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Aggregation of *Dictyostelium discoideum* Is Dependent on Myristoylation and Membrane Localization of the G Protein α-Subunit, Gα2

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Abstract The heterotrimeric G protein, G2, from the eukaryotic organism *Dictyostelium discoideum* participates in signal transduction pathways which are essential to *Dictyostelium's* developmental life cycle. G2 is activated by cell surface cAMP receptors and in turn is required for the activation of a host of effectors, including adenylyl cyclase, guanylyl cyclase, and phospholipase C. Myristoylation of G protein α-subunits is known to affect α-subunit association with the βγ subunits and membrane localization. The putative site for *N*-terminal myristoylation of Gα2 was mutated from Gly to Ala (G2A) and expressed in the *gα2*-null cell line, MYC2. Transformants expressing Gα2-G2A exhibit physiological and biochemical changes from wild-type cells. Gα2-G2A expressing cells fail to rescue the aggregation-minus phenotype of MYC2 cells on developmental agar plates. Gα2-G2A expressing cells are also not chemotactic to cAMP in a standard drop assay. Gα2-WT is found in both the pellet and supernatant fractions following lysis of the cells. Gα2-G2A however is found almost exclusively in the lysate supernatant. Gα2 is radiolabeled upon incubation of cells in [^{3}H]myristate, while Gα2-G2A is not labeled. Examination of activation of the effectors adenylyl cyclase and guanylyl cyclase reveals that Gα2-G2A expressing cells partially activate adenylyl cyclase but show no cAMP-stimulation of guanylyl cyclase. The physiological deviations from wild-type can be explained by the variations in effector activation, possibly due to improper localization of the non-myristoylated Gα2-G2A to the cytosol. J. Cell. Biochem. 74:301–311, 1999.

Key words: myristoylation; *Dictyostelium discoideum*

Following depletion of a food source, *Dictyostelium discoideum* amoebae undergo centralized aggregation utilizing pulses of cAMP as a chemoattractant [Van Haastert, 1995]. Cell aggregates then proceed through a highly regulated program of development to form spore containing fruiting bodies [Gross, 1994]. Aggregation is dependent upon G-protein mediated responses to extracellular cAMP binding to cell surface receptors. Several G-protein mediated effectors are activated by extracellular cAMP, including adenylyl cyclase, guanylyl cyclase, and phospholipase C [Caterina and Devreotes, 1991]. Activation of effectors necessary for development is expressly dependent upon Gα2 [Kumagai et al., 1989], one of eight G protein α-subunits expressed in *D. discoideum* [Wu and Devreotes, 1991]. Cells lacking Gα2 fail to show activation of cAMP-mediated responses in vivo and cells are arrested in the initial phase of the development program [Kumagai et al., 1989]. Due to the essential nature of Gα2, it presents itself as an excellent model for studying α-subunit regulation by covalent acyl modification.

Several mammalian G protein α-subunits, including $G_o\alpha$, $G_i\alpha$, $G_z\alpha$, and $G_t\alpha$, are modified at their *N*-terminus by the attachment of saturated fatty acids [reviewed in Casey, 1995]. *N*-myristoylation is the co-translational, irreversible attachment of myristic acid (C14:0) which occurs through an amide linkage to a glycine residue at the extreme *N*-terminus after removal of the initiating methionine [Gordon et al., 1991]. The *N*-terminal sequence (M)-G-X-C-X-S is a common feature of substrates myristoylated by the soluble enzyme myristoyl-CoA:protein *N*-myristoyltransferase (NMT)

Abbreviations used: cAR1, *Dictyostelium* cAMP receptor 1; G protein, guanine nucleotide binding protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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[Towler et al, 1988; Rocque et al., 1993]. It has also been suggested that the *N*-terminal recognition sequence signals dual acylation with myristoylation occurring on the N-terminal glycine and palmitoylation occurring at the nearby cysteine [Wedegaertner and Bourne, 1994]. Fatty acid attachment to the α-subunit has been shown to facilitate membrane association [Franco et al., 1996] and binding of the α-subunit to the βγ complex [Linder et al., 1991]. The full implications of G protein α-subunit *N*-myristoylation have not yet been completely elucidated.

The N-terminal sequence of Gα2, (M)-G-I-C-A-S-S-...[Pupillo et al., 1989], contains the N-terminal glycine for myristoylation as well as a cysteine for palmitoylation. Protein myristoylation has been reported in *D. discoideum* [da Silva and Klein, 1990] and we demonstrate here that Gα2 is labeled upon incubation of cells with [^{3}H]myristic acid. To examine the functional effects of G protein α-subunit myristoylation, the N-terminal glycine in Gα2 of *D. discoideum* was mutated to alanine. The mutated α-subunit, Gα2-G2A, failed to rescue the aggregation-minus phenotype of *gα2* null cells, MYC2, and the transformed cells exhibited several changes in the cAMP-induced responses. Upon cell fractionation, it was found that Gα2-G2A was neither membrane associated nor myristoylated. For *D. discoideum*, proper localization of Gα2 appears necessary for specific signaling pathways to incur early developmental programs; thus myristoylation appears essential for normal functioning and localization of Gα2.

MATERIALS AND METHODS

Cell Culture and Development

All *Dictyostelium discoideum* cell lines and mutants were derived from Ax-3 cells (wild-type). The cell lines MYC2 (*gα2* null) and LW6 (β null) were kindly supplied by Peter N. Devreotes (Johns Hopkins Medical School). All cell lines were grown axenically in modified HL-5 medium [Gundersen, 1997]. G418 was added to 20 μg/ml when required. Aggregation competent cells were generated by 4–6 h incubation in development buffer (DB) as previously described [Gundersen, 1997]. Developmental phenotypes were checked by plating 5×10^7 cells on 100 mm DB-agar (1%) plates. Cells starved 4–6 h in DB were allowed to adhere to the agar surface for 10 min and then the DB was aspirated. The plates were briefly air dried and incubated at 22°C. Initiation of aggregation was observable by 2 h and fruiting body formation was completed by 24–48 h.

