An engineered mutant of G protein α subunit that binds xanthine nucleotide and not guanine nucleotide

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To my late grandmother

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Abstract

This thesis examines the construction and characterization of mutants of G protein α subunits that bind xanthine nucleotides, but not guanine nucleotides. G proteins play a critical role in transducing extracellular signals across the cell membrane. The mechanisms of G protein-mediated signal transduction are reviewed in chapter 1.

Chapter 2 describes the characterization of the first engineered xanthine nucleotide binding mutant of a Gα subunit, GoαX (GoαD273N/Q205L). GoαX switched nucleotide binding specificity; it bound xanthine nucleotides instead of guanine nucleotides. GoαX formed an heterotrimer with βγ subunits when in the XDP form. Binding of XTP induced a conformational change in GoαX similar to that of the activated wild-type Goα and promoted its dissociation from the βγ complex.

In chapter 3, we characterized the receptor interaction of GoαX. It was able to interact with G protein-coupled receptors effectively; the stimulated m2 muscarinic acetylcholne receptor catalyzed the XTPγS binding of GoαX, and the GoαXβγ complex induced the high affinity ligand-binding state in the N-formyl peptide receptor. Interestingly, we found that the empty GoαX, in the nucleotide-free state, formed a stable complex with receptor and inhibited the activity of Go-coupled receptors in COS-7 cells.

In chapter 4, we extended this study to two other G proteins. We constructed similar xanthine nucleotide binding mutant proteins in G11α and G16α and found that G11αX (G11αD277N/Q209L) and G16αX (G16αD280N/Q213L) bound XTPγS and not

GTPγS when expressed in COS-7 cells. Empty G11α and G16α mutants also interacted with their cognate receptors and blocked their activity. Similar to GoαX, both G11αX and G16αX retained the receptor specificity of their wild-type proteins and can be used to inhibit subsets of G protein-coupled receptors.

In chapter 5, we constructed recombinant retroviruses encoding G16αX, and obtained NIH3T3 cell lines stably expressing the empty G16α mutants by viral infection. We found that G16αX blocked the activation of the endogenous thrombin and lysophophatidic acid (LPA) receptors in NIH3T3 cells. These experiments proved that retroviral gene expression can be an effective technique for delivering empty G protein mutants into cells.

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Chapter One

Introduction

A large number of hormones, neurotransmitters, chemokines and sensory stimuli transduce varieties of signals in eukaryotic cells by binding to G protein-coupled receptors on the plasma membrane. G proteins play critical roles in transducing the receptor-mediated signals into intracellular responses, such as sensory perception, neuronal activity, cell growth, and hormonal regulation (see reviews, 1-3). They are heterotrimeric complexes composed of α, β, and γ subunits. Each of the subunits belongs to a multigene protein family, containing at least 18 distinct α, 5 β, and 12 γ subunits. G proteins interact with a large family of G protein-coupled receptors, which characteristically contain seven transmembrane segments and are activated by a great variety of extracellular signaling molecules.

Signaling is normally initiated by the binding of agonist to receptor which stabilizes the receptor in an active conformation. The stimulated receptor catalyzes the exchange of GTP for GDP bound to the Gα subunits. The binding of GTP promotes a conformational change in Gα and the dissociation of the βγ subunits. Activated GTP-bound α subunits and free βγ subunits regulate a variety of cellular effectors including adenylyl cyclases, phosphodiesterases, phospholipases, ion-channels, ion transporters, and a growing number of kinases. GTP-bound G protein α subunits have an intrinsic GTPase activity which enables them to hydrolyze the bound GTP to GDP and then inactivate the α subunit. The GDP-bound Gα subunits then reassociate with free βγ subunits to form the heterotrimeric complex and complete the activation cycle (Fig.1). The signal crossing the cell membrane carried by ligands binding to G protein-coupled receptors can be amplified through several mechanisms. A single receptor can activate

multiple molecules of G proteins thus amplifying the signal tranduced by the ligand. Similarly, single G protein can regulate multiple effector molecules to further amplify the signal. Many effectors of G proteins are enzymes that generate regulatory small molecules or second messengers, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and inositol triphosphate (IP3) which in turn induce dramatic cellular effects including protein phosphorylation, gene transcription, and membrane depolarization.

In addition to G protein, both G protein-coupled receptors and effectors are specified by multigene families. There are more than one thousand different G protein-coupled receptors found in mammals, including receptors that bind the same ligand. At least five different muscarinic receptors, eight different adrenergic receptors, and five different serotonin receptors have been identified. Distinct receptor subtypes that bind the same ligand may couple with different intracellular pathway and elicit distinct physiological responses. Similarly, effectors regulated by G proteins are also encoded by multigene families e.g. the many isoforms of adenylyl cyclases, phospholipase C, phospholipase A2, and calcium, potassium, and sodium channels. The huge diversity of G proteins, G protein-coupled receptors, and effectors makes G protein-mediated signal transduction a complicated network. Furthermore, crosstalk between these different G protein-regulated pathways makes the network even more complex. Most G proteins couple specifically to certain receptors and effectors; however, individual receptors and effectors can interact with more than one family of G proteins. For example, the thrombin

receptor is known to couple to members of G_{12}, Gi and Gq families, and further physiological responses may be the result of contributions by both α and βγ subunits (5).

G protein α subunits

Heterotrimeric G protein α subunits (molecular weight from 39 to 52 kDa) can be divided into four major families: Gs, Gi, Gq, and G12 according to the similarity of their amino acid sequences that ranges from 56-95% identity (2). Table 1 lists the members of the four different classes of G protein α subunits, some of their known receptors and effectors. With a few exceptions such as transducin α and G15/16 α subunits (6,7), most G protein α subunits are widely expressed in different tissues. Individual cells usually contain at least 4 or 5 types of α subunits (8). The Gsα family was first recognized by its ability to activate adenylyl cyclase and elevate intracellular cAMP concentration, and includes the ubiquitous Gsα (four different splicing variants) and Golfα (an α subunit from olfactory neuroepithelium). The Giα family name implies that some of its members can inhibit adenylyl cyclase upon activation, and includes three Giα isoforms, Goα (a predominately neural α subunit, two different splicing variants), two Gtα variants (transducin, the retinal α subunit), Ggα (an α subunit expressed in gustatory epithelial tissue), and Gzα. However, some members of Giα family regulate ion-channels and phospholipases. The Gqα family includes Gqα, G11α, G14α, G15α, and G16α. Members of this family activate phospholipase C β isoforms which hydrolyze PIP2 and elevate cellular IP3 and DAG concentration. Among members of this family, murine G15α and its human counterpart G16α make a unique class. They are only expressed in a subset of hematopoietic cells and appear to be promiscuous G proteins which can be

activated by all kinds of G protein-coupled receptors (9). The fourth family includes G12α and G13α whose function is not clear. However, there is evidence suggesting that G12α and G13α may be involved in the regulation of the Jun-kinase/stress-activated protein kinase pathway, the Na^+/H^+ exchanger, and the Rho-dependent formation of stress fibers (10).

Most Gα subunits undergo differential posttranslational processing. Some Gα are acylated on an N-terminal glycine, with Giα and Goα myristoylated and Gtα containing either myristate or lauric acid (11). Several types of α subunits (Goα, Giα, Gzα, and Gqα) are palmitoylated on the cysteine residue nearest the N-terminus (12). In addition, G protein α subunits can also be specifically ADP-ribosylated by bacterial toxins when in complex with βγ subunits. Cholera toxin can ADP-ribosylate Gsα and Golfα in a ligand-independent manner, and Gtα and Giα in a ligand-dependent manner on the arginine located in the vicinity of the γ-phosphate group in the nucleotide-binding pocket. This modification leads to constitutive activation of the Gα due to a decrease in intrinsic GTPase activity (13). All members of Gi family, except Gzα, can be ADP-ribosylated by pertussis toxin on the cystine residue four amino acids from the C-terminus causing its inability to be activated by receptors (14).

G protein α subunits can exist in several different conformations: GDP-bound, GTP-bound, empty form, and a form that mimics the transition state for GTP hydrolysis (a complex with GDP and AlF_4^-). The GDP-bound form is the inactive form which can form an heterotrimeric complex with βγ subunits. The GTP-bound form is the active conformation which promotes the dissociation of βγ subunits, and activates a variety of

downstream effectors. In all G proteins, GTP is bound as a complex with Mg^{2+}, which is coordinated to one oxygen from the β-phosphate and one from the γ-phosphate. The binding of Mg^{2+} stabilizes the binding of GTP at least 10-fold (15,16). One of the characteristic properties of G proteins is that they can be activated by the addition of aluminum fluoride which binds GDP to the active site as a tetracoordinated AlF_4^- (17) or AlF_3OH^- (18) . It is believed that the bound AlF_4^- mimics the γ-phosphate of GTP in its pentavalent transition state during its hydrolysis (19). The empty conformation of Gα subunits is the nucleotide-free state, and is an important intermediate in receptor activation. Empty Gα has long been proposed to form a stable complex with activated receptors from studies of rhodopsin and transducin (4). However, since purified recombinant Gα subunits in the empty form are not stable (20), no study of their interaction with receptors has been reported.

G protein βγ subunits

Like the α subunits, both Gβ (~ 36 kDa) and Gγ (6-9 kDa) subunits belong to multigene families. The amino acid sequences for the five known mammalian β subunits, referred to β1, β2, β3, β4, and β5, are between 50 and 83% identical to each other. While β1, β2, and β3 are ubiquitously expressed, β4 is abundant in brain and lung tissue but is found at low levels in other tissues, and β5 appears to have tissue-specific expression present only in the retina, neuronal tissue, and platelets (2, 21). The 12 identified γ subunits which possess a narrower tissue-specific expression profile also display greater sequence divergence more than β subunits or α subunits (22). The retinal γ1 is only 38% identical to brain γ2 and equally different from other γ subunits. All γ subunits undergo

posttranslational modifications at the last cysteine residue at the C-terminus, which are believed to play important roles in forming an active dimer with β subunits and anchoring the αβγ heterotrimer in membrane. Depending on the identity of the C-terminal sequence, some γ subunits get farnesylated while others get geranylgeranylated (8).

The β and γ subunits bind very tightly to each other and can only be separated by denaturants. Not all the possible βγ combinations can form active dimers. For example, the β1 subunit is able to interact with γ1 and γ2, but the very similar β2 is able to form a dimer only with γ2 but not with γ1 (23). For a long time, the prevalent hypothesis for the mechanism of G protein activation was that the GTP-bound α subunit activated effectors, while the βγ subunit was only a negative regulator. However, in recent years, growing evidence suggested that free βγ subunits can regulate a variety of different effectors, including K^+ channels, adenylyl cyclase, phospholipase C β isoforms, phospholipase A2, β-adrenergic receptor kinase, phosphatidylinositol 3-kinase, phosducin, and Ca^{2+}/calmodulin (49).

G protein-coupled receptors

G protein-coupled receptors comprise a large family of cell surface receptors containing more than one thousand members. All of these receptors are integral membrane proteins that range in size from approximately 400 to 1000 amino acids (24,25). Although the endogenous ligands for G protein-coupled receptors include such diverse structures as amine neurotransmitters (e.g., epinephrine, dopamine, and acetylcholine), peptide hormones (e.g., N-formyl peptide, thyrotropin-releasing hormone,

and parathyroid hormone), large glycoprotein hormones (e.g., thyrotropin, follicle-stimulating hormone, and lutropin/chorionic gonadotropin), and sensory molecules including photons and odorants, they share a well-conserved structure and topology. The unifying feature of G protein-coupled receptors is the presence of seven hydrophobic domains, which are between 20 and 26 amino acids in length and assumed to be transmembrane α-helices, containing distinctive amino-acid patterns.

Binding of agonists to receptors elicits a profound change in the transmembrane α helices, thus affecting the conformation of intracellular loops, uncovering previously masked G protein binding sites (26). Although no crystal structure of G protein-coupled receptors has been solved, extensive mutagenesis studies have shown that the C-terminal region of the third intracellular loop of receptors is critical to receptor / G protein interactions (24). Most G protein-coupled receptors interact with a subset of G protein. For example, thyrotropin-releasing hormone receptor and type 1 muscarinic receptor couple only with the members of the Gq family, whereas α2-adrenergic receptor and type 2 muscarinic receptor only interact with the members of the Gi family. However, some receptors such as the thrombin receptor and lysophophatidic acid receptor are known to couple to multiple families of G proteins.

The receptor binding interface of G proteins has been mapped approximately by biochemical and mutational data. Many experiments indicate that the C-terminus of Gα is essential for receptor coupling (27,28). ADP-ribosylation by pertusis toxin, mutations, and peptide specific antibodies, all directed at this region, uncouple G proteins from receptors. Experiments with chimeric proteins containing portions of different Gα

subunits indicate that the C-terminus is important in determining G protein-receptor specificity (50). In addition, the N-terminal helix of Gα has also been implicated in receptor interaction. Peptides corresponding to this region of the transducin α subunit were found to inhibit the binding of transducin to rhodopsin (51). It is clear that βγ subunits enhance receptor interaction with α subunits (28). Direct binding interaction between receptor and βγ subunit has been reported (52). A C-terminal region of Gβ has been shown to cross-link with a receptor peptide corresponding to the third intracellular loop (53). In addition, the C-terminus of γ subunits has also been shown to be involved in receptor coupling and specificity (54,55).

G protein structures

Crystal structures of the α and βγ subunits of transducin and Gi in different conformations have been solved (19, 29-34). The structures of Gα bound to GTPγS, GDP, or GDP in addition with AlF_4^-, and of the free βγ subunit, as well as of the heterotrimeric αβγ complex are now available (Fig. 2). Gα subunits contain two domains: a Ras-like GTPase domain that contains the guanine nucleotide binding pocket as well as sites for binding receptors, effectors, and βγ subunits, and an additional α-helical domain. The guanine nucleotide binds tightly in a deep cleft between the GTPase and helical domain. This overall architecture was observed in the structures of Gα subunits in all conformations.

The nucleotide binding pocket of α subunits in different conformations is essentially the same. The α- and β-phosphate of the bound guanine nucleotide are

enfolded by a loop formed by a conserved GXXXXGK(S/T) motif. The γ-phosphate and Mg^{2+} are coordinated by the conserved DXXG motif. The conserved NKXD motif makes specific hydrogen bonds to the guanine ring of the bound nucleotide. G protein activation by receptors leads to GTP binding on the Gα subunit and a subsequent conformational change. The structural nature of the GTP-mediated activation of the Gα subunit involves a change in conformation of three flexible regions designated Switches I, II, and III to a well ordered, GTP-bound activated conformation with lowered affinity for βγ subunits.

The β subunit contains an N-terminal helix followed by a 7-membered β-propeller structure that coincides with 7 repeats of the WD40 sequence motif. The γ subunit contains two helices and has no inherent tertiary structure. The N-terminal helix of the γ subunit forms a coiled-coil with the N-terminal helix of the β subunit while the remainder of γ interacts extensively with the β-propeller domain of the β subunit. There was little conformational change observed between the free βγ subunit and the βγ in the heterotrimeric αβγ complex. The interaction between the α and βγ subunits occurs at two distinct interfaces. The most extensive interface involves residues in the switch region of Gα that interact with residues at the top of the β-propeller domain. The second interface is formed between the N-terminal helix of Gα and the side of the β-propeller domain of Gβ. However, no significant contacts between the α and γ subunits were observed.

Xanthine nucleotide binding mutants of GTP-binding proteins

The α subunits of heterotrimeric G proteins belong to a large GTPase superfamily that also includes small GTP-binding proteins such as Ras, factors involved in ribosomal

protein synthesis such as elongation factor EF-Tu, and other GTP-binding proteins (35,36). The common theme of the GTPase superfamily is that each member of the family is a precisely engineered molecular switch that can be turned on by binding GTP and off by hydrolyzing GTP to GDP. The binding of GTP promotes a conformational change leading to effector interaction and a wide range of subsequent biological effects.

Despite functional diversity, members of GTPase superfamily share distinct patterns of sequence homologies including the three most prominent conserved motifs: GXXXXGK(S/T), DXXG, and NKXD. The crystal structures of Ras (37), EF-Tu (38,39), and heterotrimeric G protein α subunits have shown that these consensus sequences lie in the vicinity of the bound guanine nucleotide and form direct contacts either with the guanine nucleotide or with the essential cofactor Mg^{2+}. Essentially, the guanine nucleotide binding pocket of these proteins is conserved.

One of the conserved feature in the guanine nucleotide binding pocket is that the carboxylate side chain of the aspartic acid in the NKXD motif interacts with the endocyclic N-1 and exocyclic 2-amino group of the guanine ring through hydrogen bonds, and such interactions provide the specificity for nucleotide binding. This hypothesis was confirmed in EF-Tu by the finding that the single mutation of Asp to Asn in the NKXD motif at position 138 of EF-Tu resulted in the mutant protein EF-TuD138N which bound and utilized xanthine nucleotides for its activities, but not guanine nucleotides (40,41). EF-Tu is a well characterized elongation factor which promotes the binding of aminoacyl-tRNA to the ribosome during polypeptide chain elongation when in complex with GTP. The EF-TuD138N mutant protein was found to bind XTP and then

possess all of the functional properties of the wild-type EF-Tu-GTP complex. The observation of the EF-TuD138N mutant activities suggests that the interaction between the aspartic acid residue and guanine ring can be fully substituted by the interaction of asparagine and the xanthine ring.

Since the interaction of Asp and the guanine ring is conserved in the members of GTPase superfamily, similar Asp to Asn mutations in the NKXD motif of other GTP-binding protein were performed in recent years. People have successfully engineered xanthine nucleotide binding mutant proteins in Ypt1 (42), rab-5 (43,44), FtsY (45), and adenylosuccinate synthetase (46). However, not all such attempts were successful. The similar D119N mutant of H-Ras still preferred GTP over XTP, and induced transformation of NIH-3T3 cells with efficiency indistinguishable from wild-type H-Ras, although the mutant protein exhibited decreased affinity for GTP and increased affinity for XTP by 2 to 3 orders of magnitude (47,48).

Prospective

G protein-mediated signal transduction is a complex network. Not only do G proteins interact with multiple G protein-coupled receptors and a variety of effectors, cross-talk between the different G protein-mediated signaling pathways has also been well documented. One way to analyze this complex network is to specifically activate or inactivate a particular G protein pathway to discern its function without interference from other G proteins. We took the approach that to switch the nucleotide specificity of G protein α subunits from guanine nucleotides to xanthine nucleotides by site-directed mutagenesis, thus allowing us to regulate a particular G protein α subunit by introducing

exogenous XTP or XDP. In addition, we believed that such a Gα mutant protein (GαX) could provide an excellent model to study empty G protein α subunits. The empty state of Gα is an important intermediate in the receptor-stimulated activation. It has been long postulated that the empty G protein forms a stable complex with the receptor. Although both Giα and Goα have been purified in the nucleotide-free condition, they are thermally labile. Therefore, the interaction between empty Gα and receptor has not been characterized. Since the concentrations of XDP or XTP in cells are relatively low, GαX expressed in cells is essentially born empty. We intended to investigate the interaction between empty GαX and G protein-coupled receptors.

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Table 1 Mammalian G protein α subunits and some of their receptors and effectors

Family	Gα	Expression	Receptors	Effectors
Gs	Gsα	Ubiquitous	β1-2 Adrenergic	AC ↑
	Golfα	Olfactory epithelium	Odorant receptors	
Gi	$Gi\alpha_{1,2,3}$	Ubiquitous	α2-Adrenergic, thrombin, m2-, 4-muscarinic, lysophophatidic acid	AC↓, K^+ Channel ↑, Ca^{2+} Channel↓
	Goα	Neuronal cells		
	$Gt\alpha_{1,2}$	Retina	Rhodopsin	cGMP PDE ↑
	Ggα	Taste cells	Taste receptors	?
	Gzα	Neuronal, platelets	Dopaminergic	AC ↓ ?
Gq	Gqα	Ubiquitous	α1-Adrenergic, thrombin, m1-, 3-, 5-muscarinic, lysophophatidic acid, thyrotropin-releasing hormone	PLC β ↑
	G11α	Almost Ubiquitous		
	G14α	Kidney, lung, spleen		
	G15/16α	Hematopoietic cells	Promiscuous	PLC β ↑
G12	G12α	Ubiquitous	Thrombin, lysophophatidic acid, thromboxane	?
	G13α	Ubiquitous		

Gsα has four spice variants. Goα has two spice variants. G16α is the human counterpart of the murine G15α. The abbreviations used are: AC, adenylyl cyclase; cGMP PDE, cGMP-phosphodiesterase; PLC β, phospholipase C β isoforms.

Figure 1 Schematic of G protein activation cycle by stimulated receptors. The binding of agonist to a G protein-coupled receptor (R) induces a conformational change leading to an activated receptor (R*). R* interacts with the heterotrimeric αβγ complex and catalyzes the release of GDP from the α subunit. Subsequent binding of GTP to the empty Gα induces a conformational change in the α subunit promoting the dissociation of the βγ subunit. The activated Gα (Gα-GTP*) and free βγ are able to regulate varieties of cellular effectors. The intrinsic GTPase activity of Gα leads to the hydrolysis of bound GTP to GDP, and Gα-GDP reassociates with free βγ subunits to complete the activation cycle.

Fig. 1 G Protein activation cycle by stimulated receptors

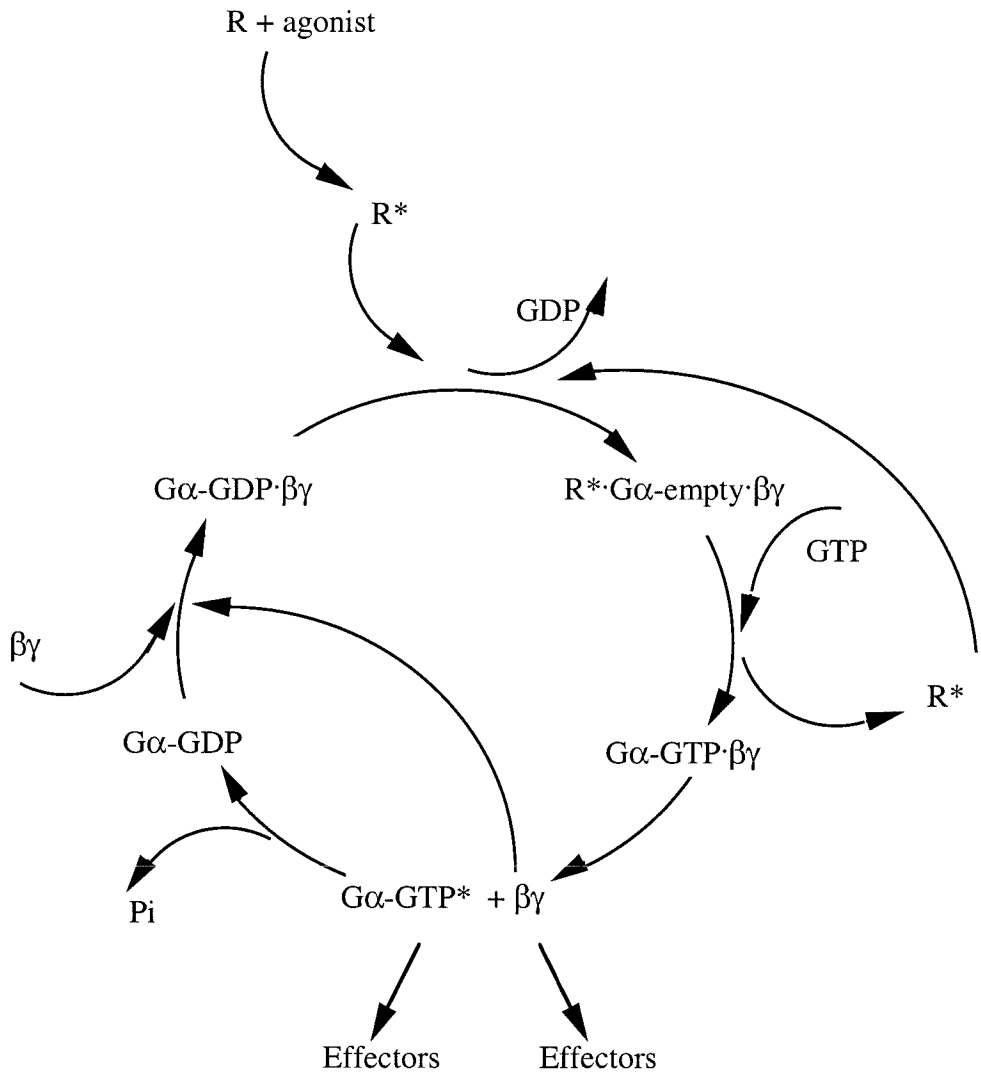


Figure 2 Crystal structure of heterotrimeric G proteins with Gα colored green, Gβ colored yellow, Gγ colored red, GDP colored magenta, and the switch I, II, III regions of Gα colored cyan. a) Ribbon drawing of the complex viewed down the axis of the β-propeller domain. b) Ribbon drawing of the complex rotated 70° about the horizontal axis compared with the view in a). Replotted from (34).

