THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 33, Issue of August 19, pp. 20925–20930, 1994
Printed in U.S.A.

Serine 113 Is the Site of Receptor-mediated Phosphorylation of the *Dictyostelium* G Protein α-Subunit Gα2*

(Received for publication, April 14, 1994, and in revised form, June 7, 1994)

Mei-Yu Chen‡, Peter N. Devreotes‡, and Robert E. Gundersen§¶

From the ‡Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185 and the §Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, Maine 04469-5735

The G protein α-subunit Gα2 is essential to the developmental program of *Dictyostelium*. Gα2 is transiently phosphorylated on a serine residue(s) following stimulation with extracellular cAMP (Gundersen, R. E., and Devreotes, P. N. (1990) *Science* 248, 591–593). To aid in defining the function of α-subunit phosphorylation, we identified the site of Gα2 phosphorylation. Comparison of the isoelectric points (pI) of the phosphorylated and nonphosphorylated forms indicated that a single mole of phosphate is added to Gα2. Cleavage at tryptophan residues and immunoprecipitation with a specific peptide antibody localized the phosphorylated serine in the N-terminal 119 residues. Analysis of a series of Gα1 and Gα2 chimeras further confined the site between amino acids 33 and 215. Site-directed mutagenesis of serines between amino acids 33 and 119 produced two mutants that were not phosphorylated, S45A and S113A. Ser^{113} was identified as the site by sequential Edman degradation of ^{32}P-radiolabeled Gα2 digested with endoproteinase Glu-C. We have expressed the Gα2 mutants S113A, S113I, S113T, and S113D in a Gα2 null cell line to examine the function of phosphorylation.

The functioning of many transmembrane receptors is mediated through the action of heterotrimeric G proteins, which couple the receptors to their signal-generating systems. Agonist stimulation of a receptor leads to activation of a distinct G protein by catalyzing the exchange of bound GDP for GTP on Gα. GTP-bound Gα dissociates from Gβγ, and either free Gα or Gβγ is then able to interact with effectors. Gα has intrinsic GTPase activity, which can be accelerated by the effector. The bound GTP is hydrolyzed, and Gα returns to its GDP-bound form, which reassociates with Gβγ to complete an activation cycle (reviewed in Refs. 1 and 2).

Two mammalian α-subunits have been shown to be phosphorylated upon agonist activation of surface receptors. $G\alpha_z$ of platelets is phosphorylated in response to thrombin (3), and $G\alpha_{i2}$ in hepatocytes in response to a variety of ligands (4, 5). Several α-subunits have also been phosphorylated *in vitro* (6–9). The function of α-subunit phosphorylation could represent an additional level of regulation in the G protein activation cycle.

* This work was supported by National Institutes of Health Grant GM28007 (to P. N. D.) and by National Science Foundation Grant MCB-9218823 and a University of Maine faculty research grant (to R. E. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: 5735 Hitchner Hall, Dept. of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469-5735. Tel.: 207-581-2802; Fax: 207-581-2801.

In *Dictyostelium*, the G protein G2 is essential for the starvation-induced processes of aggregation and cell differentiation (10). Gα2 couples surface cAMP receptors to activation of phospholipase C (11). In addition, it is required for activation of adenylyl cyclase and guanylyl cyclase as well as receptor-mediated chemotaxis, although the connection is yet to be established. Loss of Gα2 function blocks activation of these effectors *in vivo* and stalls cells at the initiation of the developmental phase of the life cycle (12). Since Gα2 controls a variety of effectors, it may be subject to multiple types of regulation.

Gα2 was found to be transiently phosphorylated on a serine residue(s) following the binding of cAMP to the surface *Dictyostelium* cAMP receptor cAR1 (13). Prior to the addition of the stimulus, there is no phosphate on Gα2. The phosphorylation, reflected in a discreet decrease in electrophoretic mobility, is detectable within 10 s of application of the stimulus. Phosphorylation peaks after 1–2 min, with 50–90% of the protein phosphorylated at maximal cAMP concentrations. Dephosphorylation occurs during the following 10–15 min, even in the continued presence of the stimulus. Further phosphorylation cannot be induced until cells have been washed free of agonist and allowed to recover. Phosphorylation occurs at physiological doses of extracellular cAMP and does not occur in cells lacking cAMP receptors. In this paper, we identify serine 113 as the site of phosphorylation on *Dictyostelium* Gα2 through a combination of techniques: peptide mapping, construction of α-subunit chimeras, site-directed mutagenesis, and manual Edman degradation sequencing.

MATERIALS AND METHODS

Cell Culture and Development—All cells were grown in HL-5 medium at 22 °C (14). Gα2 expression induced in Ax-3 cells by starvation in development buffer, ^{32}P labeling, and immunoprecipitation of Gα2 were performed as described previously (13).

Cleavage of Gα2 with N-Chlorosuccinimide (NCS)[1]—^{32}P-Labeled Gα2 was isolated as described above and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Following autoradiography, gel slices were excised, washed in distilled H_2O and then cleavage buffer, and subjected to NCS (25 mM) cleavage as described by Lischwe and Ochs (15). Immunoprecipitation from the minced NCS-treated gel slices was performed as described above with mixing at room temperature for 18 h. The digest and immunoprecipitate were separated by SDS-PAGE using a 20% polyacrylamide gel system for low molecular weight proteins (16).