Generation of Gα2-G2A Mutation

Oligonucleotide-mediated site-directed mutagenesis was performed as previously described [Kunkle et al., 1987] to create a point mutation converting Gly2 to Ala2, or Gα2-G2A. The oligonucleotide sequence was: 5'-CACAAATAGC-CATTTTTTAAG-3' (Operon, Alameda, CA). The mutated *Gα2* cDNA was cloned into the EcoRI site of pAPI, a version of pBluescript II KS$^-$ (Stratagene, La Jolla, CA) modified by the addition of a BglII site in the EcoRV, and the removal of an undesired BclI by cutting with BclI endonuclease, filling in the 5' overhang ends, and religating to create pAPI. The mutant *Gα2* cDNA was removed from pAP1 with the restriction endonucleases BglII and BamHI and cloned into the BglII site of *D. discoideum* expression vector pJKI [Pitt et al., 1992]. DNA sequencing of clones with Sequenase Version 2.0 (USB, Cleveland, OH) using internal *Gα2* cDNA primers confirmed the point mutation and orientation of the *Gα2* cDNA insert within pJKI. The plasmids containing the Gα2-G2A mutation and Gα2-WT (to serve as a control) were transformed into the *gα2*-null cell line, MYC2 [Chen et al., 1994], by electroporation [Howard et al., 1988] using a Bio-Rad gene pulser set at 1.2 mV, 200 ohms resistance and 3 μF capacitance. Transformants were selected in HL-5 supplemented with G418 at 20 μg/ml and subsequently cloned on SM/5 plates overlaid with a lawn of *Klebsiella aerogenes (Ka*) [Sussman, 1987].

Cell Fractionation and Immunoblotting

Aggregation competent cells at 5×10^7 cells/ml were fractionated by forcing cells through a Nucleopore polycarbonate 5 μ pore filter (Costar, Cambridge, MA) followed by centrifugation of the lysate at 13,000*g* for 10 min at 4°C. The 13,000*g* pellet was prepared for SDS-PAGE and the supernatant was centrifuged further at 100,000*g* for 60 min at 4°C generating the 100,000*g* pellet and supernatant. Supernatant fractions that were to undergo further separation by sucrose density gradients (5–20%) in a SW60Ti rotor at 45,000 rpm for 16 h had either GDPβS or GTPγS (100 μM) added at the time of lysis. Bovine serum albumin (BSA,

66 kD) was also added to the samples to serve as a protein size standard in the gradients. Supernatant, pellet and sucrose density gradient fractions were dissolved in SDS sample buffer for separation by SDS-PAGE (10% polyacrylamide mini gels) as previously described [Gundersen and Devreotes, 1990] with the following modifications. The gel proteins were transferred to nitrocellulose in a Hoefer mini gel transfer box at 100 mA for 90 min and stained with Ponceau S (Sigma, St. Louis, MO) to visualize molecular weight markers and the BSA which had been added to the sucrose density gradient samples. Gα2 was detected with a Gα2-specific, *N*-terminal peptide antiserum as the primary antibody. The secondary antibody was a horseradish peroxidase (HRP) anti-rabbit IgG conjugate (Sigma). Band detection was achieved using enhanced chemiluminescence (ECL). Blots were incubated in an ECL substrate solution of 0.1 M Tris-HCl, pH 8.5, 0.03% H_2O_2, 60 μM *p*-Coumeric acid, 1 mM Luminol [Matthews et al., 1985] for 1 min. Blots were then exposed to film for 20 min. Expression of the cAMP receptor, cAR1, was analyzed in membrane pellets prepared by cold ammonium sulfate lysis as described [Klein et al., 1987] using a cAR1-specific antibody [Klein et al., 1988] and detected as above. Films of cell lysate pellets and supernatants were scanned with an LKB Utroscan XL Enhanced Laser Densitometer and analyzed with GelScan XL. At least three films from separate experiments were scanned for values reported.

[^{3}H]Myristic Acid Labeling of Gα2

One hundred μl aliquots of 4 h aggregation competent cells at 1×10^8 cells/ml were incubated with 100 μCi of [^{3}H]myristic acid for 1 h. Cells were washed once in DB, resuspended in lysis buffer of 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.1 M sucrose, and a cocktail of protease inhibitors [Vaughan and Devreotes, 1988] and then immediately filter lysed. The lysate was centrifuged at 13,000*g* at 4°C for 10 min. The resulting pellet and supernatant were each subjected to immunoprecipitation with Gα2-specific antiserum as described [Gundersen and Devreotes, 1990]. Immunoprecipitated samples were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R and destained. Gels were incubated in Fluoro-Hance (Research Products International, Mount Prospect, IL) and dried. Labeled proteins were detected by fluorography at –80°C for 2–4 weeks. The fatty acid linkage was characterized by rehydrating the gel in water for 10 min and incubating the gel overnight in 1 M hydroxylamine, pH 6.7 [Buss et al., 1987]. Hydroxylamine was removed by washing the gel in 10% acetic acid/10% isopropanol. The gel was then reprocessed for fluorography. The fatty acids attached to immunoprecipitated Gα2 were identified as follows. Labeled gel bands were excised, washed for 60 min in dH_2O with three changes, and subjected to acid hydrolysis in 6N HCl at 110°C for 15–18 h. The HCl was evaporated under a stream of N_2 and the lipids were extracted from the dried material [Bligh and Dyer, 1959]. Fatty acid standards C12:0, C14:0, C14:1, and C16:0 were added just prior to extraction. Phenacyl derivatives of the fatty acids were generated as described [Wood and Lee, 1983] for improved HPLC separation and detection at A_{242}. One ml fractions were collected from the HPLC C_{18} column and counted in 10 ml of Bio-Safe II (RPI) to identify the radiolabeled fatty acids.