Fig. 2a

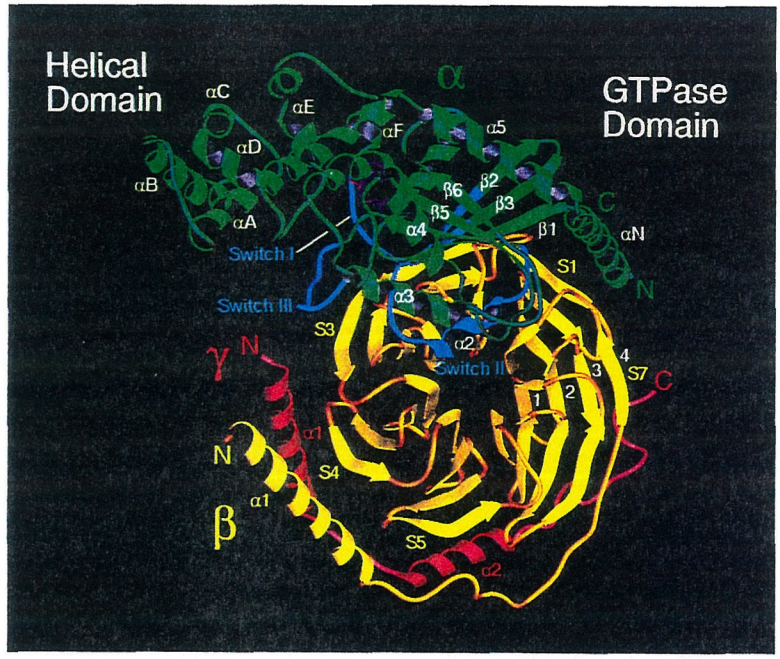
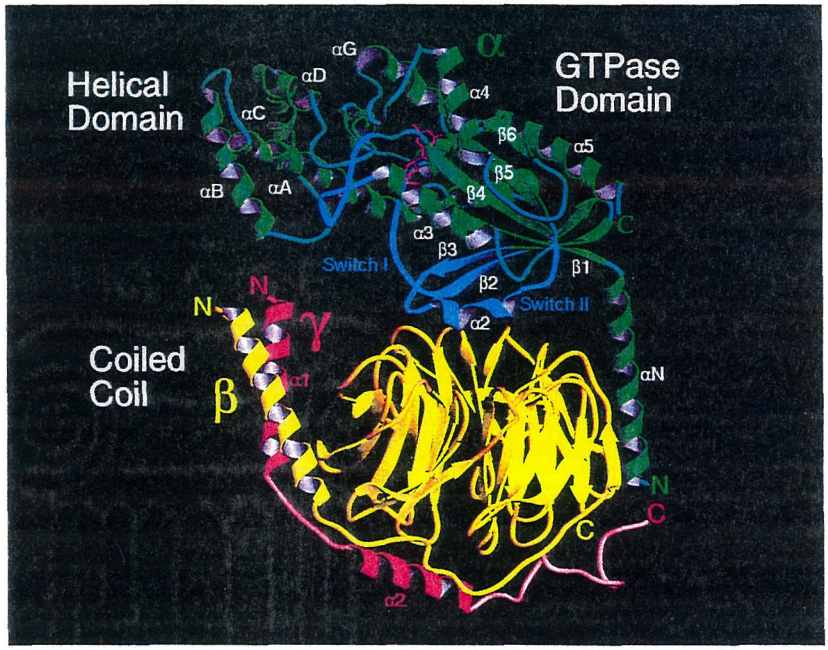

Helical
Domain
GTPase
Domain
αC
αE
αF
α5
αD
αB
β6
β2
β5
β3
αA
α4
β1
αN
Switch I
S1
Switch III
α3
S3
α2
Switch II
S7
γ
N
α1
S4
β
S5

Fig. 2b


Helical
Domain
GTPase
Domain
αG
αD
α4
αC
β6
α5
β5
αB
αA
β4
α3
β3
Switch I
β1
β2
Switch II
α2
Coiled
Coil
αN
α1
β

Chapter Two

Characterization of a Goα Mutant That Binds Xanthine Nucleotides

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Abstract

Several GTP binding proteins including EF-Tu, Ypt1, rab-5, FtsY, and adenylosuccinate synthetase have been reported to bind xanthine nucleotides when the conserved aspartate residue in the NKXD motif was changed to asparagine. However, the corresponding single Goα mutant protein (D273N) did not bind either xanthine nucleotides or guanine nucleotides. Interestingly, the introduction of a second mutation to generate the Goα subunit D273N/Q205L switched nucleotide binding specificity to xanthine nucleotide. The double mutant protein GoαD273N/Q205L (GoαX) bound xanthine triphosphate, but not guanine triphosphate. Recombinant GoαX (GoαD273N/Q205L) formed heterotrimers with βγ complexes only in the presence of xanthine diphosphate (XDP), and the binding to βγ was inhibited by xanthine triphosphate (XTP). Furthermore, as a result of binding to XTP, the GoαX protein underwent a conformational change similar to that of the activated wild-type Goα. In transfected COS-7 cells, we demonstrate that the interaction between GoαX and βγ occurred only when cell membranes were permeabilized to allow the uptake of xanthine diphosphate. This is the first example of a switch in nucleotide binding specificity from guanine to xanthine nucleotides in a heterotrimeric G protein α subunit.

G proteins transduce receptor-generated signals across the plasma membranes of eukaryotic cells. They are heterotrimeric complexes composed of α, β, and γ subunits. Each of the subunits belongs to a multigene protein family, containing at least 18 distinct α, 5 β, and 11 γ subunits. Hundreds of seven-transmembrane receptors activated by a great variety of hormones, neuromediators, and growth factors are coupled to G-proteins. Receptor-induced activation of a G protein leads to exchange of GDP for GTP bound to the α subunit. The GTP-bound α subunit is released from the αβγ trimeric complex, and both free α and βγ dimer are capable of modulating activities of effector enzymes and ion channels (1,3). G protein-mediated signaling is complicated; a single receptor can activate more than one kind of heterotrimer and both the activated α and the βγ subunits can interact with multiple effectors. For example, the thrombin receptor is known to couple to G_{12}, Gi and Gq family members (4), and physiological responses may be the result of contributions by both α and βγ subunits. Furthermore, crosstalk between these different G protein-regulated pathways makes the networks even more complex.

One way to analyze this complex network is to specifically activate a particular Gα *in vivo*, in order to discern its function without interference from other G proteins. As a first step toward this goal, we used site-specific mutagenesis to switch the nucleotide specificity of Gα from guanine to xanthine nucleotides. In cells, XMP is an intermediate in the biosynthesis of GMP; however, the steady state concentrations of XDP and XTP are relatively low (5). Thus, by subsequent introduction of XTP, we should be able to specifically activate the mutant protein. The α subunits of heterotrimeric G proteins belong to the GTPase superfamily which also includes factors involved in ribosomal

protein synthesis, such as EF-Tu, and a large number of Ras-like small guanine nucleotide binding proteins (6, 7). Crystal structures of the α subunits of transducin and Gi have been recently solved (8-11). Both Gα structures had nearly identical binding pockets for the guanine nucleotide, which was similar to the guanine nucleotide binding pocket revealed in the crystal structures of Ras (12) and EF-Tu (13, 14). One of the conserved features was the interaction between a specific Gα amino acid residue and the guanine nucleotide ring, i.e., a hydrogen bond from the side chain of a conserved aspartic acid (D268 in transducin) to the N1 nitrogen and the N2 amine of the guanine ring (Fig. 1a). D268 of transducin belongs to a conserved motif (NKXD) found in the GTPase superfamily. It has been shown that the characteristic hydrogen bond formed with the aspartic acid residue determines the specificity of guanine nucleotide binding in other GTP-binding proteins, such as EF-Tu and Ras (15, 16). A mutation of aspartate to asparagine at this position in several GTP binding proteins including EF Tu (17, 18), Ypt1 (19), rab-5 (20, 21), FtsY (22) and adenylosuccinate synthetase (23) leads to active proteins regulated by xanthine nucleotides instead of guanine nucleotides. In this report, we studied the effect of the similar D273N mutation on nucleotide binding specificity of Goα.

MATERIALS AND METHODS

Mutagenesis and Expression of the Goα. Myristoylated recombinant mouse GoAα was expressed in *E.coli* . Conditions for growth, induction and lysis of the Goα-expressing cells were described previously (24). The D273N mutation was introduced in both wild-type Goα and the activated mutant GoαQ205L by oligonucleotide-directed mutagenesis.

The oligonucleotide

TTTCTAAACAAGAAAAATTTATTTGGCGAGAAGATTAAGAAGTC was annealed to uracil-containing single-stranded DNA from the plasmids pGoα and pGoαQ205L. The resulting vectors were designated as pGoαD273N and pGoαX.

Expression and Purification of His-six-tagged Goα. We subcloned wild-type and mutant Goα cDNAs into the *E.coli* expression vector pET-15b (Novagen), which added a peptide of 20 amino acids MGSS(H)$_6$SSGLVPRGSH containing the His-six tag and a thrombin site upstream of the amino terminus of Goα. These clones were used to transform the *E.coli* strain BL21(DE3), and proteins were expressed. After harvesting the culture, cell extracts were resuspended in the binding buffer (5 mM imidazole, 0.5 M NaCl, 160 mM Tris-HCl, pH 7.9, 1 mM βMe). Binding to the Ni^{2+}-NTA resin was according to the protocol provided by Novagen. The His-six-tagged protein was eluted with a gradient of imidazole concentration (5 mM to 500 mM). The Goα and various mutant proteins eluted at about 250 mM imidazole. Proteins were then transferred to TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) with 0.1 mM $MgCl_2$ and 0.1 mM nucleotide diphosphate [GDP or XDP as appropriate] by gel filtration. Purified proteins were stored in 50% glycerol at -70°C.

Synthesis of XTPγS. XTPγS was synthesized from XDP and ATPγS with Nucleotide Diphosphate Kinase (NDK) as described previously (25). To produce ^{35}S-labeled XTPγS, the reaction contained 10 μM XDP, 1 μM [^{35}S]ATPγS, and 10 units NDK (Sigma) in 100 μl NDK buffer (1 mM $MgCl_2$, 5 mM DTT, 20 mM Tris-HCl, pH 8.0).

The mixture was incubated at room temperature for 2 hours. The resulting concentration of [^{35}S]XTPγS was about 1 μM (1 μCi/pmol). The radiochemical purity of XTPγS was monitored by TLC on Avicel/PEAE plates (Analtech) in 0.07 N HCl.

Nucleotide Binding. Binding of [^{35}S]GTPγS and [^{35}S]XTPγS to the recombinant Goα and the mutant proteins was performed as described (24). The binding reaction contained 0.5 μg of purified protein or 200 μg crude *E.coli* protein in TED buffer, with 0.1 mM $MgCl_2$, 1 μM ATP, 0.1 μM GTPγS or XTPγS (20,000 cpm/pmol). For the time course experiments, 20 μl aliquots were withdrawn form a 200 μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM $MgCl_2$, filtered through a 0.45-μm nitrocellulose filter, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

Proteolysis with Trypsin. Approximately 0.1 μg of purified recombinant Goα was preincubated with nucleotide at room temperature for 30 minutes in the TED buffer. 10 ng of trypsin was then added to the mixture, and the reaction was terminated after 10 minutes by addition of an equal volume of 2X SDS-PAGE sample buffer and heating for 3 minutes at 100°C. The proteolytic pattern was subsequently analyzed by Western Blot using antibodies against Goα.

ADP-ribosylation by Pertussis Toxin. Pertussis toxin-catalyzed ADP-ribosylation was performed as described (24). Briefly, 0.1 μg of recombinant Goα was mixed with 0.1 μg of purified retinal βγ subunit complex in the presence of the appropriate nucleotide and incubated for 10 minutes at room temperature before addition of the reaction mixture

[final concentration of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, 0.5 μM [^{32}P]NAD (20,000 cpm/pmol) and 10 μg/ml pertussis toxin (List Biologicals)]. Reactions were incubated for 30 minutes at room temperature and terminated by the addition of 5X SDS-PAGE sample buffer. Samples were resolved on SDS-PAGE gel. Gels were stained with Coomassie Blue, dried, and exposed to X-ray film.

Cell Culture and Transfection. COS-7 cells were cultured in DMEM containing 10% FCS. $1x10^5$ cells/well were seeded in 12-well plates 1 day before transfection. All transfection assays contained a total amount of 1 μg of DNA; the plasmid pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg of DNA was mixed with 5 μl of lipofectamin (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and five hours later, 0.5 ml of 20% FCS in DMEM was added to the cells. After 48 hours, cells were assayed for inositol phosphate levels as described previously (26, 27).

Permeabilization of COS-7 Cell Membranes. Transfected COS-7 cells were washed twice with phosphate-buffered saline, and incubated in 200 μl of permeabilization solution consisting of 115 mM KCl, 15 mM NaCl, 0.5 mM $MgCl_2$, 20 mM Hepes·NaOH (pH 7), 1 mM EGTA, 100 μM ATP, 0.37 mM $CaCl_2$ (to give a free Ca^{2+} concentration of 100 nM), and 200 units/ml α-toxin with or without 0.1 mM XDP for 10 minutes at 37°C. Then 2 μl of 1 M LiCl was added before the inositol phosphate assay.

RESULTS

In order to change the binding specificity of Goα from guanine nucleotides to xanthine nucleotides, we replaced Asp273 by an asparagine residue which was expected on the basis of structural analysis to coordinate with xanthine instead of guanine (Fig. 1b). This mutation was introduced into both the wild-type Goα subunit and the GTPase-deficient Goα mutant (Q205L). We chose Goα because myristoylated Goα can be expressed in *E.coli* , and it has been shown that many of the characteristics of the recombinant Goα protein are similar to those of the protein isolated from brain. To further characterize the function of XTP-bound Goα mutants, we purified these proteins in the form of non-myristoylated His-six-tagged Goα by affinity chromatography on a Ni^{2+}-NTA column. It has been shown that the non-myristoylated form of Goα has identical nucleotide binding properties comparing with the myristoylated form, and it also forms trimeric complexes with βγ subunits although the affinity to βγ is much less than the myristoylated form.

Binding of GTPγS and XTPγS

The nucleotide binding of Goα, GoαD273N and GoαX (GoαD273N/Q205L) was assayed with ^{35}S-labeled GTPγS and XTPγS. In *E.coli* crude extracts, Goα reached maximum binding of GTPγS in about 30 minutes (Fig. 2a). As expected, Goα showed no affinity for XTPγS. However, GoαX revealed a switch in nucleotide specificity. As shown in Fig. 2b GoαX had high affinity for XTPγS, but not for GTPγS. Interestingly, only the double mutant was active, while GoαD273N did not bind either GTPγS or XTPγS (data not shown). Goα binds GTPγS very tightly in the presence of 1 μM Mg^{2+}

(28, 29). Both Goα (Fig. 2c) and GoαX (Fig. 2d) did not exchange bound [^{35}S]NTPγS when excess non-radioactive nucleotides was subsequently added.

The purified His-six-tagged proteins in general retained the properties of the untagged myristoylated α subunits. However, we detected some differences in nucleotide binding. His-six-tagged Goα or GoαX bound GTPγS or XTPγS respectively, but the binding was less stable than with the untagged myristoylated protein. In the case of His-six-tagged Goα, the bound GTPγS could be exchanged after excess non-radioactive GTPγS was added (Fig. 2c). Similar behavior was observed in the XTPγS binding of pure His-six-tagged GoαX, which also showed distinct nucleotide exchange after non-radioactive XDP or XTP were added to the binding reaction (Fig. 2d). The decrease in nucleotide affinity was apparently the result of the presence of the His-six-tag. Although the nucleotide binding of His-six-tagged proteins was less stable, the specificity of binding was clearly maintained and the mutant bound the xanthine nucleotides rather than the guanine nucleotides. As expected, the purified single mutant GoαD273N did not show any nucleotide binding activity (data not shown).

Activation Conformational Change as Assessed by Limited Proteolysis

Guanine nucleotides protect G protein α subunits, including Goα, from complete proteolytic degradation (30-32). The pattern of fragments derived from partial tryptic digestion can be used as an indicator of the conformation of the protein. In the presence of GDP, Goα is hydrolyzed by trypsin resulting in two products, a stable 25-kDa and an unstable 17-kDa peptide. Binding of non-hydrolyzable analogs of GTP can induce an

active conformation of the Goα subunit, which is resistant to proteolytic degradation, and protects a stable 37-kDa polypeptide from further degradation. In the case of the activated mutant GoαQ205L, GTP can also protect the remaining 37-kDa polypeptide from complete proteolytic digestion by trypsin, because GoαQ205L lacks GTPase activity. Figure 3a shows that XTP protects GoαX from proteolysis by trypsin (lanes 4 and 5), whereas in the control experiment, GTPγS protected wild-type Goα(lane 8). This experiment indicates that GoαX binds XTP without hydrolyzing it. After binding to XTP, GoαX must have assumed a conformation similar to that of GTPγS-bound wild-type Goα. In this experiment, wild-type Goα needed only 1 μM GTPγS to prevent complete proteolysis. Similarly, GoαX was sufficiently protected in the presence of 1 μM XTP. It is noteworthy that GTPγS but not GTP was also able to protect GoαX from complete tryptic digestion although this pretection required GTPγS concentrations above 100 μM (lanes 1, 2 and 3). Thus GoαX has a much lower affinity for GTPγS than for XTP. We did not detect any of GTPγS binding activity of GoαX in our nucleotide binding assay because the highest concentrations of [^{35}S]GTPγS used in the reaction were micromolar. Consistent with the results of the nucleotide binding experiments, the single mutant GoαD273N was not protected by any nucleotides including GTP, GTPγS and XTP up to millimolar concentrations (data not shown).

Pertussis Toxin induced ADP-ribosylation

The interaction of Goα with the βγ complex can be assayed by ADP-ribosylation of the α subunit induced by pertussis toxin (PTX), because ADP-ribosylation requires the formation of the heterotrimeric complex (33, 34). Modification (by ADP-ribosylation)

of recombinant Goα catalyzed by PTX is the same in the presence of GTP or GDP because of the GTPase activity of Goα. However, GTPγS strongly inhibits the modification, since Goα can not hydrolyze GTPγS. GTPγS binding thus promotes the dissociation of the trimeric αβγ complex and prevents the ADP-ribosylation of the Goα subunit. The activated GoαQ205L mutant lacks GTPase activity, and the effect of GTP on ADP-ribosylation is similar to that of GTPγS on the wild-type Goα. Therefore, PTX labeling can be used not only to examine βγ binding but also GTPase activity. Fig. 3b shows that purified Goα was ADP-ribosylated by pertussis toxin (lane 7), and the labeling was strongly inhibited by GTPγS (lane 6). In contrast, GoαX was modified by pertussis toxin only in the presence of XDP (lane 4), but not with GDP (lane 5), and as expected, the reaction was strongly inhibited by XTP (lane 2), whereas GTP had no effect (lane 3). Therefore, only XDP-bound GoαX can form trimeric complexes with βγ, and binding of XTP induces dissociation of the trimeric complex. As a control, we did not detect any ADP-ribosylation of GoαX when GTPγS, GTP, or XTP alone was present (data not shown). Consistent with the results of trypsin digestion, this experiment indicated that XTP was not hydrolyzed by GoαX. The quantitation of [^{32}P] ADP-ribose incorporation revealed that the labeling of GoαX was proportional to the amount of βγ used and reached a maximum at a GoαX : βγ ratio of 1:1, similar to wild-type Goα (data not shown). Interestingly, high concentrations (over 100 μM) of GTPγS also inhibited the ADP-ribosylation of GoαX (Fig. 3b lane 1), offering further evidence that GoαX was able to bind GTPγS with low affinity. As expected, GoαD273N did not interact with βγ

and was not modified by pertussis toxin in the presence of either GDP or XDP (data not shown).

XDP-dependent βγ interaction in transfected COS-7 cells

In transfected COS-7 cells, $\beta_1\gamma_2$ is able to activate PLCβ_2, and the activation of PLCβ_2 can be inhibited by cotransfection with Goα because of competition for βγ (35). We cotransfected COS-7 cells with PLCβ2, β1, γ2, and GoαD273N or GoαX, and found that both Goα mutants did not inhibit PLCβ_2 activity, whereas wild-type Goα did. This experiment indicates that both mutants do not bind βγ in COS-7 cells, and is consistent with the *in vitro* experiments on PTX-induced ADP-ribosylation. GoαX bound βγ only in the presence of XDP, and because XDP concentration is negligible inside the cell, the interaction did not occur. In order to deliver XDP into cells, we tried to permeabilize COS-7 cells by several methods including digitonin treatment, electroporation, and α-toxin (36). We found that only α-toxin gave us consistent results and had no effect on the PLCβ2 activities stimulated by βγ. After incubating cells with α-toxin in the presence of XDP, we found that GoαX inhibited PLCβ2 activity, whereas GoαD273N was not affected by XDP (Fig. 4). In the control experiments, we found that adding GDP or GTP to the permeabilization buffer had no effect on the PLCβ2 activity of cells transfected with the Goα mutants (data not shown). This experiment shows that the Goα mutants behave similarly *in vitro* and in cultured cells; GoαX binds βγ only when exogenous XDP is available.

