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Regulation of Hematopoietic-Specific G-Protein $G\alpha_{15}$ and $G\alpha_{16}$ by Protein Kinase C

Jennifer L. Gu,[1] Wei Lu,[2] Chunzhi Xia,[2] Xiushan Wu,[3] and Mingyao Liu[2]*

[1]Division of Biology, California Institute of Technology, Pasadena, California 91125
[2]Center for Cancer Biology, Alkek Institute of Biosciences and Technology, and Department of Medical Biochemistry and Genetics, Texas A&M University System Health Science Center, Houston, Texas 77030
[3]College of Life Sciences, Hunan Normal University, Changsha, Hunan 410081, China

Abstract Heterotrimeric G proteins mediate cell growth and differentiation by coupling cell surface receptors to intracellular effector enzymes. The G-protein α subunit, $G\alpha_{16}$, and its murine homologue $G\alpha_{15}$, are expressed specifically in hematopoietic cells and their expression is highly regulated during differentiation of normal and leukemic cells. In this study, we examined the phosphorylation of $G\alpha_{15}/G\alpha_{16}$ and its role in receptor and effector coupling. We observed a PMA-stimulated intact cell phosphorylation of $G\alpha_{15}$ in COS7 cells transfected with $G\alpha_{15}$ and protein kinase Cα (PKCα), and phosphorylation of endogenous $G\alpha_{16}$ in HL60 cells. We also showed that peptides derived from the two G-proteins were phosphorylated in vitro using purified brain PKC. Furthermore, we identified the putative phosphorylation site and showed that mutation or deletion of this PKC phosphorylation site inhibited phospholipase C (PLC) activation. The behavior of double mutants with the constitutively active G-protein mutation (QL-mutant) and mutation in the putative phosphorylation site suggests that the phosphorylation site of $G\alpha_{15/16}$ is essential for receptor-coupled activation of PLC, but not for direct interaction of the G-protein with PLC-β. J. Cell. Biochem. 88: 1101–1111, 2003. © 2003 Wiley-Liss, Inc.

Key words: protein phosphorylation; heterotrimeric G proteins; $G\alpha_{15/16}$; GPCR; phospholipase C-β (PLC-β)

The G-protein coupled signal transduction pathways represent important targets for a variety of therapeutic applications, ranging from the control of blood pressure, allergic response, kidney function, and hormonal disorders, to neurological diseases and chronic pain. Based on sequence homology and sensitivity to bacterial toxins, the G-protein α subunits have been grouped into four subfamilies, Gαs, Gαi, Gαq, and $G\alpha_{12/13}$ [Simon et al., 1991]. Two subfamilies of G-proteins, Gαs and Gαi, activate or inhibit adenylyl cyclases, and are sensitive to ADP-ribosylation catalyzed by cholera toxin or pertussis toxin (PTX), respectively. The Gαq family of G-proteins consists of four members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$) with different expression patterns. $G\alpha_q$ and $G\alpha_{11}$, which are 88% identical, are ubiquitously expressed and are primarily responsible for coupling receptors in a PTX-insensitive manner to phospholipase C (PLC) isoforms [Strathmann and Simon, 1990; Rhee and Choi, 1992; Sternweis and Smrcka, 1992]. The expression of $G\alpha_{14}$, which is 81% identical with Gαq, is more restricted [Wilkie et al., 1991]. The human $G\alpha_{16}$ and its murine counterpart $G\alpha_{15}$ are only expressed in a subset of hematopoietic cells [Wilkie et al., 1991]. These two G-protein α subunits ($G\alpha_{15}$ and $G\alpha_{16}$) share 57% homology with Gαq and have a unique domain inserted in the C-terminal region of the protein [Amatruda et al., 1991]. All members of the $G\alpha_q$ family share functional properties such as coupling to the activation of PLC-β, leading to the hydrolysis of phosphotidyl-4, 5-bisphosphate, and the production of diacylglycerol (DAG) and $InsP_3$. These two molecules play important roles as intracellular second messengers by activating protein kinase C (PKC) and by evoking intracellular Ca^{2+} release, respectively [Berridge et al., 1998].

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*Correspondence to: Mingyao Liu, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, The Texas Medical Center, 2121 W. Holcombe Blvd., Houston, TX 77030.
E-mail: mliu@ibt.tamu.edu

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A variety of factors have been shown to regulate G-protein coupled signal transduction pathways. Examples include bacterial toxins, the family of RGS proteins, and post-translational modifications, such as myristoylation, palmitoylation, and protein phosphorylation [Casey et al., 1991; Casey, 1994; Neer, 1994; Koelle, 1997]. The stimulation of receptors coupled to $G\alpha_{q/11}$ induces phosphorylation on a tyrosine residue of the $G\alpha_{q/11}$ subunit, and this tyrosine phosphorylation event is essential for $G\alpha_{q/11}$ activation [Umemori et al., 1997]. In addition, PKC has been shown to directly phosphorylate G-proteins Gαi, Gαz, $G\alpha_{12}$, and $G\alpha_{13}$. Phosphorylation of these proteins by PKC regulates G-protein signaling by disrupting the association of the Gα with Gβγ subunits [Fields and Casey, 1995; Kozasa and Gilman, 1996; Offermanns et al., 1996]. In *Xenopus* oocytes, PKC activators reduced both the magnitude and the time course of thyrotropin-releasing hormone (TRH)-mediated responses when TRH receptor and $G\alpha_{15}$ or $G\alpha_{16}$ were co-expressed. Repeated TRH-stimulation of oocytes expressing $G\alpha_{16}$ mimics the effects of PKC activators, suggesting the possibility of phosphorylation upon receptor activation [Aragay and Quick, 1999]. These data point to a shared mechanism for regulating G-protein signaling through G-protein phosphorylation.