Proteolytic Digestion of Gα2 from Nitrocellulose—^{32}P-Labeled Gα2 was isolated by SDS-PAGE and transferred to nitrocellulose as described previously (13). Following autoradiography, ^{32}P-radiolabeled Gα2 bands were cut out, and the nitrocellulose pieces were blocked with 0.5% polyvinylpyrrolidone (M_r 40,000) in 0.1 M acetic acid for 30 min at 37 °C, followed by several washes with deionized water (17). For proteolytic digestion, the pieces of nitrocellulose were incubated in 50 mM

[1] The abbreviations used are: NCS, *N*-chlorosuccinimide; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GTPγS, guanosine 5′-*O*-(thiotriphosphate).

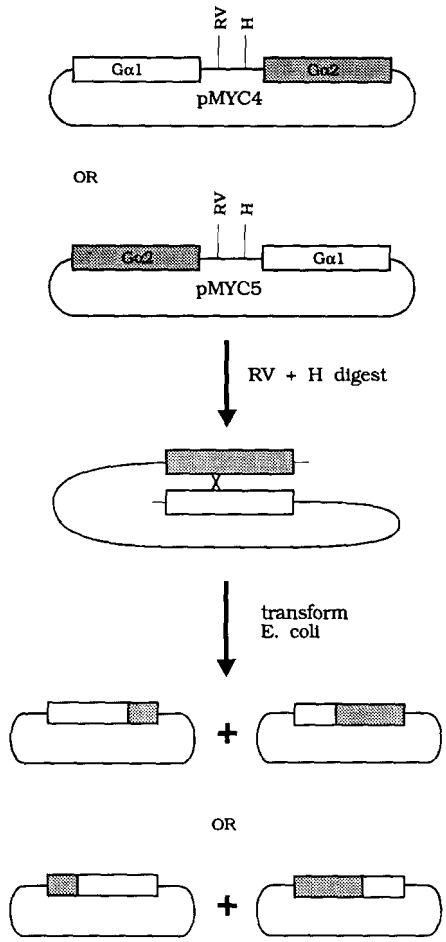


FIG. 1. **Random chimeragenesis.** See "Materials and Methods" for a detailed description. *RV*, *Eco*RV; *H*, *Hin*dIII.

ammonium bicarbonate, 5% acetonitrile, and 2–5 μg of protease (Glu-C or trypsin; Boehringer Mannheim) for 6–18 h at 22 °C for Glu-C or at 37 °C for trypsin. The liquid fraction was then removed, and the release of ^{32}P radiolabel was determined by Cerenkov counting. The digests were dried in a SpeedVac with a second evaporation from distilled H_2O. Peptides were dissolved in the appropriate buffers for TLC or manual sequencing. For manual sequencing, the proteolytic digests of Gα2 were coupled to Sequelon discs (Millipore). Cycles of phenyl isothiocyanate modification and trifluoroacetic acid cleavage were performed as described by Sullivan and Wong (18). ^{32}P-Labeled Kemptide (LRRASLG) was used as a standard for the manual sequencing.

Two-dimensional Gel Electrophoresis—Aggregation-competent cells at 5 × 10^7 cells/ml in development buffer were incubated with 1 mCi/ml Tran^{35}S-label (ICN) for 2 h. Saturated ammonium sulfate cell lysates were prepared (19) from unstimulated and 1-min cAMP-stimulated cells. ^{35}S-Labeled Gα2 was immunoprecipitated from the resulting pellets and isolated by SDS-PAGE and autoradiography. For the first dimension, gel pieces containing ^{35}S-labeled Gα2 were incubated in isoelectric focusing (IEF) sample buffer (9 M urea, 2% Chaps) and loaded onto 10-cm IEF tube gels (1.5-cm inner diameter; 9 M urea, 2% Chaps, 2% ampholytes pH 6–8/pH 3–10 (2.5:1), 4% acrylamide). Carbamylation was induced by heating the samples at 100 °C for 2 min. Tube gels were focused at 400 V for 16 h. For the second dimension, tube gels were incubated in SDS gel buffer (0.1 M Tris (pH 8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol plus bromphenol blue) and layered onto 10% SDS-polyacrylamide gels. Following electrophoresis, gels were soaked in 1 M sodium salicylate (to enhance the ^{35}S signal), dried, and placed at −80 °C with Kodak XAR-5 film for 14 days. The pH gradient in the IEF gels was determined by cutting a tube gel into 0.5-cm segments and incubating each in 2 ml of degassed 0.1 M NaCl for 30 min prior to measuring the pH.

Random Chimeragenesis—Fig. 1 illustrates this technique, which was first explored in Dr. Randall Reed's laboratory[2] and has recently been used to examine functional domains of the *Dictyostelium* cAMP receptor.[3] Two homologous genes, in this case Gα1 and Gα2, were cloned in tandem into Bluescript KS$^-$ (Stratagene). The two plasmids, pMYC4 for generating Gα1/Gα2 chimeras and pMYC5 for generating Gα2/Gα1 chimeras, were then linearized between the two inserts with *Eco*RV and *Hin*dIII digestion and transformed into *Escherichia coli* strain JM101. The transformants were screened for chimeras of these two genes by polymerase chain reactions using a sense Gα1 5′-primer (CGGGATCCATAAATAATGGGTAATATTTGTG) and an antisense Gα2 3′-primer (GCGGATCCTTAAGAATATAAACCAGC) or a sense Gα2 5′-primer (CGGGATCCTTAAAAAATGGGTATTTG) and an antisense Gα1 3′-primer (GCGGATCCTTAAAGAATCATACCAGCTTCAC). The junctions of sequences from the two genes in each chimera were then identified by the Sanger method of DNA sequencing (20). Each Gα1/Gα2 chimera is designated by a name beginning with a number followed by the letter "C," indicating that the Gα2 segment in the chimera starts from that number of amino acid residue and ends at the C terminus of Gα2. Similarly, each Gα2/Gα1 chimera is designated by a name beginning with the letter "N," followed by a number, indicating that the Gα2 segment in the chimera is from the N terminus to that number of amino acid residue of Gα2. In our experiments, a high portion of the transformants were found to be chimeras, and the crossover points occurred randomly at stretches of nucleotide identity. All the chimeras examined retained the correct reading frame after crossover points.