DISCUSSION

We engineered a mutant of Goα which switched nucleotide binding activity from guanine nucleotides to xanthine nucleotides. The mutation (D273N) was at a conserved residue of the NKXD motif which appears in all GTPase superfamily proteins. Crystal structures of transducin and Gi showed that this aspartic acid residue participated in hydrogen bonding to the guanine ring (Fig. 1a). The proposed interaction between the mutagenized Asn and the xanthine ring is shown in Fig. 1b in which the hydrogen bond is "flipped" when compared with wild-type Gα. Similar single DN mutations have been made in other GTP binding proteins including EF-Tu (17,18), Ypt1 (19), rab-5 (20,21), FtsY (22) and *E.coli* adenylosuccinate synthetase (23), resulting in active proteins regulated by xanthine nucleotides instead of guanine nucleotides. However, the similar D119N mutant of H-Ras induced transformation of NIH 3T3 cells with efficiency indistinguishable from wild-type H-Ras (16, 37). Although the mutant D119N Ras exhibited decreased affinity for GTP and increased affinity for XTP (by 2 to 3 orders of magnitude), the high intracellular concentration of GTP (millimolar) probably ensures that the protein is still bound to the guanine nucleotides in the cell. Interestingly, we found the corresponding D273N mutation in Goα did not result in binding of either GTPγS or XTPγS, whereas the D273N/Q205L double mutant, GoαX, switched nucleotide binding ability. Examining the crystal structure of transducin, it is not clear why the QL mutation (position 200 in transducin α), which is at the opposite side of the nucleotide binding pocket from the DN mutation (position 268 in transducin α), rescued the xanthine nucleotide binding of GoαD273N. It is interesting to note that GoαX binds GTPγS at concentrations higher than 100 μM. In our nucleotide binding experiments, we could not observe this binding because the affinity was weak requiring concentrations

higher than 1 μM [^{35}S]GTPγS which was the highest concentration that we could use. The P-S bond of the γ phosphate in GTPγS is longer than the P-O bond in GTP, which not only prevents nucleotide hydrolysis when binding to G protein α subunits, but also results in qualitatively different interactions and different affinities.

In vitro experiments using limited trypsin digestion and PTX-induced ADP-ribosylation showed that GoαX retained the characteristic properties of wild-type Goα in the presence of XDP or XTP. In addition, our data confirm the assumption that diphosphate nucleotides are required for the interaction of G protein α subunits with βγ subunits. XTP-bound GoαX assumed a trypsin-resistant conformation similar to that of the activated wild-type Goα and stimulated βγ dissociation from the trimeric complex, suggesting that GoαX can be activated by XTP. In transfected COS-7 cells, PLCβ2 is activated by G protein βγ subunits, and the activity is inhibited when cotransfecting with Goα because of the competition for βγ. To study βγ binding of the mutant GoαX *in vivo*, we looked for inhibition of PLCβ2 activity as an indication of βγ binding. We found that GoαX did not affect βγ stimulated PLCβ2 activity because of the absence of XDP. In order to turn on βγ binding, we used α-toxin to make cell membranes permeable to XDP, and indeed under these conditions GoαX attenuated PLCβ2 activity. G protein derived βγ subunits are shown to be able to bind many proteins other than Gα, and may be involved in many signal transduction pathways. We demonstrated that XDP can be delivered into cells and GoαX may be used as a "βγ quencher" that can make the cellular βγ pool unavailable to other βγ effectors. The ability to turn on and off βγ *in vivo* could be useful in order to better understand the physiological function of βγ.

Goα is one of the G protein α subunits whose functions are not well understood although there is some evidence supporting a role in the regulation of calcium channels (38-42). Since βγ subunits are also proposed as regulators of calcium channels (43), it is difficult to differentiate the activities of Goα and βγ in some situations when activated receptors release both Goα and βγ subunits. This is one of the problems that the GoαX mutant might be used to address. The channel may be activated directly by adding XTP without releasing free βγ in cells that have been transfected with cDNA expressing the mutant protein. Cross-talk between the different G protein-mediated signaling pathways has been well demonstrated. Activating GoαX directly and instantly by XTP would avoid the interference of other pathways, and help us to differentiate individual pathways. Introducing this mutation into other G protein α subunits may be used to study their functions as well.

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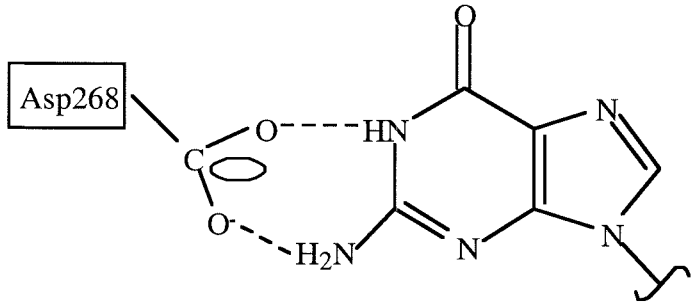
Figure 1 a) Interaction between the aspartic acid side chain at position 268 in the α subunit of transducin with the guanine ring of GTPγS, revealed by the solved crystal structure. b) A proposed model for the interaction between the substituted asparagine residue at position 273 in Goα and the xanthine ring.

Fig. 1

a



b

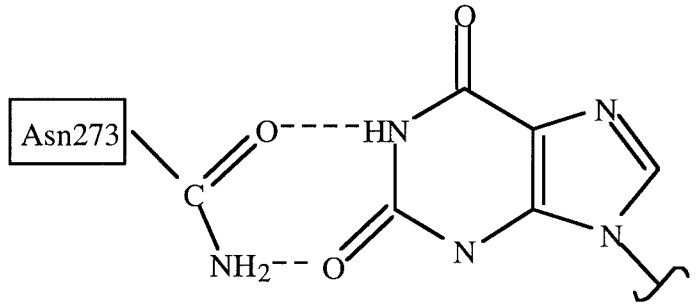


Figure 2 GoαX binds XTPγS but not GTPγS. 20 μl of the *E.coli* extract containing wild-type (a) Goα or (b) GoαX was diluted 10-fold with TEDM buffer containing either 0.1 μM [^{35}S]GTPγS or [^{35}S]XTPγS (20,000 cpm/pmol) and incubated at room temperature. At the indicated times, 20 μl aliquots were withdrawn and assayed for the bound nucleotides. GTPγS binding of the purified (c) His-six-tagged Goα and XTPγS binding of the purified (d) His-six-tagged GoαX were compared with those of untagged Goα and GoαX in the *E.coli* extract. After 40 minutes, 1 mM unlabeled (c) GTPγS or (d) XTP was introduced into the reaction.

Fig. 2 Xanthine nucleotide binding of GoαX

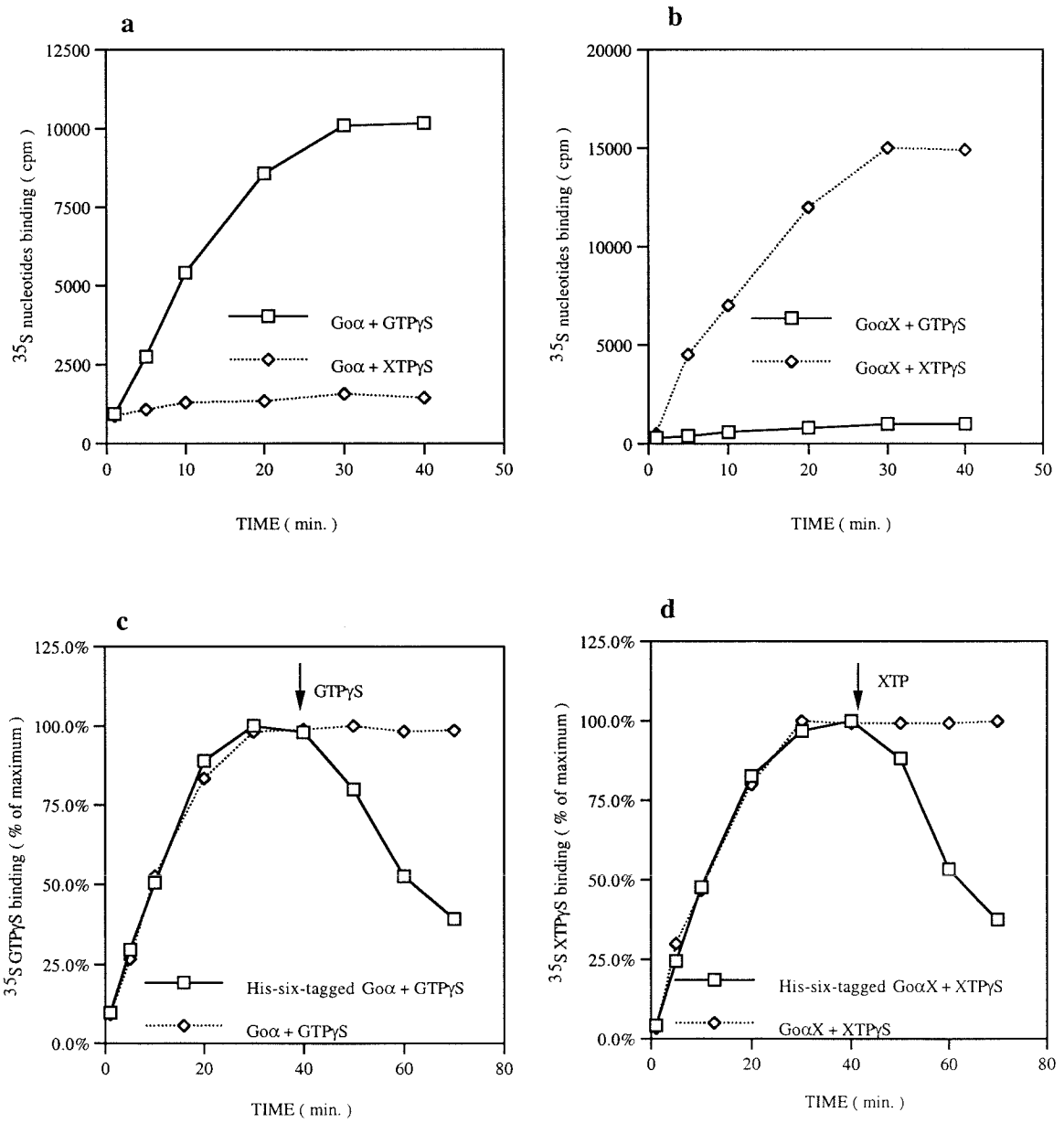
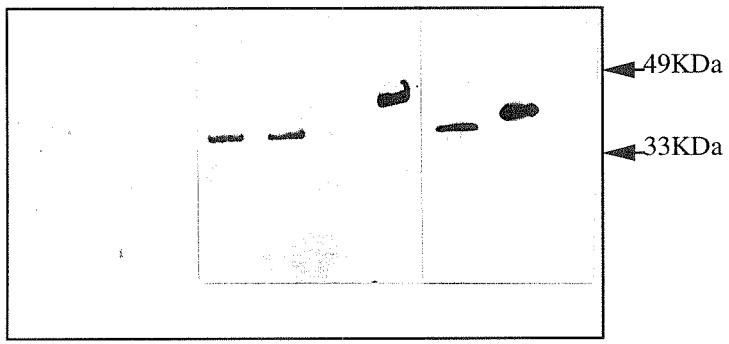


Figure 3 Functional Regulation of Goα by Xanthine Nucleotides. a) XTP protects the proteolysis of GoαX with trypsin. 0.1 μg of purified recombinant Goα or GoαX was incubated with indicated nucleotides at room temperature for 30 minutes. 10 ng of trypsin was then added to the mixture, and the reaction was terminated by addition of an equal volume of 2X SDS-PAGE sample buffer. The proteolytic pattern was visualized by Western Blot using an antibody against a C-terminal peptide of Goα. **b) PTX-induced ADP-ribosylation of GoαX requires XDP, and is inhibited by XTP.** 0.1 μg of purified recombinant Goα or GoαX was mixed with 0.1 μg of purified bovine retinal βγ complex in the presence of indicated nucleotides (100 μM of each, including the carry over GDP or XDP from the protein storage buffer) and incubated for 10 minutes at room temperature. Then the reaction mixture containing 10 μg/ml pertussis toxin, 0.5 μM [^{32}P]NAD (20,000 cpm/pmol), and other necessary components was added. Reactions were incubated for 30 minutes at room temperature and terminated by the addition of 10 μl 5X SDS-PAGE sample buffer. The samples were then resolved on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. The arrows indicate the positions of molecular weight markers.

Fig. 3 Functional Regulation of Goα by Xanthine Nucleotides

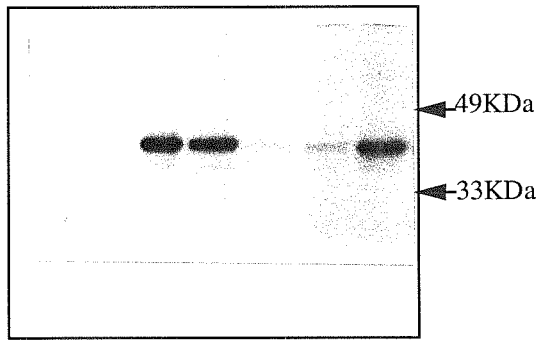
a. Proteolysis of Goα with Trypsin



GoαX Goα

	1	2	3	4	5	6	7	8	9	10
Trypsin	+	+	+	+	+	+	−	+	−	+
GTP (M)	−	−	10^{-4}	−	−	−	−	−	−	−
GTPγS (M)	10^{-4}	10^{-6}	−	−	−	−	−	10^{-6}	−	−
XTP (M)	−	−	−	10^{-4}	10^{-6}	−	−	−	−	−

b. PTX-Induced ADP-ribosylation of Goα

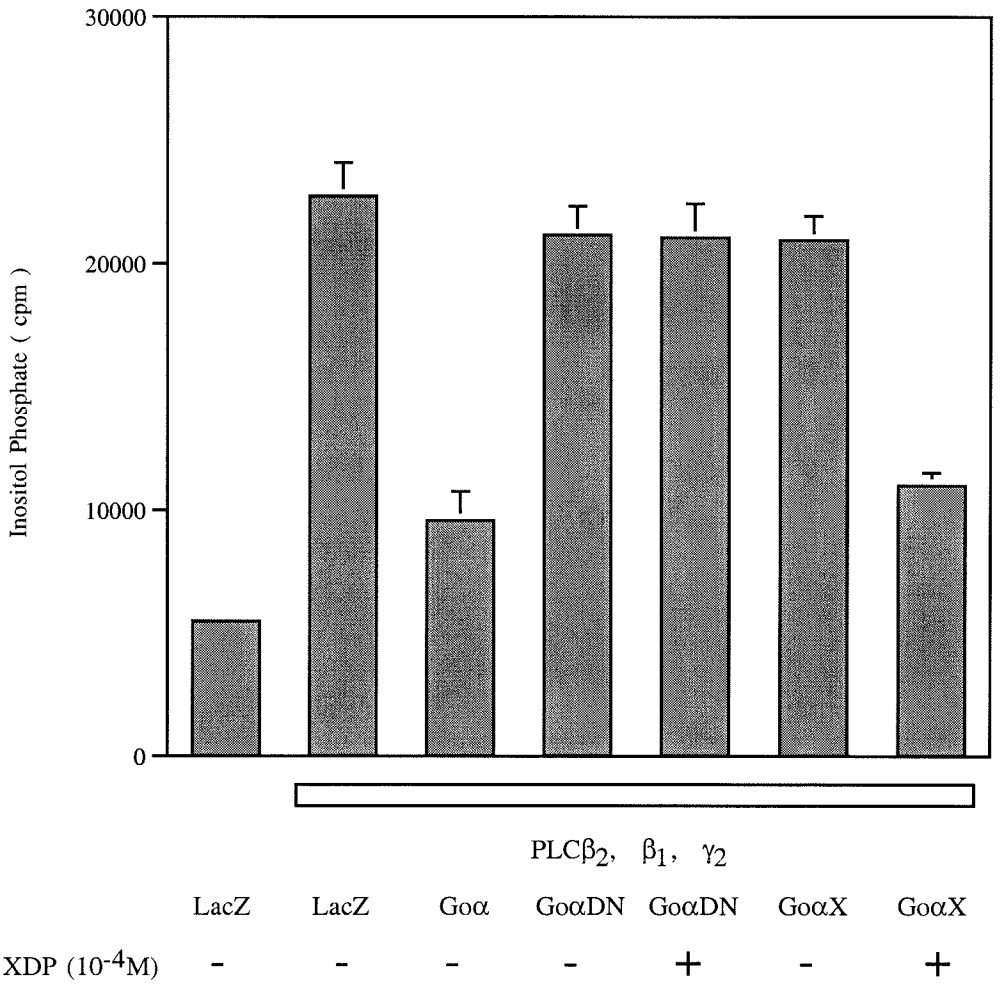


GoαX Goα

	1	2	3	4	5	6	7
GDP	−	−	+	−	+	+	+
GTP	−	−	+	−	−	−	−
GTPγS	+	−	−	−	−	+	−
XDP	+	+	+	+	−	−	−
XTP	−	+	−	−	−	−	−

Figure 4 The interaction of GoαX with βγ in transfected COS-7 cells is XDP-dependent. $1x10^5$ cells/well were seeded in a 12-well plate, and then were transfected with cDNAs encoding the indicated proteins the next day. The total amount of cDNA for each well was adjusted to 1.0 μg by addition of CMV-LacZ cDNA. Cells were labeled with [^{3}H]inositol, and the levels of inositol phosphates were determined after incubating cells with 200 U/ml α-toxin with or without 10^{-4} M XDP.

Fig. 4 βγ Coupling in Transfected COS-7 Cells



Chapter Three

Interaction of the Xanthine Nucleotide Binding Goα Mutant with G-protein-coupled Receptors

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Abstract

We constructed a double mutant version of the alpha-subunit of Go, that was regulated by xanthine nucleotides instead of guanine nucleotides (GoαX). We investigated the interaction between GoαX and G-protein-coupled receptors *in vitro*. First, we found that the activated m2 muscarinic cholingeric receptor (MAChR) could facilitate the exchange of XTPγS for XDP in the GoαXβγ heterotrimer, and second, the GoαXβγ complex was able to induce the high affinity ligand-binding state in the N-formyl peptide receptor (NFPR). These experiments demonstrated that GoαX was able to interact effectively with G-protein-coupled receptors. Third, we found that the empty form of GoαX, lacking a bound nucleotide and βγ, formed a stable complex with the m2 muscarinic cholingeric receptor associated with the plasma membrane. Finally, we investigated the interaction of GoαX with receptor in COS-7 cells. The empty form of GoαX bound tightly to the receptor and was not activated because XTP was not available intracellularly. We tested the ability of GoαX to inhibit the activities of several different G-protein-coupled receptors in transfected COS-7 cells, and found that GoαX specifically inhibited Go-coupled receptors. Thus the modified G proteins may act as dominant negative mutants to trap and inactivate specific subsets of receptors.

Hundreds of seven-transmembrane receptors activate heterotrimeric G proteins and transduce signals across cell membranes in eukaryotic cells. The stimulated receptors catalyze the exchange of GTP for GDP bound to G protein α subunits. Activated GTP bound α subunits and free βγ subunits regulate a variety of cellular effectors including enzymes and ion-channels (1-3). Signaling is normally initiated by the binding of agonist to receptor which stabilizes the receptor in an active conformation. Receptors function to stimulate the dissociation of GDP bound to the G protein α subunits. The subsequent binding of GTP to the empty α subunit promotes the conformational change of Gα and dissociation of the βγ subunits. The G protein α subunit in the nucleotide free state appears to be an important intermediate in the activation. From studies of rhodopsin and transducin, it has been postulated that the empty G protein (nucleotide free) forms a stable complex with the receptor (4). Both empty forms of Gi and Go α subunits have been purified under harsh conditions (1 M $(NH_4)_2SO_4$ and 20% glycerol), and they were unstable (5).

We recently reported that a mutant version of Goα, GoαX (GoαD273N/Q205L), was regulated by xanthine nucleotides, not by guanine nucleotides (6). GoαX bound XDP and XTP instead of GDP or GTP. GoαX bound G protein βγ subunits only in the presence of XDP, and XTP stimulated dissociation of the GoαXβγ heterotrimer. XTP-bound GoαX underwent a conformational change similar to the activated wild-type Goα. In the present study, we investigated the interaction between GoαX and G-protein-coupled receptors. We found that GoαX mutant proteins retained the receptor binding specificity of the wild-type Goα and were able to interact with Go-coupled receptors, such as the m2 muscarinic cholinergic receptor (MAChR), N-formyl peptide receptor (NFPR), and thrombin receptor, but not with m1 MAChR or thyrotropin-releasing hormone (TRH) receptor which do not couple to wild-type Go. Since the concentrations

of XDP and XTP are relatively low *in vivo* (7), GoαX mutant proteins are essentially nucleotide-free unless exogenous xanthine nucleotides are provided. Thus, GoαX provides an excellent model to study the receptor interaction of empty G protein α subunits. Consistent with the previously reported studies on the empty form of transducin (4), our data are most readily interpreted as showing that "empty" GoαX can form a stable complex with appropriate receptors on the membrane.

MATERIALS AND METHODS

Materials. Purified bovine retinal transducin βγ were generous gifts from Dr. O. Nakanishi. Xanthine and guanine nucleotides were from Sigma. All the radioactive ligands including [^{35}S]ATPγS, [^{35}S]GTPγS, [^{3}H]DNB, and fML[^{3}H]P were from DuPont NEN.

Expression and Purification of His$_6$-tagged Goα. Both wild-type Goα and mutant GoαX were subcloned into the *E.coli* expression vector pET-15b (Novagen) with His$_6$ tag at the N terminus. These clones were used to transform the *E.coli* strain BL21(DE3), and proteins were expressed. Expression and purification of these proteins was described previously (6, 8). After harvesting the culture, cell extracts were resuspended in the binding buffer (5 mM imidazole, 0.5 M NaCl, 160 mM Tris-HCl, pH 7.9, and 1 mM βMe). The His$_6$-tagged proteins were purified from Ni^{2+}-NTA column according to the protocol provided by Novagen. Purified proteins were stored in TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT) with 0.1 mM $MgCl_2$ and 0.1 mM nucleotide diphosphate (GDP or XDP as appropriate).

Membrane Preparation from Baculovirus Infected Sf9 Cells. Sf9 cells were grown and infected with recombinant baculoviruses encoding either m2 MAChR or NFPR (9-11). Infected cells were centrifuged and resuspended at $< 10^7$ cells/ml in HME/PI buffer

(20 mM NaHepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspension was homogenized by 10 strokes in a glass homogenizer followed by passing through a 27 gauge hypodermic needle several times. The homogenate was briefly centrifuged at 3,000 x g for 10 min., and then the supernatant was collected and centrifuged at 30,000 x g for 30 min. The pellet was washed once with HME/PI and the final pellet was resuspended in HME/PI at a protein concentration of 5 mg/ml.

Synthesis of XTPγS. XTPγS was synthesized from XDP and ATPγS with Nucleotide Diphosphate Kinase (NDK) as described previously (12). To produce ^{35}S labeled XTPγS, the reaction contained 10 μM XDP, 1 μM [^{35}S]ATPγS, and 10 units NDK (Sigma) in 100 μl NDK buffer (1 mM $MgCl_2$, 5 mM DTT, and 20 mM Tris-HCl, pH 8.0). The mixture was incubated at room temperature for 2 hours. The resulting concentration of [^{35}S]XTPγS was about 1 μM (1 μCi /pmol). The radiochemical purity of XTPγS was monitored by TLC on Avicel/PEAE plates (Analtech) in 0.07 N HCl.