The signaling pathways that engage $G\alpha_{15/16}$ in hematopoietic cells are poorly defined. Recent studies suggest that chemotactic receptors, such as C5a, formylmethionyl-leucyl-phenylalanine (fMLP), and interleukin-8 receptors, interact specifically with $G\alpha_{16}$ to stimulate the production of $InsP_3$ and mobilization of intracellular Ca^{2+} in transfection assays [Wu et al., 1993]. In a human erythroleukemia (HEL) cell line, $G\alpha_{16}$ specifically couples to the P2Y2 (P2U) receptor [Baltensperger and Porzig, 1997]. On the other hand, a number of receptors ordinarily coupled to different G-protein signaling pathways have been found to interact promiscuously with $G\alpha_{15}$ or $G\alpha_{16}$ when reconstituted into the COS-7 cells [Offermanns and Simon 1995; Zhu and Birnbaumer, 1996]. The precise mechanisms for receptor-mediated $G\alpha_{15/16}$ activation remain to be elucidated, and the molecular basis for the promiscuious ability of $G\alpha_{15/16}$ to couple to different G-protein receptors needs to be understood. In this report, we investigated the phosphorylation and activation of hematopoietic-specific G-protein $G\alpha_{15}$ and $G\alpha_{16}$ by PKC. Our results show that activation of G-protein coupled receptors induced the phosphorylation of $G\alpha_{15}$ and $G\alpha_{16}$ expressed in COS-7 cells. Furthermore, we demonstrated that the phosphorylation site of $G\alpha_{15/16}$ is essential for receptor-coupled activation of PLC, since mutation or deletion of this site in G-proteins disrupts receptor coupled PLC signaling, whereas phosphorylation of $G\alpha_{15/16}$ or mutation at the phosphorylation site has little effect on the interactions of Gα subunits with PLC-β or G-protein βγ subunits, respectively. These results suggest an important role for PKC phosphorylation in the regulation of G-protein interaction with receptor.

MATERIALS AND METHODS

Materials

Carbachol and isoproterenol were from Sigma Chemicals (Sigma, St. Louis, MO). Tissue culture media and media supplements were purchased from Life Technologies, Inc. (Invitrogen, Carlsbad, CA). Purified PKC from rat brain was purchased from Calbiochem, Inc. (San Diego, CA). Unless otherwise mentioned, all chemicals (analytical grade) are from Sigma Chemicals.

The antibodies for $G\alpha_{15/16}$ used in the experiments were produced in Dr. Melvin Simon's lab at Caltech (Pasadena, CA). These antibodies are rabbit polyclonal antibodies against the specific peptide regions of the two G-proteins [Amatruda et al., 1991]. The antibodies are unique and do not cross-react with other Gq family members.

Transient Transfection of COS-7 Cells and Determination of Inositol Phosphate Levels

COS-7 cells were purchased from American Type Culture Collection (Manassas, VA and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were not tested for mycoplasmal contamination. For transient transfection experiments, cells were seeded in 12-well plates at a density of 1×10^5 cells per well and grown overnight. Transfection of COS-7 cells was performed as previously described [Wu et al., 1992a; Xia et al., 2001]. Briefly, cells were washed with phosphate-buffered saline, and 1 μg of DNA mixed with 5 μl of lipofectamine (Invitrogen)

in 0.5 ml of Opti-MEM was added to each well. In cotransfection experiments with two different plasmids, 0.5 μg of each plasmid was added. The total amount of DNA was maintained constant by adding cDNA vector encoding β-galactosidase. The transfection efficiency of the COS-7 cells was about 50% using the cDNA vector containing β-galactosidase. After 5 h at 37°C, 0.5 ml of DMEM containing 20% (v/v) fetal bovine serum was added to each well. Twenty-four hours after transfection, cells were labeled for 20–24 h with 120 pmol of myo-[2-^{3}H]inositol (758.5 GBq/mmol; Du Pont NEN, Wilmington, DE) per well as described [Wu et al., 1992b]. Labeled cells were washed with phosphate-buffered saline and then incubated for 10 min at 37°C with 0.5 ml of inositol-free DMEM containing 10 mM LiCl. Thereafter, medium was aspirated, and the indicated agents were added in DMEM containing 10 mM LiCl. Inositol phosphate formation was stopped after 20 min by removing the medium and adding 0.2 ml of 10-mM ice-cold formic acid. After keeping the samples on ice for 20 min, 0.45 ml of 10 mM NH_4OH was added, and the whole sample was loaded onto a column containing 0.75 ml of anion exchange resin (AG 1-X8; Bio-Rad, Hercules, CA) equilibrated with 5 mM borax and 60 mM sodium formate. Total inositol phosphates ($InsP_3$) were then separated and measured as described [Wu et al., 1992a].

All experiments were repeated at least three times and each experimental point was done in triplicate. Results were plotted using mean values and the bars representing the standard deviation.

Peptide Phosphorylation by PKC

Peptides corresponding to the unique domain of $G\alpha_{15}$ and $G\alpha_{16}$ were synthesized and purified by HPLC. Peptide phosphorylation was performed with rat brain PKC (specific activity, 1,000 U/mg proteins, Calbiochem) in 100 μl of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 125 μM $CaCl_2$, 1 mM DTT, 10 μM [^{32}-P]ATP (6,000 cpm/pmol), and 10 μg/ml phosphatidylserine-diolein (Sigma) at 30°C for 30 min. Phosphorylation reactions were stopped by addition of 10 mM EDTA. Phosphorylated peptides were separated by phosphocellulose spin columns (Pierce, Rockford, IL) and washed by 5% trichloroacetic acid. Peptide phosphorylation was counted by liquid scintillation.

For labeling with either [^{35}S]methionine or [^{32}P]orthphosphate, cells were incubated with methionine- or phosphate-free DMEM (Invitrogen) for 1 h, followed by incubation with medium supplemented with [^{35}S]methionine (50 μCi/ml) or [^{32}P]orthophosphate (0.5 mCi/ml) for 3 h. Cells were washed twice with PBS, harvested, resuspended in 500 μl of RIPA buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1% sodium cholate, 1% Triton X-100, and 0.5% SDS). Phosphorylated G-proteins were separated and precipitated by specific antibodies against $G\alpha_{15/16}$. Immuno-precipitated proteins were extracted with SDS sample buffer, separated by SDS–PAGE, and followed by autoradiography.