TABLE I
Oligonucleotides used for site-directed mutagenesis of Gα2

Sequence[a]	Mutation
GATTTACCAGCTTCACCAGC[b]	S42A
GAAATTGTTGCTTTACCAG[b]	S45A
TTTGTTTTGCAATTGTTG[b]	S48A
ATTCATCAAGGTGGTTAC	S57G
GTGGTTAC(G/A)(C/A)TAATGAAG[c]	S60A
CAATTGACCCA(G/A)GTAAT(G/T)CAAACGCAGC[c]	S96G or S98A
GAATTAACAGCATTACAAG	S109A
TTACAAGCAGCAATTGTTAC	S113A

[a] All of the oligonucleotides used are shown here in their 5′ → 3′ direction. All the oligonucleotides except for the ones indicated (Footnote *b*) are sense Gα2 sequences. Underlined nucleotides represent substitutions.

[b] Antisense Gα2 oligonucleotides.

[c] (G/A), (C/A), and (G/T) indicate degeneracy in these oligonucleotides.

Site-directed Mutagenesis—An *Eco*RI cDNA fragment containing the full length of Gα2 sequence was subcloned into M13mp19. Single-stranded DNA prepared from the recombinant bacteriophage was used as template in oligonucleotide-directed mutagenesis reactions performed as described previously (21). Table I summarizes the oligonucleotides used. Each mutation was confirmed by sequencing the single-stranded DNA isolated from individual plaques.

Expression and Analysis of α-Subunit Chimeras and Site-directed Gα2 Mutants in Dictyostelium—Gα1/Gα2 or Gα2/Gα1 chimeras generated in random chimeragenesis were moved as a *Bam*HI-*Bam*HI fragment into the *Bgl*II site of a G418^r marker containing *Dictyostelium* extrachromosomal vector pJK1 (22). Each site-directed Gα2 mutant was first moved as an *Eco*RI-*Eco*RI fragment into the *Eco*RI site of pPL1 (Bluescript KS$^-$ modified by the insertion of a *Bgl*II linker into the *Eco*RV site of the multiple cloning sequence) and then moved as a *Bam*HI-*Bgl*II fragment into the *Bgl*II site of pJK1. Expression constructs were introduced into *Dictyostelium* cells by electroporation as described (23). Stably transformed clones were selected in the presence of G418 (20 μg/ml).

Antisera—Specific anti-peptide antisera against Gα1 and Gα2 sequences near the N termini have been described previously (10). A peptide (Gα1C) corresponding to the C-terminal 13 amino acid residues was synthesized with a cysteine added to its amino end to enable cross-linking. The sequence of Gα1C was H_2N-CVLNLTLGEAGMIL-COOH. This was coupled to keyhole limpet hemocyanin (Sigma) and used to immunize a rabbit. High titer antiserum was obtained following the second boost.

Assays—The mobility shift experiments were done essentially as described (13). For analysis of developmental phenotypes, cells grown axenically in shaking cultures to 2–5 × 10^6 cells/ml were plated on Na^+/K^+ phosphate-buffered (pH 6.2) 2% agar plates as described previously (24). For cAMP chemotaxis assay (25), adenylate cyclase assay

[2] R. Reed, personal communication.

[3] J.-Y. Kim and P. N. Devreotes, submitted for publication.

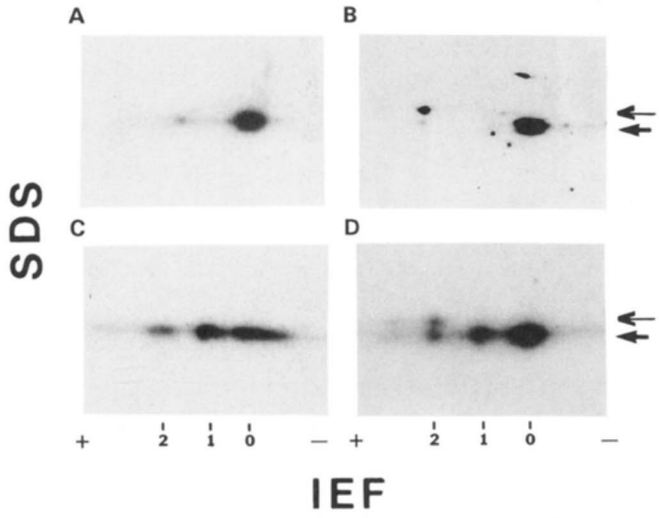


FIG. 2. **Two-dimensional gel electrophoresis of ^{35}S-labeled Gα2.** Gels were run as described under "Materials and Methods." The SDS and IEF dimensions are marked. The acidic (+) and basic (–) directions are indicated. *A*, Gα2 from unstimulated cells; *B*, Gα2 from cells stimulated with cAMP for 1 min; *C*, Gα2 (as in *A*) heated at 100 °C for 2 min to induce carbamylation; *D*, Gα2 (as in *B*) also heated. Nonphosphorylated Gα2 has a pI of ~7.4–7.5 and is marked by a *thick arrow* in the SDS dimension and above 0 in the IEF dimension. Phosphorylated Gα2 with a pI of ~7.0–7.1 is marked by a *thin arrow* in the SDS dimension and occurs above 2 in the IEF dimension. The *0*, *1*, and *2* in the IEF dimension identify unit changes in Gα2 charge due to carbamylation.

