Journal of Cellular Biochemistry 66:268–276 (1997)

Phosphorylation of the G Protein α-Subunit, Gα2, of *Dictyostelium discoideum* Requires a Functional and Activated Gα2

Robert E. Gundersen*

Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, Maine 04469

Abstract The Gα2-subunit of *Dictyostelium discoideum* is essential to the initial stage of the cell's developmental life cycle. In response to the extracellular chemoattractant, cAMP, Gα2 is activated and transiently phosphorylated on serine-113 [Chen et al. (1994): J Biol Chem 269:20925–20930]. The role of Gα2 phosphorylation remains elusive; cells expressing the S113A, nonphosphorylated mutation of Gα2 appear to proceed through the developmental phase normally. To gain insight into the function of Gα2 phosphorylation, the conditions for Gα2 phosphorylation were examined using a variety of α-subunit point mutations and chimeras. Mutations that block the G protein activation cycle prior to or at the hydrolysis of GTP (Gα2-S45A, Gα2-G207A, and Gα2-Q208L) preclude Gα2 phosphorylation in vivo. Phosphorylation of the Gα2-Q208L mutation does however occur in an in vitro phosphorylation assay. It appears that Gα2 phosphorylation, shown previously in vivo to require the cAMP receptor, also requires signaling through the G2 pathway. Results from the in vitro assay suggest that the substrate for phosphorylation is the α-subunit monomer. J. Cell. Biochem. 66:268–276, 1997.

Key words: Gα2; phosphorylation; S113A; *Dictyostelium discoideum;* cAMP

The heterotrimeric, guanine nucleotide binding proteins (G proteins) function by coupling plasma membrane receptors to their signal generating systems. Ligand binding to a receptor activates a distinct G protein, by catalyzing the exchange of bound GDP for GTP on the Gα-subunit. The activated G α-subunit dissociates from Gβγ complex, and the free Gα-subunit and/or Gβγ complex then interact with effectors. An intrinsic GTPase activity of the G α-subunit hydrolyses the bound GTP to GDP and the Gα-subunit re-associates with the Gβγ complex to complete an activation cycle [reviewed in Simon et al., 1991; Neer, 1995].

Crystal structures for two G protein α-subunits [Lambright et al., 1994; Coleman et al., 1994] and recently the heterotrimer [Wall et al., 1995; Lambright et al., 1996] provide insight into how heterotrimeric G proteins function and are regulated. However, many unresolved questions remain. G protein α-subunits have been shown to be myristoylated, palmitoylated, and phosphorylated, yet how these covalent modifications affect G protein function is unclear. Much is already know about α-subunit myristoylation [Wedegaertner et al., 1995], but relatively less is understood for α-subunit palmitoylation and phosphorylation. The mammalian α-subunits, $G_z\alpha$ and $G_{i2}\alpha$, and *Dictyostelium* Gα2 are known to be phosphorylated upon activation [Carlson et al., 1989; Bushfield et al., 1990; Gundersen and Devreotes, 1990]. Several α-subunits have also been phosphorylated in vitro [Katada et al., 1985; Zick et al., 1986; Lounsbury et al., 1991; Pyne et al., 1992]. The function of α-subunit phosphorylation appears to represent an additional level of regulation in the G protein activation cycle [Fields and Casey, 1995; Strassheim and Malbon, 1994].

In *Dictyostelium discoideum*, G protein-mediated signal transduction is essential to the multicellular, developmental life cycle of this soil ameba [Chen et al., 1996; Gross, 1994]. The developmental phase is initiated by starvation of a population of amoebae, which induces the cells to aggregate. This process is driven by a chemotactic response of the cells to secreted

Contract grant sponsor: National Science Foundation, contract grant number MCB-9218823.

*Correspondence to: Robert E. Gundersen, Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469-5735.

Received 18 March 1997; accepted 21 April 1997

cAMP. The 'aggregation' phase is dependent on a G protein signal transduction pathway, using the Gα2-subunit [Kumagai et al., 1989]. The G2 signaling pathway in *Dictyostelium* is responsible for the cAMP-induced responses of cAMP signaling, chemotaxis, and changes in gene expression, all of which contribute to aggregation. Deletion of the gα2 gene results in the loss of cAMP-induced responses and cell aggregation [Kumagai et al., 1989]. Activated Gα2 directly stimulates phospholipase C in *Dictyostelium*. However, the significance of this response is unclear, since cells deleted of the only PLC gene retain a wild-type phenotype [Drayer et al., 1994]. cAMP signaling is mediated by activation of adenylyl cyclase, which occurs through the βγ complex and the cytosolic protein, CRAC [Lilly and Devreotes, 1994]. The chemotactic response is at least in part regulated through the activation of guanylyl cyclase. However the events leading from Gα2 to guanylyl cyclase activation remain unidentified.