Phosphoamino Acid Analysis

$G\alpha_{15}$ protein was metabolically radiolabeled with [^{32}P]orthophosphate after stimulation of the cells by PMA. Then, the phosphorylated protein was immuno-purified using specific anti-$G\alpha_{15}$ antibody, separated by SDS–PAGE, and transferred to a PVDF membrane. The phosphorylated $G\alpha_{15}$ protein was excised, washed, and subjected to standard phosphoamino acid analysis as previously described [Liu and Simon, 1996; Xia et al., 2001]. Briefly, the protein was digested with 6 N HCl for 1 h at 110°C. The sample was lyophilized and dissolved in 5 mM Tris-Cl, pH 8.0. The phosphoamino acids were spotted on a silica gel plate (Eastman Kodak Co., Rochester, NY) along with phosphoamino acid standards and separated by two-dimensional thin layer liquid chromatography (TLC). Phosphoamino acid standards were detected using 0.2% ninhydrin solution spray (Sigma). The results were visualized by autoradiography at −80°C for overnight.

Construction of $G\alpha_{15}$ and $G\alpha_{16}$ Mutations

PCR was used to generate the mutations of $G\alpha_{16}$ and $G\alpha_{15}$ using the full-length human $G\alpha_{16}$ and murine $G\alpha_{15}$ as templates [Wilkie et al., 1991]. Pfu (Strategene, La Jolla, CA) was used in all PCR subcloning and the cDNAs were inserted into the mammalian expression vector pcDNA3 (Invitrogen). The sequences of different mutant constructs were confirmed by DNA sequencing. The cDNAs encoding the PKC isozymes are gifts of Dr. PJ Parker (Imperial Cancer Research Fund, London).

RESULTS

Phosphorylation of Gα15 and Gα16 by PKC

To define the role of PKC in G-protein mediated intracellular Ca^{2+} signaling, we asked whether PKC could phosphorylate hematopoietic-specific $G\alpha_{16}$ and $G\alpha_{15}$. COS-7 cells were cotransfected with $G\alpha_{15}$ and with PKC-α. The cells were labeled with [^{32}P]orthophosphate and stimulated with the phorbol ester, PMA. After immunoprecipitation with specific antibodies against $G\alpha_{15/16}$, phosphorylated proteins were separated on SDS gel and visualized by autoradiography. As shown in Figure 1A, activation of PKC dramatically increased the [^{32}P]-labeling of the $G\alpha_{15}$ protein, indicating that $G\alpha_{15}$ can be phosphorylated by PKC in the cells. Phosphorylation of $G\alpha_{16}$ was also examined in human HL60 cells. Since HL60 cells express endogenous $G\alpha_{16}$, we directly labeled the cells with [^{32}P]orthophosphate and stimulated with PMA. [^{32}P]-labeled $G\alpha_{16}$-protein was isolated by immunoprecipitation with specific anti-$G\alpha_{16}$ antibody. As shown in Figure 1B, treatment of the cell with PMA substantially increased the incorporation of [^{32}P]orthophosphate to the $G\alpha_{16}$-protein. Immunoprecipitation with anti-Gαq showed no labeling of the Gαq protein in both experiments (data not shown), suggesting that phosphorylation of $G\alpha_{16}$ is specific upon activation of PKC.

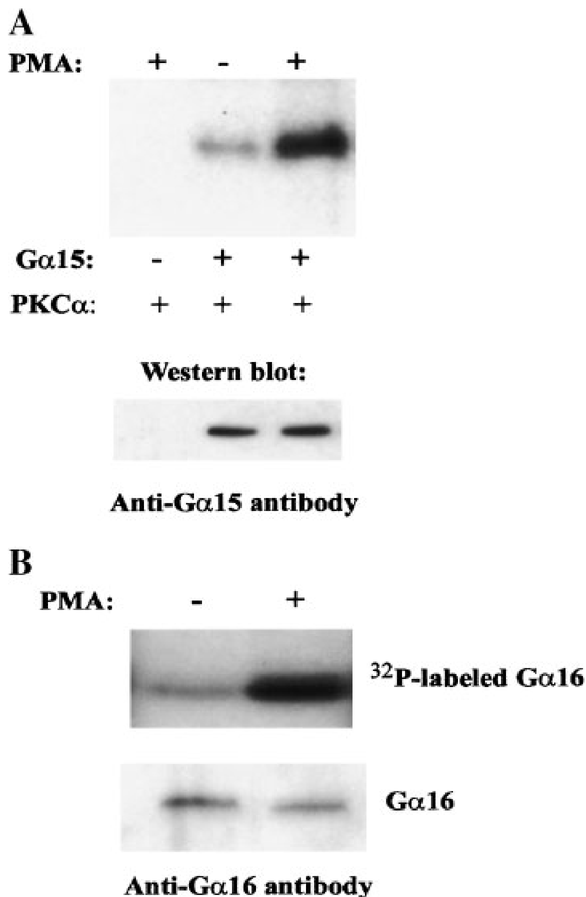
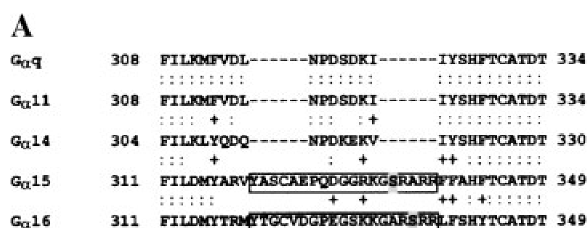
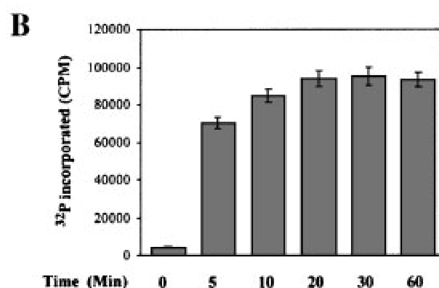


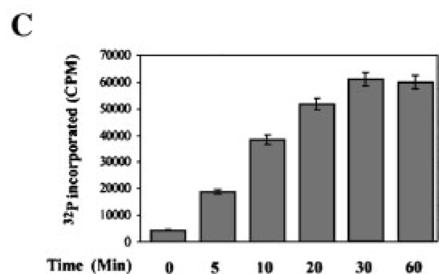
Fig. 1. Phosphorylation of $G\alpha_{15}$ and $G\alpha_{16}$ in vivo. **A**: COS-7 cells transfected with $G\alpha_{15}$ and protein kinase Cα (PKCα) were metabolically labeled with [^{32}P]H_3PO_4 and subsequently incubated in the presence or absence of 1 μM PMA at 37°C for 10 min. **B**: Phosphorylation of $G\alpha_{16}$ in human promyelocytic leukemia cell line (HL-60). HL-60 cells were metabolically labeled with [^{32}P]orthophosphates and stimulated with or without 1 μM PMA for 10 min. Following solubilization of the cells, $G\alpha_{16}$ was precipitated with an anti-$G\alpha_{16}$ antibody. Shown are autoradiograms of SDS–PAGE. Western blot shows similar proteins were loaded in the experiments.