Nucleotide Binding of Purified Goα. Binding of [^{35}S]GTPγS and [^{35}S]XTPγS to the recombinant Goα or the mutant proteins was performed as described (6). The binding reaction contained 0.5 μg of purified protein in TED buffer, with 0.1 mM $MgCl_2$, 1 μM ATP, and 0.1 μM GTPγS or XTPγS (20,000 cpm/pmol). For the time course experiments, 20-μl aliquots were withdrawn from a 200 μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM $MgCl_2$, filtered through 45-μm nitrocellulose, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

Radioligand Binding of Receptors. The ligand binding assays of membrane-bound receptors were performed as described (9-11). The total concentration of m2 MAChR and the affinities of NFPR were determined by incubating membranes with 2 nM [^{3}H]QNB

or various concentrations of fML[^{3}H]P for 1 hour in 20 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1 mM EDTA at 30°C in a final volume of 0.5 ml. Nonspecific binding was defined as binding that was not displaced by 10 μM atropine for m2 MAChR or 10 μM fMLP for NFPR. Unbound ligands were removed by filtration through Whatman GF/C filters and washing 4 times using ice-cold binding buffer. The amount of bound radioactivity was determined by scintillation counting.

Binding of Goα on Sf9 Cell Membranes. 0.2 μg of purified Goα or Goα mutant proteins were incubated with 100 μg of Sf9 cell membranes in TED buffer of a final volume of 100 μl at room temperature for 1 hour. The membranes were centrifuged and subjected to Western Blot using antibodies against Goα.

Cell Culture and Transfection. COS-7 cells were cultured in DMEM containing 10% FCS. $1x10^5$ cells/well were seeded in 12-well plates 1 day before transfection. All transfection assays contained a total amount of 1 μg DNA, and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg DNA was mixed with 5 μl lipofectamin (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and 5 hours later, 0.5 ml of 20% FCS in DMEM was added to the medium. After 48 hours, cells were assayed for inositol phosphate levels as described previously (13, 14).

RESULTS

Stimulation of XTPγS Binding of GoαX by Activated M2 Muscarinic Receptor

In order to test if GoαX could interact with G-protein-coupled receptors, we investigated the receptor stimulated nucleotide binding of GoαX. Activated G-protein-coupled receptors are known to facilitate the binding of GTPγS to G protein α subunits. It

has been reported that recombinant m2 MAChR from Sf9 cells stimulated the binding of GTPγS to wild-type Goα two to three-fold in response to muscarinic agonists (9, 10). We infected Sf9 cells with recombinant baculoviruses encoding m2 MAChR and prepared membranes. The concentration of receptor was about 20 pmol/mg membrane protein, determined from [^{3}H]QNB binding. We have previously shown that GoαX mutant proteins bind only xanthine nucleotides, but not guanine nucleotides. In this experiment, we reconstituted purified GoαX with Sf9 cell membranes containing m2 MAChR in the presence of XDP and G protein βγ subunits purified from bovine retina, and followed agonist dependent stimulation of XTPγS binding to GoαX. We found that carbachol accelerated the XTPγS binding of GoαX, in a fashion similar to the acceleration of GTPγS binding observed with wild-type Goα (Fig. 1a). In control experiments using wild-type Sf9 cell membranes, both atropine and carbachol had no effect on the XTPγS binding of GoαX (Fig. 1a). βγ subunits were required for the carbachol dependent stimulation of nucleotide binding (Fig. 1b), suggesting that only the trimeric complex of GoαX with βγ can be activated to exchange XDP for XTPγS by the ligand bound receptors.

High Affinity Ligand Binding of N-formyl Peptide Receptor Induced by GoαX

Another well-documented indication of receptor-G protein interaction is that GTP or GTPγS inhibits the high affinity binding of G-protein-coupled receptors to their agonists. NFPR receptors expressed in Sf9 cells are known to be in the low ligand affinity state (~60 nM fMLP) (11), presumably because of the lack of mammalian Gi-like G proteins in Sf9 cells, whereas NFPR in neutrophils and NFPR expressed in mouse L cells exhibited high affinity ligand binding (0.5-3 nM fMLP) (15, 16). Therefore, we reconstituted GoαX with NFPR from Sf9 cells and investigated if GoαX could induce the high affinity ligand binding state in NFPR receptors. Sf9 cell membranes containing

NFPR receptors were prepared as described in Materials and Methods. The expression level of NFPR was about 20 pmol/mg membrane protein, determined by fML[^{3}H]P binding. We incubated the NFPR with wild-type Goα or mutant GoαX in the presence of βγ and varieties of nucleotides, and determined their affinities for the agonist fMLP. As expected, NFPR expressed in Sf9 cells showed low ligand affinity binding of fMLP (~100 nM), and GoαX alone did not affect ligand binding (Fig. 2a). More interestingly, NFPR exhibited high affinity ligand binding (~10 nM) when GoαX, βγ and XDP were present (Fig. 2a). Both βγ and XDP were required to induce the high ligand affinity state of NFPR, and XTP inhibited the fMLP binding of the receptors (Fig. 2b). In the control experiments, wild-type Go αβγ heterotrimer was also found to convert the NFPR to the high affinity ligand binding state, which was inhibited by GTPγS (Fig. 2b). These experiments demonstrated that the heterotrimeric complex of GoαXβγ can interact efficiently with NFPR.

Binding of GoαX with M2 Muscarinic Receptor on Sf9 Cell Membranes

The previous two experiments showed that GoαXβγ heterotrimer could interact with the G-protein-coupled receptors efficiently, and that the interaction was similar to the interaction between wild-type Go and receptors. To investigate receptor interaction of GoαX more directly, we studied binding of GoαX directly to receptor containing Sf9 cell membranes. Purified wild-type Goα or GoαX were incubated with Sf9 cell membranes containing m2 MAChR in the presence of different reagents. The membranes were then pelleted and subjected to Western Blotting using antibodies against Goα to see if Goα remained bound to the membrane. In the control experiments using wild-type Sf9 cell membranes without m2 MAChR, both wild-type Goα and GoαX did not remain associated with the membrane. However, wild-type Goα was bound to membrane when it was coincubated with βγ. Similarly, GoαX stayed on the membrane when in complex

with βγ in the presence of XDP (data not shown). These experiments using wild-type Sf9 cell membranes showed that Goα bound to the membranes only in the αβγ complex form, presumably because βγ facilitates membrane association. In the experiments using membranes containing m2 MAChR, we found somewhat surprisingly that GoαX bound to receptor containing membranes even in the absence of carbachol and without βγ (Fig. 3 lane 12), whereas wild-type Goα did not (Fig. 3 lane 1), suggesting GoαX alone was able to bind to receptor. Interestingly, both XDP and XTP abolished the interaction between GoαX and m2 MAChR containing membranes and released GoαX from the membrane fraction (Fig. 3 lane 7 and 8), whereas GDP or GTP had no effect (data not shown), suggesting that the nucleotide free form of GoαX can recognize and bind to Go-mediated receptor. As expected, GoαX stayed on the membrane when both XDP and βγ were present (Fig. 3 lane 10), and XTP promoted dissociation of the GoαXβγ complex (Fig. 3 lane 9). In the case of wild-type Goα, the binding pattern was the same between membranes with or without the receptors, and XDP or XTP had no effect on binding (Fig. 3 lane 1-5). In a titration experiment, quantitation of GoαX revealed that the amount of GoαX bound to the membrane increased linearly until it reached saturation, and the level of saturation was proportional to the amount of receptor incubated in the reaction (Fig. 4a, b and c). Furthermore, similar experiments using Sf9 cell membranes containing NFPR were also performed and the results were similar (data not shown). These experiments indicated that the empty form of GoαX, without a bound nucleotide and βγ, could form a stable complex with receptor. In summary, these data suggest that GoαX with XDP bound and βγ bind to membranes while the XTP form is found to be cytoplasmic. The nucleotide free form is able to bind to Go-mediated receptors.

Dominant Negative Effect of GoαX on Receptor Activation in COS-7 Cells

Since our experiments suggested that empty GoαX was able to bind to the receptor *in vitro*, we went on to test for this interaction in intact cells. Indeed, we found that GoαX was able to interact with receptors and inhibit their activities in COS-7 consistent with the observation that GoαX did not dissociate from the receptors without xanthine nucleotides. Thrombin receptors are known to couple with G proteins from both the Gi and Gq families (17). In COS-7 cells transfected with the thrombin receptor, endogenous Gq is activated by the addition of thrombin, and stimulates PLC β isoforms to elevate cellular IP_3 concentration. Inhibition of receptor activation in transfected cells by wild-type G proteins was observed before (18). Thus if cells are cotransfected with both the thrombin receptor and wild-type Goα subunit, the activation of Gq is inhibited because of the competition of Goα for the receptor or endogenous G protein βγ subunits (Fig. 5a). We cotransfected thrombin receptor and GoαX to determine if GoαX could compete with endogenous Gq for the receptors. Indeed, we found that GoαX inhibited Gq activity stimulated by thrombin, and the inhibition was proportional to the amount of GoαX cDNA used in the transfection (Fig. 5a). Since GoαX in the absence of XDP does not interact with βγ and does not affect the βγ stimulated PLCβ2 activity in COS-7 cells (6), the inhibition by GoαX of Gq activation stimulated by the thrombin receptor must come from the competitive binding of GoαX to the receptor. Similar experiments were performed with m1 MAChR and TRH receptor which were known to couple only to the Gq family of Gα proteins, and not to the Goα family (10, 19, 20). GoαX had no effect on the activation of m1 MAChR or TRH receptor (Fig. 5b and 5c). On the other hand wild-type Goα which can compete for endogenous βγ inhibited both m1 MAChR and TRH receptor stimulation as expected. In COS-7 cells, the activation of thrombin receptor, m1 MAChR, and TRH receptor share the same Gq pathway downstream of the receptor. Since GoαX inhibited only the thrombin receptor activity, but had no effect on m1

MAChR or TRH receptor, we concluded that GoαX inhibited thrombin receptor stimulation by competitive binding to the receptor.

In order to test if GoαX could bind to other Go-coupled receptors in cells, we looked into the interaction between GoαX and m2 MAChR. Since m2 MAChR couples only to the Gi family of Gα proteins and not to the Gq family (9, 21), we could not assay their interaction in the same way as the thrombin receptor by monitoring PLC activities in COS-7 cells transfected with the receptor and GoαX. Therefore, we constructed an artificial pathway by cotransfecting both m2 MAChR and G15α into COS-7 cells. G15 is known as a promiscuous G protein which can be activated by all kinds of G-protein-coupled receptors, and G15 also actives PLC β isoforms (21). In cells cotransfected with both m2 MAChR and G15α, we were able to activate endogenous PLC β isoforms by the addition of the muscarinic agonist carbachol. We found that this m2 MAChR stimulation pathway could also be inhibited by GoαX (Fig. 5d). All these experiments suggested that GoαX was able to interact with G-protein-coupled receptors in cells and retained the receptor specificity of wild-type Goα; it coupled with thrombin receptor and m2 MAChR, but not with m1 MAChR or TRH receptor. Furthermore, GoαX exhibited dominant negative inhibitory effects against these receptors in cells.

DISCUSSION

GoαX (GoαD273N/Q205L) was the first reported mutant of heterotrimeric G protein α subunits that bound xanthine nucleotides, not guanine nucleotides (6). It bound βγ only in the presence of XDP, and could be activated by XTP. We continued to study the interaction of GoαX with G-protein-coupled receptors in this report. The interaction of G proteins and their receptors is best demonstrated in two experiments: agonist stimulated GTPγS binding of G protein α subunits and inhibition of high affinity ligand

binding of the receptors by GTPγS. To test if GoαX can interact with G-protein-coupled receptors and be activated by their agonists, we reconstituted purified GoαX with Sf9 cell membranes containing m2 MAChR or NFPR. First, we found that binding of XTPγS to GoαX was stimulated by the muscarinic agonist carbachol in the presence of m2 MAChR. In similar experiments using wild-type Goα, GTPγS binding was also stimulated by carbachol. In both cases, βγ was required for the carbachol dependent nucleotide binding, suggesting that only GoαXβγ heterotrimer could interact with the receptors effectively. Second, we tested GoαX to determine if it could induce the high affinity state in NFPR receptors expressed in Sf9 cells. The NFPR expressed in these cells is known to be in the low affinity state probably because of lack of mammalian Gi-like proteins in Sf9 cells (11). In our experiments, we found that GoαX could convert NFPR into the high affinity state in the presence of βγ and XDP, and this effect was inhibited by XTP. These two experiments demonstrated that GoαX, when in complex with βγ and XDP, could interact with G-protein-coupled receptors effectively and be activated by the agonists.

Because cells lack xanthine nucleotides, GoαX provides an excellent model to study empty G protein α subunits. The empty form of Gα is an important intermediate in receptor activation, and has long been proposed to form a stable complex with activated receptors. However, stable interaction between empty G proteins and their receptors was only reported in the transducin-rhodopsin system. Empty transducin apparently formed stable complex with light-activated rhodopsin and stayed on the ROS membrane. Interestingly, deactivation of the rhodopsin did not lead to the dissociation of transducin from the complex (4). In this report, we showed that empty GoαX was able to bind to the receptor on the membrane in the absence of βγ subunits and without agonists, and the interaction could be abolished by either XDP or XTP. The amount of GoαX

associated on the membranes with m2 MAChR was proportional to the amount of receptor at saturation. Interestingly, binding of GoαX alone did not convert the receptor to the high ligand affinity conformation, which required the αβγ complex. Therefore, the binding of GoαX alone to the receptor is not functional in contrast with the binding in presence of βγ and XDP.

Since GoαX appears to form a stable complex with the receptor, we explored this aspect to test if GoαX could inhibit receptor activation in cells. In transfected COS-7 cells, we showed that GoαX was able to inhibit thrombin receptor or m2 MAChR stimulated PLC β activities via Gq or G15 pathway, but had no effect on m1 MAChR or TRH receptor stimulation. Since both thrombin receptor and m2 MAChR are known to couple with wild-type Go, and m1 MAChR and TRH receptor only couple with Gq, we interpret the data to mean that GoαX retained the receptor specificity of wild-type Go and was able to interact with Go-coupled receptors in cells. The inhibitory binding of GoαX enables us to specifically block Go-coupled receptors in certain systems. This could be a useful means to analyze different receptor stimulated signal transduction pathways, and could perhaps be useful in drug screening associated with G-protein-coupled receptors.

In the previous report (6), we showed that the single Goα mutant, GoαD273N, lost the ability to bind either guanine nucleotides or xanthine nucleotides, and could not bind βγ under any conditions. Surprisingly, GoαD273N can still bind to receptors. In transfected COS-7 cells, we found that GoαD273N inhibited thrombin receptor and m2 MAChR activation, in a fashion similar to GoαX (Fig. 5a and d). GoαD273N also retained the same receptor specificity as wild-type Goα; i.e., it had no effect on m1 MAChR or TRH receptor stimulated pathways (Fig. 5b and c). In the Sf9 cell membrane binding assay, it only bound to the m2 MAChR membranes, not to the control wild-type

Sf9 cell membranes. However, in contrast to GoαX, GoαD273N was not released from the m2 MAChR membranes by XDP or XTP, consistent with its inability to bind nucleotides (data not shown). The reason that GoαD273N mutant proteins do not bind xanthine nucleotides is not clear. Apparently it must have attained a profile structure similar to that of the empty Goα which enables it to bind receptors, but the structure is probably not stable locally around the nucleotide binding pocket. Nevertheless, GoαD273N can also be used as dominant negative inhibitor of receptor functions.

Acknowledgments--The recombinant baculovirus encoding m2 MAChR was a generous gift from Dr. E. Ross. We thank members of the Simon lab for helpful discussions, and Dr. Tau-Mu Yi for comments on the manuscript.

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Figure 1 M2 MAChR stimulated XTPγS binding of GoαX. a) 0.5 μg of purified GoαX was incubated with 1 μg of βγ, 100 μg of m2 MAChR membranes or control Sf9 cell membranes, and 10 μM XDP in TEDM buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1mM DTT, and 1 mM $MgCl_2$) for 30 min. at room temperature, then the mixture was diluted 10-fold with TEDM buffer containing 0.1 μM [^{35}S]XTPγS (20,000 cpm/pmol) and 100 μM carbachol or 2 μM atropine at time 0. 20-μl aliquots were withdrawn and assayed for the bound nucleotides at the indicated times. b) 0.5 μg of purified wild-type Goα or GoαX were subjected to the similar nucleotide binding assay as in a) under indicated conditions. Only data at 20 min. were shown as the percentage of maximum binding.

Fig.1a XTPγS binding of GoαX

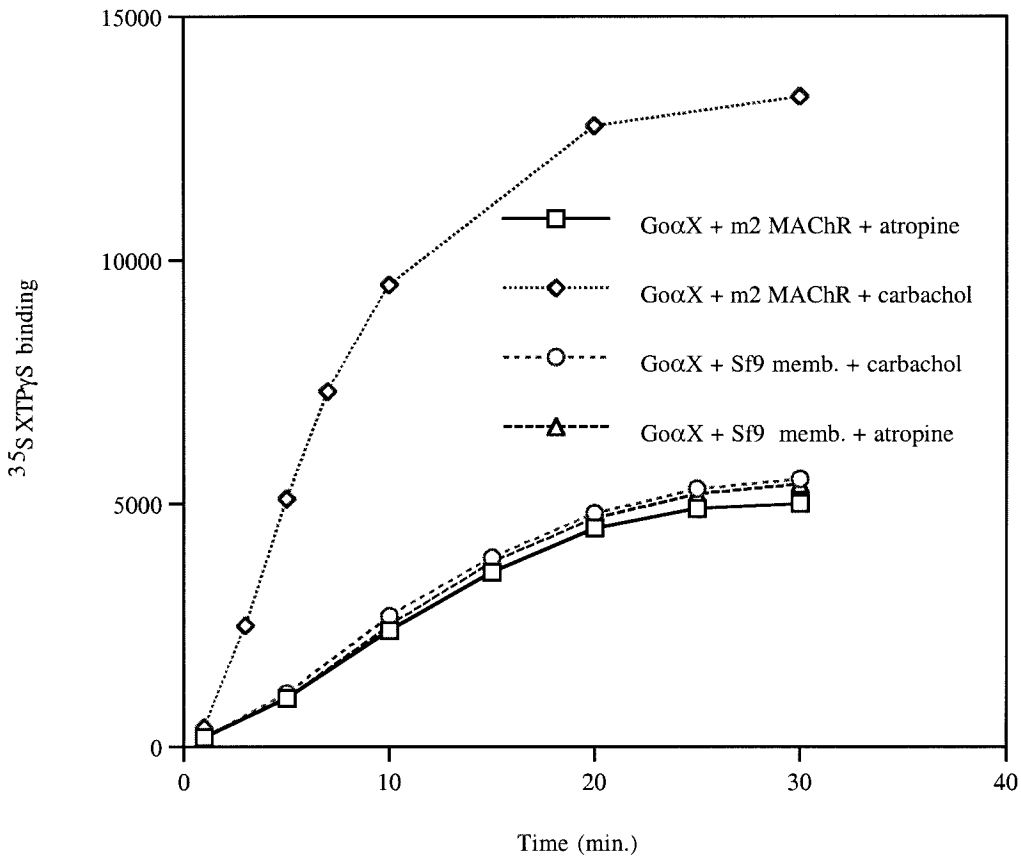


Fig.1b Receptor stimulated nucleotide binding of GoαX at 20 min.

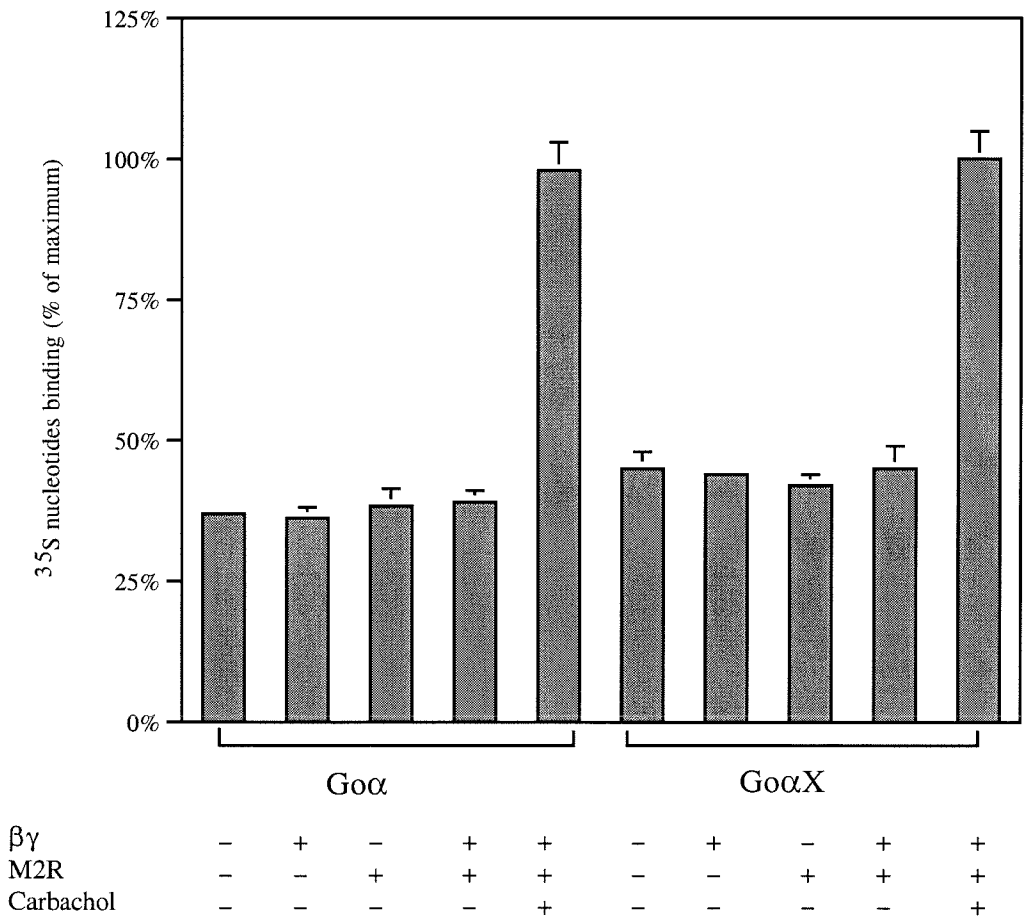


Figure 2 High affinity ligand binding of NFPR induced by GoαX. 10 μg of NFPR membranes or wild-type Sf9 cell membranes were incubated with various concentrations of fML[^{3}H]P for 1 hour in 20 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1mM EDTA at 30°C in a final volume of 0.5 ml, in the presence of 0.1 μg of GoαX, 0.2 μg of βγ, and 100 μM XDP, or GoαX alone (a). Amount of bound radioligand then was determined. Nonspecific binding was defined as binding in the presence of 10 μM cold fMLP which was less than 10% of total ligand binding, and was subtracted before analyzing. In (b), NFPR was incubated with 50 nM fML[^{3}H]P and various reagents under the same conditions as (a).