While the essential nature of Gα2 has been clearly demonstrated, important questions remain concerning regulation of its activity. Gα2 is transiently phosphorylated on a single serine residue, S113, in response to the extracellular cAMP stimulus [Chen et al., 1994]. However, site-directed mutation of serine 113 to an alanine does not result in any obvious change to the developmental phenotype, leaving the significance of this modification unclear [Chen et al., 1994]. To examine the requirements for Gα2 phosphorylation, several Gα2 mutations were generated based on analogous mammalian α-subunit mutations. These mutations have been shown to block the G protein activation cycle at various stages. In $G_o\alpha$, mutation of Ser-47 inhibits the binding of GTP [Slepak et al., 1993]. In $G_s\alpha$ mutation of Gly-226 blocks the release of the βγ complex upon activation [Lee et al., 1992] and mutation of Gln-227 dramatically reduces GTPase activity [Graziano and Gilman, 1989]. These mutations occur at highly conserved amino acid residues in the α-subunit sequence within motifs required for GTP binding and hydrolysis. The corresponding residues in *Dictyostelium* Gα2 are Ser-45, Gly-207, and Gln-208. In addition, two α-subunit chimeras N236 and 33C [Chen et al., 1994] were examined. The chimeras are combinations of *Dictyostelium* Gα1 and Gα2. The letter refers to whether Gα2 is the N-terminus or the C-terminus portion and the number refers to the crossover amino acid on Gα2. Both chimeras were shown to be phosphorylated when expressed in the presence of wild-type Gα2 [Chen et al., 1994]. Each of the Gα2 mutations was transformed into a cell line deleted of the gα2 gene, MYC2 [Chen et al., 1994], and the resulting transformants were observed for their cell phenotype and for their ability to phosphorylate the expressed Gα2 both in vivo and in vitro. Results suggest that Gα2 phosphorylation requires a functioning Gα2 signaling pathway and that the substrate for phosphorylation is the monomeric form of the α-subunit.

METHODS

Cell Culture and Development

All cell lines were grown in a modified HL-5 medium at 22°C [Ashworth and Watts, 1970]; 1 L of growth medium contains, 10 g proteose peptone (Difco, Detroit, MI), 5 g yeast extract (Difco), 10 g dextrose, 3.6 mM KH_2PO4, 3.6 mM Na_2HPO4, and 30 mg dihydrostreptomycin. For all experiments, cells were made aggregation competent as previously described [Devreotes et al., 1987]. Briefly, cells at 2–6 $\times$ 10^6 cells/ml in HL-5 were pelleted by centrifugation at 700*g* for 4 min, resuspended in development buffer (DB, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, pH 6.2), and recentrifuged. The washed cells were resuspended in DB at 2 $\times$ 10^7 cells/ml, and cAMP (50 nM final concentration) was added every 6 min during hours 2–6 of starvation in DB.

Mutations

The site-specific mutations Gα2-G207A and Gα2-Q208L were generated by oligonucleotide-directed mutagenesis as described [Kunkel et al., 1987; Chen et al., 1994]. Generation of the Gα2-S45A, the Gα chimeras, N236 and 33C and transformation and expression of the mutations in *Dictyostelium* has been previously described [Chen et al., 1994].

Assays

The Gα2 mobility shift assay in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots were performed as described [Gundersen and Devreotes, 1990]. GTPγS inhibition of cAMP binding to membrane cAMP receptors was performed according to a standard protocol [Snaar-Jagalska and Van-Haastert, 1994]. Briefly, aggregation

competent cells were diluted from 2×10^7 cells/ml to 5×10^6 cells/ml with DB and shaken at 200 rpm for 30 min. Cells were centrifuged and washed once in DB followed by a second wash in AC buffer (40 mM Hepes-NaOH, 0.5 mM EDTA, pH 7.7) and finally resuspended in AC buffer supplemented with 250 mM sucrose to a density of 1×10^8 cells/ml. The cells were lysed by forcing them through two layers of a Nucleopore filter (5-µm pore size) and centrifuged at 13,000*g* for 10 min at 4°C. The pellet was washed once in AC buffer plus sucrose and once in phosphate buffer (PB, 10 mM Na_2HPO_4/KH_2PO_4, pH 6.5). The pellet was resuspended in PB at 4°C in a volume equivalent to 1×10^8 cells/ml. The assay contained 10 µl of [^{3}H]cAMP (5 nM final concentration; Amersham, 38 Ci/mmol), 10 µl of either dH_2O, GTPγS (100 µM final concentration) or cAMP (10 µM final concentration) plus 80 µl of the resuspended lysate pellet. Following incubation on ice for 5 min, the tubes were centrifuged at 14,000*g* for 3 min at 4°C, and the supernatant was carefully aspirated. The pellet was dissolved in 0.1 ml of 1% SDS. Scintillation fluid was added and [^{3}H]cAMP bound to the pellet was determined. All samples were done in triplicate. Radioactive cpm obtained in the presence of H_2O or GTPγS represents "total" cAMP bound and is subtracted from cpm in the presence of 10 µM cAMP (nonspecific) to give "specific" cAMP bound. The in vitro assay was performed with whole cell lysates. Aggregation competent cells were resuspended at 1×10^8 cells/ml in DB plus 4 mM caffeine to inhibit endogenous cAMP production [Brenner and Thoms, 1984] and shaken at 200 rpm. After 30 min, an aliquot of cells was mixed 1:1 with ice-cold lysis buffer (20 mM MES pH 6.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 M NaCl and a cocktail of protease inhibitors [Klein et al., 1987] and forced through two layers of a 5-µm-pore Nucleopore filter [Devreotes et al., 1987] into a microcentrifuge tube on ice. To start the assay, 40 µl of lysate was transferred to a set of tubes at room temperature that contained 10 µl of ATP (1 mM final concentration) and Na_3VO_4 (0.1 mM final concentration) and incubated for 0, 1, and 5 minutes. The reactions were stopped by the addition of an equal volume of 100°C, 2X-SDS sample buffer. Samples were separated by SDS-PAGE and analyzed for phosphorylation (gel shift) by immunoblotting with the Gα2 peptide antiserum. For radiolabeling experiments, the ATP concentration was reduced to 0.1 mM and 0.5 µCi of [γ-^{32}P]ATP (Amersham, >5,000 Ci/mmol) was added. Following the incubations, Gα2 was immunoprecipitated and subject to SDS-PAGE and autoradiography as previously described [Gundersen and Devreotes, 1990].