Identification of the Phosphorylation Sites in $G\alpha_{15}$ and $G\alpha_{16}$

Comparison of the amino acid sequences of the Gαq subfamily members revealed that $G\alpha_{15}$ and $G\alpha_{16}$ contain unique insertion domains in their C-terminal regions (Fig. 2A). To determine, whether the phosphorylation sites of $G\alpha_{15}$ and $G\alpha_{16}$ were contained within the peptides corresponding to the unique domains, these two peptides were synthesized and purified by HPLC column. In vitro phosphorylation experiments indicated that these unique domains in $G\alpha_{15}$ and $G\alpha_{16}$ are good substrates for PKC in vitro (Fig. 2B,C). Sequence analysis revealed that this unique domain contains serine^{334} (RKGSR) of $G\alpha_{15}$ and serine^{336} (KKGARSRR) of $G\alpha_{16}$. Both serines represent consensus phosphorylation sites for PKC [Newton, 1995]. To determine whether these two serines are the potential phosphorylation sites in the two proteins, site-specific mutations were performed to change serine^{334} of $G\alpha_{15}$ and serine^{336} of $G\alpha_{16}$ to alanines, respectively. cDNAs encoding the mutant proteins were then transfected into COS-7 cells together with PKC-α. The cells were then labeled with [^{32}P]orthophosphate and stimulated with PMA. Phosphorylation of these two mutant proteins by PKC was significantly reduced (Fig. 3), suggesting that these two serines are the key PKC phosphorylation sites in $G\alpha_{15}$ and $G\alpha_{16}$. To rule out the possibility that $G\alpha_{15}$ can also be phosphorylated at other sites, we performed a phospho-amino acid analysis of $G\alpha_{15}$ phosphorylated in intact cells. As shown in Figure 2D, serine was strongly phosphorylated







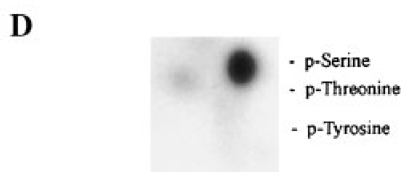


Fig. 2. Identification of the phosphorylation domains of $G\alpha_{15}$ and $G\alpha_{16}$ by PKC. **A**: Amino acid comparison of the Gαq subfamily members (Gαq, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$). Both $G\alpha_{15}$ and $G\alpha_{16}$ have a unique insert domain in this region. **B** and **C**: Phosphorylation of peptides containing consensus PKC phosphorylation site from $G\alpha_{15}$ (YASCAEPQDGGRKGSRARR) and $G\alpha_{16}$ (YTGCVDGPEGSKKGARSRR), respectively, by purified rat brain PKC in vitro. Peptides corresponding to the unique domains of $G\alpha_{15}$ and $G\alpha_{16}$ were synthesized and purified by HPLC. Purified peptides were phosphorylated by purified rat brain PKC (25 ng) in the presence of $[^{32}P]\gamma$-ATP for indicated times (min). The Km for the G-protein peptides is estimated to be 9 μM for $G\alpha_{15}$ and 12 μM for $G\alpha_{16}$ with comparable phosphorylation rate to other known PKC peptides. **D**: Phospho-amino acid analysis of $G\alpha_{15}$ phosphorylated in cells.

while little or no phosphorylation was detected at threonine or tyrosine residues.

Roles of $G\alpha_{15/16}$ Phosphorylation in G-Protein Coupled PLC Activation

A variety of G-protein coupled receptors, including β_2 adrenergic receptor (β_2AR), M_2 muscarinic receptor (M_2R), vasopressin V_2 receptor, dopamine D_1 receptor, and adenosine A receptor [Offermanns and Simon, 1995], can activate $G\alpha_{15}$ and $G\alpha_{16}$, suggesting that there are unique determinants in $G\alpha_{15}$ and $G\alpha_{16}$ that are responsible for their ability to become activated by different receptors. The carboxyl-terminal 55 amino acids of $G\alpha_{15}$ and $G\alpha_{16}$ are most divergent from other members of the Gαq family (G_q, G_{11}, and G_{14}) [Strathmann and Simon, 1990; Amatruda et al., 1991; Wilkie et al., 1991]. This region includes the very carboxyl terminus of the α-subunit that has been shown to affect receptor specificity [Dratz et al., 1993; Noel et al., 1993]. Interestingly, $G\alpha_{15}$ and $G\alpha_{16}$ possess a unique insert of several amino acids including differently charged residues between helix 4 and 5 that are adjacent to a region homologous to residues 311–329 of transducin [Hamm et al., 1988; Amatruda et al., 1991; Wilkie et al., 1991]. This region of transducin has been implicated in the interaction with receptor [Lee et al., 1995]. As shown in our experiments, this unique domain in $G\alpha_{15}$ and $G\alpha_{16}$ can be phoshorylated by PKC (Figs. 2 and 3). To determine whether phosphorylation of $G\alpha_{15}$ and $G\alpha_{16}$ is correlated with receptor activation, COS-7 cells were cotransfected with M_2R and $G\alpha_{15}$ or $G\alpha_{16}$, respectively. The cells were preincubated with $[^{32}P]$orthophosphate