(26), cAMP secretion assay (24), and cGMP accumulation assay (27), cells were first developed at 2×10^7 cells/ml in development buffer for 4–6 h, and then assays were performed as described above.

RESULTS

Cyclic AMP stimulation triggers a rapid phosphorylation of Gα2 and a concomitant increase in its apparent molecular mass from 40 to 42 kDa on SDS-PAGE. This is a relatively common feature for phosphorylated proteins; removal of the phosphate reverses the effect. The discreet shift of the protein indicates that at least 1 mol of phosphate is added per mol of Gα2. Two-dimensional gel electrophoresis was used to further define the stoichiometry. When ^{35}S-labeled Gα2 from unstimulated cells was immunoprecipitated and subjected to analysis, it ran as a single spot with a pI of ~7.4–7.5 in 9.5 M urea (Fig. 2*A*). When it was prepared from cAMP-stimulated cells, the phosphorylated species displayed the expected higher molecular mass and a lower pI (Fig. 2*B*). The decrease in pI is expected to be proportional to the number of phosphates added. The extent of lowering of the pI per charge added, which is unique for each protein, can be determined by modifying the protein in single charge units. In this case, Gα2 was carbamylated by heating in the presence of urea (Fig. 2, *C* and *D*). A series of spots appeared extending toward the acidic end of the IEF gel resulting from the modification of individual lysine residues and the sequential loss of single positive charges (identified in Fig. 2 with *0*, *1*, and *2*). The phosphorylated form of Gα2 was shifted 2 charge units in the acidic direction (Fig. 2*D*), which is the charge expected for a single phosphate at a pH of 7.5–7.6 (28).

The determination that Gα2 is modified at a single serine simplified the task of identifying the residue. Gα2 contains 26 serine residues in its amino acid sequence. To narrow down the number of possible candidates, Gα2 was treated with NCS, which is predicted to cleave at its 5 tryptophan residues, yielding six peptides of varied masses (Fig. 3*A*). NCS digestion of ^{32}P-labeled Gα2 produced a labeled band of ~18 kDa (Fig. 3*B*). The minor bands of larger molecular masses presumably reflect incomplete digestion based on the sizes of the expected products. Immunoprecipitation of ^{32}P-labeled Gα2 (*lane 1*) and the ^{32}P-labeled NCS-generated fragment (*lane 2*) with a N terminus-specific antiserum identified the labeled peptide as the N terminus of Gα2. No labeled peptides remained in either immunoprecipitation supernatant (*lanes 3* and *4*). The preimmune serum also failed to precipitate labeled peptides (*lane 5*). The N-terminal fragment produced by NCS digestion presumably was generated by cleavage at Trp^{119}, although cleavage might instead have occurred at Trp^{134}. In either case, both peptides contain 12 serines as possible sites for the cAMP-induced phosphorylation.

To more closely localize the substrate for the Gα2 kinase, a series of Gα2/Gα1 or Gα1/Gα2 chimeras was generated using the "random chimeragenesis" technique as described under "Materials and Methods." Gα1, another *Dictyostelium* G protein α-subunit, does not undergo receptor-mediated phosphorylation.[4] Since we did not know whether a functional Gα2 protein is required for the phosphorylation response, we chose to express these chimeras in a gα1$^-$ cell line. We monitored the receptor-mediated shift in electrophoretic mobility of the Gα2/Gα1 and Gα1/Gα2 series by immunoblotting with either a C-terminal or an N-terminal Gα1 peptide antiserum, respectively.

Fig. 4 illustrates the nine different chimeras tested and summarizes the results of mobility shift experiments on these chimeras. Among the Gα2/Gα1 chimeras analyzed, N215 and N236 responded to the stimulus, while N47, N55, and N101 did not. Among the Gα1/Gα2 chimeras, only 33C responded; none carrying shorter Gα2 segments displayed a shift. These data position the substrate for the Gα2 kinase in the region between amino acids 33 and 215. If only a few residues surrounding the target serine are required for phosphorylation, the results suggest that the substrate lies between amino acids 101 and 154 (N101 and 154C) (Fig. 4). Taking into account the results of NCS experiments, the possible C-terminal boundary of the region can be adjusted from amino acid 154 to amino acid 119. Together, these results suggest the phosphorylated serine to be either Ser^{109} or Ser^{113}.