RESULTS

Mutant Phenotype and In Vivo Phosphorylation

Wild-type and each mutated gα2 sequence were transformed into the gα2-minus cell line MYC2. Clonal transformants were isolated and the resulting developmental phenotype noted (Table I). Re-introduction of the wild-type sequence (Gα2-wt) rescued the aggregation phenotype in MYC2 cells, as did the phosphorylation-blocked mutant Gα2-S113A and the Gα1/Gα2 chimera 33C. The Gα2 point mutants Gα2-S45A, Gα2-G207A, and Gα2-Q208L, as well as the Gα2/Gα1 chimera N236, all failed to rescue the aggregation phenotype in the MYC2. Each cell line was examined for phosphorylation by in vivo cAMP-induced mobility shift of the Gα2 protein on SDS-PAGE. Cells expressing wild-type Gα2 demonstrated a normal shift in mobility. The chimera 33C also showed a mobility shift, but to a lesser degree than wild type (Fig. 1A; Table I). The chimera N236 also showed a minimal amount of gel shift; however, the time course differed from that of the wild type. The shift of N236 is delayed being first apparent around 5 min. A similar delay in the onset of the gel shift was also observed when N236 was expressed in the presence of wild type Gα2 [Chen

TABLE I. Analysis of Gα2 Mutants*

Cell lines	Aggregation	Phosphorylation in vivo	Phosphorylation in vitro
Ax-3	+	+	+
MYC2 (Gα2−)	−	(−)	(−)
Gα2-wt	+	+	+
Gα2-S113A	+	−	−
Gα2-S45A	−	NS	−
Gα2-G207A	−	−	−
Gα2-Q208L	−	−	+
N236 (chimera)	−	+ (delayed)	−
33C (chimera)	+	+	ND

*Cells that aggregate into mounds within 24–48 h were designated +, while those that failed to aggregate after 48 h were −. Phosphorylation in vivo and in vitro are summarized from Figure 1. NS, not shown [refer to Chen et al., 1994]. ND, not determined. For MYC2, no Gα2 protein is present, so phosphorylation is absent (−).

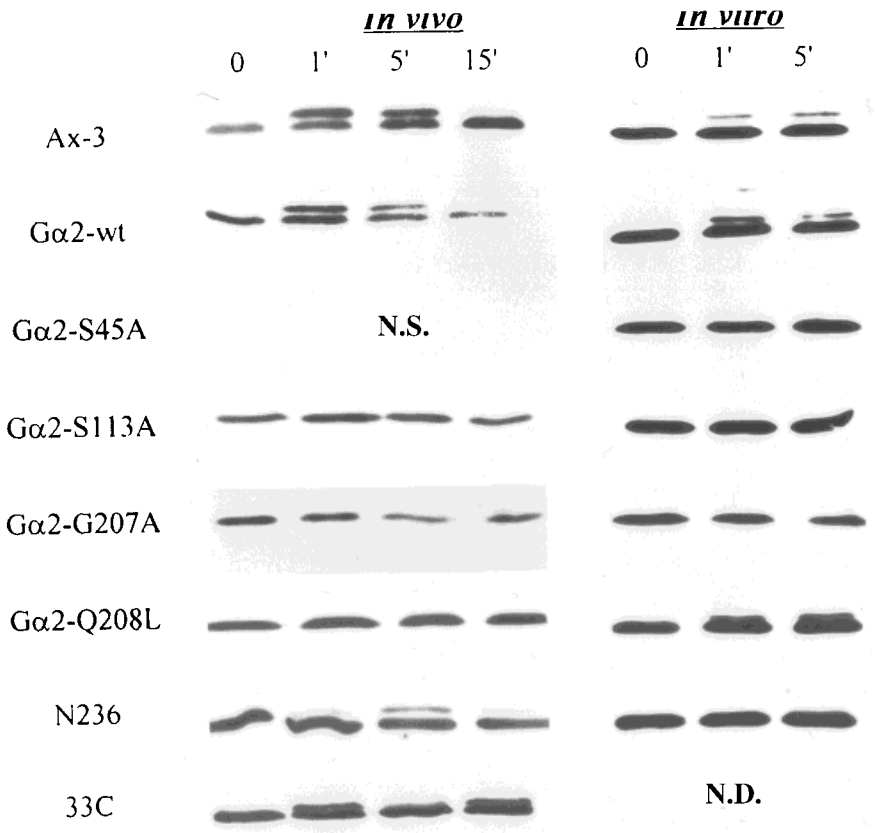

Fig. 1. Phosphorylation of various mutant Gα2 proteins as determined by mobility on SDS-PAGE in vivo and in vitro. Cells expressing Gα2-wt, the various mutant Gα2 proteins, or the chimeras were made aggregation competent and sampled under either in vivo or in vitro assay conditions. Samples were examined for mobility shift (phosphorylation) on SDS-PAGE by immunoblotting for Gα2, as described under Methods. The phosphorylation-induced mobility shift of Gα2 (approx. 40 kDa) on SDS-PAGE is seen in the appearance of a higher-MW band (approx. 42 kDa) in the 1 and 5 min lanes in both the in vivo and in vitro samples.