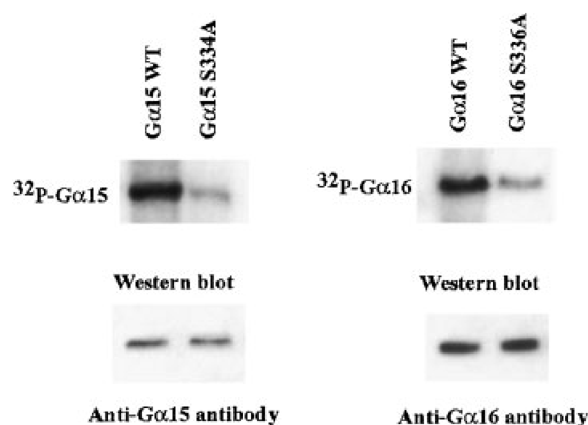


Fig. 3. Phosphorylation of $G\alpha_{15}$ and $G\alpha_{16}$ mutants by PKC. COS-7 cells were cotransfected with cDNAs encoding wild type or mutant G-protein α subunits together with PKCα. The cells were labeled with $[^{32}P]$orthophosphates and stimulated with PMA. G-proteins were precipitated with anti-$G\alpha_{15}$ or anti-$G\alpha_{16}$ antibodies. Proteins were separated by SDS–PAGE and phosphorylated proteins were detected by autoradiography. A representative result from one of at least three experiments are shown. **Bottom** shows that equal amounts of protein were loaded in the experiments.

and stimulated with carbachol. Compared to unstimulated cells, phosphorylation of $G\alpha_{15}$ was increased upon stimulation of the cell (Fig. 4A), suggesting that the phosphorylation of $G_{15/16}$ is influenced by receptor activation.

If phosphorylation of $G_{15/16}$ by PKC is involved in the functional coupling of receptor to G-protein signaling, mutant $G\alpha_{15}$ or $G\alpha_{16}$ that lacks the phosphorylation domain or site should have defects in coupling the receptor to PLC activation. To test this hypothesis, we constructed a chimeric protein with the N-terminus from $G\alpha_{15}$ and the C-terminal half from the $G\alpha_q$, as well as a deletion mutant of $G\alpha_{15}$ (del 317–335) (Fig. 4B,C). The chimeric protein lacks the unique domain from the $G\alpha_{15}$ protein. Cotransfection of cDNAs encoding the chimeric protein or the deletion mutant protein together with the receptors was not able to couple the activation of M_2R or β_2AR (data not shown) to PLC activation (Fig. 4C). On the other hand, cotransfection with the M1 muscarinic receptor showed that the chimeric G-protein was functional since it was able to couple the activation of the M1 receptor which normally interacts with Gαq mediated PLC-β activation (data not shown). Together, these data imply that the unique domains in these two hematopoietic G-proteins are involved in the interactions with receptors. We also found that when $G\alpha_{15}$ was cofransfected with M_2R, carbocol stimulated PLC-β activation much more strongly than when the M_2R alone was transfected. This is a reproducible observation. We reasoned that endogenous G-proteins might be limiting. After transfecting the cell with G-proteins, the amplification effect of G-proteins on activated receptors leads to a strong increase in the signal observed as would be predicted if G-proteins were limiting.

To further define the role of the phosphorylation sites of $G\alpha_{15}$ and $G\alpha_{16}$ in receptor-coupled activation, we cotransfected the single amino acid mutants, $G\alpha_{15}$ (S334A) or $G\alpha_{16}$ (S336A), together with different receptors into COS-7 cells. Activation of PLC-β was assayed by measuring the release of $InsP_3$. Unlike the wild type G-proteins, activation of the M_2R no longer stimulated the mutant G-protein mediated PLC-β activation (Fig. 5A). Similar results were obtained with the β_2AR. Western blots show that the expression levels of wild type and mutant proteins are similar in COS-7 cells (Fig. 5B). These data strongly support the hypothesis that

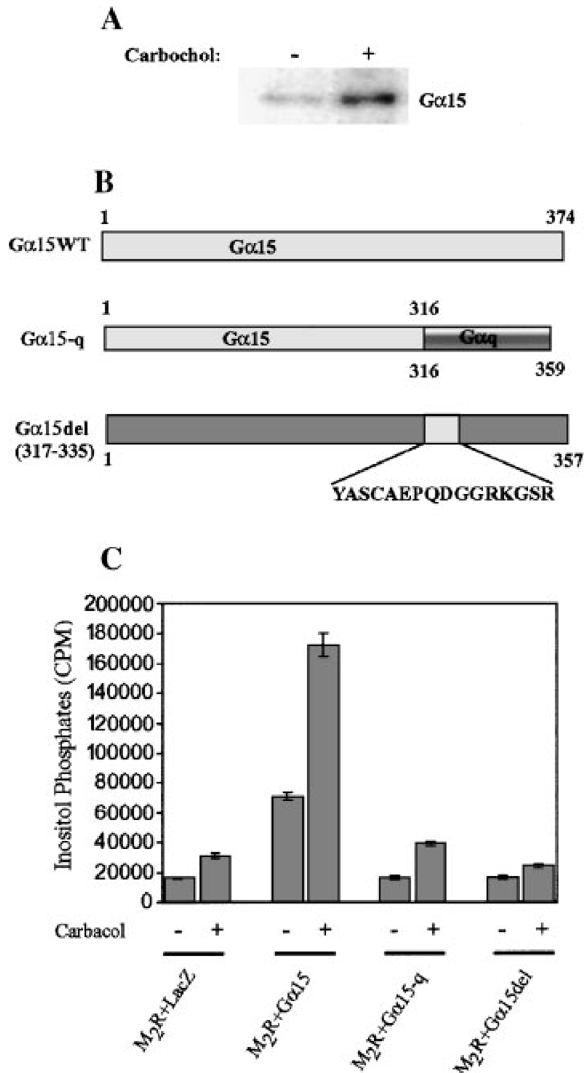


Fig. 4. Role of the unique domain of $G\alpha_{15}$ in receptor coupled G-protein mediated PLC-β activation. **A**: Activation of muscarinic receptor stimulates the phosphorylation of $G\alpha_{15}$. COS-7 cells were cotransfected with cDNAs encoding $G\alpha_{15}$ and the M_2 muscarinic receptor (M_2R), labeled with [^{32}P]orthophosphates, and stimulated with (+) or without (–) 100 μM carbachol. Proteins were precipitated using specific anti-$G\alpha_{15}$ antibody and separated by SDS–PAGE. Phosphorylated protein was detected by autoradiography. One representative result from three independent experiments is shown. **B**: Chimeric protein with the N-terminal region from $G\alpha_{15}$ and the C-terminal domain from Gαq protein, and $G\alpha_{15}$ deletion construct. Amino acids 317–335 were deleted in the cDNA of $G\alpha_{15}$. **C**: Functional assay of $G\alpha_{15}$ chimeric protein and deletion mutant. cDNAs encoding wild type and mutant $G\alpha_{15}$ proteins were cotransfected into COS-7 cells with M_2 muscaric receptor (M_2R). The cells were stimulated with 100 μM carbachol for 10 min. The production of inositol phosphates ($InsP_3$) was measured as described. Shown are mean values of triplicates ± SD.