Chemotaxis Assay

The chemotactic response of cells to cAMP was determined by the small drop method [Konijn, 1970; Devreotes et al., 1987] using aggregation competent cells. Drops of 1 μl containing roughly 5000 cells were placed on DB agar plates (0.5% agar) next to 1 μl drops of 10 μM cAMP. Drops containing either Ax-3 or cells expressing Gα2-WT were placed on the opposite side of the cAMP drop to Gα2-G2A as positive controls. Plates were incubated at 22°C and observed from 30 min to 2 h. Movement of cells toward the drop perimeter nearest the cAMP was indicative of a positive chemotactic response.

Gα2 Mobility Shift on SDS-PAGE

The mutant Gα2-G2A protein was tested for cAMP-induced shift in mobility on SDS-PAGE as described. The shift in electrophoretic mobility was observed by separation under modified Laemmli conditions as previously described [Gundersen and Devreotes, 1990]. Gα2 was detected by immunoblot as described above.

In Vivo cAMP and cGMP Assays

Aggregation competent cells starved 4–5 h were used to measure the cAMP-induced cAMP and cGMP responses using isotope dilution assays as described previously [Snaar-Jagalska

and Van- Haastert, 1994]. For measurement of cGMP production, cells were stimulated with 1 μM cAMP and for cAMP production cells were stimulated with 2 μM 2′-deoxycAMP. Cell samples were withdrawn at the indicated times and added to an equal volume of 3% perchloric acid. The solution was then neutralized with 50% saturated $KHCO_3$ and centrifuged. cAMP and cGMP in the sample supernatants were quantified using cAMP and cGMP radioimmunoassay kits following the manufacturer's instructions (Amersham Life Sciences, Arlington Heights, IL).

GTPγS Inhibition of cAMP Binding

Aggregation competent cells starved for 5 h were used to examine GTPγS inhibition of cAMP binding to *Dictyostelium* membranes to examine Gα2-cAR1 coupling as previously described [Van Haastert, 1984; Gundersen, 1997].

RESULTS

Phenotype of Non-Myristoylated Gα2-G2A

MYC2, a *gα2*-null cell line, fails to undergo aggregation and development under normal starvation conditions. Transformation of MYC2 with *Gα2*-WT cDNA rescued the developmental phenotype; normal fruiting bodies formed within 24 h of starvation. Phenotypic rescue occurred on both non-nutrient agar (DB) plates (Fig. 1) or when cells cleared plaques on a bacteria lawn. MYC2 cells expressing the Gα2-G2A mutation failed to undergo aggregation and development and remained as a cell monolayer when placed on DB plates (Fig. 1). When grown on bacteria lawns, the plaques produced by the Gα2-G2A clones were considerably smaller than plaques cleared by the Gα2-WT clones. This observation has been reported previously for G protein gene deletions in *Dictyostelium* [Lilly et al., 1993]. Gα2-G2A expressing clones also exhibited a variable developmental phenotype on the bacteria lawns. Roughly 10% of the plaques formed mounds after 24 h, with approximately 2% culminating in fruiting bodies. This same percentage of mounds and fruiting bodies was generated whether starting with one of the aggregation-minus or aggregating Gα2-G2A expressing clones.

Wild-type *D. discoideum* cells undergo coordinated, directional movement or chemotaxis in response to extracellular cAMP. Mutant and wild-type cells were tested for their chemotactic response to cAMP using a small drop assay (Fig. 2). MYC2 cells expressing Gα2-WT demonstrated typical migration within the drop toward the perimeter closest to a drop of cAMP within 1 h. Ax-3 cells showed a similar response (not shown). MYC2 cells expressing Gα2-G2A remained random within the drop indicating a chemotaxis-minus phenotype.

Cellular Location and Subunit Composition of Gα2-WT and Gα2-G2A

The cellular location of Gα2-WT and Gα2-G2A was determined following lysis of aggregation competent cells. The presence of Gα2 was examined in the 13,000*g* pellet and the 100,000*g* pellet and supernatant (Fig. 3). The 13,000*g* pellet contained 61 ± 8% of Gα2-WT with the remaining 39 ± 8% in the 100,000*g* supernatant. Similar results were obtained with the parental cell line, Ax-3 (not shown). Gα2-G2A however was found almost exclusively in the 100,000*g* supernatant, 96 ± 2%, with the remaining 4 ± 2% in the 13,000*g* pellet. Neither

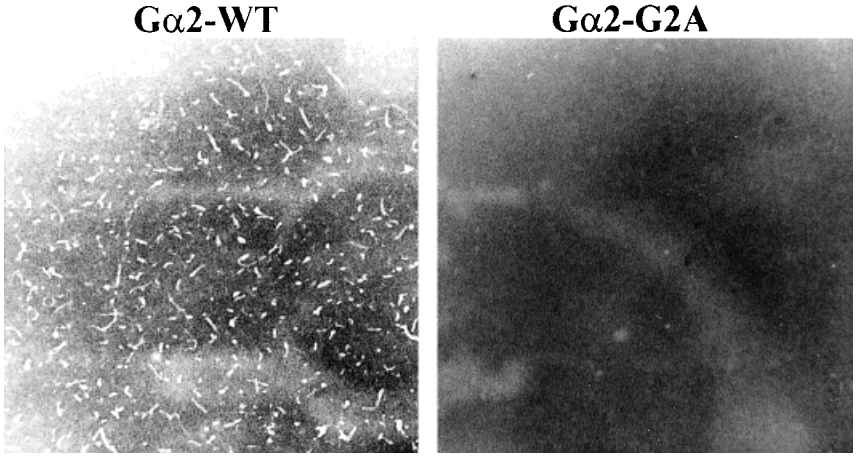

Fig. 1. Phenotypes of Gα2-WT and Gα2-G2A on development buffer (DB) agar plates. Cells expressing either Gα2-WT or Gα2-G2A were plated onto the surface of DB agar (1%) plates as described in Materials and Methods. After 24 h at 22°C the plates were photographed. The rough appearance of the Gα2-WT plate is due to cell aggregation and the formation of slugs and fruiting bodies. The Gα2-G2A plate appears smooth. Cells have failed to aggregate and remain as a cell monolayer.