et al., 1994]. Mutations Gα2-G207A, Gα2-Q208L, and Gα2-S113A all failed to shift mobility following cAMP stimulation. Gα2-S45A is also not phosphorylated [Chen et al., 1994]. Since the site for phosphorylation is present in all the Gα2 mutants except Gα2-S113A, it is assumed that a failure to be phosphorylated results from an inability of the Gα2 mutants to serve as substrates for the responsible kinase, or that the receptor-mediated signal for Gα2 phosphorylation requires an activated G2 pathway.

Monomeric Form of Gα2 Is Required for Phosphorylation

To distinguish between these two possibilities, each mutant Gα2 was examined for phosphorylation using an in vitro assay. Following cell lysis, we have observed that a small but significant portion of Gα2 is phosphorylated without the requirement for exogenous cAMP. In vitro, Gα2 phosphorylation also appears within 1 minute and remains stable over 5 min (Fig. 1). In vitro labeling with [γ-^{32}P]ATP revealed that the phosphorylation of Gα2 was stable for at least 5 min (Fig. 2A, lanes 1–4), yet ^{32}P-labeling is lost in the presence of 5 mM unlabeled ATP (Fig. 2A, lanes 5–8). Also the ^{32}P-label is not rapidly tuning over, since a 1-min chase with 5 mM ATP did not decrease the level of ^{32}P-label on Gα2 (Fig. 2B). The results of the in vitro assay for each of the Gα2 mutations are shown in Figure 1 and summarized in Table I. In addition to the wild-type protein, only Gα2-Q208L became phosphorylated following cell lysis. Gα2-S113A, Gα2-S45A, Gα2-G207A, and the N236 chimera were not phosphorylated. The absence of Gα2-S113A phosphorylation in vitro suggests that the correct site, Ser-113, is being phosphorylated in the lysates. What form of Gα2-wt is being phosphorylated in vitro is uncertain but phosphorylation of Gα2-Q208L suggests that the kinase

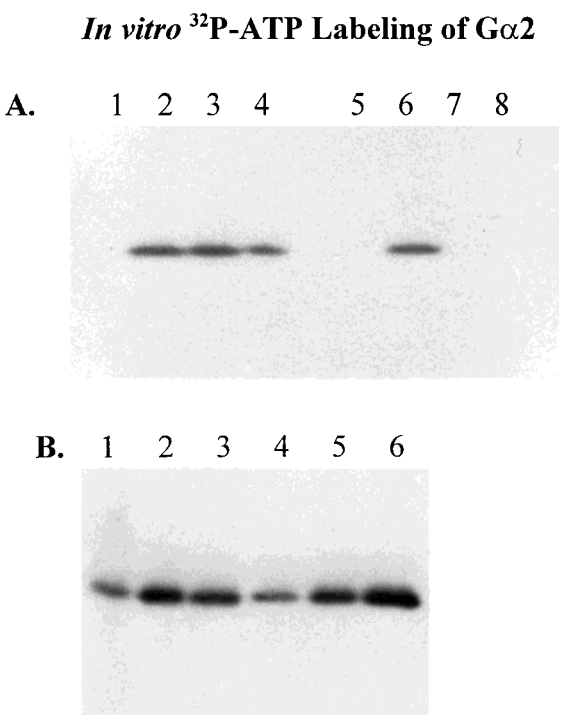

Fig. 2. In vitro Gα2 phosphorylation detected by ^{32}P-incorporation. Cells expressing Gα2-wild type (wt) were made aggregation competent and lysed for the in vitro Gα2 mobility shift assay as described in the Methods section. **A:** *Lanes 1–4,* lysates were incubated with [^{32}P-γ-]ATP for 0, 1, 2, and 5 min; *lanes 5, 6,* same as lanes 1 and 2; *lanes 7, 8,* also contained 5 mM unlabeled ATP. **B:** In a second experiment, Gα2 was ^{32}P-labeled as in A for 0, 1, and 2 min (*lanes 1–3*). For the chase, lysate labeled for 0 and 1 min (*lanes 4, 5*) had 5 mM unlabeled ATP added after 1 min of ^{32}P-labeling for an additional minute prior to immunoprecipitation (*lane 6*). ^{32}P-labeled Gα2 was isolated from the lysates by immunoprecipitation, separated on SDS-PAGE and subject to autoradiography.

recognizes the monomeric form of Gα2. Sucrose-density centrifugation gradients of membrane extracts confirmed that Gα2-Q208L is monomeric (data not shown). No phosphorylation occurred on Gα2-G207A. Its mammalian counterpart has been shown to bind GTP, but not to release the βγ complex and thus remains heterotrimic. Presumably, the G2 heterotrimer with GTP bound is not a substrate for the kinase in *Dictyostelium*. Sucrose density centrifugation gradients of Gα2-G207A confirmed that this mutant protein indeed exists as the heterotrimer even in the presence of GTPγS (data not shown). To determine whether the kinase that phosphorylates Gα2 is affected by cAMP, cGMP, Ca^{2+} or EGTA, each compound was added to wild-type lysates in the in vitro assay, but in each instance no difference was observed in the degree of Gα2 phosphorylation (data not shown).