Fig. 2a Binding of fML[^{3}H]P by NFPR

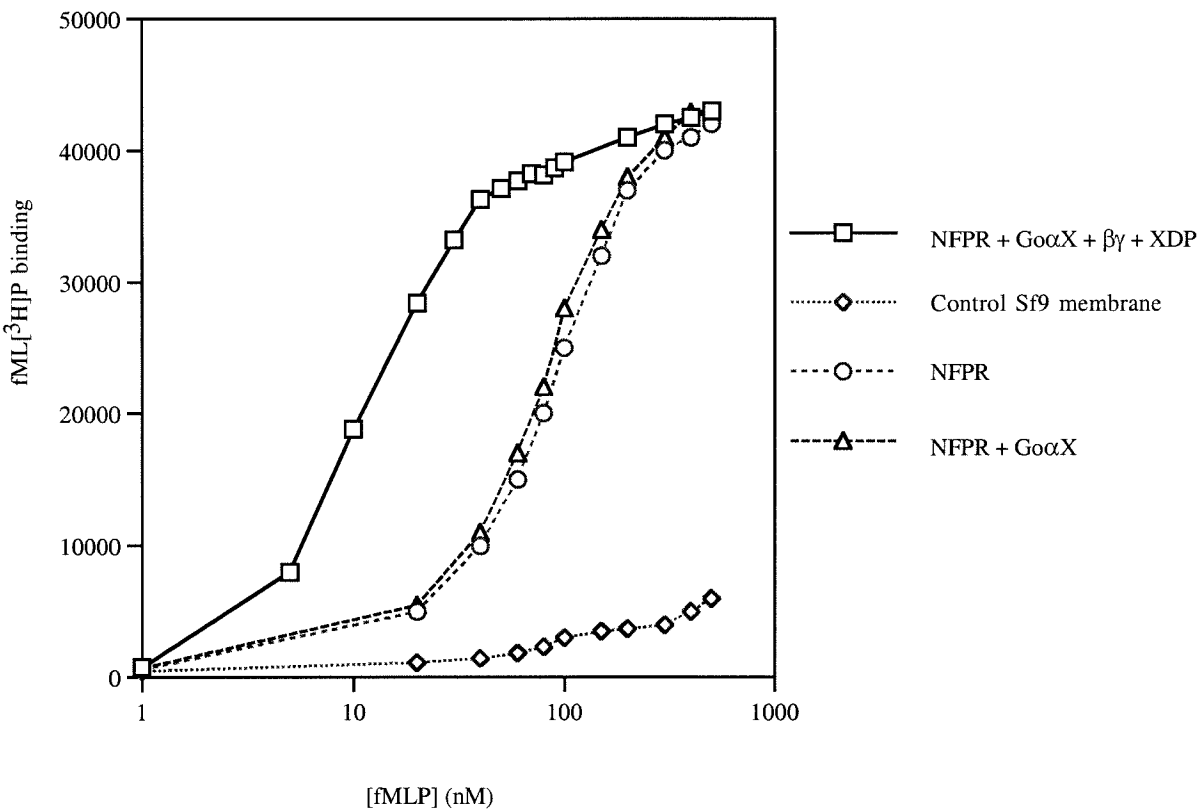
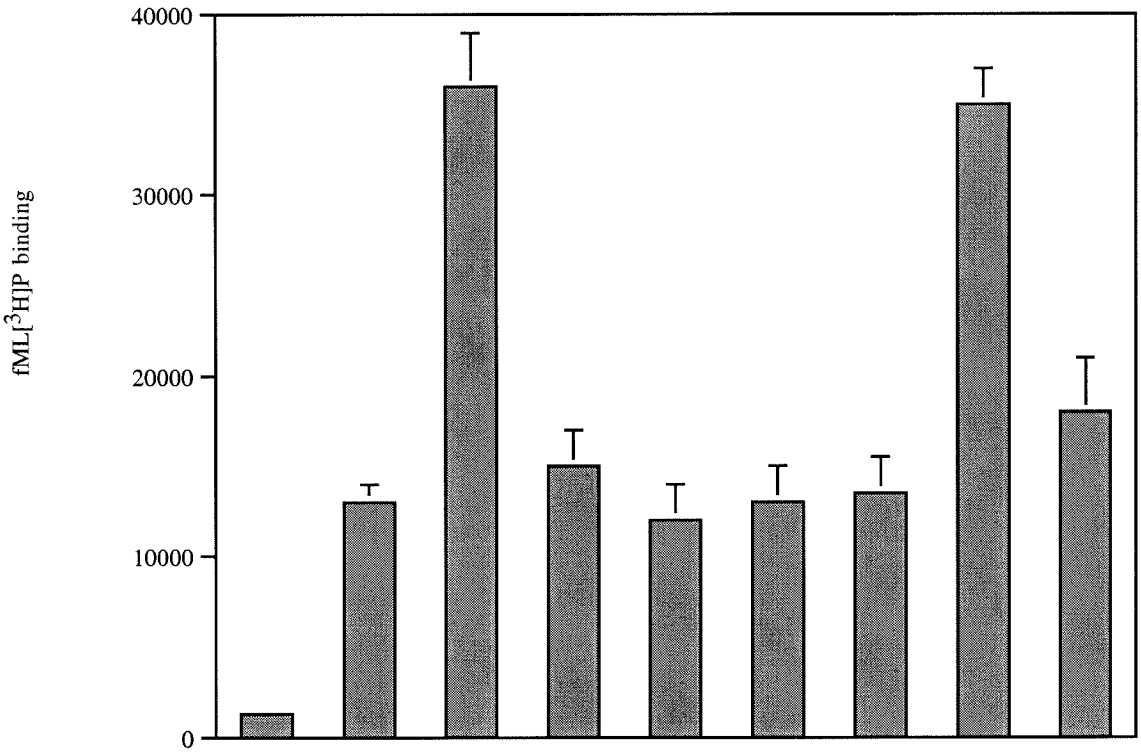


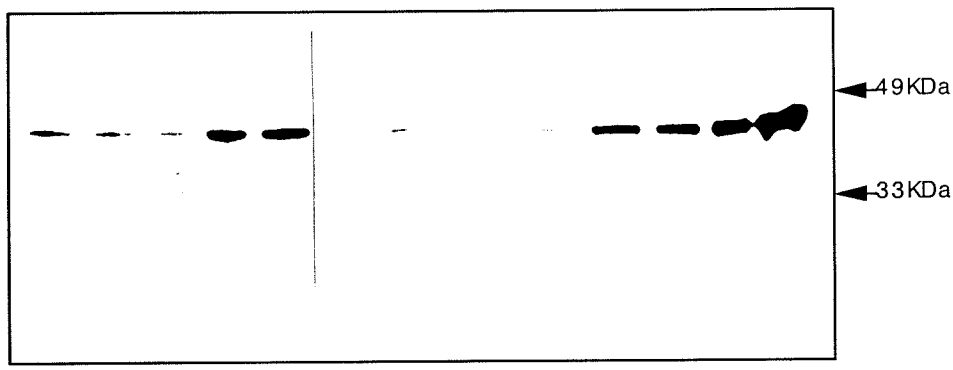
Fig. 2b Ligand binding of NFPR at 50 nM [fMLP]



NFPR Memb. (10 mg)	–	+	+	+	+	+	+	+	+
Goα (0.1 μg)	–	–	+	+	+	–	–	–	–
βγ (0.2 μg)	–	–	+	+	–	–	+	+	+
GTPγS (100 μM)	–	–	–	+	–	–	–	–	–
GoαX (0.1 μg)	–	–	–	–	+	+	+	+	+
XDP (100 μM)	–	–	–	–	–	+	–	+	+
XTP (100 μM)	–	–	–	–	–	–	–	–	+

Figure 3 Binding of GoαX to m2 MAChR on Sf9 cell membranes. 0.2 μg of wild-type Goα (with 100 μM GDP) or GoαX were incubated with 100 μg of m2 MAChR membranes in TED buffer of a final volume of 100 μl at room temperature for 1 hour with indicated reagents. The membrane then was centrifuged and subjected to Western Blot using antibodies against Goα. All nucleotide concentrations were 100 μM and the amount of βγ was 0.5 μg. The lane 13 showed the total amount of Goα used in each assay.

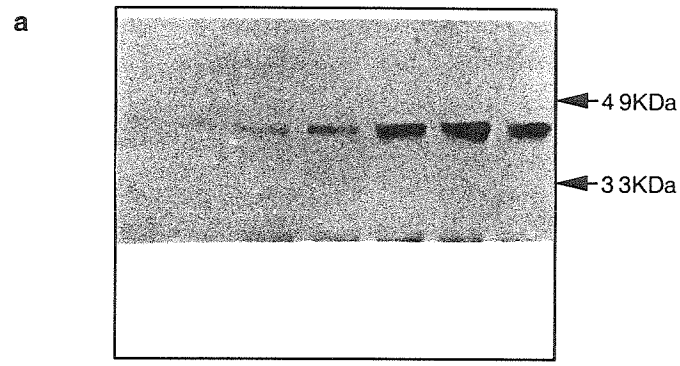
Fig. 3 Binding of GoαX to m2 MAChR membrane

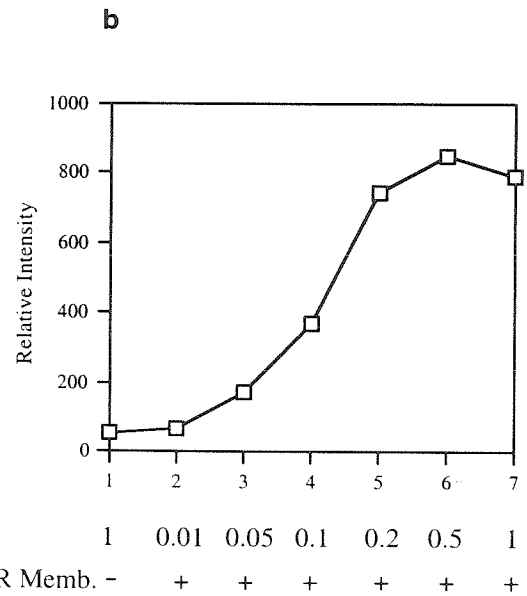


	Goα					GoαX							
	1	2	3	4	5	6	7	8	9	10	11	12	13
M2R Memb.	+	+	+	+	+	−	+	+	+	+	+	+	
βγ	−	−	+	+	+	−	−	−	+	+	+	−	
GTPγS	−	−	+	−	−	−	−	−	−	−	−	−	
XDP	−	+	−	+	−	−	−	+	+	+	−	−	
XTP	−	+	−	+	−	−	+	−	+	−	−	−	

Figure 4 Titration of GoαX bound to m2 MAChR on Sf9 cell membranes The binding assays were done at the same conditions described in Fig. 3 legend. (a) Indicated amount of GoαX was incubated with 100 μg of m2 MAChR membranes in TED buffer. (b) The relative intensities of bands in (a) were quantitated. (c) Quantitated binding of 0.2 μg of GoαX with indicated amount of m2 MAChR membranes. Wild-type Sf9 cell membranes were used to maintain a constant amount of membranes of 200 μg in each binding reaction.

Fig. 4 Quatitation of GoαX bound to M2 MAChR membrane





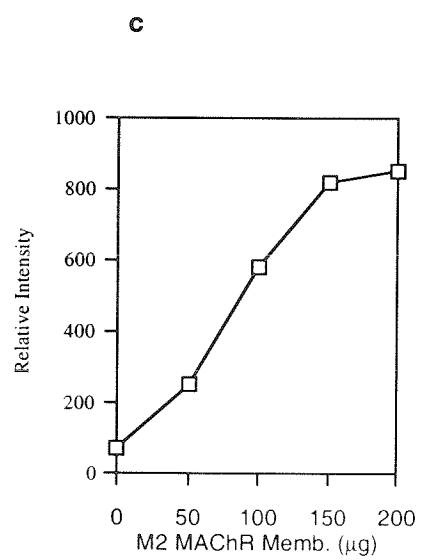


Figure 5 The negative inhibitory effects of GoαX on receptor-stimulated PLCβ activation in COS-7 cells. 1 x 10^5 cells/well were seeded in a 12-well plate and then were transfected with cDNAs encoding the indicated G proteins (GoαDN designates GoαD273N) and thrombin receptor (a), or m1 MAChR (b), or TRH receptor (c), or m2 MAChR (d). In (a), (b) and (c), the amount of receptor cDNA used in each well was 0.25 μg, and the amount of Goα cDNA was 0.75 μg per well unless otherwise indicated. In (d), amount of both m2 MAChR and G15α cDNA was 0.2 μg per well, and that of Goα was 0.6 μg per well. The total amount of cDNA for each well was adjusted to 1.0 μg by addition of CMV-LacZ cDNA. After cells were labeled with [^{3}H]inositol overnight, they were incubated in the medium containing 0.1 unit/ml thrombin (a), or 1 μM carbachol (b and d), or 1 μM TRH (c) before levels of inositol phosphates were determined.

Fig. 5a Inhibition of thrombin receptor activation by GoαX

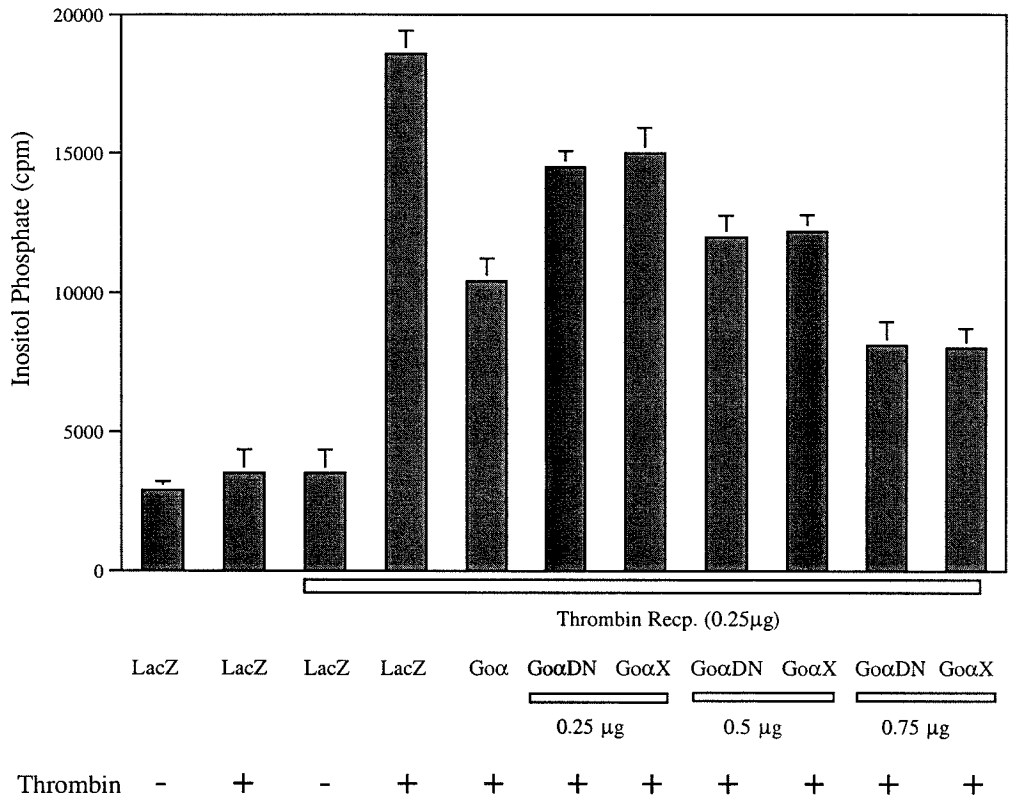


Fig. 5b M1 MAChR stimulated PLC activities

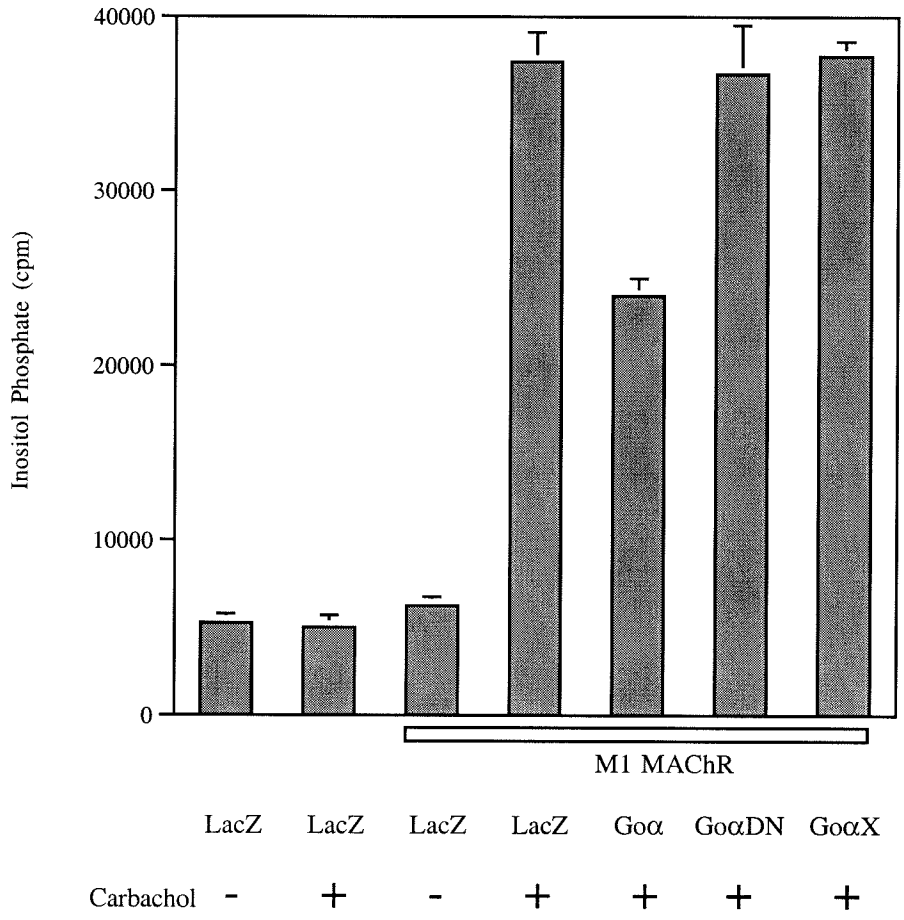


Fig. 5c TRH receptor stimulated PLC activities

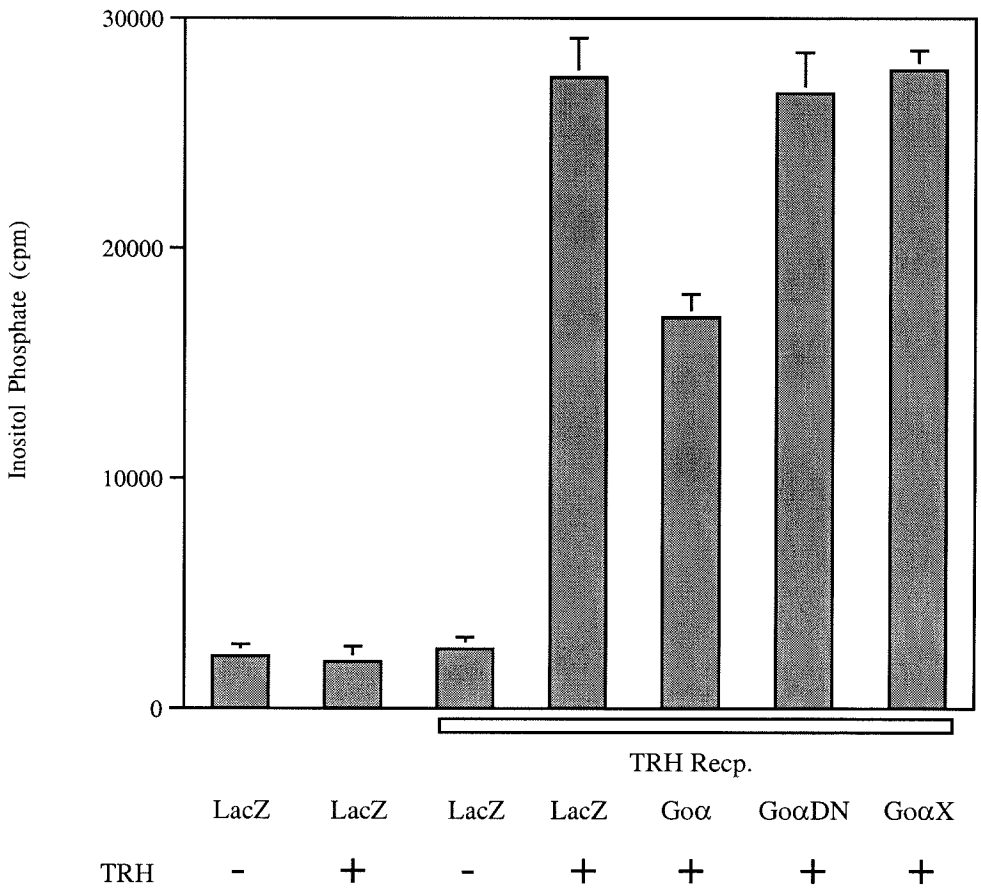
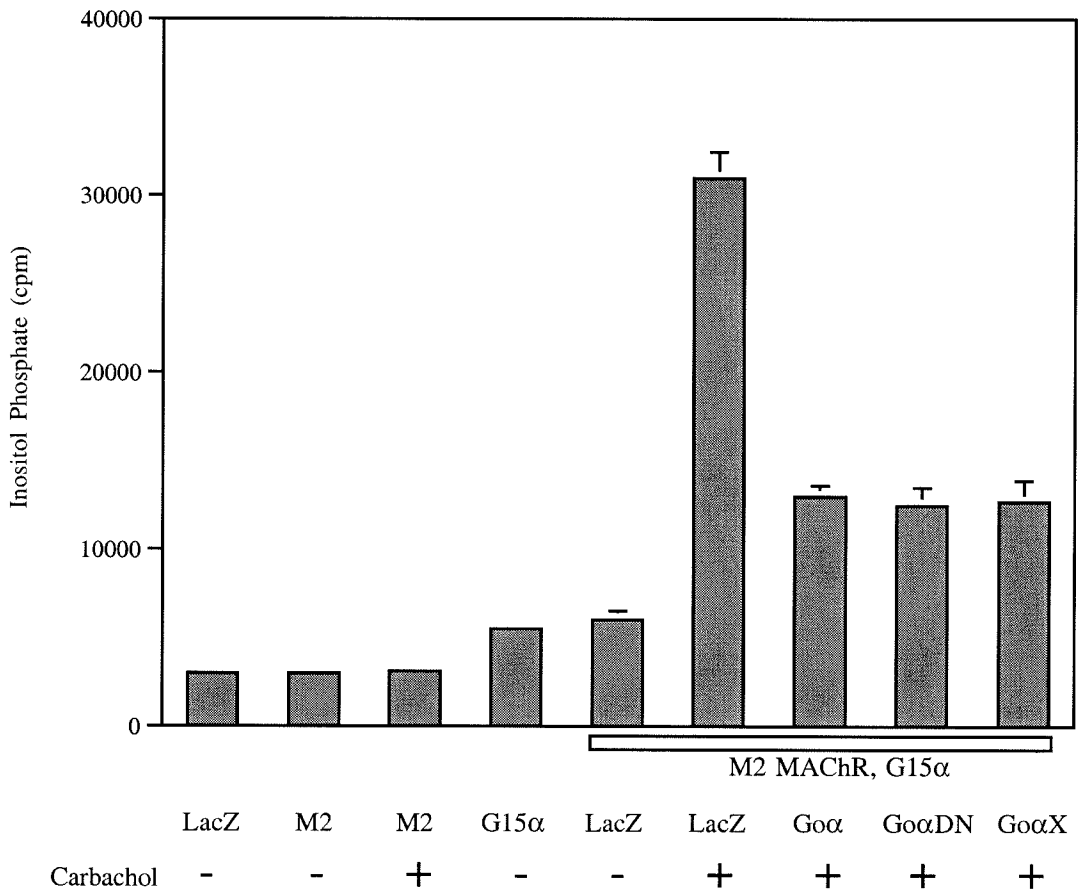


Fig. 5d Inhibition of GoαX on M2 MAChR activated G15 activities



Chapter Four

Inhibition of Subsets of G-protein-coupled Receptors by Empty Mutants of G Protein α Subunits in Go, G11, and G16

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Abstract

We previously reported that the xanthine nucleotide binding Goα mutant, GoαX (GoαD273N/Q205L), inhibited the activation of Gi-coupled receptors. In this study we constructed similar mutations in G11α and G16α and characterized their nucleotide binding and receptor interaction. First, we found that G11αX (G11αD277N/Q209L) and G16αX (G16αD280N/Q213L) expressed in COS-7 cells bound XTPγS instead of GTPγS. Second, we found that G11αX and G16αX interacted with βγ subunits in the presence of XDP. These experiments demonstrated that G11αX and G16αX were xanthine nucleotide binding proteins, similar to GoαX. Third, in COS-7 cells, both G11αX and G16αX inhibited the activation of Gq-coupled receptors, whereas only G16αX inhibited the activation of Gi-coupled receptors. Therefore, when in the nucleotide-free state, empty G11αX and G16αX appeared to retain the same receptor binding specificity as their wild-type counterparts. Finally, we found that GoαX, G11αX and G16αX all inhibited the endogenous thrombin receptor and lysophophatidic acid receptor in NIH3T3 cells, whereas G11αX and G16αX, but not GoαX, inhibited the activation of transfected m1 muscarinic receptor in these cells. We conclude that these empty G protein mutants of Goα, G11α, and G16α can act as dominant negative inhibitors against specific subsets of G protein-coupled receptors.