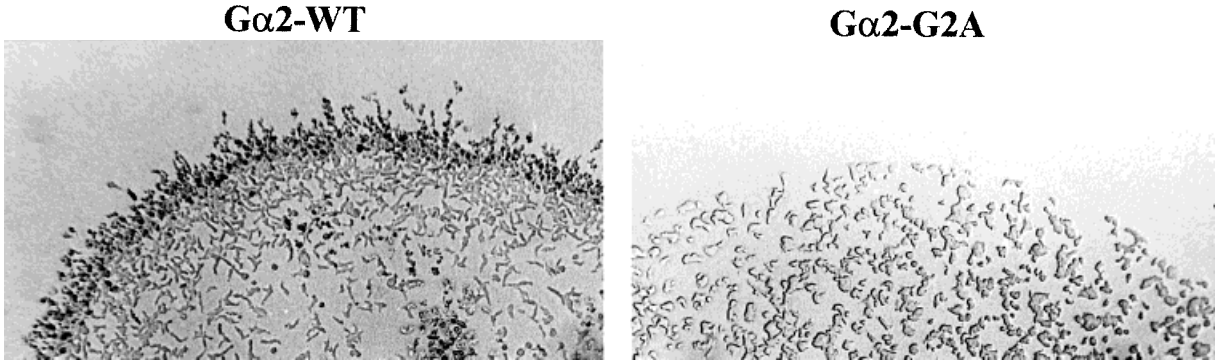

Fig. 2. Chemotactic response of Gα2-WT and Gα2-G2A to cAMP. Cells expressing either Gα2-WT or Gα2-G2A were subjected to a simple chemotactic drop assay as described in Materials and Methods. Roughly 5,000 cells in a 1 µl drop were placed within 2–5 mm of a 1 µl drop of 10 µM cAMP. Cells were allowed to migrate at 22°C and then the edge of the drop closest to the cAMP was photographed after 40 min. Accumulation of the Gα2-WT expressing cells at the edge of the drop is a positive indication of chemotaxis, while the Gα2-G2A expressing cells remain randomly distributed within the drop and are nonchemotactic to cAMP.

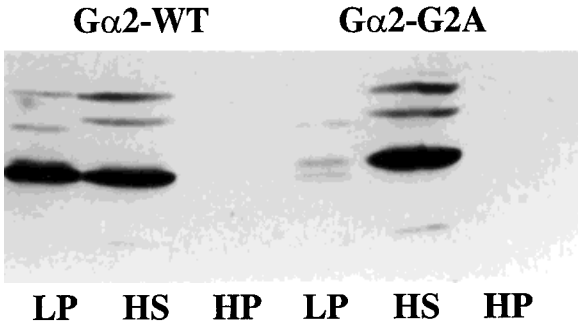

Fig. 3. Localization of Gα2-WT and Gα2-G2A to membrane and supernatant fractions following cells lysis. Cells expressing either Gα2-WT or Gα2-G2A were lysed and fractions separated by centrifugation; first at 13,000*g* followed by a 100,000*g* centrifugation of the first supernatant. Fractions were prepared for SDS-PAGE and immunoblots as described in Materials and Methods. Gα2 was identified on immunoblots using a Gα2-specific peptide antiserum. Each lane contains protein from the equivalent of 3.75×10^5 cells. LP, 13,000*g* pellet; HP, 100,000*g* pellet; HS, 100,000*g* supernatant.

Gα2-WT nor Gα2-G2A was found in significant amounts in the 100,000*g* pellet which allowed us to treat Gα2 found in the 13,000*g* supernatant as soluble. These results clearly suggest that Gα2-G2A is localized to the cell cytoplasm and is not membrane associated.

The heterotrimer subunit composition of the two G proteins was analyzed by sucrose density gradient centrifugation (Fig. 4). In the presence of GDPβS, cytosolic Gα2-WT was spread through fractions 6–12 with two peaks, one in fractions 7 and 8 and a second in fractions 10 and 11. By Ponceau S staining, bovine serum albumin (BSA) peaked in fractions 8 and 9 (not shown). The Gα2-WT peak further down in the gradient in relation to BSA (7 and 8) suggests a heterotrimeric, αβγ, subunit composition, while the peak above BSA (10 and 11) suggests a monomeric form for Gα2-WT. In the presence of GTPγS which causes the α-subunit to dissociate from the βγ complex, Gα2-WT is located in fractions above BSA in the gradient suggesting a complete conversion to the α-subunit monomer. In the presence of GDPβS, cytosolic Gα2-G2A already appears in fractions above BSA in the gradient (10 and 11) suggesting that it is monomeric and not associated with the βγcomplex. Identification of the β-subunit in the gradient fractions for all three samples revealed the β-subunit as a single peak around fraction 8 when samples were prepared in GDPβS or GTPγS (Fig. 4).

[^{3}H]myristic Acid Labeling

Cells expressing either Gα2-WT or Gα2-G2A were labeled with [^{3}H]myristic acid under starvation conditions. Fluorograms of [^{3}H]myristic acid labeled pellet and supernatant fractions prior to immunoprecipitation showed numerous labeled bands (Fig. 5A, lanes 1–4) confirming a previous observation [da Silva and Klein, 1990]. The difference in labeling patterns seen in some bands for the two cell lines is likely due to the expression of different sets of proteins in the two cell lines since signaling through Gα2-G2A appears severely disrupted. Immunoprecipitation with Gα2-specific antisera revealed ^{3}H-label in both the pellet and supernatant fractions of Gα2-WT (Fig. 5A, lanes 5,7). Neither the pellet nor supernatant fractions from Gα2-G2A exhibited fatty acid labeling (Fig. 5A,