Coupling to the cAMP Receptor

To determine which Gα2 mutant proteins were coupling to the cAMP receptor and thus initiating the G2 signaling pathways, the effect of GTPγS on cAMP binding was examined. G protein interaction with its receptor affects the receptor's ligand binding affinity [reviewed in Gilman, 1987]. G protein-coupled receptors generally show both high-affinity and low-affinity components in their ligand binding curves. The high-affinity state of the receptor indicates receptor-G protein coupling (interaction). Ligand binding stimulates release of GDP from the α-subunit and replacement with GTP. This results in release of the G protein from the receptor and separation of the α-subunit from the βγ complex. One consequence of the dissociation is the loss of receptor high-affinity binding. This general observation for G protein-coupled receptors holds true for the cAMP receptor of *Dictyostelium*. Scatchard analysis of cAMP binding to *Dictyostelium* membranes has revealed both high- and low-affinity binding components. The addition of GTPγS to the cAMP binding assay results in the loss of high-affinity cAMP binding [Van Haastert, 1985]. The effect of GTPγS on cAMP binding affinity in the various Gα2 mutants is presented in Table II. At 5 nM cAMP binding to membranes from wild type cells (Ax-3) is reduced 81% in the presence of GTPγS. The reduction is slightly less for Gα2-wt, Gα2-S113A, and the chimera 33C, being 70%, 66% and 76%, respectively. The mutations Gα2-S45A, Gα2-G207A, and Gα2-Q208L each show a smaller but significant reduction (approx. 50%) in cAMP binding in the presence of GTPγS

TABLE II. GTPγS Inhibition of cAMP Binding*

Cell lines	cAMP binding (% remaining)
Ax-3	19 ± 4 (n = 3)
MYC2 (Gα2−)	84 ± 11 (n = 5)
Gα2-wt	30 ± 3 (n = 5)
Gα2-S113A	34 ± 3 (n = 3)
Gα2-S45A	58 ± 6 (n = 4)
Gα2-G207A	55 ± 5 (n = 3)
Gα2-Q208L	53 ± 8 (n = 3)
N236 (chimera)	99 ± 19 (n = 5)
33C (chimera)	24 ± 6 (n = 5)

*GTPγS inhibition of high-affinity cAMP binding was performed on cell lysate pellets as described under Methods. Values reported are the % cpm remaining bound to membranes in the presence of GTPγS and are the mean ± SEM. n, the number of individual experiments.

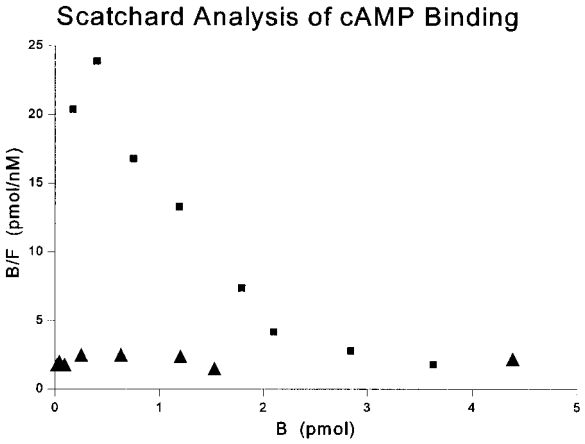

Fig. 3. Scatchard Analysis of cAMP binding to membranes from cells expressing Gα2-wt and the Gα2/Gα1 chimera N236. Cell lysate pellets were prepared from aggregation competent Gα2-wt (■) and N236 (▲) for the GTPγS inhibition of cAMP binding assay as described in the Methods section. For Scatchard analysis cAMP binding was carried out by varying the concentration of [^{3}H]cAMP from 1 nM to 200 nM. Specific cAMP binding was determined by subtracting Nonspecific binding (10 μM cAMP added) from total binding (dH_2O added). Each sample was done in triplicate.

in comparison with wild type. MYC2 and the chimera N236 each show no reduction in cAMP binding in the presence of GTPγS. The lack of an effect in the assay for MYC2 cells is thought to reflect the loss of the interaction of the G2 heterotrimer with the cAMP receptor. The absence of an effect for N236 suggests that this chimeric G protein does not interact with the cAMP receptor. To further define the state of the cAMP receptor in the N236 cell line, we examined cAMP binding by Scatchard analysis and found that N236 contains no high affinity sites in comparison to the Gα2-wt (Fig. 3). Immunoblots for cAR1 on the two cells lines revealed essentially equal levels of the receptor (data not shown).

