser Ser Tyr Lys Thr Ile Tyr Asn Asn Thr Val Gly Ser MET Arg Val Leu |
| 391 | GTA AAG GCC GCT GTA GAA GTA TTA AAC ATT GTA ATC GAT GAA AAT AAA GAA GAC CAA ACC TAT AAA ACC TCT GCC GTA CAA |
| 434 | Val Asn Ala Ala Glu Glu Leu Lys Ile Ile Ser Glu Asn Asn Lys Ala Ala Ser Arg Ile Ser Asn Asn Arg Ala Gly Asp His |
| 478 | GTC ATT GCT GGT TGT ACT GCA GAG TGG TCA GTA CAA CAT ATT AAA GCC CTT TGG GTA CAT CCA GAT ATT CAA AAT ACC TCT CAA ACA TCT |
| 521 | Phe Asn Gly Val Leu Val Ala Ala Gin Asp Lys Ala Tyr Lys Thr Gly Ile Ile Thr Thr Ile Gly Ser Ser Gly Thr Ph Gln Gin Arg Ser |
| 565 | GTA AAG GCC GCT GTA GAA GTA CTA AAT GTA GCA GCC GCT TAC TAT TTT GAT GAT ATC GAT AGA ATT GCT GTA CCA TAT TCA TATA TTA CCA TCT GCC GTA CAA |
| 608 | Ser Gin Phe Gin Leu Asn Asp Ser Ala Ile Tyr Tyr Phe Ser Asp Ser Gin Gin Ser Pro Leu Tyr Leu Pro Ser Gin Ser |
| 652 | GAT GCT GTA CTA AGA ACT AAA ACA ACT GTC ATC ATT AGA ACA TTT TTC TGG AAA ATT CAA CAA ACT CAA TAG GCT GAT GTC |
| 705 | Asp Arg Val Arg Leu Arg Ser Lys Thr Tyr Lys Thr Gly Ile Ile Thr Val Phe Gin Gin Ser Ser Thr Phe Arg Met Val Asp Val |
| 739 | GGC GCT CAA GAG AAG AAA AAC TGG ATC ATG CTT TCA GTA CAA GTA GCT GTA CCT TGG GCC CTT AGT GTA CAA |
| 782 | Gln Gly Gin Gin Ser Gin Gin Ser Pro Gly Leu Thr Ala Val Ile Phe Cys Gin Gin Val Thr Ala Leu Ser Gin Gly |
| 826 | GAT TTT AAA ATG CAT GCT GAT ACT ACA AAT AGA ATG CAA GAG TCA TTT AAA CTC TTT AAA GAA GTA TAT AAC ACC AAA TGG TTT |
| 879 | Asp Leu Leu Leu Tyr Gin Asp Thr Thr Arg Asp Arg Asp Met Gin Gin Gin Ser Leu Leu Phe Leu Phe Gin Ile Cys Gin Thr Tsp Phe |
| 913 | GCA ATT GCT GAT ATG ATT CTT TTA ATT AAA GAA CAT ATT TCT TCA GAT GTA AAC ATT AAA GCC CAA CTA ATT AAA CCA |
| 966 | Asp Asn Thr Ala Met Ile Thr Asp Leu Asn Arg Ser Gin Gin Ser Gin Gin Ser Gin Ser Gin Gin Gin Gin Gin Gin |
| 1000 | GTA CCA CTA ATC GAC GTA GCT GTA GTC TCT AAA CAA CAA TAT ATT ACA AAT CAT AAA AAC AAT CAA AAA TGG AAA |
| 1053 | Ser Tyr Gin Gin Ser Tyr Gin Gin Ser Tyr Gin Gin Ser Gin Ser Gin Ser Gin Ser Gin Ser Gin Gin |
| 1087 | TAC ATC CAA ACT TAT AAT GCC GAT CAT AAT AAT ATT CTT TTA AAT GTA GTA GTA AAT GTA AAT GTA GTA AAT |
| 1140 | Val Ile Thr Pro His Leu Thr Cys Ala Thr Asp Thr Arg Asn Asn Ile Leu Val Phe Asn Asp Ala Val Asp Lys Val Asp Leu Leu |
| 1174 | TGT GAA GTA GTG ATT CTT AAA AGA ATT AAA AAT ACA AAA ACT TCA AAG CAC TAT TCA ATT AAA TAA TAA TAA TAA TAA TAA TAA AAA |
| 1217 | Ser Leu Gly Ala Gin Met Ile Leu |

**Fig. 2.** Nucleotide and deduced amino acid sequence of G1. EcoRI inserts were subcloned into pSP73 or pGEM2 and sequenced directly with the dideoxynucleotide method using Sequenase.
agreed completely with each other and with partial sequence of FR7 and RF3 (one of the larger class of clones). The longest open reading frame begins at the first AUG at nucleotide 130 and ends at nucleotide 1197. It encodes a polypeptide of 356 amino acids with a calculated molecular mass of 40,621 daltons. The internal EcoRI restriction site is at nucleotide 568.