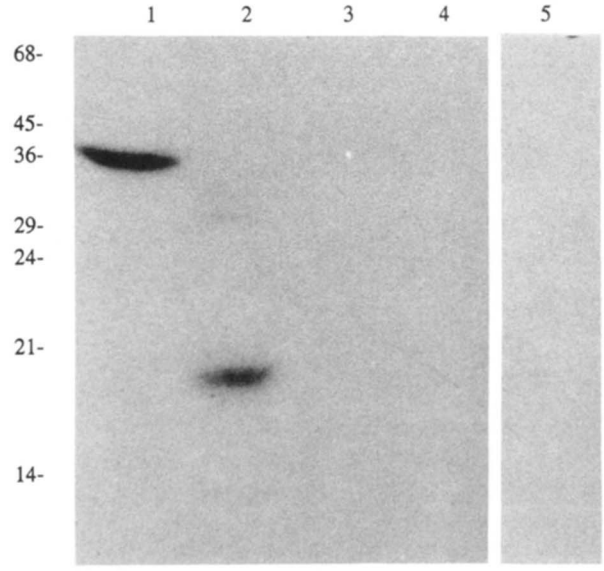
Since it was theoretically possible that 1 of the 9 serines between amino acids 33 and 119 was the target, each was changed by site-directed mutagenesis to either glycine or alanine. The mutated Gα2 sequences were then expressed in a gα2$^-$ cell line, and the phosphorylation of the mutant proteins was examined by electrophoretic mobility shift assay. Fig. 5*A* shows an example of the mobility shift assay using mutants S96G, S98A, S109A, and S113A, and the results for all of the mutants are summarized in Fig. 5*B*. The mutants S42A, S48A, S57G, S60A, S96G, S98A, and S109A each responded to some degree and were eliminated as candidates for the phosphorylation site. Two mutants, S45A and S113A, completely failed to undergo mobility shifts. Furthermore, cells expressing S113A were labeled *in vivo* with $^{32}P_i$ and incorporated no label in response to cAMP stimulation (data not shown).

Ser^{113} was identified as the site of phosphorylation through sequential Edman degradation. ^{32}P-Labeled Gα2 was treated with endoproteinase Glu-C, followed by manual sequencing of the digest. Manual Edman degradation released the majority of counts at cycle 7 (Fig. 6). Following Glu-C digestion, Ser^{45} is the fourth residue in its peptide, while Ser^{113} is the seventh amino acid in its peptide. Three other serine residues (Ser^7, Ser^{48}, and Ser^{155}) would also be at position 7 in the Glu-C-generated peptides (Fig. 2*A*), but none are candidates for the site of phosphorylation (see "Discussion"). Digestion of ^{32}P-labeled Gα2 first with trypsin followed by endoproteinase Glu-C also released the ^{32}P label after seven cycles of manual Edman degradation, again consistent with Ser^{113} as the phosphorylation site (data not shown).

[4] R. E. Gundersen and P. N. Devreotes, unpublished data.

	AA#'s	Sequence	MW
1.	1-119	MGICASS•MEGEKTNTDINLSIEKRKKKHNEVKLLLLGAGE**S**GK**S**TI**S***K QMKIIHQ**S**GY**S**NEERKEFKPIITRNILDNMRVLLDGMGRLGMTIDP**S**N**S** DAAVMIKELT**S**LQA**S***IVTDCW	13,212
2.	120-134	GELNEDQGKKIKALW	1,729
3.	135-215	TDPGVKQAMRRANEFSTLPDS•APYFFDSIDRMTSPVYIPTDQDILHT RVMTRGVHETNFEIGKIKFRLVDVGGQRSERKKW	9,387
4.	216-261	LSCFDDVTAVVFCVALSEYDLLLYEDNSTNRMLESLRVFSDVCNSW	5,284
5.	262-311	FVNTPIILFLNKSDLFREKIKHVDLSETFPEYKGGRDYERASNYIKERFW	6,112
6.	312-357	QINKTEQKAIYSHITCATDTNNIRVVFEAVKDIIFTQCVMKAGLYS	5,175

FIG. 3. **Identification of NCS peptide containing cAMP-induced ^{32}P label of Gα2.** *A*, shown are the six potential NCS peptides of Gα2 and their calculated molecular weights (*MW*). The 9 serines changed by site-directed mutagenesis are *enlarged* and in *boldface*. Ser^{113}, the site of Gα2 phosphorylation, is also *double-underlined*. The *asterisks* following Ser^{7}, Ser^{48}, Ser^{113}, and Ser^{155} identify the serines that occur as a seventh residue in peptides generated by endoproteinase Glu-C digestion. *B*, Gα2 was ^{32}P-labeled and then treated with NCS, subjected to immunoprecipitation, and separated by SDS-PAGE. *Lane 1*, immunoprecipitation of undigested Gα2; *lane 2*, immunoprecipitation of NCS-digested Gα2; *lane 3*, immunoprecipitation supernatant of *lane 1*; *lane 4*, immunoprecipitation supernatant of *lane 2*; *lane 5*, immunoprecipitation of NCS-digested Gα2 with preimmune serum.