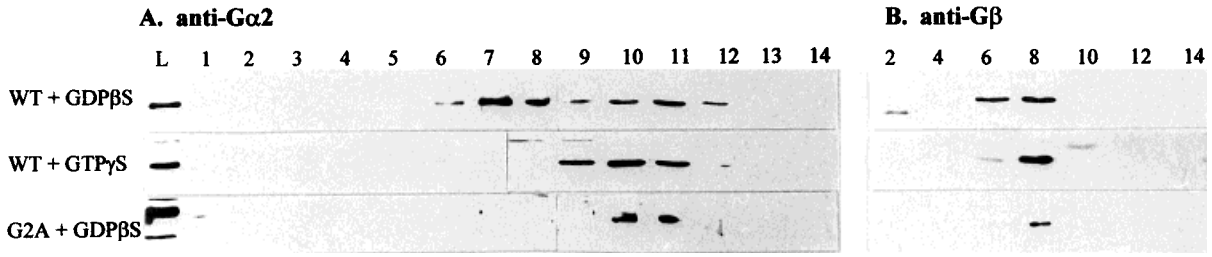

Fig. 4. Sucrose density gradient fractionation of 100,000 xg cell lysate supernatants. The 100,000*g* supernatants from cells expressing either Gα2-WT or Gα2-G2A were prepared in the presence of GDPβS (100 μM). Gα2-WT was also prepared in GTPγS (100 μM). Samples (0.1 ml) were loaded (L) onto 4.5 ml 5–20% sucrose density gradients as described in Materials and Methods. Fractions were collected starting from the bottom of the gradient (Fraction #1). Fraction were subjected to SDS-PAGE, transferred to nitrocellulose and probed for the presence of **(A)** Gα2 (approx. 42 kD) and **(B)** the β-subunit (approx. 40 kD) by immunoblot. BSA (66 kD) peaked in fractions 8 & 9 as determined by Ponceau S staining (not shown).

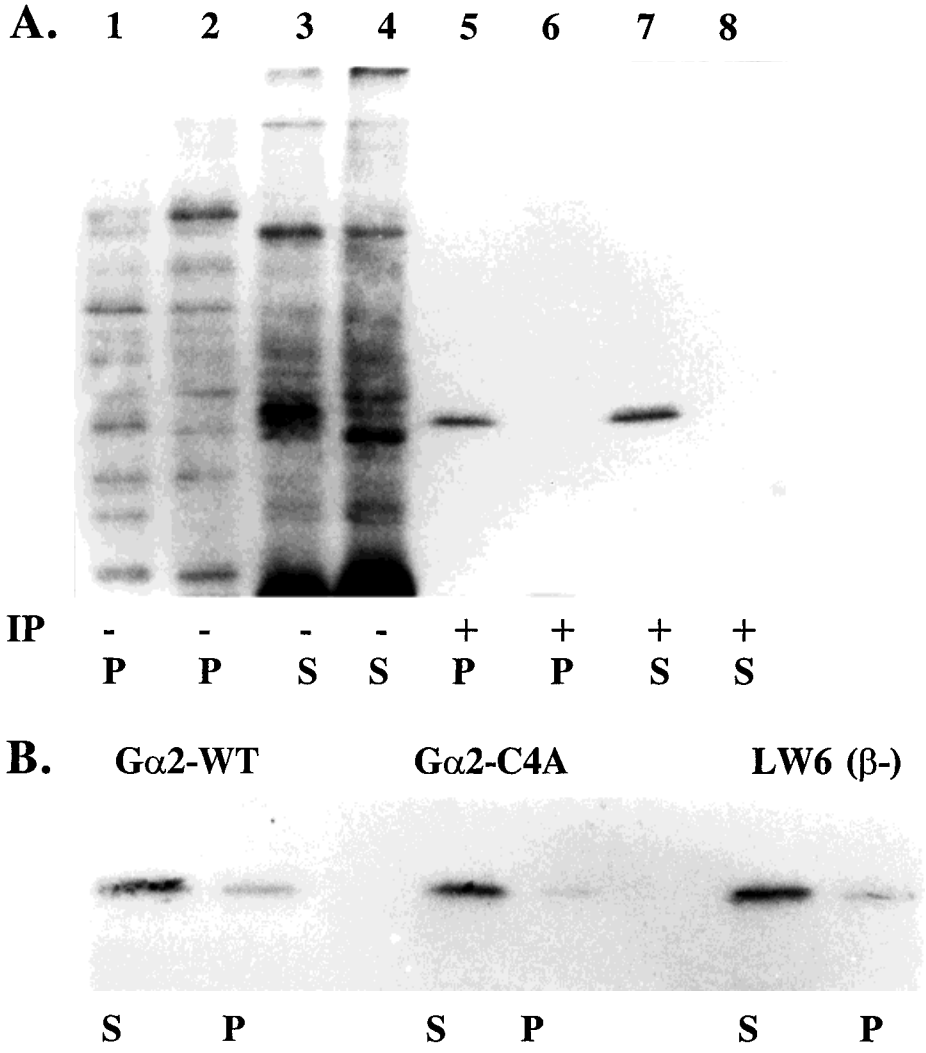

Fig. 5. [^{3}H]Myristic acid labeling. Cells expressing **A:** Gα2-WT or Gα2-G2A; **B:** Gα2-WT, Gα2-C4A or LW6 (β null) were incubated with [^{3}H]myristic acid at 1 mCi/ml and samples prepared as described in Materials and Methods. **A:** Gα2-WT **(lanes 1, 3, 5, 7)** and Gα2-G2A **(lanes 2, 4, 6, 8)**. Aliquots of each sample, 13,000*g* pellet (P) or 13,000*g* supernatant (S) were applied directly to gel lanes 1–4 (IP-) while equivalent samples were immunoprecipitated using a Gα2-specific peptide antiserum (IP + ; lanes 5–8). **B:** The supernatant (S) and pellet (P) fractions were immunoprecipitated for the three cell lines.

lanes 6,8). Two additional cell lines were tested for myristic acid labeling, the Gα2-C4A point mutation which blocks Gα2 palmitoylation and a β-subunit null cell line, LW6 (Fig. 5B). Radiolabel was observed in Gα2 immunoprecipitated from both supernatant and pellet fractions of these cells.