DISCUSSION

Understanding the conditions required for Gα2 phosphorylation may help define the role of Gα2 phosphorylation in *Dictyostelium*. Phosphorylation of the *Dictyostelium* Gα2 occurs on Ser-113 [Chen et al., 1994], which according to the crystal structures of $G_t\alpha$ and $G_i\alpha$ [Lambright et al., 1994; Coleman et al., 1994] lies within the helical domain of the G protein α-subunit. No function or similar modification has been confirmed for the helical domain, although two reports have suggested a role for this domain in effector binding [Antonelli et al., 1994; Mixon et al., 1995]. Antonelli et al. suggest that *Xenopus* $G_s\alpha$ activation of adenylyl cyclase requires a sequence within the helical domain. The comparable sequence in *Dictyostelium* Gα2 contains Ser-113.

Does the signal for Gα2 phosphorylation require activation of the G2 signaling pathway, or is it independent of Gα2 function? Failure to detect Gα2 phosphorylation in a variety of Gα2 mutants that are unable to undergo cell aggregation, a response requiring Gα2 function, suggests that the signal for phosphorylation is dependent upon prior activation of the G2 heterotrimer. The mutations Gα2-G207A and Gα2-Q208L are analogous to constitutively activated mammalian α-subunits, yet they fail to be phosphorylated upon cAMP stimulation. Previous work with these mutations in *Dictyostelium* suggests that their aggregation-minus phenotype, which seems contrary to constitutive activation of the α-subunit, stems from adaptation of the signaling pathway [Okaichi et al., 1992]. Thus it seems that activation of the phosphorylation response must lie downstream of the adaptation. Additional evidence suggesting the requirement for an active G2 signaling pathway comes from observation that monomeric Gα2 in the β-minus cell line is not phosphorylated (data not shown). No Gα2-dependent responses are seen in the absence of the β-subunit in vivo [Wu et al., 1995]. The β-minus cell line LW6, which continues to express Gα2, fails to undergo aggregation [Lilly et al., 1993] and lacks G protein coupling to the cAMP receptor [Wu et al., 1995]. Whether Gα2 in the β-minus cell line has any guanine nucleotide bound is unknown. An additional consideration for Gα2 phosphorylation may also be its cellular location. Gα2 from the β-minus cells appears predominantly in a lysate supernatant rather than the pellet fraction as in wild-type cells and a nonmyristoylated mutant, Gα2-G2A is also not membrane localized and is not phosphorylated in vivo [Root and Gundersen, 1997]. However the mutant proteins produced and described here were all membrane associated (Fig. 1A).

An alternative interpretation of the data is that the mutated α-subunits are no longer suitable substrates for the kinase. In vitro phosphorylation of purified transducin by protein kinase C suggests that the substrate for phosphorylation is the monomeric GDP-bound form of transducin, since GTPγS blocked phosphorylation by

protein kinase C [Sagi-Eisenberg, 1989]. In the in vitro assay, phosphorylation of the Gα2-wt is independent of exogenous cAMP addition and is not affected by the addition of GTPγS. What form of Gα2-wt is being phosphorylated in vitro is uncertain. The observation that Gα2-Q208L is the only mutation phosphorylated in vitro suggests that the kinase can recognize the monomeric form of Gα2. No phosphorylation occurred on Gα2-G207A, which remains heterotrimic.

From these results, it is concluded that for Gα2 phosphorylation to occur in vivo, an active Gα2 signaling pathway is required and that Gα2 must be the activated monomer. Why, then, is the chimera N236 phosphorylated? Clearly N236 is not coupled to the cAMP receptor based on a lack of GTPγS inhibition of cAMP binding (Table II). N236 should remain as a heterotrimer in the cell and would not be phosphorylated if the kinase requires a monomeric substrate. A weak gel shift of N236 is observed although the time course is delayed. One possible explanation is that N236 phosphorylation does not occur on Ser-113, but on another serine, possibly a serine in the Gα1 portion of the chimera. We have been unable to identify the phosphorylation site on N236 by the same methods used to identify the phosphorylation site in wild-type Gα2 [Chen et al., 1994], due to very low levels of ^{32}P incorporation during attempts to radiolabel N236. Phosphorylation of Gα1 has not been observed using a variety of conditions (unpublished observations). One possible explanation for N236 phosphorylation requires an understanding of α-subunit–receptor coupling in *Dictyostelium*. A vast majority of the experimental evidence suggests that Gα2 is coupled to the cAMP receptor, cAR1 during the aggregation stage. Cells lacking a functional Gα2 show a clear loss of cAMP high-affinity binding and there is no effect of GTPγS on cAMP binding [Kumagai et al., 1989]. Gα1 may couple to cAR1 [Bominaar and Van Haastert, 1994] and/or to the CMF (conditioned media factor) receptor [R. Gomer, personal communication]. The C-terminal region of G protein α-subunits is essential for receptor coupling [Conklin and Bourne, 1993]. The results from the GTPγS inhibition of cAMP binding demonstrate that N236 does not affect cAMP binding and implies that Gα1 is not coupled to cAR1. The question remains, how does signaling occur through the chimera N236, which results in its phosphorylation? Usual activation events obviously do not occur, because N236 expression does not rescue the aggregation-minus phenotype of MYC2 (gα2−). N236 may become activated through coupling to the CMF receptor, activating the kinase and subsequent phosphorylation of S113 of N236. Presumably activation of the kinase occurs through the Gα2 portion (the N-terminal half) of N236. Alternatively an undetectable interaction of the cAMP receptor with the Gα1 (C-terminal portion) of N236 may lead to activation of the kinase. We are currently attempting to resolve this question.