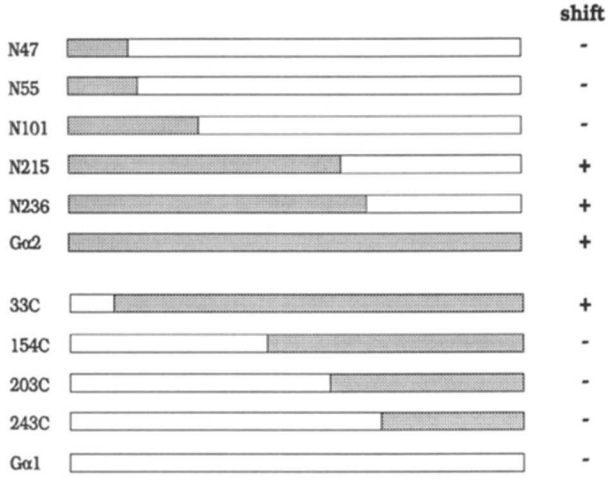


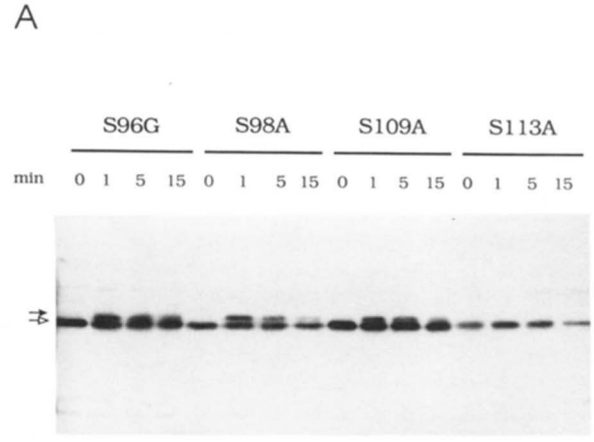
FIG. 4. **Gel mobility shift assay of Gα2/Gα1 and Gα1/Gα2 chimeras generated by random chimeragenesis.** All chimeras were expressed in a gα1$^-$ cell line. Gel mobility shift experiments were performed as described under "Materials and Methods." Protein samples were electrophoresed on 10% SDS-polyacrylamide gels and analyzed by immunoblots. Gα2/Gα1 chimeras were blotted by a Gα1 C terminus-specific peptide antiserum, while Gα1/Gα2 chimeras were blotted by a Gα1 N terminus-specific peptide antiserum. The presence (+) and absence (–) of shifting of the chimera proteins in response to cAMP stimulation are indicated.

The developmental phenotype of each serine mutant was examined by expressing each in a Gα2 null background (gα2$^-$ cells). All were wild-type, demonstrating normal aggregation and fruiting body formation, except S42A and S45A. S42A was able to aggregate and form fruiting bodies, but was slower by ~12–24 h, while S45A failed to aggregate. The inability of S45A to aggregate suggests that this mutation produces a major functional defect in the α-subunit and will be discussed below. The S113A mutant expressed in gα2$^-$ cells showed no obvious difference in its pattern of aggregation and morphogenesis when compared in parallel with gα2$^-$ cells expressing wild-type Gα2. Analyses of chemotaxis to cAMP, accumulation of cAMP and cGMP, and cAMP secretion in a perfusion assay have also not revealed any obvious defect in the S113A mutant. The substitution mutants S113D, S113I, and S113T were also expressed in the gα2$^-$ cells and displayed essentially wild-type aggregation patterns.[5]

DISCUSSION

The data presented illustrate that *Dictyostelium* Gα2 is phosphorylated on a single serine residue (Ser^{113}) in response to cAMP stimulation. IEF electrophoresis of phosphorylated Gα2 suggests that a single phosphate is covalently attached to Gα2, although it is possible that a second phosphorylation site of 5% or less may be undetectable. The site was mapped to the N-terminal region by chemical cleavage and by analysis of Gα2/Gα1 and Gα1/Gα2 chimeras. Site-directed mutagenesis gener-

[5] M.-Y. Chen and P. N. Devreotes, unpublished data.



B

	shift
S42A	+
S45A	-
S48A	+
S57G	+
S60A	+
S96G	+
S98A	+
S109A	+
S113A	-

FIG. 5. **Gel mobility shift assay of Gα2 serine mutants.** *A*, an example of the gel mobility shift experiment using mutants S96G, S98A, S109A, and S113A. These mutants were expressed in a $g\alpha2^-$ cell line. Gel shift assays were performed as described under "Materials and Methods," and the protein samples were subjected to immunoblots using the Gα2 N terminus-specific antiserum. Samples prior to cAMP stimulation (*0*) and after cAMP stimulation for 1 min (*1*), 5 min (*5*), and 15 min (*15*) are shown for each mutant. *Open arrow*, the unshifted chimera protein band; *close arrow*, the shifted chimera protein band. *B*, a summary of the results of gel mobility shift assays for all the Gα2 serine mutants analyzed.