Heterotrimeric G protein signaling pathways are commonly used to transduce signals across cell membranes in eukaryotic cells. G proteins contain three subunits α, β, and γ, and can be activated by hundreds of seven-transmembrane receptors. Binding of agonist to receptor activates the receptor which then catalyzes the exchange of GTP for GDP bound to G protein α subunits. Activated GTP bound α subunits and free βγ subunits regulate a variety of cellular effectors including enzymes and ion-channels (1-3). G protein α subunits can be divided into four families: Gs, Gi (Gi, Go, and transducin), Gq (Gq, G14, and G16), and G12 (G12 and G13). Some G protein-coupled receptors activate only one family of G proteins, whereas other receptors may activate multiple families of G proteins. G16 and its mouse homologue G15 behave promiscuously; they can be activated by all classes of G protein-coupled receptors (4).

We recently reported that the xanthine nucleotide binding Goα mutant, GoαX (a double mutant of Goα, D273N/Q205L), can interact with appropriate receptors on the membrane (5, 6). GoαX was regulated by xanthine nucleotides instead of guanine nucleotides. The empty form (nucleotide free) of GoαX has been shown to form a stable complex with Go-coupled receptors, and to inhibit the cognate receptor by competing with endogenous wild-type G proteins. In the present study, we investigated the functions of similar mutants in another G protein family. We found that both G11αX (G11αD277N/Q209L) and G16αX (G16αD280N/Q213L) were xanthine nucleotide binding proteins. They bound XTPγS, but not GTPγS. These mutant proteins were also able to bind βγ subunits only in the presence of XDP. In the nucleotide free state, they interacted with their appropriate receptors and inhibited activation. Furthermore, G11αX

and G16αX retained the same receptor binding specificity of the wild-type proteins. G11αX only inhibited Gq-coupled receptors, but not Gi-coupled receptors, whereas G16αX was able to inhibit receptors from both families. These results suggest that as with GoαX, G11αX and G16αX can be used as dominant inhibitors against a subset of receptors.

MATERIALS AND METHODS

Materials—Purified bovine retinal transducin βγ were generous gifts from Dr. O. Nakanishi (Division of Biology, Caltech). Xanthine nucleotides and guanine nucleotides were from Sigma. Radioactive [^{35}S]ATPγS, [^{35}S]GTPγS and [^{3}H] QNB were from NEN Life Science Products.

Mutagenesis of G11α and G16α—The D277N mutation was introduced in both wild-type G11α and the activated mutant G11αQ209L. The D280N mutation was introduced in both wild-type G16α and the activated G16αQ213L. The site-specific mutagenesis was conducted by PCR using oligonucleotide TTCCTCAACAAGAAGGACCTTCTAGAAGAC for G11α and TTTCTCAACAAAACCGACATCCTGGAGGAGAAAATCCC for G16α. The cDNAs were subcloned into the pCIS vector under the control of a CMV promoter.

Expression and Purification of His$_6$-tagged Goα--Both wild-type Goα and mutant GoαX were subcloned into the *E.coli* expression vector pET-15b (Novagen) with a His$_6$ tag at the N terminus (5). The recombinant proteins were expressed and purified as described previously. The His$_6$-tagged proteins were purified over a Ni^{2+}-NTA column

according to the protocol provided by the manufacturer (Novagen, Inc.). Purified proteins were stored in TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT) with 0.1 mM $MgCl_2$.

Membrane Preparation from Baculovirus Infected Sf9 Cells--Sf9 cells were grown and infected with recombinant baculoviruses encoding m2 MAChR (7, 8). Membranes of the infected cells were prepared as described. Infected cells were centrifuged and resuspended at $< 10^7$ cells/ml in HME/PI buffer (20 mM NaHepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 2 μg/ml aprotinin, and 10 μg/ml leupeptin). The cell suspension was homogenized by 10 strokes in a glass homogenizer followed by passing through a 27 gauge hypodermic needle several times. The homogenate was briefly centrifuged at 3,000 x g for 10 min., and then the supernatant was collected and centrifuged at 30,000 x g for 30 min. The pellet was washed once with HME/PI and the final pellet was resuspended in HME/PI at a protein concentration of 5 mg/ml.

Synthesis of XTPγS--XTPγS was synthesized from XDP and ATPγS with Nucleotide Diphosphate Kinase (NDK) as described previously (9). To produce ^{35}S labeled XTPγS, the reaction contained 10 μM XDP, 1 μM [^{35}S]ATPγS, and 10 units NDK (Sigma) in 100 μl NDK buffer (1 mM $MgCl_2$, 5 mM DTT, and 20 mM Tris-HCl, pH 8.0). The mixture was incubated at room temperature for 2 hours. The resulting concentration of [^{35}S]XTPγS was about 1 μ (1 μCi /pmol). The radiochemical purity of XTPγS was monitored by TLC on Avicel/PEAE plates (Analtech) in 0.07 N HCl.

Receptor-stimulated GTPγS Binding of Purified Goα--Binding of [^{35}S]GTPγS to recombinant Goα was performed as previously described (5, 6). 0.5 μg of purified Goα was first incubated with 1μg of transducin βγ and 100 μg of m2 MAChR membrane in TED buffer with 10 μM GDP, 0.1 mM $MgCl_2$ and 1 μM ATP for a half of an hour. The reaction was started with the addition of 0.1 μM GTPγS (20,000 cpm/pmol) and 100 μM carbachol. For the time course experiments, 20-μl aliquots were withdrawn from a 200 μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM $MgCl_2$, filtered through 45-μm nitrocellulose, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

COS-7 Cell Culture and Transfection--COS-7 cells were cultured in DMEM containing 10% fetal bovine serum. $1x10^5$ cells/well were seeded in 12-well plates one day before transfection. All transfection assays contained a total amount of 1 μg DNA, and pCIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg DNA was mixed with 5 μl lipofectamin (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and 5 hours later, 0.5 ml of 20% FCS in DMEM was added to the medium. After 48 hours, cells were assayed for inositol phosphate levels as described previously (10, 11).

Immunoprecipitation of XTPγS-bound G11αX and G16αX—COS-7 cells were transfected with G11αX and G16αX two days before being lysed in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) with 2 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors (0.1 mM PMSF, 2 μg/ml aprotinin,

and 10 μg/ml leupeptin). The lysate was centrifuged at 12,000 x g for 10 min. and the supernatant was incubated with 0.1 μM [^{35}S]XTPγS or [^{35}S]GTPγS (20,000 cpm/ml) for 1 hour at room temperature. G11αX and G16αX proteins were then immunoprecipitated using appropriate antibodies and protein-A Sepharose (Sigma). The amount of radioactive nucleotide in the immunoprecipitates was determined by scintillation counting.

Permeabilization of COS-7 cell Membranes—Membranes of transfected COS-7 cells were permeabilized as described (5). Cells were washed and incubated in 200 ml of permeabilization solution consisting of 115 mM KCl, 15 mM NaCl, 0.5 mM $MgCl_2$, 20 mM Hepes—NaOH, pH 7, 1mM EGTA, 100 mM ATP, 0.37 mM $CaCl_2$ (to give a free Ca^{2+} concentration of 100 nM), and 200 units/ml a-toxin with or without 0.1 mM XDP for 10 min. at 37°C. Then 2 ul of 1M LiCl was added before the inositol phosphate assay.

NIH3T3 Cell Culture and Transfection—NIH3T3 cells were maintained in DMEM containing 10% calf serum. $1x10^5$ cells/well were seeded into 24-well plates one day before transfection. Total of 1μg/well of DNA including 0.2 μg of pSRF-Luc reporter plasmid (Strategene, Inc.) were used to transfect cells with Superfect (Life Technologies, Inc.), following the manufacturer's recommendations. Plasmid of pCIS encoding β-galactosidase was used to maintain a constant amount of DNA for each well.

Luciferase Assay—Transfected NIT3T3 cells were maintained in DMEM containing 0.05% calf serum overnight and then were stimulated with 500 nM of LPA or 1 unit/ml of thrombin in the same medium for 6 hours before cell extracts were collected to

determine the activity of luciferase. The luciferase assay was performed following the protocol of Luciferase Assay System from Promega. The activity of luciferase was determined by measuring luminescence intensity using a luminometer (Monolight 2010 from Analytical Luminescence Laboratory).

RESULTS

Inhibition of GoαX to GTPγS binding of wild-type Goα stimulated by m2 muscarinic receptors

We have previously shown that GoαX inhibited the activation of m2 muscarinic receptors (m2 MAChR) in transfected COS-7 cells (6). To test inhibition of m2 MAChR more directly, we asked whether preincubation of receptor with GoαX inhibited the binding of GTPγS to wild-type Goα facilitated by activated m2 MAChR *in vitro*. Recombinant m2 MAChR from Sf9 cells has been shown to stimulate the binding of GTPγS to wild-type Goα 2-3 fold in response to muscarinic agonists (7, 8). We infected Sf9 cells with recombinant baculoviruses encoding m2 MAChR and prepared membranes. The concentration of receptor in isolated membranes was about 20 pmol/mg of membrane protein, determined from [^{3}H]QNB binding (6). In the control experiments, we reconstituted purified Goα with the transducin β1γ1 subunits and Sf9 cell membranes containing m2 MAChR, and then assayed the binding of GTPγS to Goα upon activation with the muscarinic agonist carbachol. We found that carbachol accelerated the binding of GTPγS to Goα (Fig. 1a). Then we coincubated different amounts of purified GoαX in similar experiments, and found that GoαX attenuated the m2 MAChR catalyzed

activation of GTPγS binding to Goα. The inhibitory effect of GoαX was proportional to the amount of GoαX added (Fig.1a). This result is consistent with our previous finding that GoαX forms a stable complex with m2 MAChR. Since we have also demonstrated that the interaction between GoαX and m2 MAChR can be abolished by either XDP or XTP, we then tested whether XDP or XTP could relieve the inhibitory effect of GoαX. As expected, GoαX did not inhibit the activation of m2 MAChR in the presence of XTP. However, GoαX was able to inhibit the binding of GTPγS to Goα when XDP was present (Fig.1b). This is not surprising because GoαX is able to bind βγ in the presence of XDP. We conclude that GoαX competes with wild-type Goα for βγ subunits and inhibits the activation of Goα by receptor. This is also consistent with the fact that the activation of Goα by m2 MAChR requires βγ (Fig. 1b).

Binding of XTPγS to G11αX and G16αX

To test whether the DN mutation of the conserved NKXD motif in other G protein α subunits also generates xanthine nucleotide binding proteins, we introduced the mutation in both wild-type G11α and G16α and into their activated QL mutant cDNA, and expressed the mutant proteins G11αDN (G11αD277N), G11αX (G11αD277N/Q209L), G16αDN (G16αD280N), and G16αX (G16αD280N/Q213L) in COS-7 cells (Fig. 2a,b). Unlike Goα, large quantities of active recombinant proteins of G11α and G16α are not easily expressed and purified from *E.coli*. Thus we decided to test the nucleotide binding of the mutant proteins in COS-7 cell lysates. After incubating with the radioactive GTPγS or XTPγS, we immunoprecipitated the mutant proteins and assayed bound radioactive nucleotide. We found that G11αX and G16αX bound XTPγS

instead of GTPγS, whereas wild-type G11α and G16α preferred GTPγS (Fig. 2c,d). However, consistent with our pervious finding that GoαX bound xanthine nucleotides but not guanine nucleotides, whereas GoαDN did not bind either nucleotides, We found that both G11αDN and G16αDN did not show strong binding of either [^{35}S]GTPγS or [^{35}S]XTPγS.

XDP-dependent βγ interaction

We previously showed that GoαX was able to bind βγ subunits in the presence of XDP (5). To test whether G11αX and G16αX also shows XDP-dependent βγ interaction, we assayed their binding with βγ in transfected COS-7 cells. In COS-7 cells, β1γ2 is able to activate PLCβ2, and the activation of PLCβ2 can be inhibited by cotransfection with wild-type Goα because of competition for βγ (5, 12). We cotransfected COS-7 cells with PLCβ2, β1, γ2, and G11αX or G16αX, and found that neither mutant inhibited PLCβ2 activity, whereas wild-type Goα did inhibit. This experiment suggests that G11αX and G16αX do not bind βγ presumably because the intracellular concentration of XDP is negligible (13) (Fig. 3). To deliver XDP into cells, we permeabilized the cell membrane with α-toxin. After incubating transfected COS-7 cells with α-toxin in the presence of XDP, we found that both G11αX and G16αX inhibited PLCβ2 activity stimulated by βγ (Fig. 3). In the similar experiments with G11αDN and G16αDN, we did not see inhibition of the activation of PLCβ2, even when XDP was present (Fig. 3). In the control experiments, we found that α-toxin alone or α-toxin followed by GDP or GTP addition did not affect the activity of PLCβ2 (data not shown). These experiments show that G11αX and G16αX do not bind βγ and interfere with its activation of PLCβ2 in the

nucleotide free state. However, when in the XDP bound form, G11αX and G16αX are able to sequester cellular βγ and inhibit its ability to activate on PLCβ2.

Dominant negative inhibition of G protein-coupled receptor by empty G11αX and G16αX

GoαX binds to members of the Gi-coupled receptor family and has been shown to act as a dominant inhibitor of Gi-coupled receptors (6). To test receptor interaction of G11αX and G16αX, we assayed their ability to inhibit the activation of G protein-coupled receptors in COS-7 cells. M1 muscarinic receptors (m1 MAChR) have been shown to primarily activate the Gq family of G proteins (10). Activated Gqα then stimulates PLC β isoforms to elevate cellular IP3 concentration. We cotransfected COS-7 cells with m1 MAChR and G11αX or G16αX and tested whether the mutant proteins inhibited the activation of m1 MAChR by competing with endogenous Gqα. We found that both G11αX and G16αX were able to inhibit the activity of endogenous PLC β isoforms stimulated by activated m1 MAChR (Fig. 4a). Since G11αX and G16αX did not affect the activation of PLCβ2 by βγ in COS-7 cells, the inhibition of m1 MAChR-stimulation PLCβ activation by G11αX and G16αX most probably results from the competitive binding of the mutant proteins to the receptor. In similar experiments using G11αDN and G16αDN, we found that they inhibited the activation of m1 MAChR as well as G11αX and G16αX (Fig. 4a), suggesting that G11αDN and G16αDN could also bind to m1 MAChR although they do not bind either guanine nucleotides or xanthine nucleotides. In the experiments with two other Gq-coupled receptors, TRH receptor and thrombin receptor (14-16), we found that all four mutant proteins were able to inhibit

activation by receptors (Fig. 4, b and c). These experiments agree with our previous observation that the empty Goα mutant protein forms a stable complex with appropriate receptor. Thus our working hypothesis is that in the absence of xanthine nucleotides sufficient levels of mutant proteins are made to interact with the appropriate receptors.

Empty GoαX mutants appear to retain the receptor binding specificity of wild-type Goα (6). To test whether empty G11α and G16α mutants also behave similarly, we assayed for their inhibition of activation by the m2 MAChR, a member of Gi-coupled receptor family. Since m2 MAChR couples only to the Gi family of Gα proteins but not to the Gq family (4, 9), we could not assay the activity of m1 MAChR by monitoring Gqα-stimulated PLCβ activities in COS-7 cells. Therefore, we constructed an artificial pathway by cotransfecting both m2 MAChR and G15α into COS-7 cells. G15α and G16α are homologous proteins (G15 mouse and G16 human) that behave as a promiscuous G protein which can be activated by all kinds of G protein-coupled receptors, and activated G15 stimulates the activity of PLC β (4). In cells cotransfected with both m2 MAChR and G15α, we were able to activate endogenous PLC β isoforms by the addition of the muscarinic agonist carbachol. In the cotransfection experiments with m2 MAChR, G15α, and the empty mutants of G11α and G16α, we found that only G16αX and G16αDN inhibited activation by m2 MAChR, whereas G11αX and G11αDN had no effect (Fig. 4d). This is consistent with the fact that G11α does not couple to the m2 MAChR and G16α does. These experiments suggest that both empty mutants of G11α and G16α retain the binding specificity of their wild-type counterparts;

G11αX and G11αDN only interact with Gq-coupled receptors, but not with Gi-coupled receptor, whereas G16αX and G16αDN can interact with both families of receptors.

Inhibition of endogenous thrombin receptor and LPA receptor in NIH3T3 cells

NIH3T3 cells express endogenous thrombin receptor and lysophophatidic acid (LPA) receptor (17). These two receptors couple to a variety of G proteins including Gi, Gq and G12/13 (16, 18). Activation of these two receptors leads to the activation of serum response factor (SRF) and SRF-mediated gene transcription through RhoA, *via* both the Gq and G12/13 pathways. To investigate whether empty Gα mutants inhibit thrombin receptor and LPA receptor in NIH3T3 cells, we determined the activity of luciferase, whose expression was under the regulation of SRE.L, when the cells were cotransfected with the mutant G proteins and the reporter gene plasmid. SRE.L is a derivative of the c-Fos serum response element (SRE) to which SRF binds (19) and activate luciferase transcription. We found that the empty mutants of all three types of a subunits, Goα, G11α, and G16α, were able to inhibit the activation of both thrombin receptor (Fig. 5a) and LPA receptor (Fig.5b). These results are consistent with the experiments in COS-7 cells showing that the empty G proteins bind tightly to their cognate receptors. To exclude the possibility that empty Gα mutants interfere with the downstream components of the signaling pathway, we cotransfected the cells with the activated Gq mutant, GqαQL, and the empty Gα mutants. We found that the empty Gα mutants did not affect GqαQL-stimulated SRF activation (data not shown), indicating that the inhibition of receptor-stimulated SRF activation by empty Gα mutants must

come from their competitive binding to the receptor, not from direct Gα activation of downstream effectors.

NIH3T3 cells apparently do not have endogenous muscarinic receptors since addition of carbachol did not lead to the activation of SRF (17). We transfected the cells with m1 MAChR and found that its activation resulted in stimulated luciferase activity, presumably through the endogenous Gq pathway. Coexpression of the Gα mutants showed that G11αX, G11αDN, G16αX, and G16αDN inhibited the activation of m1 MAChR, but GoαX and GoαDN did not. These experiments indicate that the presumptive empty forms of G11α and G16α bound m1 MAChR whereas the empty form of Goα did not, consistent with the results from COS-7 cell experiments.

DISCUSSION

We previously reported that GoαX, the xanthine nucleotide binding mutant protein of Goα, formed stable complexes with their appropriate receptors and inhibited the activation of cognate receptors because of competitive binding (6). In this study, we reconstituted GoαX, Goα, βγ, and m2 MAChR and sf9 cell membranes. We monitored the GTPγS binding of Goα facilitated by m2 MAChR upon the activation of its agonist carbachol. Not surprisingly, we found that GoαX inhibited the nucleotide exchange of wild-type Goα catalyzed by the activated m2 MAChR. Therefore, we demonstrated that GoαX was able to inhibit the activation of m2 MAChR *in vitro*.

To extend this work to other families of G proteins, we introduced the similar DN mutation in wild-type G11α and G16α, as well as activated G11αQL and G16αQL, and

expressed the mutant proteins in COS-7 cells. After immunoprecipitating the mutant proteins incubated with radioactive nucleotides in COS-7 cell lysates, we found that G11αX and G16αX bound XTPγS instead of GTPγS, whereas wild-type G11α and G16α preferred GTPγS. However, G11αDN and G16αDN did not appear to bind either nucleotides. We also showed that the mutant proteins of G11αX and G16αX expressed in COS-7 cells interacted with βγ subunits in a XDP-dependent fashion; they only bound βγ when XDP was available, whereas G11αDN and G16αDN did not. These results are consistent with previous findings using GoαX and GoαDN; the single DN mutation resulted in a loss of ability to bind nucleotides whereas the double DN/QL mutation lead to xanthine nucleotide binding (5). Although the mutation of Asp → Asn in the conserved NKXD motif of G protein α subunits was expected to switch the nucleotide specificity of the mutated protein from guanine nucleotide to xanthine nucleotide, according to the available crystal structures of G protein α subunits and other GTP-binding proteins, we observed that the single DN mutation resulted in a protein not able to bind either nucleotides in three G protein α subunits: Goα, G11α, and G16α. It is not apparent from the crystal structures why the second QL mutation, a well-characterized GTPase deficient mutation, restored the xanthine nucleotide binding of the mutant proteins; the conserved Gln (position 200 in transducin α) resides at the opposite side of the nucleotide binding pocket from the DN mutation (position 268 in transducin α).

To test whether empty mutants of G11α and G16α interacted with G protein-coupled receptors and inhibited the activation of appropriate receptors, we assayed the stimulated PLC β activity by transfected receptors in COS-7 cells and the activation of

SRF by endogenous thrombin receptors and LPA receptors in NIH3T3 cells. We found that G11αX and G16αX inhibited the activation of m1 MAChR and TRH receptor but not m2 MAChR, whereas GoαX and GoαDN inhibited the activation of m2 MAChR but not m1 MAChR or TRH receptor. Furthermore, G16αX and G16αDN were found to inhibit the activation of m1 MAChR, TRH receptor, and m2 MAChR, in addition to that GoαX, GoαDN, G11αX, G11αDN, G16αX, and G16αDN were all able to inhibit the activation of thrombin receptor and LPA receptor. Therefore, we conclude that these empty mutants of G protein α subunits retain the same receptor binding specificity of their wild-type counterparts. Empty Goα interacts with only Gi-coupled receptors, and empty G11α interacts with only Gq-coupled receptors, while G16α can interact with both families of G protein-coupled receptors. It is interesting to note that G11αDN and G16αDN were able to inhibit the activation of their appropriate receptors as effectively as G11αX and G16αX, although G11αDN and G16αDN did not bind xanthine nucleotides. Similarly, GoαDN was shown to interact with receptors but not able to bind nucleotides (5,6). These experiments proved that the empty mutant form of these G protein α subunits can act as effective dominant negative inhibitors against a subset of G protein-coupled receptors. They can be very useful tools to dissect signaling pathways of different G protein-coupled receptors by specifically blocking one family of receptors.