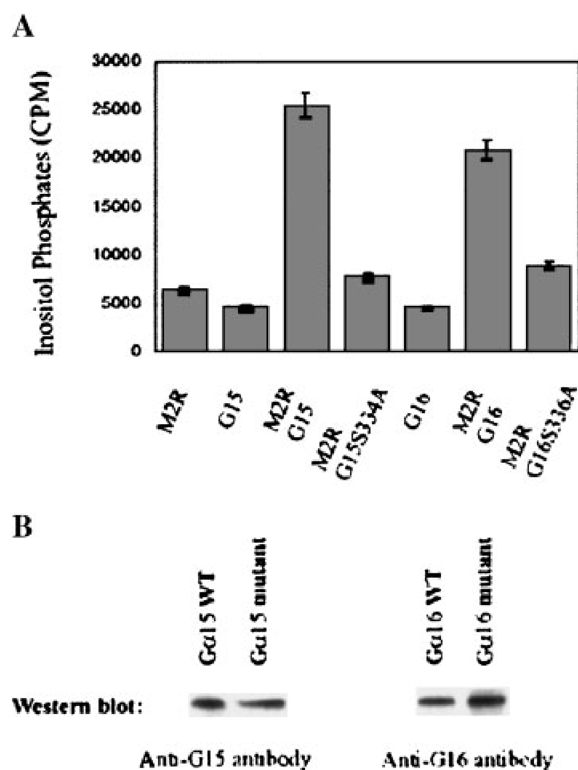


Fig. 5. Role of $G\alpha_{15}$ and $G\alpha_{16}$ phosphorylation in the activation of receptor mediated PLC activation. **A**: Mutation at phosphorylation sites (Ser^{334} of $G\alpha_{15}$ and Ser^{336} of $G\alpha_{16}$) blocks the receptor stimulated PLC-β activation. Results were plotted using mean values of triplicate wells and bars represent standard deviation from a representative experiment. **B**: Western blot analysis of the expression levels of $G\alpha_{15}$ and $G\alpha_{16}$ proteins used in (A), showing similar amount of wild-type and mutant G-proteins were expressed in the experiments. cDNAs encoding the wild type and mutant $G\alpha_{15}$ and $G\alpha_{16}$ were transfected into COS-7 cells. Proteins were separated by SDS–PAGE and detected by Western blot using specific anti-$G\alpha_{15}$ and $G\alpha_{16}$ antibodies.

the unique phosphorylation domain (sites) of $G\alpha_{15}$ and $G\alpha_{16}$ is involved in the functional regulation of receptor activity.

$G\alpha_{15/16}$ Phosphorylation has Little or no Effect on the Activation of PLC-β by Gα Proteins

GTPase deficient mutant G-proteins can activate PLC-β in the absence of receptors. We have demonstrated that mutation at the PKC phosphorylation sites ($serine^{334}$ of $G\alpha_{15}$ and $serine^{336}$ of $G\alpha_{16}$) disrupted the receptor-coupled PLC activation by G-proteins in our transfection assays. To determine whether the serine mutations affect receptor–G-protein coupling or G-protein–PLC interaction in the signaling pathway, we constructed double mutations of $G\alpha_{15}$ (S334A and Q212L) and $G\alpha_{16}$ (S336A and Q212L). These double mutant proteins are deficient in both serine phosphorylation and GTPase activity. To examine the effects of the mutant G-protein α subunits in PLC-β activation, we transiently transfected cDNAs encoding the mutant $G\alpha_{15}$ or the mutant $G\alpha_{16}$ into COS-7 cells and assayed for the activation of PLC-β by the production of $InsP_3$. A single mutation at Ser^{334} of $G\alpha_{15}$ or Ser^{336} of $G\alpha_{16}$ inactivated its ability to modulate receptor-coupled PLC-β activation. Mutation at Q212L has been shown to activate PLC-β enzymes independent of receptor stimulation. As shown in Figure 6, the double mutants of $G\alpha_{15}$ and $G\alpha_{16}$ activated endogenous PLC-β (Fig. 6), indicating that mutation or lack of phosphorylation at the serine residues has little effect on the activation of PLC-β isozymes by $G\alpha_{15/16}$. Thus, these results suggest that the unique domain in $G\alpha_{15}$ and $G\alpha_{16}$ could be involved in the coupling of receptor with G-protein since mutation at this domain disrupted the agonist-stimulated PLC activation, but not the activation of PLC-β by the dominant-active G-protein mutant (Q212L).

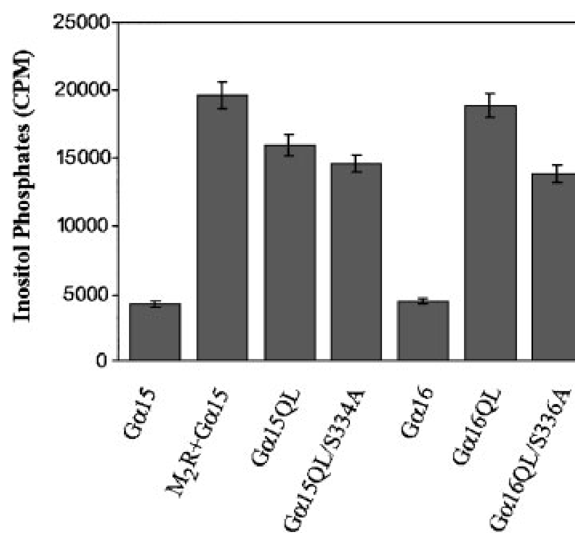


Fig. 6. Mutation at the phosphorylation sites has little effect on the activation of PLC-β by dominant active Gα mutants ($G\alpha_{15}$QL and $G\alpha_{16}$QL). COS-7 cells were transfected with cDNAs encoding wild-type $G\alpha_{15}$, and $G\alpha_{16}$, dominant active $G\alpha_{15}$QL and $G\alpha_{16}$QL, and double mutants of $G\alpha_{15}$ and $G\alpha_{16}$ ($G\alpha_{15}$QL/S334A and $G\alpha_{16}$QL/S336A). M_2R and $G\alpha_{15}$ were co-transfected into the cells and used as a positive control. G-protein induced PLC-β activity was determined by the production of $InsP_3$. Shown are mean values of triplicates.