CMF Is Not Required for Gα2 Phosphorylation

It was recently shown that a secreted protein, CMF, is required at a specific threshold concentration for in vivo cAMP-induced activation of adenylyl cyclase and guanylyl cyclase [Yuen et al., 1995]. This protein may function as a sensor of cell density and thus regulates initiation of the development phase of the *Dictyostelium* life cycle [Yuen et al., 1991]. To determine whether Gα2 phosphorylation is regulated by CMF, Gα2 gel mobility shift was tested at three different cell densities. The lowest cell density used (1×10^6 cells/ml for 30 min) should not accumulate sufficient CMF to activate the CMF-dependent pathways. Regardless of the cell density, cAMP addition induced a shift in mobility of Gα2 [Brazill et al., 1997]. This result suggests that the kinase responsible for Gα2 phosphorylation is not under the control of adenylyl cyclase or guanylyl cyclase, both of which are CMF-regulated pathways.

A role for Gα2 phosphorylation is yet to be determined, but the apparent need for an active G2 signaling pathway suggests that a feedback mechanism is involved. A series of cAMP receptor mutations has recently been described that separates Gα2-dependent and Gα2-independent signaling pathways [Milne et al., 1997]. Examination of these mutants in conjunction with Gα2 phosphorylation will help to confirm a requirement for Gα2 signaling in the phosphorylation event. In addition, a search for the kinase responsible for Gα2 phosphorylation may help define a role for the helical domain of the G protein α-subunit.

ACKNOWLEDGMENTS

I thank Alison Prince for her excellent technical assistance and two undergraduates, Krista Buldoc for her analysis of Gα2-S45A, and

Joshua Sparling for generating Gα2-G207A and Gα2-Q208L. Their work was supported by supplements from the NSF-Undergraduate Research Program.

REFERENCES

Ashworth JM, Watts DJ (1970): Metabolism of the cellular slime mould *Dictyostelium discoideum* grown in axenic culture. Biochem J 119:175–182.

Antonelli M, Birnbaumer L, Allende JE, Olate J (1994): Human-*Xenopus* chimeras of $G_{s\alpha}$ reveal a new region important for its activation of adenylyl cyclase. FEBS Lett 340:249–254.

Bominaar AA, Van Haastert PJM (1994): Phospholipase C in *Dictyostelium discoideum:* Identification of stimulatory and inhibitory surface receptors and G-proteins. Biochem J 297:189–193.

Brazill DT, Gundersen RE, Gomer RH (1997): A cell-density sensing factors regulates the lifetime of a chemoattractant-induced Gα-GTP conformation. FEBS Lett 404: 100–104.

Brenner M, Thoms SD (1984): Caffeine blocks activation of cyclic AMP synthesis in *Dictyostelium discoideum*. Dev Biol 101:136–146.

Bushfield M, Murphy GJ, Lavan BE, Parker PJ, Hruby VJ, Milligan G, Houslay MD (1990): Hormonal regulation of G_{i2} α-subunit phosphorylation in intact hepatocytes. Biochem J 268:449–457.

Carlson KE, Brass LF, Manning DR (1989): Thrombin and phorbol esters cause selective phosphorylation of a G protein other than G_i in human platlets. J Biol Chem 264:13298–13305.

Chen MY, Devreotes PN, Gundersen RE (1994): Serine 113 is the site of receptor-mediated phosphorylation of the *Dictyostelium* G protein α-subunit Gα2. J Biol Chem 269:20925–20930.

Chen MY, Insall RH, Devreotes PN (1996): Signaling through chemoattractant receptors in *Dictyostelium*. Trends Genet 12:52–57.

Coleman DE, Berghuis AM, Lee E, Linder ME, Gilman AG, Sprang SR (1994): Structures of active conformation of $G_{i\alpha1}$ and the mechanism of GTP hydrolysis. Science 265: 1405–1412.

Conklin RB, Bourne HR (1993): Structural elements of Gα subunits that interact with Gβγ, receptors, and effectors. Cell 73:631–641.

Devreotes P, Fontana D, Klein P, Sherring J, Theibert A (1987): Transmembrane signaling in *Dictyostelium*. Methods Cell Biol 28:299–331.

Drayer AL, Van der Kaay J, Mayr GW, Van Haastert PJM (1994): Role of phospholipase C in *Dictyostelium:* Formation of inositol 1,4,5-trisphosphate and normal development in cells lacking phospholipase C activity. EMBO J 13:1601–1609.

Fields TA, Casey PJ (1995): Phosphorylation of $G_{z\alpha}$ by protein kinase C blocks interaction with the βγ complex. J Biol Chem 270:23119–23125.

Gilman AG (1987): G proteins: transducers of receptor-generated signals. Annu Rev Biochem 85:615–649.

Graziano MP, Gilman AG (1989): Synthesis in *Escherichia coli* of GTPase-deficient mutants of $G_{s\alpha}$. J Biol Chem 264:15475–15482.

Gross JD (1994): Developmental decisions in *Dictyostelium discoideum*. Microbiol Rev 58:330–351.