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Figure 1 GoαX inhibits the GTPγS binding of wild-type Goα stimulated by m2 MAChR. a) 100 μg of m2 MAChR membranes was incubated with 0.5 μg of Goα, 1 μg of βγ, 10 μM GDP and indicated amount of GoαX in TEDM buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1mM dithiothreitol, and 1 mM $MgCl_2$) for 20 min. at room temperature, and then the mixture was diluted 10-fold with TEDM buffer containing 0.1 μM [^{35}S]GTPγS (20,000 cpm/pmol) and 100 μM carbachol at time 0. 20-μl aliquots were withdrawn and assayed for the bound nucleotides at the indicated times. b) 0.5 μg of wild-type Goα was preincubated with 100 μg of m2 MAChR membranes and 3 μg of GoαX under indicated conditions, and then subjected to the similar GTPγS binding assay as in panel a). Only data at 20 min. were shown as the percentage of maximum binding.

Fig. 1a GoαX inhibits M2 MAChR-stimulated Goα activation

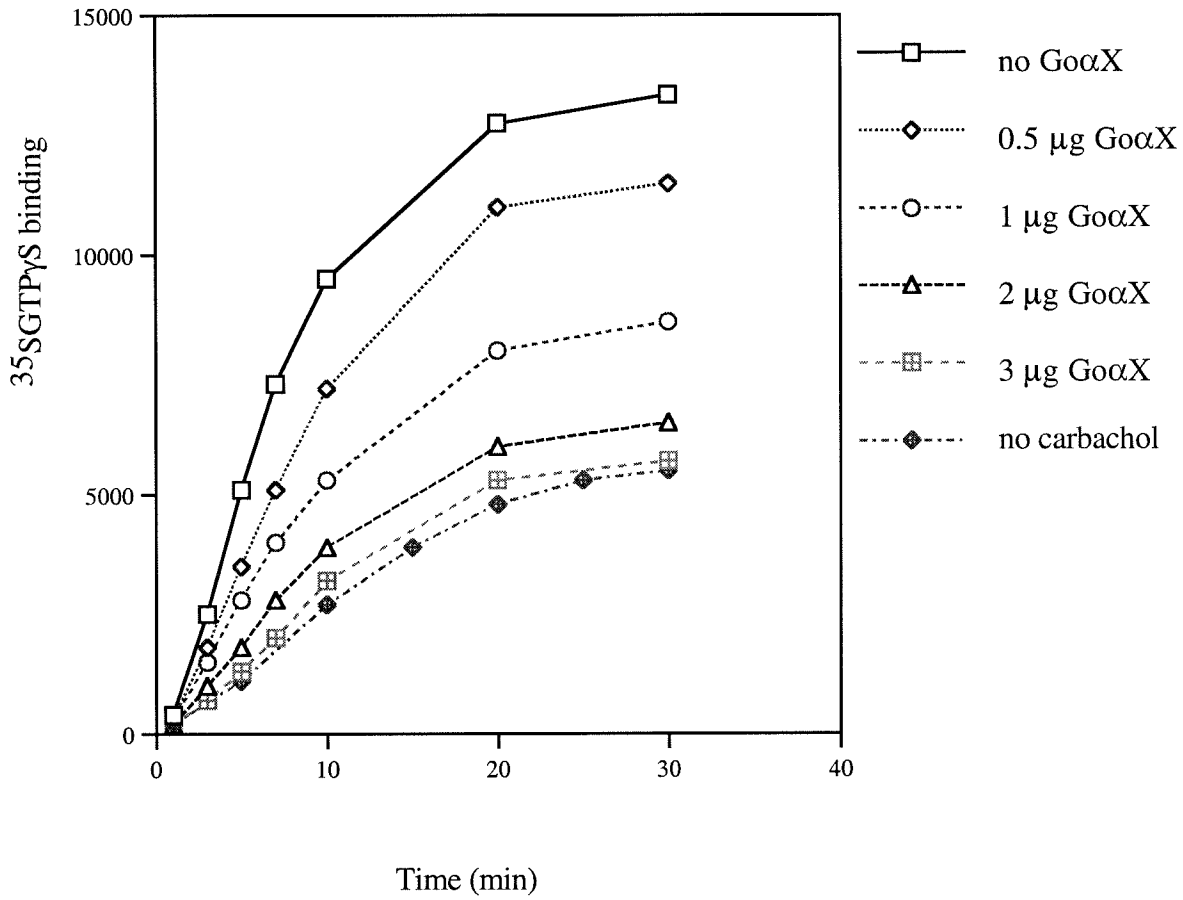
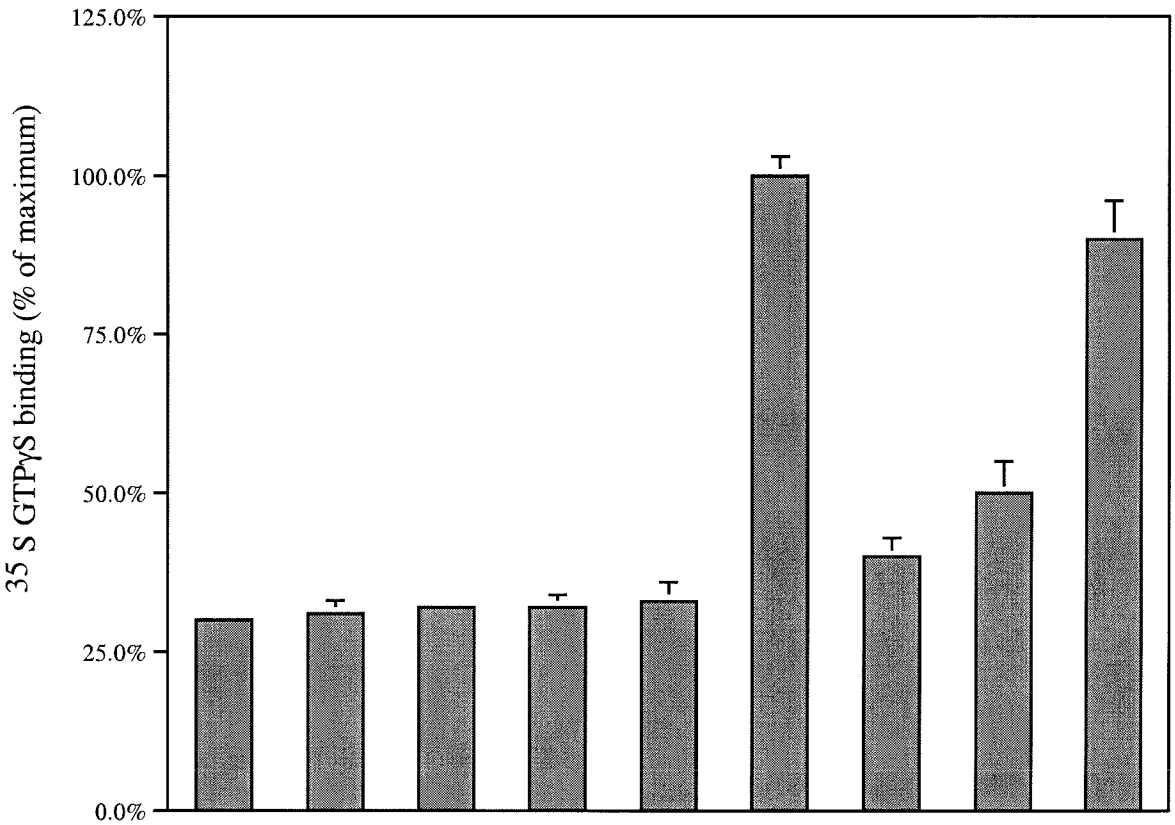


Fig. 1b XTP relieves the inhibitory effect of GoαX



Goα	+	+	+	+	+	+	+	+	+
GoαX	–	+	–	–	–	–	+	+	+
βγ	–	–	+	–	+	+	+	+	+
M2 MAChR	–	–	–	–	+	+	+	+	+
Carbachol	–	–	–	–	–	+	+	+	+
XDP	–	–	–	+	–	–	–	+	–
XTP	–	–	–	+	–	–	–	–	+

Figure 2 XTPγS binding of G11αX and G16αX. In panel a) and b), mutant proteins of G11αX, G11αDN, G16αX, and G16αDN were expressed in COS-7 cell and subjected to Western blots using antibodies against G11α and G16α, respectively. In panel c) and d), the lysates of transfected COS-7 cells were incubated with indicated radioactive nucleotide for 1 hour at room temperature. The mutant protein was then immunoprecipitated using appropriate antibodies and the amount of radioactive nucleotide was determined.

Fig. 2 Expression of G11αX and G16αX in COS-7 cells

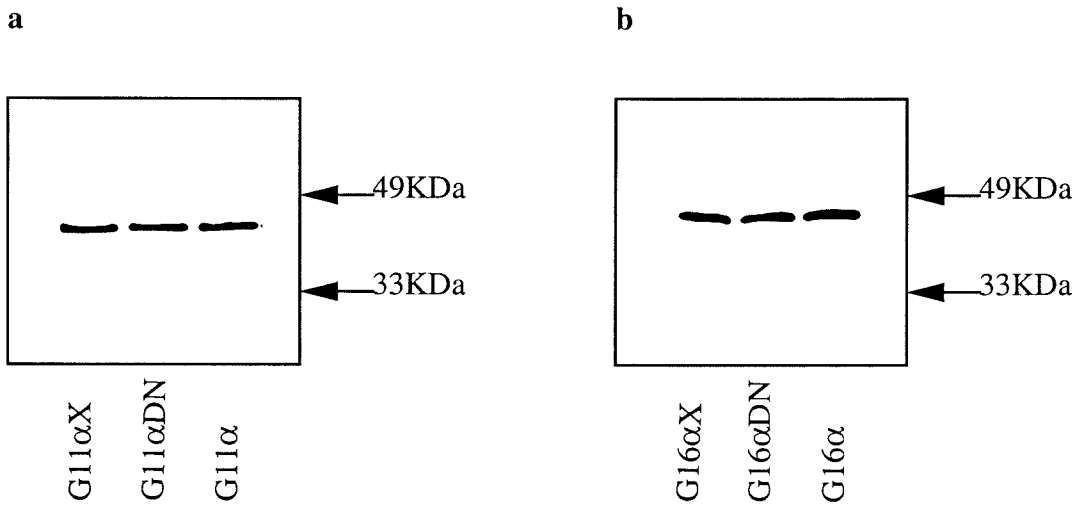


Fig. 2c XTPγS binding of G11αX

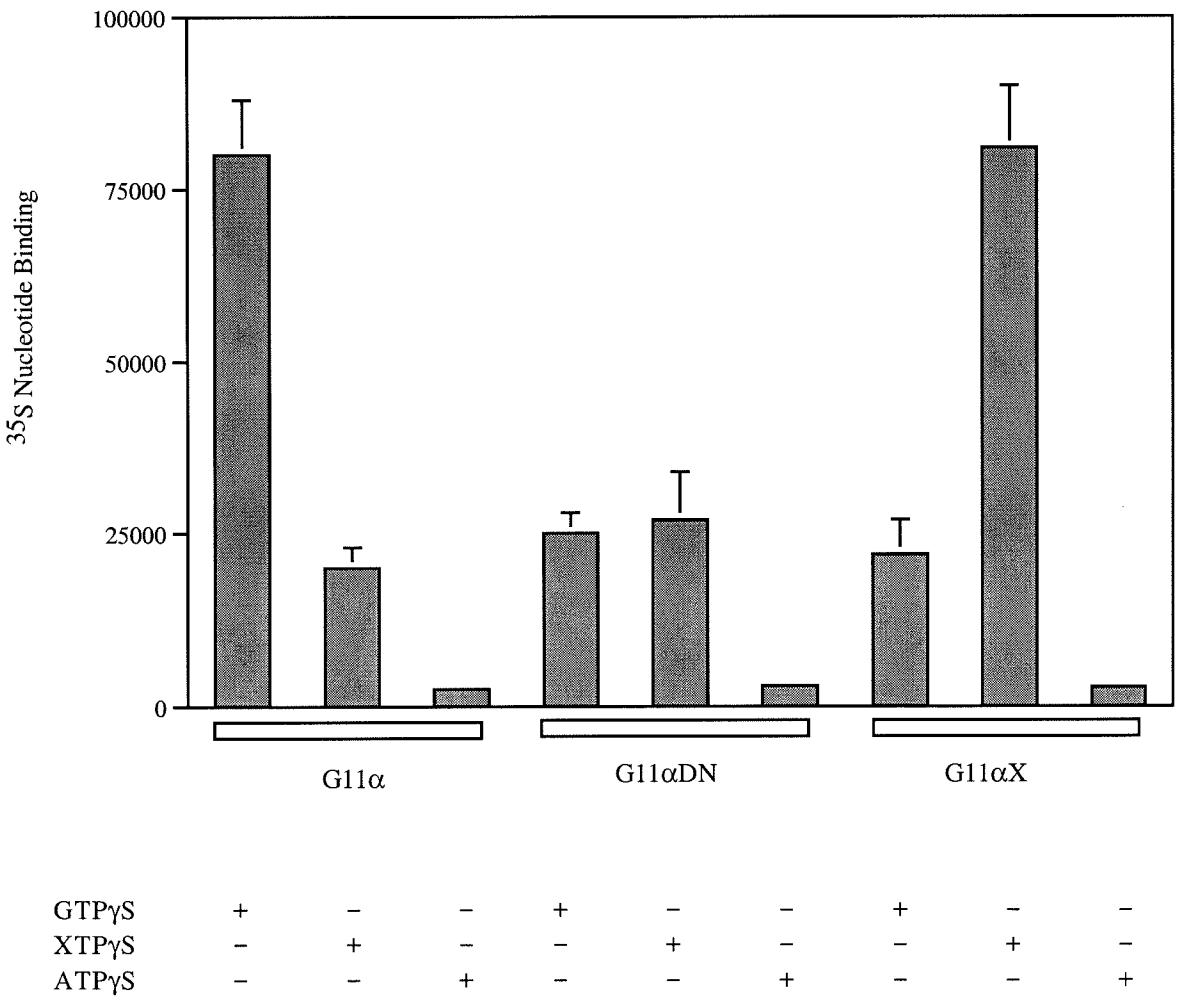


Fig. 2d XTPγS binding of G16αX

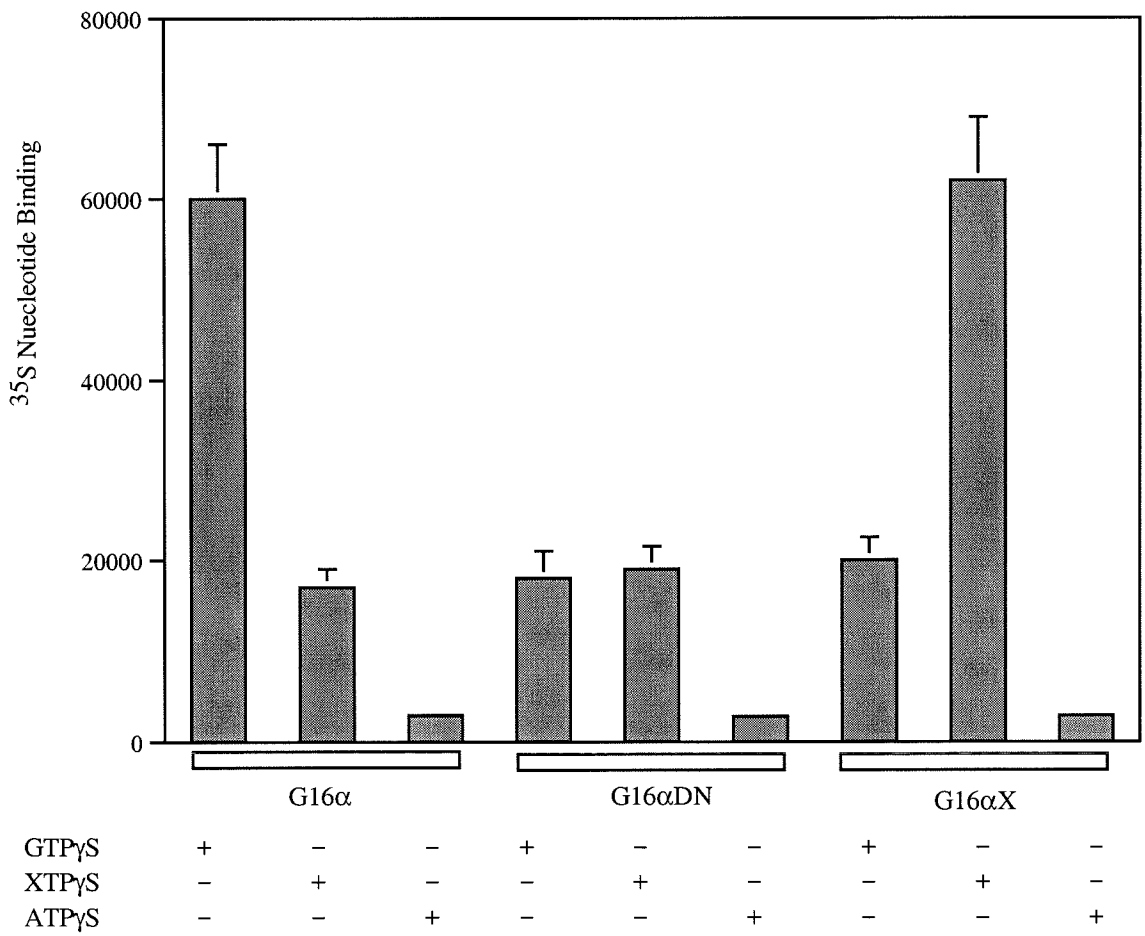


Figure 3 The interaction of G11αX and G16αX with βγ in transfected COS-7 cells is XDP-dependent. $1x10^5$ cells/well were seeded in a 12-well plate and then were transfected with cDNAs encoding the indicated proteins the next day. The total amount of cDNA for each well was adjusted to 1.0 μg by addition of CMV-LacZ cDNA. Cells were labeled with [^{3}H]inositol, and the levels of inositol phosphates were determined after incubating cells with 200 units/ml of α-toxin with or without 10^{-4} M XDP.

Fig. 3 Effects of G11 and G16 mutants on βγ-stimulated PLCβ2 activation

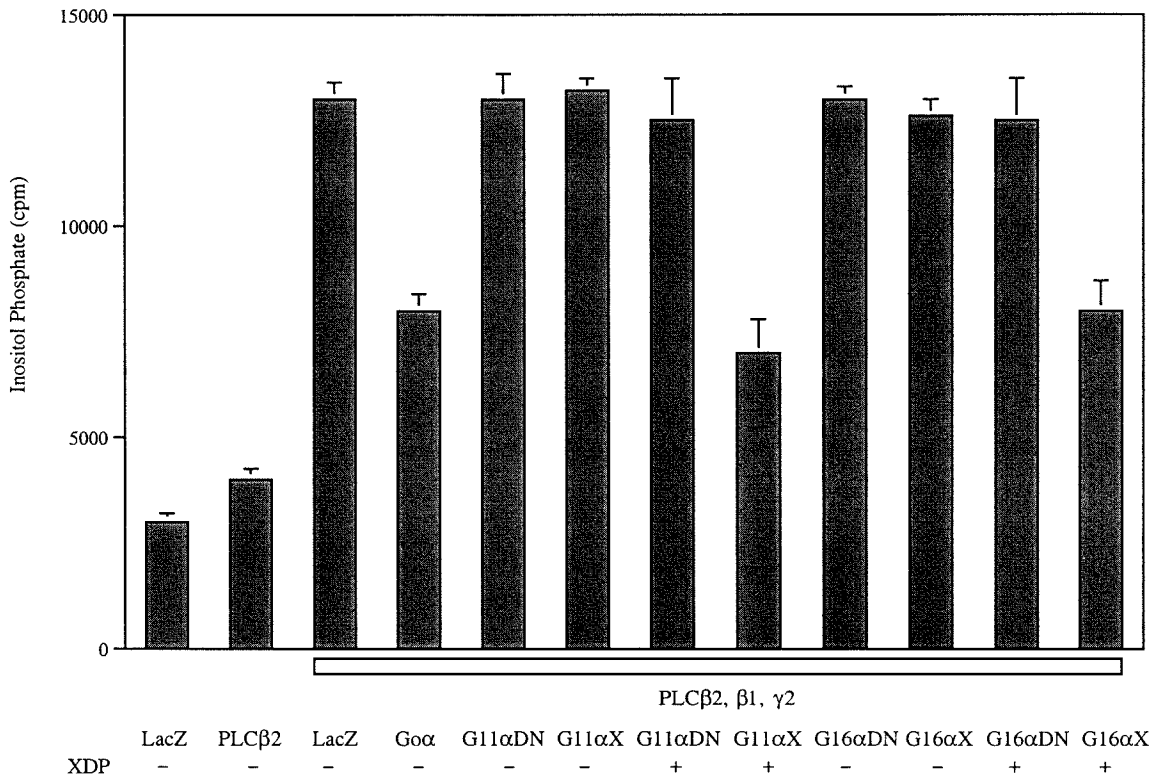


Figure 4 Empty mutants of G11α and G16α inhibited appropriate receptors in COS-7 cells. 1 x 10^5 cells/well were seeded and then transfected with m1 MAChR (a), or TRH receptor (b), or thrombin receptor (c), or m2 MAChR (d) and indicated Gα. In panel a-c, the amount of receptor cDNA for each well was 0.25 μg, and the amount of Gα was 0.75 μg. In panel d, the amount of both m2 MAChR and G15α cDNA was 0.2 μg/well and that of mutant Gα was 0.6 μg/well. After cells were labeled with [^{3}H]inositol overnight, they were incubated in the medium containing 1 μM carbachol (a and d), or 1 μM TRH (b), or 0.1 unit/ml thrombin (c) before levels of inositol phosphates were determined.