Phosphorylation Mutants of $G\alpha_{15}$ and $G\alpha_{16}$ are Still Able to Inhibit $G\beta\gamma$-Mediated PLC-β Activation

Our results suggest that mutations at serine[334] of $G\alpha_{15}$ and serine[336] of $G\alpha_{16}$ could disrupt the receptor-coupled PLC-β activation by G-protein subunits. To rule out the possibility of the overall structural change after the mutation of the prospective Gα subunits, we assayed the ability of the mutant $G\alpha_{15}$ and $G\alpha_{16}$ to interact with Gβγ subunits. In transfected COS-7 cells, G-protein βγ subunits are able to activate PLC-β_2, and the activation of PLC-β_2 can be inhibited by cotransfection with wild type Gα subunit because of competitive binding of Gα with the βγ subunits [Yu and Simon, 1998]. To examine whether mutation at the phosphorylation sites (serine[334] of $G\alpha_{15}$ or serine[336] of $G\alpha_{16}$) disrupts the overall structure of the Gα subunits and the formation of G-protein αβγ heterotrimers, we cotransfected COS-7 cells with PLC-β_2, Gβγ subunits, wild-type $G\alpha_{15}$ or $G\alpha_{16}$, or mutant $G\alpha_{15}$ and $G\alpha_{16}$ ($G\alpha_{15}$S334A or $G\alpha_{16}$S336A), respectively. The activation of PLC-β_2 by Gβγ subunits was measured in the absence or presence of different Gα subunits. As shown in Figure 7A, both the wild type and the mutant Gα subunits ($G\alpha_{15}$S334A and $G\alpha_{16}$S336A) inhibited the Gβγ stimulated-PLC-β_2 activity, presumably by competing for the available Gβγ subunits in the COS-7 cell assays. To role out the possibility that the expression level of Gβγ is affected by the cotransfection of Gα subunit, we performed Western analysis using specific anti-Gβ1 antibody to determine the level of Gβγ proteins in the cotransfected cells. As shown in Figure 7B, coexpression of $G\alpha_{15}$ or $G\alpha_{16}$ has little or no effect on the level of Gβγ protein expression, suggesting the observed effect is not due to the expression level of Gβγ proteins.

In summary, these experiments indicate that, like the wild type Gα proteins, the mutant proteins could still interfere with Gβγ stimulated PLC-β activity in COS-7 cells, and that mutation or phosphorylation did not disrupt the overall structure of the Gα protein.

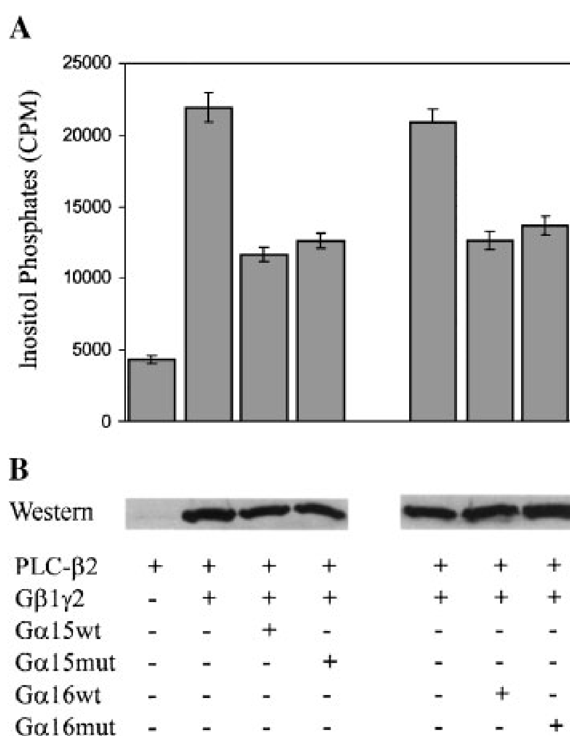


Fig. 7. Inhibition of Gβγ activated PLC-β2 activity by $G\alpha_{15}$ and $G\alpha_{16}$ mutants. **A**: COS-7 cells were cotransfected with PLC-β2 and Gβ1γ2 in the presence of $G\alpha_{15}$, $G\alpha_{16}$, or mutant proteins, respectively. The amount of $InsP_3$ was measured as described in the experimental procedure and shown are mean values of triplicates ± SD. **B**: Expression of Gβ1γ2 is not affected by coexpression of Gα protein. The protein expression level of Gβ1γ2 protein was examined by specific anti-Gβ1 antibody (sc-379 Santa Cruz) using Western analysis. Coexpression of $G\alpha_{15}$ and $G\alpha_{16}$ has little or no effect on the level of Gβγ expression.