To characterize the nature of the bond between [^{3}H]-label and Gα2-WT, the sensitivity of the labeled protein to hydroxylamine treat-

ment was investigated. After hydroxylamine treatment, densitometric evaluation determined that 94% of the ^{3}H-label remained attached to Gα2-WT samples (data not shown). Since ester or thioester bonds hydrolyze under these conditions, the fatty acid label is most likely linked to Gα2-WT via an amide bond to the *N*-terminal glycine. To confirm that the label attached to Gα2 was indeed myristic acid, labeled gel bands from pellet and supernatant were excised and processed as described. HPLC separation and identification of the fatty acid label revealed two major peaks of ^{3}H-labeled fatty acids for both fractions (Fig. 6). The first peak eluted just ahead of the C12:0 standard and remains unidentified, but clearly matches a peak comprising roughly 8% of the [^{3}H]myristic acid label. The second major peak, roughly 50% of the recovered label, eluted with the myristic acid (C14:0) standard. Several minor peaks were also observed each containing less than 5% of the total recovered counts. The counts eluted at 21–22 min match palmitic acid (C16:0), while the other small peaks remain unidentified.

Biochemical Analysis of the Non-Myristoylated Gα2-G2A

Activation of guanylyl cyclase and the production of cGMP has been implicated as a requirement for chemotactic movement of *D. discoideum* amoebae [Ross and Newell, 1981]. A typical cGMP response in *D. discoideum* was seen when Gα2-WT expressing cells were stimulated with 1 uM cAMP. Cyclic-GMP levels peaked within 10 sec and reverted to basal levels by 1 min. Gα2-G2A expressing cells failed to show guanylyl cyclase activation (Fig. 7A). Aggregation of *D. discoideum* cells is also dependent upon production of cAMP by adenylyl cyclase. Cyclic-AMP production normally peaks at 1–2 min following stimulation and declines to basal levels by 10 min. Gα2-G2A and Gα2-WT cells displayed parallel responses in cAMP production, although cAMP levels were significantly reduced in Gα2-G2A cells (Fig. 7B).

GTPγS binding to the G protein α-subunit causes uncoupling of the G protein from the receptor and results in a loss of high-affinity

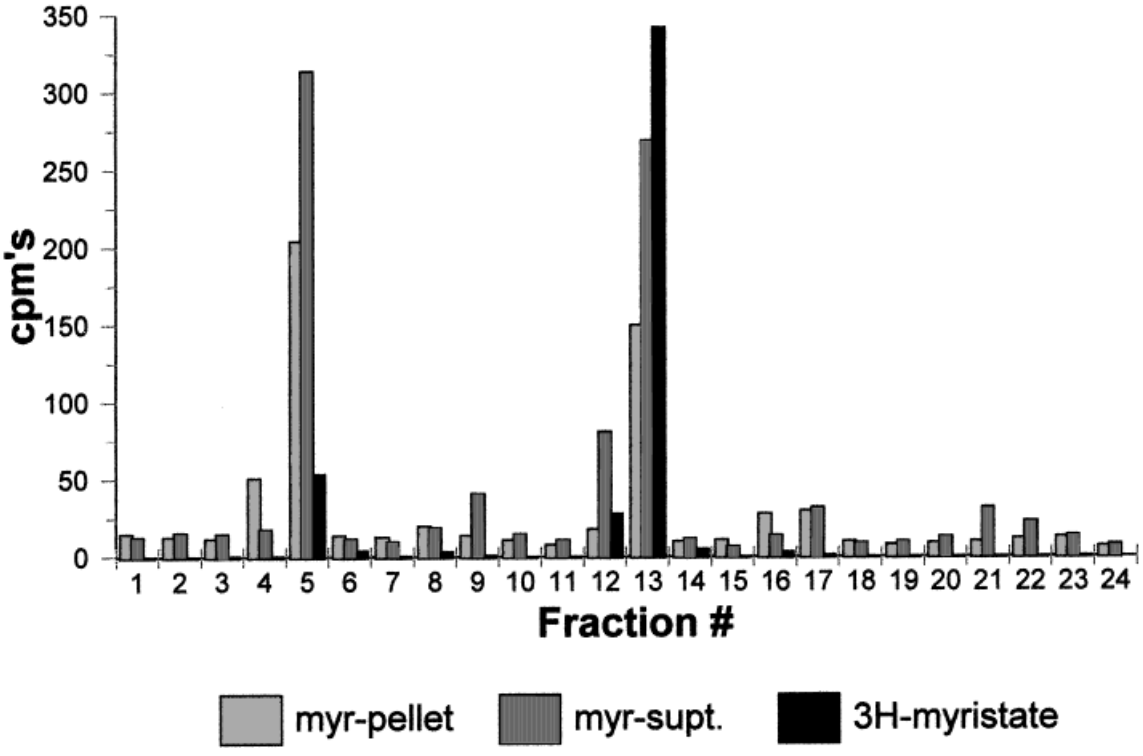

Fig. 6. Identification of ^{3}H-labeled fatty acids on Gα2 by HPLC following [^{3}H]myristic acid labeling. Radiolabeled Gα2 bands from pellet and supernatant fractions were cut from gels and processed as described in Materials and Methods. [^{3}H]mryistate label was also processed for an HPLC run. Fraction numbering starts 1 min into the run. Data is one representative experiment. Standard peak retention times (peak fraction) were; C12:0, 7.7 min (fraction 7); C14:1, 8.2 min (fraction 8); C14:0, 13.2 min (fraction 13); C16:0, 21.8 min (fraction 22). No significant counts were recovered in fractions 25 to 45 min (not shown) which make up the complete gradient.