The nucleotide and deduced amino acid sequences for G2 are shown in Fig. 3. These were determined by overlapping clones D2 and AD6 and a polymerase chain reaction-generated copy of genomic DNA (15). The clones encompass 1174 base pairs (bp) and a complete coding sequence. The open reading frame begins at the first AUG at nucleotide 72 and ends at nucleotide 1142. It encodes a polypeptide of 357 amino acids with a calculated molecular mass of 41,323 daltons.

**Comparison of Primary Sequence of α Subunits.** Fig. 4 shows an amino acid sequence comparison between the G1 and G2 subunits of Dictyostelium, yeast GPA1, mammalian G protein of unknown function (Gαi), inhibitory G protein (Gβ), stimulatory G protein (Gγ), and transducin (Tr). G1 and G2 are 45% identical to each other and 45% identical to mammalian G proteins, Gαi, and Tr. They are 35% identical to Gγ, and GP91 (18, 19). The predicted molecular masses in the range of mammalian G proteins (39–45 kilodaltons) since the 107 amino acid “insert” found in GPA1 is not present in Dictyostelium α subunits.

Five regions of the α subunits are highly identical. Four of these regions have been proposed as the sites for binding GTP based on analogy to c-Ha-ras and elongation factor Tu and are designated A, C, E, and G (19). The fifth region near the carboxyl terminus, which we designate region T, has not been previously described.

**Region A.** This region is located at amino acids 36–52 in G2 and is proposed to be the site of GTPase activity. A large portion of this region is 100% identical among all seven α subunits compared in Fig. 4. The sequence GAGEGSK, from which one of the oligonucleotides was prepared, is included in the stretch of 100% identity.

**Region C.** Region C is located at amino acids 201–207 in G1 and 203–209 in G2. With the exception of the initial position where F is replaced with V, G31 and G2 are identical to mammalian α subunits. GPA1 has two substitutions in this stretch. The stretch of highest identity among all seven α subunits includes the 3’ half of this proposed site and 6 amino acids on the 3’ side of the C site. This sequence, GQQRERK, is the position of the second oligonucleotide.

**Region E.** The E region includes amino acids 224–232 in G1 and 226–234 in G2. The differences between the α subunits in this stretch are clustered in five positions. By comparing the first four positions to G1 (amino acids 224, 226, 227, and 229), it is found that yeast differs in all four, Tr and G differ in two positions, and G2 differs in one position. In the fifth position (amino acid 231 in G1 and 233 in G2), G1, G2, and yeast are identical, whereas all of the mammalian α subunits are different.

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FIG. 3. Nucleotide sequence and deduced amino acid sequence of G2. EcoRI inserts were subcloned into pSP73 or pGEM2 and sequenced directly with the dideoxynucleotide method using Sequenase.
Region G. The proposed G region contains residues 266–278 in G₁ and 267–279 in G₂. Again the nonidentical residues are clustered in a few positions. In positions 273 and 277 of G₁, all of the α subunits differ from each other. In the remaining positions, G₁, Tr, and G₂ have two substitutions and yeast has one.

Region T. The fifth stretch of nearly complete identity is the sequence TCATDT near the carboxyl terminus of the polypeptides. This is located at positions 325–330 in G₁ and 326–331 in G₂. Only G₁ has one substitution in this stretch.

Two areas in which the α subunits diverge significantly from each other are the amino-terminal 30–35 amino acids and the region between the A and the C sites. In G₁ and G₂ this second divergent area includes residues 62–197 and 57–199, respectively. This area can be subdivided into two regions with different degrees of identity. The 5' half (roughly amino acids 62–129 in G₁ and 52–130 in G₂) contains very few identities among the α subunits. However, the 3' half (amino acids 130–197 in G₁ and 131–199 in G₂) contains patches of identity which are conserved within the α subunits.

Both G₁ and G₂ contain the arginine (position 180 in G₁ and 182 in G₂), which, in mammalian Gα, is the site for ADP-ribosylation by cholera toxin (20). Neither α subunit has a cysteine as the fourth residue from the carboxyl terminus, which is the consensus site for ADP-ribosylation by pertussis toxin (21).