DISCUSSION

An important aspect of G-protein mediated signaling cascades is the regulation and coordination of the activation of different receptors and the regulation of second messenger signaling pathways. In our previous research, we have shown that the signaling pathway coupled to cAMP-dependent protein kinase could phosphorylate and regulate the G-protein activated PLC-β, and thus, modulate intracellular Ca^{2+} signaling [Liu and Simon, 1996]. Increases in intracellular Ca^{2+} could lead to the binding of the Ca^{2+}-sensitive protein calmodulin with G-protein beta subunits, regulating the interaction of the heterotrimeric G-protein subunits [Liu et al., 1997]. In this study, our data shows that $G\alpha_{15}$ and $G\alpha_{16}$ are two members of the $G\alpha_q$ family that can be phosphorylated and functionally regulated by PKC. These two Gα-proteins have a number of interesting features. First, they can be activated by a variety of functionally different receptors [Offermanns et al., 1996]. The potential for these G proteins to couple to a broad spectrum of receptors makes them good targets for feedback regulation in

order to prevent aberrant signaling. Second, $G\alpha_{15}$ and $G\alpha_{16}$ are specifically expressed in hematopoietic cells and are highly regulated during differentiation of normal and leukemia cells. In HEL cells, suppression of $G\alpha_{16}$ inhibited cellular growth rates [Ghose et al., 1999]. Regulation of the expression and activity of $G\alpha_{16}$ may be important in controlling cell activation and proliferation. Third, these two G-protein α subunits possess a unique domain in their C-terminal region compared to other members of $G\alpha q$ family of proteins.

The unique insert of several amino acids in $G\alpha_{15}$ and $G\alpha_{16}$ includes different charged residues and a putative phosphorylation site between helix 4 and 5, just adjacent to a region homologous to residues 311–329 of transducin [Hamm et al., 1988; Amatruda et al., 1991; Wilkie et al., 1991], which have been implicated in the interaction with receptor [Hamm et al., 1988; Lee et al., 1995]. Using deletion and site-specific mutagenesis, we have shown that the unique C-terminal domain in $G\alpha_{15}$ and $G\alpha_{16}$ contains a PKC phosphorylation site. Deletion of this domain not only prevented the protein from being phosphorylated by PKC, but also impaired the coupling of receptor to G-protein signaling. Single amino acid mutation at serine^{334} of $G\alpha_{15}$ and serine^{336} of $G\alpha_{16}$ inhibited the receptor-mediated PLC-β activation, indicating the involvement of this amino acid in receptor/G-protein coupling. We propose that phosphorylation of the serine residues in the unique domain of $G\alpha_{15}$ and $G\alpha_{16}$ plays a role in the interaction of receptor and G-protein.

Previous studies have demonstrated the phosphorylation of certain G-protein α subunits by PKC. For example, $G\alpha z$ was reported to be phosphorylated in vitro by PKC and in platelets after PMA treatment [Carlson et al., 1989; Lounsbury et al., 1991]. Phosphorylation of $G\alpha_{12}$ and $G\alpha_{13}$ was found to occur by purified PKC, in NIH 3T3 cells, and in platelets treated with PMA [Kozasa and Gilman, 1996; Offermanns et al., 1996]. The PKC phosphorylation sites in these systems were identified to be in the amino-terminal region that interacts with the $G\beta\gamma$ subunits. Phosphorylation of both $G\alpha z$ and $G\alpha_{12}$ inhibits G-protein signaling by preventing heterotrimer association of $G\alpha\beta\gamma$ [Fields and Casey, 1995; Kozasa and Gilman, 1996]. More recently, phosphorylation of $G\alpha z$ has been reported to reduce the ability of an RGS to accelerate GTPase activity [Glick et al., 1998]. Our findings that the hematopoietic-specific G-proteins, $G\alpha_{15}$ and $G\alpha_{16}$, are phosphorylated by PKC support the possibility that feedback regulation of G-protein signaling occurs by protein phosphorylation. In the current study, we have identified the phosphorylation site located in the unique domain of $G\alpha_{15}$ and $G\alpha_{16}$. Structural and functional analysis of the corresponding region in transducin have revealed that it is involved in the interaction with receptors. Unlike the phosphorylation of $G\alpha z$ and $G\alpha_{12}$, phosphorylation of $G\alpha_{15}$ and $G\alpha_{16}$ does not appear to affect the formation of G-protein heterotrimers and the interaction of G-protein with the effector molecule, PLC-β. Phosphorylation or mutation in this unique domain may modulate the receptor–G-protein coupling.

A previous report by Aragay and Quick [1999] examined the role of PKC in modulating $G\alpha_{16}$-mediated signaling in *Xenopus* oocytes and demonstrated that $G\alpha_{16}$ was phosphorylated in oocytes upon stimulation by either PMA or TRH. In the present study, we not only confirmed the phosphorylation of $G\alpha_{16}$ by PKC in vivo, we also examined the detailed phosphorylation sites in the proteins and provided evidence for interference at the receptor/G protein level instead of G-protein/effector level. An interesting observation in Aragay and Quick's studies, which differs from ours, is that mutations at the putative $G\alpha_{16}$ phosphorylation sites did not affect TRH-mediated Cl^- channel response, while we observed attenuated IP_3 response with our $G\alpha_{16}$ phosphorylation mutants when receptors normally coupled to Gi were activated. Although we do not know the mechanism contributing to this difference, it could have resulted from different cell systems used in the two studies. In addition, the strong coupling with receptors normally mediated by endogenous $G\alpha q$ could override the effects of the mutant G proteins in *Xenopus* oocytes.

In hematopoietic cells, the expression of $G\alpha_{16}$ is highly regulated [Wilkie et al., 1991]. An increase of $G\alpha_{16}$ function or its down-regulation effects on cell proliferation may cause erythroid differentiation in specific cell lines [Ghose et al., 1999]. However, the role of $G\alpha_{16}$ in hematopoietic cell differentiation and proliferation remains to be determined. In Jurkat T cells, permanent disruption of regulated $G\alpha_{16}$ inhibited Leuco A-induced interleukin-2 production, CD69 up-regulation and cell apoptosis,

suggesting that coordinated regulation of $G\alpha_{16}$ is necessary for T-cell responses and that $G\alpha_{16}$ protein is involved in the negative regulation of T-cell receptor signaling. On the other hand, deletion of the $G\alpha_{15}$ gene in mice does not have profound phenotypes [Davignon et al., 2000]. Thus there may be multiple cellular controls and interactions that contribute to the function of $G\alpha_{15}$. The results presented here illustrate that PKC can directly phosphorylate the $G\alpha_{15/16}$ protein and regulate receptor coupled G-protein signal transduction. Further study will likely shed more light on the potential role of the hematopoietic-specific G-protein in lymphocyte activation, differentiation, and development.

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