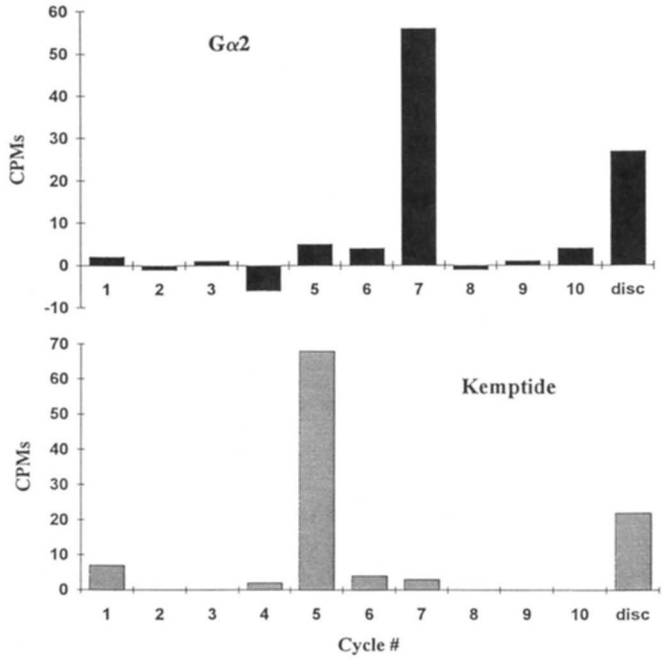


FIG. 6. **Manual Edman degradation sequencing of ^{32}P-labeled Gα2 and Kemptide.** Edman degradation was performed as outlined under "Materials and Methods." The radioactivity released after each hydrolysis step is plotted. In this experiment, the counts for Gα2 represent ~42% of the counts transferred to the nitrocellulose. Release from nitrocellulose by Glu-C digestion was 68%, while ~62% of that coupled to the Sequelon disc. A roughly equivalent number of counts from ^{32}P-labeled Kemptide were coupled to the Sequelon discs and processed to compare recovery efficiency.

ated two mutants (S45A and S113A) that failed to be phosphorylated in response to cAMP stimulation. Edman degradation sequencing of endoproteinase Glu-C digests released the ^{32}P label after cycle 7, consistent with phosphorylation at Ser^{113} rather than Ser^{45}. Following Glu-C digestion, 3 other serine residues (Ser^7, Ser^{48}, and Ser^{155}) within the Gα2 sequence would be at position 7 in their peptides (see Fig. 3*A*), but each can be eliminated as the phosphorylation site. Ser^{155} is beyond position 119, the site of NCS cleavage (Fig. 3); the Gα1/Gα2 chimera that crosses over at amino acid 33 is phosphorylated and does not contain Ser^7 (Fig. 4); and the S48A mutant is still phosphorylated in response to cAMP (Fig. 5*B*). A double digest of ^{32}P-labeled Gα2 with trypsin and endoproteinase Glu-C also released the ^{32}P label after cycle 7, inconsistent with Ser^{48}, which in this case would be at position 4.

Although our data suggest that Ser^{45} is not the site of cAMP-induced phosphorylation of Gα2, the Gα2 S45A mutant is not phosphorylated upon cAMP stimulation. In addition, cells expressing the S45A mutation have an aggregation-minus phenotype. A possible explanation for these observations is that S45A mutation produces an inactive α-subunit that can neither carry out Gα2 functions nor serve as a substrate for the Gα2 kinase. Ser^{45} lies within a highly conserved α-subunit sequence. In Ras and transducin, the backbone amide of the equivalent serine is involved in binding the β-phosphate of GTP, and the hydroxyl group serves in coordinating the essential Mg^{2+} ion (29, 30). A mammalian Gαo mutation (S47C; equivalent to Ser^{45} in Gα2) is unable to bind GTPγS and produces a dominant negative phenotype when expressed in *Xenopus* oocytes (31). Gα2 S45A is being tested for its level of function. It appears that this Gα2 mutant is also unable to be activated by GTPγS since it, unlike wild-type Gα2 and α-subunits in general (32), fails to be protected from tryptic digestion in the presence of GTPγS.[6] It is possible that this defect in S45A prevents it from serving as a substrate for the Gα2 kinase.

An examination of the point in the G protein cycle at which phosphorylation occurs will be important in determining the criteria for Gα2 phosphorylation. It has been shown that isolated transducin, the α-subunit that couples rhodopsin to cGMP phosphodiesterase in rod cells, was phosphorylated by purified protein kinase C only after GTP was added to the reaction (33). GTP hydrolysis to GDP was also required since transducin was not phosphorylated in the presence of GTPγS (33). In Gα2, the results from mutant S45A likewise suggest that the protein must be able to bind GTP to be phosphorylated. Two Gα2 mutants, G40D and Q208L (34), produce an aggregation-minus phenotype when substituted for the wild type, which has been attributed to a constitutive adaptation induced by the activated proteins. The cognate mutations in $G\alpha_s$, G49V and Q227L (35), reduce the rate of GTP hydrolysis by 4- and 100-fold, respectively. We have noted that G40D can be partially phosphorylated despite the cell line's aggregation-minus phenotype.[4] We are currently testing Q208L to definitively determine whether GTP hydrolysis is required for Gα2 phosphorylation.

It is unclear whether or not signal transduction through Gα2 is required to activate the phosphorylation response. While cAR1 is absolutely required and does couple to Gα2, some evidence suggests that a functional Gα2 is not necessary. For example, even though the Gα2/Gα1 chimera N215 fails to rescue the aggregation-minus phenotype of $g\alpha2^-$ cells,[5] it is phosphorylated in response to the stimulus, *i.e.* N215 is activated by cAR1, but is unable to rescue the aggregation-minus cells probably because it does not activate the proper effectors as Gα2 does. This suggests that Gα2 may not be in the pathway leading to the activation of Gα2 kinase, unless the "pathway" con-

[6] K. Bolduc and R. E. Gundersen, manuscript in preparation.

sists merely of the α-subunit being activated and thereby becoming a substrate. These questions should be answered through identification of the kinase and its regulation by the cAMP receptor.