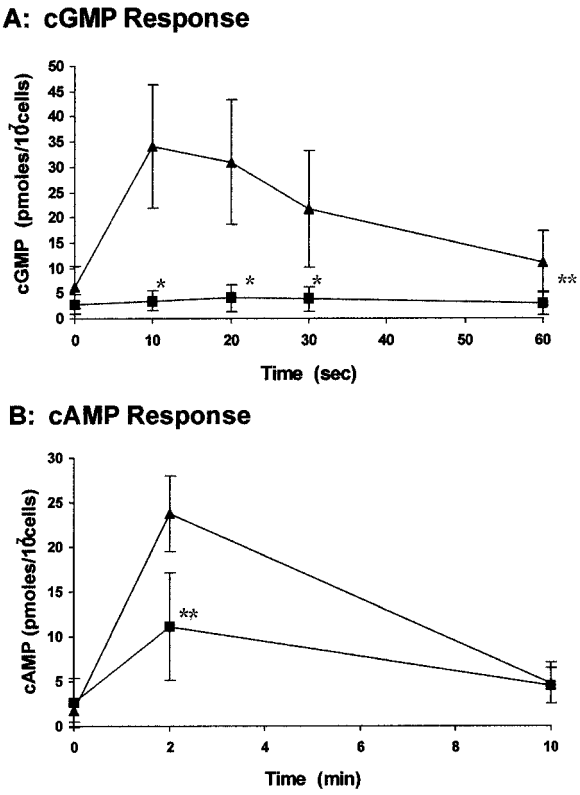

Fig. 7. cGMP and cAMP responses of cells expressing Gα2-WT or Gα2-G2A. Aggregation-competent Gα2-WT (▲) or Gα2-G2A (■) expressing cells were stimulated with either 1 μM cAMP for the cGMP response **(A)** or with 2 μM 2'-deoxy-cAMP for cAMP response **(B)**. Cells were removed for processing as described in Materials and Methods at 0, 10, 20, 30, and 60 sec for cGMP and at 0, 2, and 10 min for cAMP. The cGMP and cAMP levels in cell samples was determined by radioimmunoassays. All values are the average of three experiments and error bars are standard deviations. By Student *t*-test analysis, G2A data significantly different from WT is marked: $*P < 0.01$, $**P < 0.05$.

ligand binding by the receptor [Van Haastert, 1984]. Coupling of the G2 protein to the cAMP receptor, cAR1, was determined by measuring the effect of GTPγS on binding of cAMP to cell membranes (Table I). Wild-type, Ax3, cells retained only 20% of [^{3}H]cAMP binding in the presence of GTPγS, whereas [^{3}H]cAMP binding to membranes from MYC2 cells, which lack Gα2, were relatively unaffected by the addition of GTPγS. However, [^{3}H]cAMP binding to MYC2 membranes was extremely low and of low affinity (data not shown). Membranes from Gα2-WT expressing cells also demonstrated significant loss of [^{3}H]cAMP binding in the presence of GTPγS. The Gα2-G2A cell line revealed a significant reduction in the level of GTPγS inhibition with a level of [^{3}H]cAMP binding intermediate to that seen in wild-type and MYC2. The reduced level of G protein-receptor coupling in the Gα2-G2A expressing cells probably results from the loss of Gα2-G2A from the membrane fraction but may in part be due to lower cAR1 expression. Immunoblots of cAR1 in Gα2-G2A cells showed reduced cAR1 expression in comparison to the Gα2-WT cell line (Fig. 8).

Cyclic-AMP binding to cAR1 induces transient phosphorylation of Gα2 on serine-113 [Chen et al., 1994] which peaks at 1 to 2 min and returns to a non-phosphorylated state by 15 min. Phosphorylation reduces the mobility of Gα2 on SDS/PAGE gels from 40 kD to 43 kD. Aggregation competent Gα2-G2A and Gα2-WT cells were stimulated with cAMP, sampled over 15 min and examined for Gα2 mobility shift. Gα2-WT displayed a characteristic shift in mobility while Gα2-G2A failed to shift upon stimulation with cAMP, indicating that Gα2-G2A is not phosphorylated (Fig. 9).

TABLE I. GTPγS Inhibition of cAMP Binding

Cell line	% [^{3}H]cAMP remaining bound[a]
Ax-3	19 ± 4%
Myc2 (Gα2 null)	84 ± 11%
Gα2-WT	30 ± 3%
Gα2-G2A	56 ± 5%

[a]% [^{3}H]cAMP remaining bound to cell lysate membranes was determined by subtracting counts bound in the presence of GTPγS (50 μM) from the Total bound (H_2O) dividing by Total bound and multiplying by 100. Nonspecific binding (100 μM cAMP) was subtracted from both GTPγS and Total bound. Average of three or more experiments; Mean ± S.E.

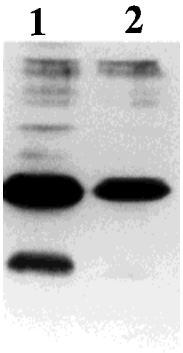

Fig. 8. Cyclic-AMP receptor, cAR1, expression in Gα2-WT and Gα2-G2A expressing cells. Membranes from aggregation-competent Gα2-WT (**lane 1**) or Gα2-G2A (**lane 2**) expressing cells were prepared by ammonium sulfate lysis [Klein et al., 1987]. Sample proteins were separated by SDS-PAGE and subject to immunoblot using a cAR1-specific antiserum. Samples are a 13,000*g* lysis pellet and contain protein from the equivalent of 2.5×10^5 cells.

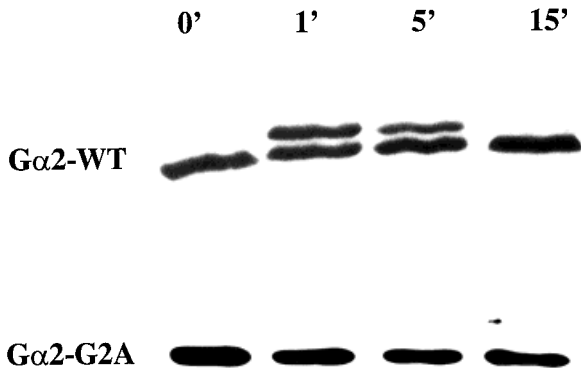

Fig. 9. Cyclic-AMP induced phosphorylation (gel shift) of Gα2-WT and Gα2-G2A. Aggregation competent cells expressing Gα2-WT or Gα2-G2A were stimulated with 1 μM cAMP and sampled after 0, 1, 5, and 15 min. Gα2-WT expressing cell samples (ammonium sulfate lysis pellets) and Gα2-G2A expressing cells (whole cells) were mixed with hot SDS sample buffer and separated by modified SDS-PAGE (see Materials and Methods). Both samples contain protein from the equivalent of 2.5×10^5 cells. Immunoblots were performed with the Gα2-specific antiserum.