Gundersen RE, Devreotes PN (1990): In vivo receptor-mediated phosphorylation of a G-protein in *Dictyostelium*. Science 248:591–593.

Katada T, Gilman AG, Watanabe Y, Bauer S, Jakobs KH (1985): Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenlate cyclase. Eur J Biochem 151:431–437.

Klein P, Knox B, Borleis J, Devreotes PN (1987): Purification of the surface cAMP receptor in *Dictyostelium*. J Biol Chem 262:352–357.

Kumagai A, Pupillo M, Gundersen R, Miake-Lye R, Devreotes PN, Firtel RA (1989): Regulation and function of Gα protein subunits in *Dictyostelium*. Cell 57:265–275.

Kunkle TA, Roberts JD, Zakour RA (1987): Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 154:367–383.

Lambright DG, Noel JP, Hamm HE, Sigler PB (1994): Structural determinants for activation of the α-subunit of a heterotrimeric G protein. Nature 369:621–628.

Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE, Sigler PB (1996): The 2.0A crystal structure of a heterotrimeric G protein. Nature 379:311–319.

Lee E, Taussig R, Gilman AG (1992): The G226A mutant of Gs-α highlights the requirement for dissociation of G-protein subunits. J Biol Chem 267:1212–1218.

Lilly PJ, Devreotes PN (1994): Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium*. J Biol Chem 269:14123–14129.

Lilly P, Wu LJ, Welker DL, Devreotes PN (1993): A G-protein β-subunit is essential for *Dictyostelium* development. Genes Dev 7:986–995.

Lounsbury KM, Casey PJ, Brass LF, Manning DR (1991): Phosphorylation of Gz in human platelets. J Biol Chem 266:22051–22056.

Milne JLS, Caterina MJ, Devreotes PN (1997): Random mutagenesis of the chemoattractant receptor, cAR1, of *Dictyostelium:* Evidence for multiple states of activation. J Biol Chem 272:2069–2076.

Mixon MB, Lee E, Coleman DE, Berghuis AM, Gilman AG, Sprang SR (1995): Tertiary and quaternary structural changes in $G_{i\alpha1}$ induced by GTP hydrolysis. Science 270: 954–960.

Neer EJ (1995): Heterotrimeric G proteins: Organizers of transmembrane signals. Cell 80:249–257.

Okaichi K, Cubitt AB, Pitt GS, Firtel RA (1992): Amino acid substitutions in the *Dictyostelium* Gα-subunit Gα2 produce dominant negative phenotypes and inhibit the activation of adenylyl cyclase, guanylyl cyclase, and phospholipase-C. Mol Biol Cell 3:735–747.

Pyne NJ, Freissmuth M, Pyne S (1992): Phosphorylation of the recombinant spliced variants of the a subunit of the stimulatory guanine nucleotide binding regulatory protein (Gs) by the catalytic subunit of protein kinase A. Biochem Biophys Res Commun 186:1081–1086.

Root P, Gundersen RE (1997): Myristoylation of Gα2-subunit in *Dictyostelium* is required for cell aggregation. In preparation.

Sagi-Eisenberg R (1989): GTP-binding proteins as a possible target for protein kinase C action. Trends Biochem Sci 14:355–357.

Simon MI, Strathmann MP, Gautam N (1991): Diversity of G proteins in signal transduction. Science 252:802–808.

Slepak VZ, Quick MW, Aragay AM, Davidson N, Lester HA, Simon MI (1993): Random mutagenesis of G protein α-subunit $G_o\alpha$. J Biol Chem 268:21889–21894.

Snaar-Jagalska BE, Van-Haastert PJM (1994): G-protein assays in *Dictyostelium*. Methods Enzymol 237:387–408.

Strassheim D, Malbon CC (1994): Phosphorylation of $G_{i\alpha 2}$ attenuates inhibitory adenylyl cyclase in neuroblastoma/glioma hybrid (NG-108-15) cells. J Biol Chem 269:14307–14313.

Van Haastert PJM (1985): The modulation of cell surface cAMP receptors from *Dictyostelium discoideum* by ammonium sulfate. Biochim Biophys Acta 845:254–260.

Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, Sprang SR (1995): The structure of the G protein heterotrimer $G_i\alpha 1\beta 1\gamma 2$. Cell 83:1047–1058.

Wedegaertner PB, Wilson PT, Bourne HR (1995): Lipid modifications of trimeric G proteins. J Biol Chem 270:503–506.

Wu LJ, Valkema R, van Haastert PJM, Devreotes PN (1995): The G protein β subunit is essential for multiple responses to chemoattractants in *Dictyostelium*. J Cell Biol 129:1667–1675.

Yuen IS, Taphouse C, Halfant KA, Gomer RH (1991): Regulation and processing of a secreted protein that mediates sensing of cell density in *Dictyostelium*. Development 113:1375–1385.

Yuen IS, Jain R, Bishop JD, Lindsey DF, Deery WJ, van Haastert PJM, Gomer RH (1995): A density-sensing factor regulates signal transduction in *Dictyostelium*. J Cell Biol 129:1251–1262.

Zick Y, Sagi-Eisenberg R, Pines M, Gierschik P, Spiegel AM (1986): Multisite phosphorylation of the α subunit of transducin by the insulin receptor kinase and protein kinase C. Proc Natl Acad Sci USA 83:9294–9297.