The phosphorylation site of $G\alpha_z$ from platelets has been identified primarily as serine 27, with possible secondary sites being serines 16 and 25. The N-terminal location of these sites led to the speculation that phosphorylation of Gαz may play a role in βγ complex interaction (36). The only equivalent serine in Gα2 is Ser^{20}, which is not the site of Gα2 phosphorylation (Fig. 4). Thus, a similar function for Gα2 phosphorylation seems unlikely since Ser^{113} is not positioned near the N terminus as defined by the recently published three-dimensional structure of transducin (30).

Ser^{113} is located in what Noel *et al.* (30) have termed the "helical domain" between αB and αC in the three-dimensional structure of transducin. No function has been definitively assigned to this domain, although it has been speculated to act as an endogenous GTPase-activating protein or as a lid for the GTP-binding pocket. It appears that Ser^{113} would be exposed on the surface at the base of the helical domain, ideally positioned to behave as a substrate for a kinase. What role phosphorylation in this domain might play is unclear, but it does suggest that functioning of the helical domain can be regulated. One possible function for Ser^{113} phosphorylation may be to enhance the on/off state of the cycling α-subunit. Phosphorylation on the lid to the GTP-binding pocket may serve to keep the pocket either opened or closed, thus enhancing activity or inactivity. We are in the process of isolating phosphorylated and nonphosphorylated Gα2 in order to analyze GTP loading and GTPase activity of the two forms to address this possibility. Obviously, the effect of Gα2 phosphorylation is subtle and will require this more detailed biochemical analysis.

Acknowledgments—We thank Ji-Yun Kim for providing pJK1 and Pamela Lilly for providing pPL1.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56,** 615–649
2. Bourne, H. R., Sanders, D. A., and McCormick, F. (1990) *Nature* **348,** 125–131
3. Carlson, K. E., Brass, L. F., and Manning, D. R. (1989) *J. Biol. Chem.* **264,** 13298–13305
4. Pyne, N. J., Murphy, G. J., Milligan, G., and Houslay, M. D. (1989) *FEBS Lett.* **243,** 77–82
5. Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G., and Houslay, M. D. (1990) *Biochem. J.* **268,** 449–457
6. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S., and Jakobs, K. H. (1985) *Eur. J. Biochem.* **151,** 431–437
7. Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschik, P., and Spiegel, A. M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9294–9297
8. Lounsbury, K. M., Casey, P. J., Brass, L. F., and Manning, D. R. (1991) *J. Biol. Chem.* **266,** 22051–22056
9. Pyne, N. J., Freissmuth, M., and Pyne, S. (1992) *Biochem. Biophy. Res. Commun.* **186,** 1081–1086
10. Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1989) *Cell* **57,** 265–275
11. Bominaar, A. A., and van Haastert, P. J. M. (1994) *Biochem. J.* **297,** 189–193
12. Kumagai, A., Hadwiger, J. A., Pupillo, M., and Firtel, R. A. (1991) *J. Biol. Chem.* **266,** 1220–1228
13. Gundersen, R. E., and Devreotes, P. N. (1990) *Science* **248,** 591–593
14. Ashworth, J., and Watts, D. (1970) *Biochem. J.* **119,** 171–174
15. Lischwe, M. A., and Ochs, D. (1982) *Anal. Biochem.* **127,** 453–457
16. Giulian, G. G., Shanahan, M. F., Graham, J. M., and Moss, R. L. (1985) *Fed. Proc.* **44,** 686
17. Aebersold, R. (1993) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., ed) 2nd Ed., pp. 103–124, Academic Press, Inc., San Diego, CA
18. Sullivan, S., and Wong, T. W. (1991) *Anal. Biochem.* **197,** 65–68
19. Vaughan, R. A., and Devreotes, P. N. (1988) *J. Biol. Chem.* **263,** 14538–14543
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–383
22. Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., and Devreotes, P. N. (1992) *Cell* **69,** 305–315
23. Dynes, J. L., and Firtel, R. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7966–7970
24. Devreotes, P., Fontana, D., Klein, P., Sherring, J., and Theibert, A. (1987) *Methods Cell Biol.* **28,** 299–331
25. Konijn, T. M., and Van Haastert, P. J. M. (1987) *Methods Cell Biol.* **28,** 283–298
26. Roos, W., and Gerisch, G. (1976) *FEBS Lett.* **68,** 170–172
27. Kesbeke, F., Snaar-Jagalska, B. E., and van Haastert, P. J. M. (1988) *J. Cell Biol.* **107,** 521–528
28. Cooper, J. (1991) *Methods Enzymol.* **201,** 251–261
29. Pai, E. F., Kabsch, W., Krengel, U., Holmes, K. C., John, J., and Wittinghofer, A. (1989) *Nature* **341,** 209–214
30. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) *Nature* **366,** 654–663
31. Slepak, V. Z., Quick, M. W., Aragay, A. M., Davidson, N., Lester, H. A., and Simon M. I. (1993) *J. Biol. Chem.* **268,** 21889–21894
32. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem.* **259,** 13806–13813
33. Sagi-Eisenberg, R. (1989) *Trends Biochem. Sci.* **14,** 355–357
34. Okaichi, K., Cubitt, A. B., Pitt, G. S., and Firtel, R. A. (1992) *Mol. Biol. Cell* **3,** 735–747
35. Graziano, M. P., and Gilman, A. G. (1989) *J. Biol. Chem.* **264,** 15475–15482
36. Lounsbury, K. M., Schlegel, B., Poncz, M., Brass, L. F., and Manning, D. R. (1993) *J. Biol. Chem.* **268,** 3494–3498