Developmental Expression of G₁ and G₂. RNA isolated from wild-type Dictyostelium cells (strain NC-4) was probed with G₁ and G₂ cDNA (Fig. 5). G₁ hybridizes to multiple mRNAs. The predominant species of 1.7 kb is expressed at moderate levels in vegetative cells and increases to a maximal level at 10–12 hr, corresponding to the time of loose aggregate formation in these experiments. Thereafter, RNA levels rapidly decrease. The 2.6-kb species has a similar pattern of regulation. The 2.2-kb RNA is preferentially expressed in multicellular aggregates.

G₂ also hybridizes to multiple RNA bands having different patterns of expression. The predominant 2.7-kb RNA is not expressed or expressed at very low levels in vegetative cells. Upon initiation of development, RNA levels increase, reaching a maximum level during aggregation and then declining. The 2.9-kb mRNA is present briefly very early in development. The two other mRNAs (1.9 kb and 2.3 kb) appear in sequence during the multicellular aggregate stage.

Genomic Structure of α Subunits. Genomic mapping and isolation of genomic clones suggest that there is a single G₂ gene and a single G₂ gene (data not shown; ref. 22). This suggests that the multiple transcripts with dramatically different developmental time courses result from alternate splicing events or nested promoters.

DISCUSSION

D. discoideum has at least two G protein α subunits, designated G₁ and G₂. They are closely related to the mammalian G protein α subunits, being most similar in amino acid sequence with G₁ and G₂.
sequence to $G_{\alpha 1}$. Both subunits are potential substrates for ADP-ribosylation by cholera toxin. No physiological effects of cholera toxin on *D. discoideum* have been found, although ADP-ribose acceptors have been reported (23). Although pertussis toxin exerts its effects most frequently on G-like G proteins, such as $G_{\alpha 1}$ and $G_{\alpha 2}$, neither sequence contains the cysteine, which is the consensus site for ADP-ribosylation. However, biochemical effects of pertussis toxin on signaling in *D. discoideum* have been reported, suggesting that there may be at least one more $G_{\alpha}$ subunit (24).

The previously unidentified T region (325–330) appears to be important since it is completely identical in all $G_{\alpha}$ subunits. The carboxy terminus of $G_{\alpha}$ subunits has been implicated in receptor binding based on antibody blocking experiments (25). A recent report demonstrates that a peptide that encompasses this sequence blocks binding of Tr to rhodopsin (26). The most critical residue for the blocking effect was cysteine 321, which is the C of TCATDT.

The A region is flanked by highly divergent areas (amino acids 3–35 and 60–125). The amino terminus of $G_{\alpha}$ subunits has been implicated in $\beta$ subunits binding based on reconstitution assays with proteolytically cleaved Tr (27). At least two mammalian $\beta$ subunits and two $\gamma$ subunits have been identified (28). Neer and Clapham (28) suggest that different combinations of $\beta$ and $\gamma$ may be functionally different. If this is the case, the site of $\beta$ interaction with $\gamma$ may be expected to be a divergent area. The stretch 60–125 has been proposed to confer effector specificity. However, a chimeric $\alpha$ subunit containing the amino half of murine $G_{\alpha 1}$ and the carboxyl half of murine $G_{\alpha 2}$ stimulated adenylate cyclase (29). Either the effector interaction site is not contained in this portion of the molecule or only part of the site is contained in this region. In addition, the stretch 60–125 corresponds to residues 73–245 in GPA1, which includes the 107 amino acid insert not found in *Dictyostelium* or mammalian $\alpha$ subunits.

Both $G_{\alpha 1}$ and $G_{\alpha 2}$ probes are complementary to multiple, developmentally expressed RNAs, but DNA blot data suggest that both are encoded by single genes. At present, we do not know if the multiple mRNAs result from differential splicing or multiple transcription initiation sites. It is possible that some of the less abundant mRNA species complementary to these probes could be the products of other related $G_{\alpha}$ protein genes.

Two lines of evidence strongly suggest that $G_{\alpha 1}$ and $G_{\alpha 2}$ have distinct functions. (i) $G_{\alpha 1}$ and $G_{\alpha 2}$ are as divergent from each other as they are from the $\alpha$ subunits of other organisms. (ii) The mRNAs encoding these proteins have very different developmental patterns of expression. We have examined the regulation and function of $G_{\alpha 1}$ and $G_{\alpha 2}$ during growth and development (22). Our results indicate that the two proteins have distinct functions and may regulate different signal transduction systems. 

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