DISCUSSION

Mutation of the putative *N*-myristoylation site on Gα2 results in physiological changes in *Dictyostelium discoideum*. The *N*-terminal sequence of the wild-type Gα2 contains recognition sequence for myristoylation plus a site for potential modification by palmitate at Cys4. The data clearly demonstrate that Gα2-WT is labeled by myristic acid most likely through an amide linkage at an N-terminal glycine. Cells expressing Gα2-G2A fail to aggregate on developmental (DB) agar plates, even when the cells are preconditioned with exogenous pulses of cAMP. The non-myristoylated Gα2-G2A is almost exclusively cytosolic and fails to activate guanylyl cyclase. How Gα2 activation of guanylyl cyclase is accomplished in *D. discoideum* is unknown, but this data suggest it may be dependent on membrane bound Gα2 or the presence of glycine or myristoylated glycine at position 2. Interaction with the cAMP receptor, cAR1, is markedly decreased though not completely absent (Table I). The low level of receptor coupling still present may account for the reduced level of adenylyl cyclase activation (Fig. 7B). It is important to note that activation of adenylyl cyclase in *D. discoideum* occurs via the βγ complex [Wu et al., 1995] and thus membrane bound α-subunit may not be a strict requirement. The partial activation of adenylyl cyclase may in part explain the observed aggregation and formation of fruiting bodies by a small percentage of cells grown on bacteria lawns. What exact conditions are necessary for this phenotype is unclear since the majority of Gα2-G2A cells do not aggregate. The use of DB agar plates presumably does not regenerate the conditions that occur on the bacterial lawn plates since only the aggregation-minus phenotype is observed.

Fatty acid attachment facilitates membrane association in a manner that is still unclear. Several mechanisms for lipid involvement in membrane adherence have been examined [Casey, 1995]. Traditionally, hydrophobic lipids are believed to insert into the bilayer, affording membrane association of the protein. Current views speculate that lipid moieties on proteins may target protein to the membrane where protein-protein interactions may stabilize membrane association [Wedegaertner and Bourne, 1995]. A current hypothesis suggests myristic acid supplies initial membrane binding at the endoplasmic reticulum, while localization to the plasma membrane is brought about by palmitoylation [Morales et al., 1998]. Binding of the myristoylated α-subunit to the βγ complex is currently thought to favor α-subunit palmitoylation and stronger membrane association. Turnover of the palmitoyl group upon G protein activation has been suggested as an additional means of G protein regulation [Wedegaertner et al., 1994]. Upon cell fractionation, Gα2-WT is localized in both pellet and cytosolic fractions. This difference is not the result of differential myristoylation since both fractions are labeled by [^{3}H]myristate. Cytosolic Gα2-WT appears to exist in part as a heterotrimer as demonstrated by its migration with the β-subunit in the presence of GDPβS and as a size larger than BSA in sucrose density gradients. The β-subunit is also found in both pellet and supernatant fractions in *D. discoideum* [Gundersen, Lilly, and Devreotes, unpublished observation]. GTPγS addition to the lysate supernatant should cause the dissociation of G protein heterotrimer and the monomeric α-subunit and the βγ complex should move up in the sucrose density gradient to reflect the change in size. Cytosolic Gα2-WT clearly changes peak fractions in the presence of GTPγS moving to fractions above or smaller in size than BSA. However, the β-subunit remains in the same fractions as when run with

GDPβS; the reason for this is unclear. In comparison, Gα2-G2A is found only in the cytosolic fraction (Fig. 3) and its migration in sucrose density gradient suggest that the majority of Gα2-G2A is not associated with the βγ complex. Gα2 in the β-null cell line LW6 is also clearly labeled when cells are incubated in [^{3}H]myristate (Fig. 5B) and is primarily cytosolic, although not completely. In addition, the biochemical responses of LW6 parallel those of Gα2-G2A [Wu et al., 1995]. These results indicate that N-terminal lipid modification may facilitate membrane association of Gα2 through interaction with the βγ complex found in the membrane. However, the observation that myristoylated Gα2-WT is localized to both the membrane pellet and the high-speed supernatant suggests that myristoylation of Gα2 alone is not responsible for membrane attachment.

Myristoylation of Gα2 in *Dictyostelium* is required for the cells to enter their developmental life cycle. Both the cytosolic and membrane forms of the G2 protein appear to exist as the heterotrimer as shown by co-migration of Gα2 with the β-subunit in sucrose density gradients. However, direct interaction between Gα2 and the βγ complex in both fractions has to be confirmed. We are currently attempting to generate a serum usable for co-immunoprecipitation experiments. The loss of the N-terminal glycine as the myristoylation site shifts G2 completely to the cytoplasmic fraction. More important, it seems to disrupt the G protein heterotrimer. While the loss of G2 function involved in cell aggregation is likely the result of dissociation from the membrane, it can not be ruled out that important functions of the cytoplasmic form of G2 are also lost by disruption of the heterotrimer. G protein α-subunit myristoylation appears to be key to the protein-protein interaction between the α-subunit and the βγ complex. What factors are involved in membrane association are less clear since myristoylated Gα2 can be identified in both membrane and soluble fractions. Possibly the answer lies in the palmitoylation of Gα2, although our preliminary data suggest this may not be the case and that additional unknown factors are involved (Gundersen, in preparation). We are also examining the hydrophobic properties of the membrane bound and cytosolic Gα2 in an attempt to understand the difference between these two Gα2 forms.

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