Fig. 4a Inhibition of G11 and G16 mutants on the Gq pathway regulated by M1 receptors

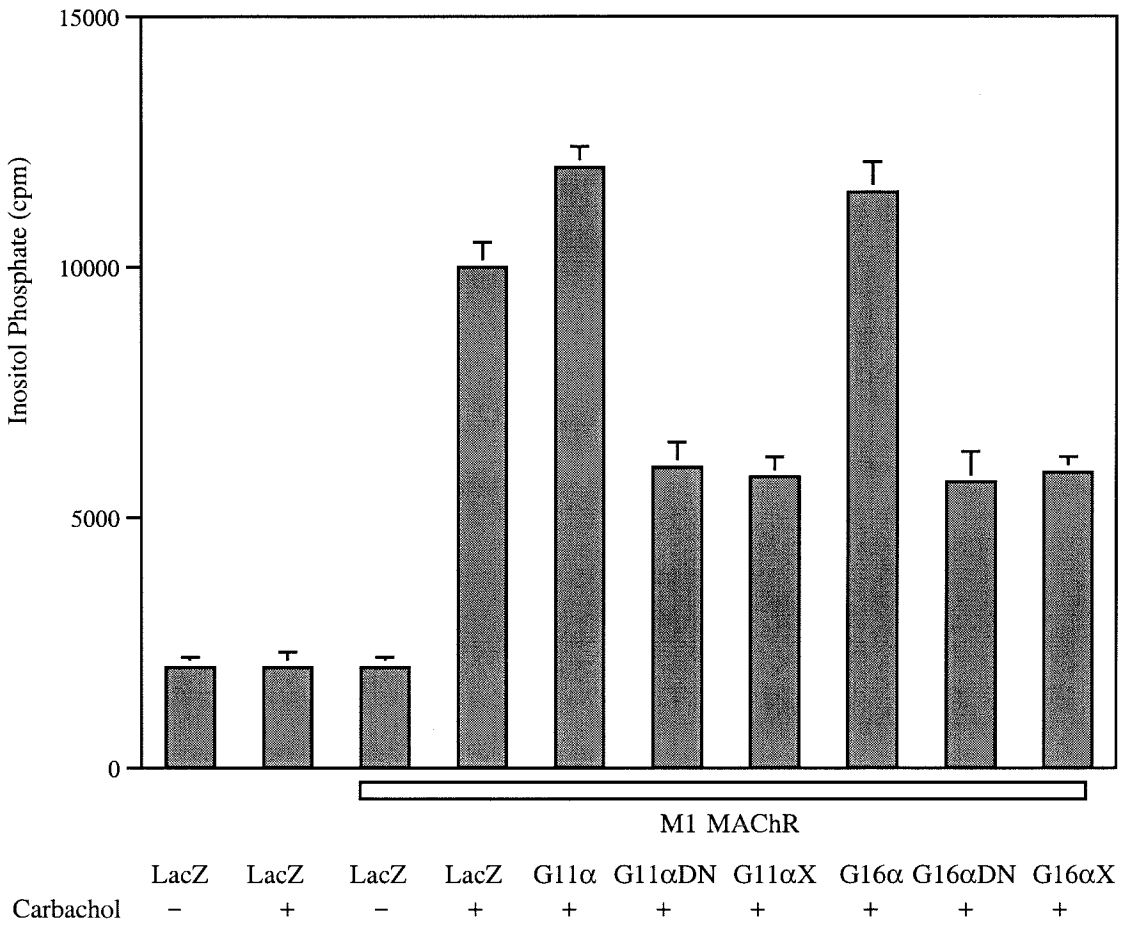


Fig. 4b G11 and G16 mutants inhibits TRH receptor activation

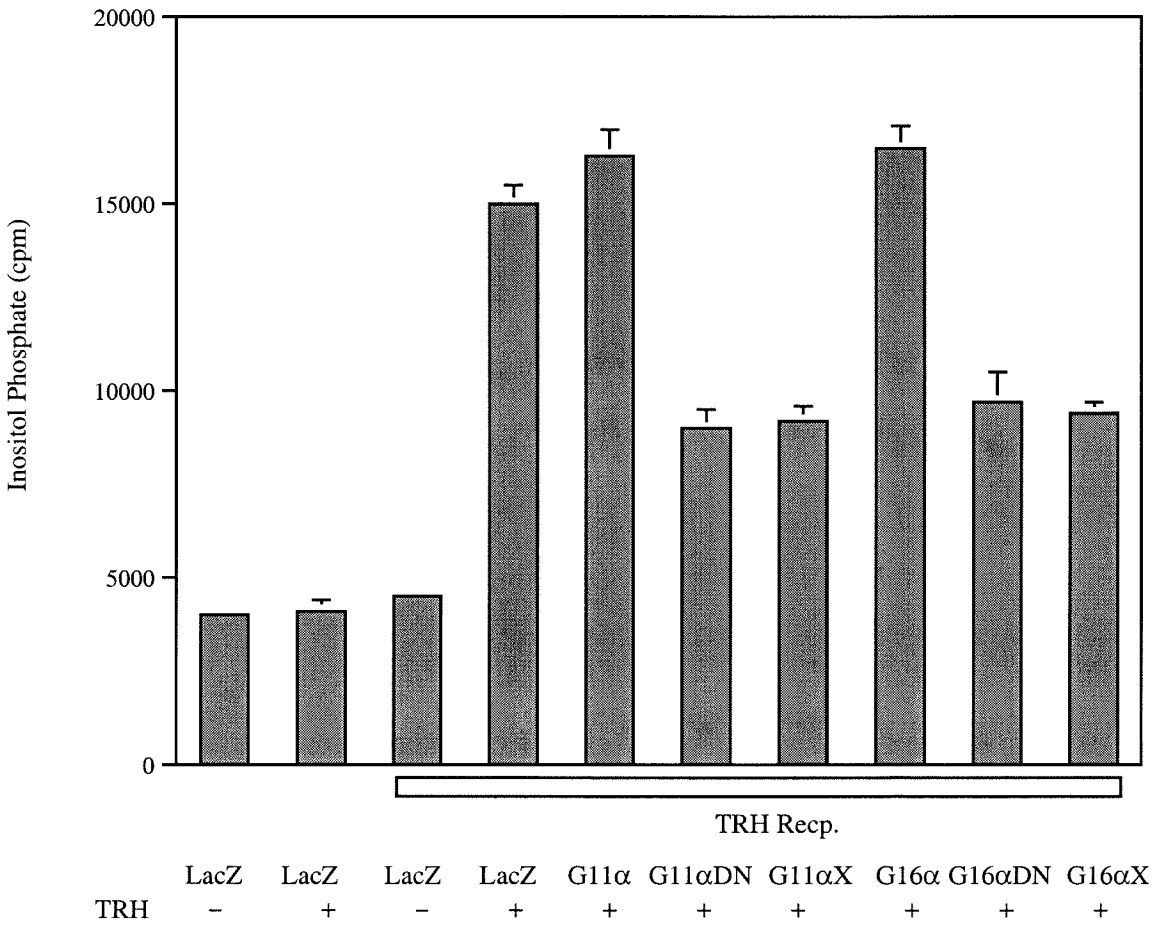


Fig. 4c G11 and G16 mutants inhibits thrombin receptor activation

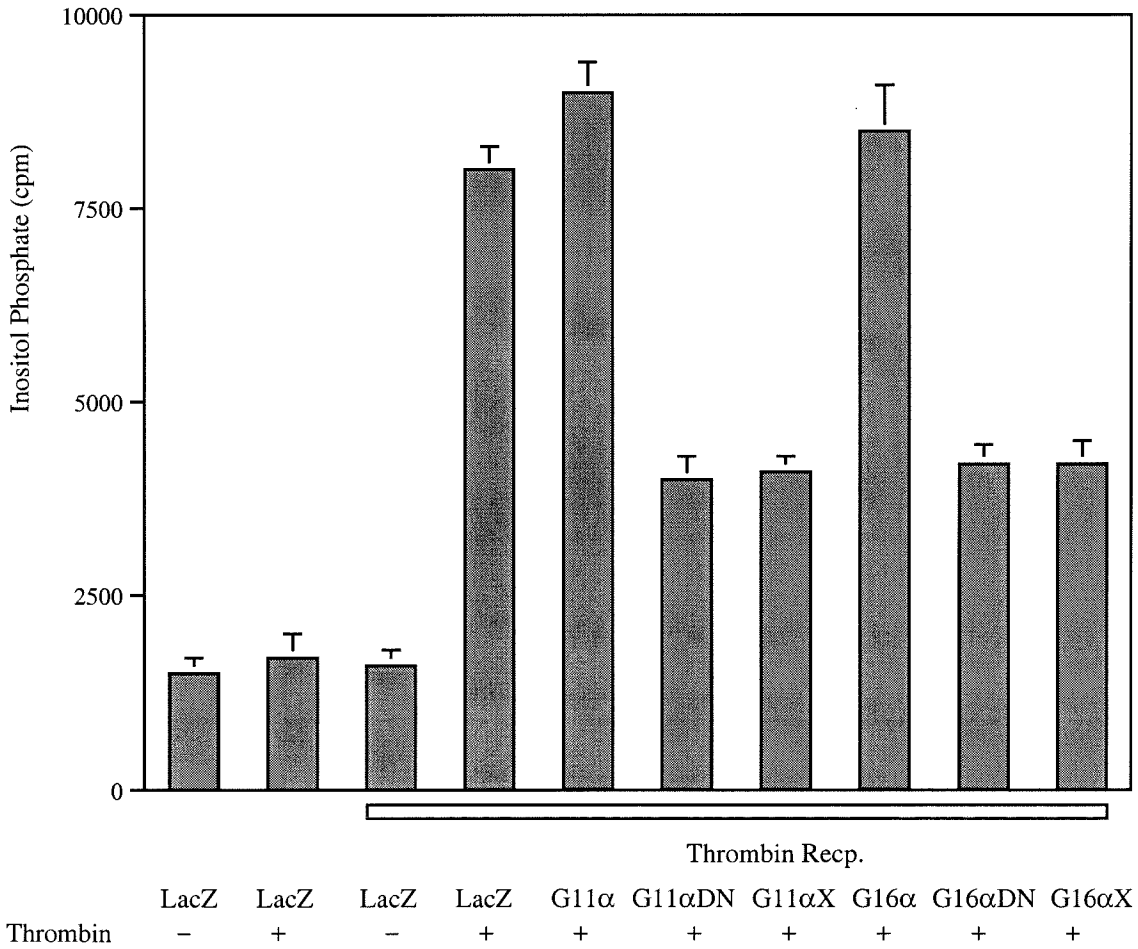


Fig. 4d G16 mutants inhibit M2 receptor stimulated G15 activites

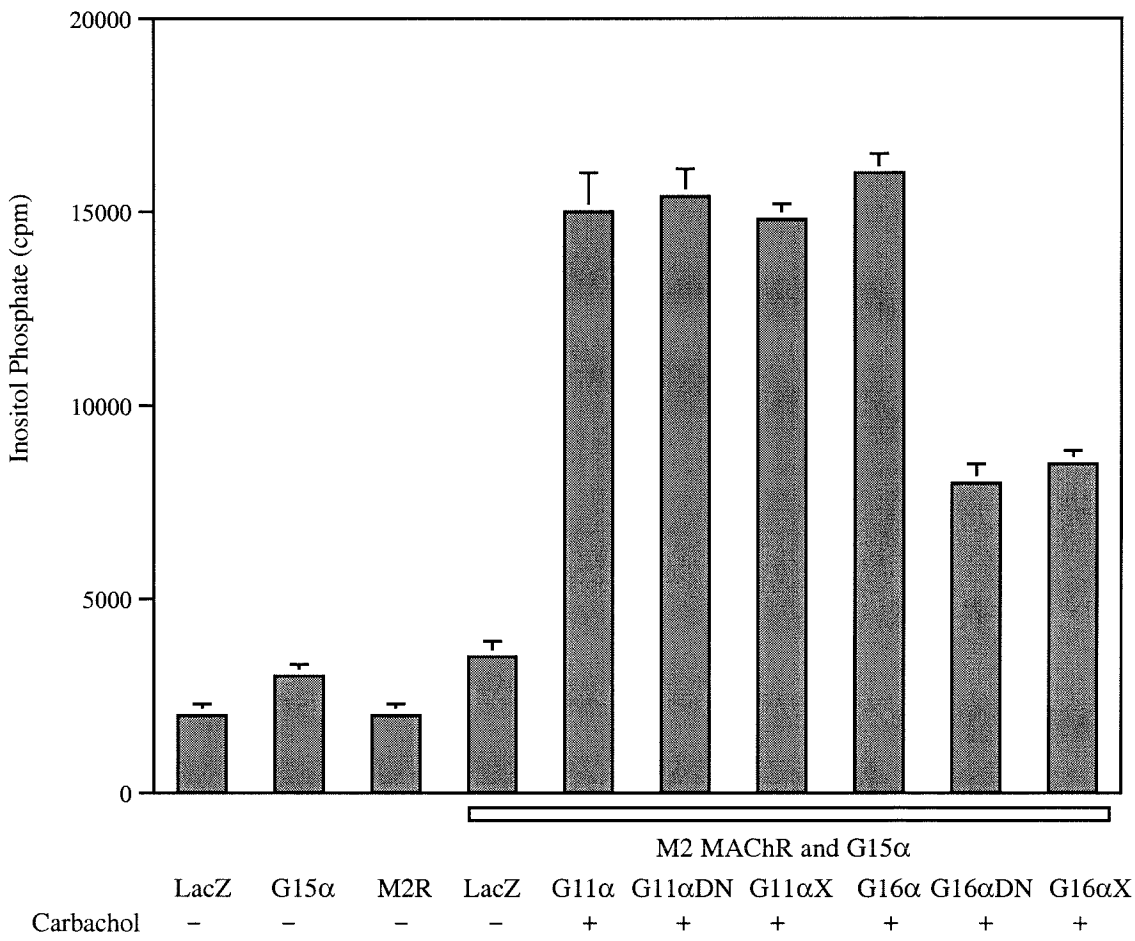


Figure 5 Empty mutants of Goα, G11α, and G16α inhibited endogenous thrombin and LPA receptors in NIH3T3 cells. 1 x 10^5 cells/well in a 24-well plate were seeded and then transfected with 0.2 μg of luciferase reporter plasmid cDNA and 0.75 μg of indicated Gα mutant cDNA (a and b), or 0.2 μg of luciferase reporter plasmid cDNA, 0.2 μg of m1 MAChR cDNA and 0.5 μg of indicated Gα mutant cDNA (c). After starvation overnight, cells were stimulated with 0.5 μM of LPA (a), or 1 unit/ml of thrombin, or 1 μM of carbachol (c) for 6 hours before the activity of luciferase was determined.

Fig. 5a Inhibition of LPA receptor activation by empty Gα mutants

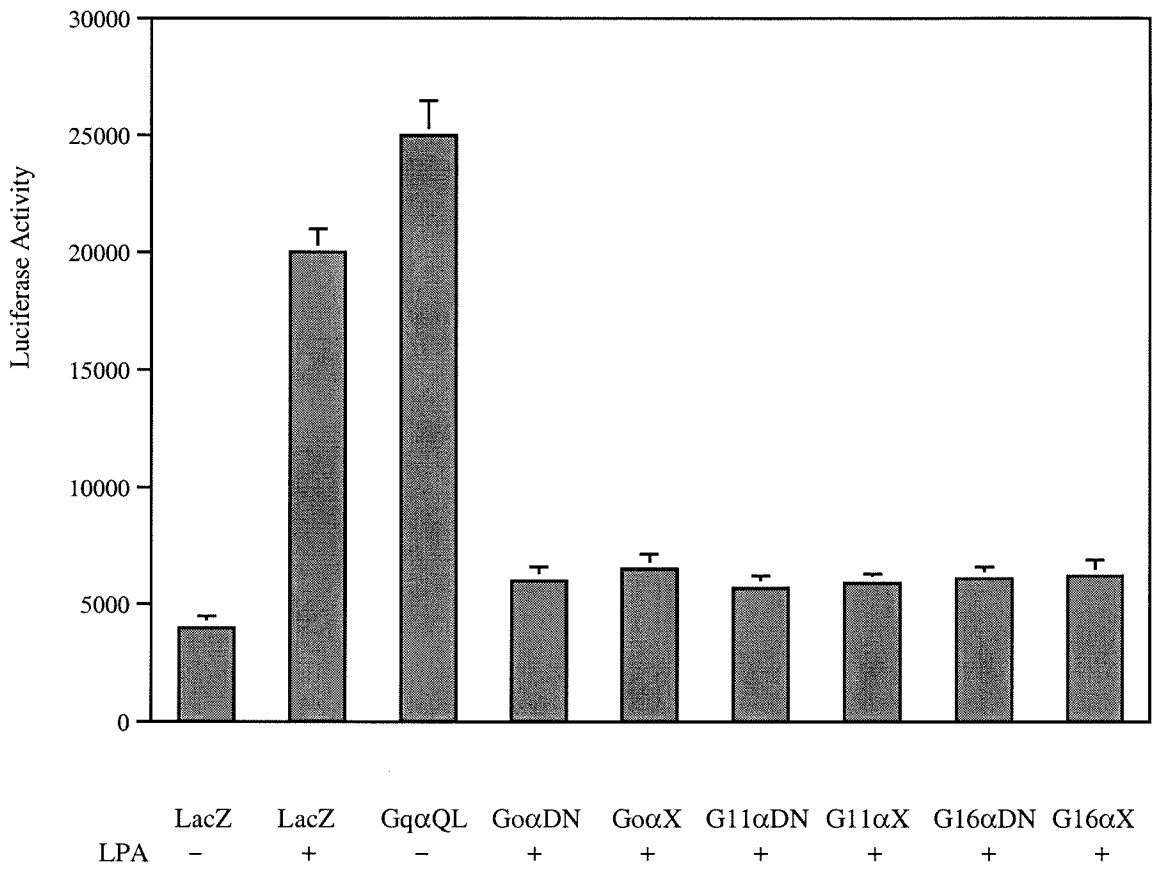


Fig. 5b Inhibition of thrombin receptor by empty Ga mutants

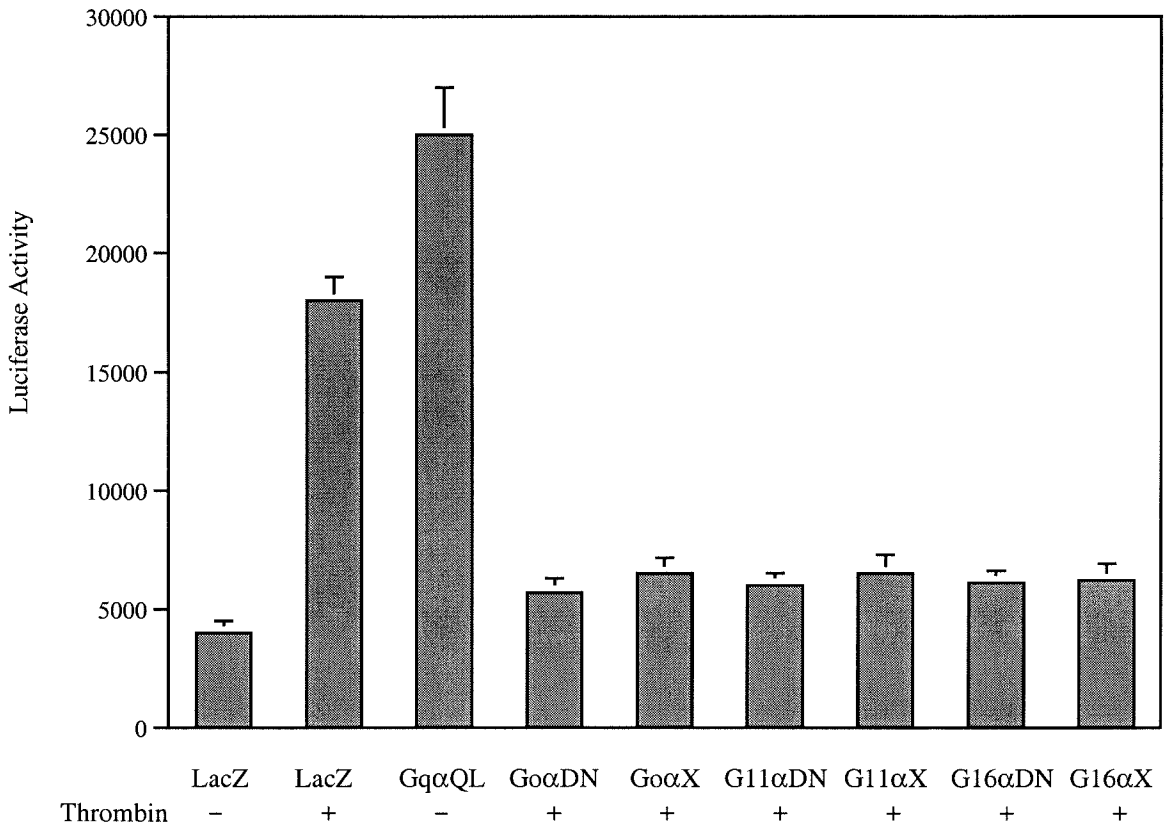
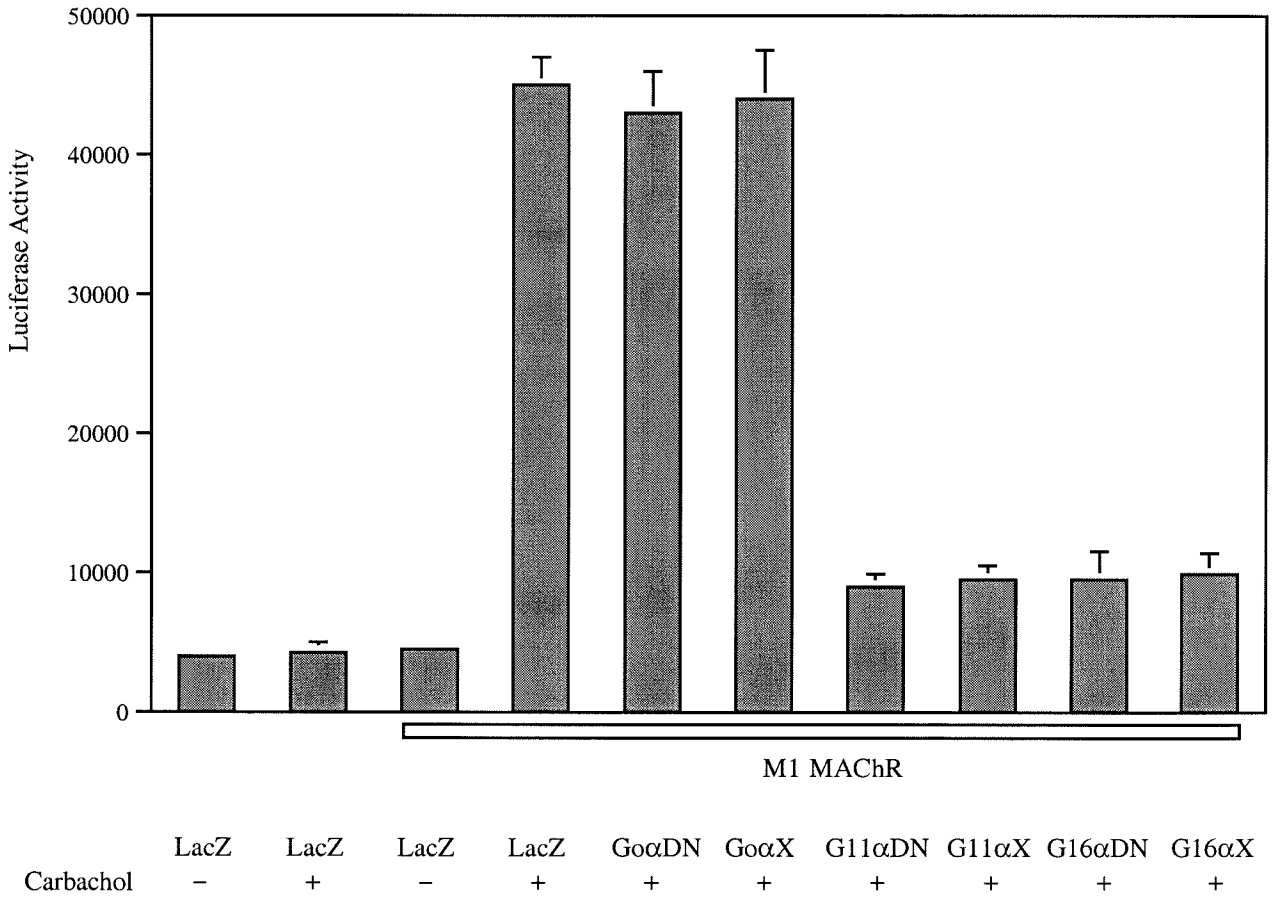


Fig. 5c Empty G11and G16 mutants inhibit M1 MAChR activation



Chapter Five

Expression of Empty G16α Mutants in NIH3T3 Cells Using Retroviruses

Abstract

We constructed recombinant retroviruses encoding empty mutant forms (nucleotide-free) of G16α, G16αD280N and G16αX (G16αD280N/Q213L), and obtained NIH3T3 cell lines stably expressing the empty G16α mutants by viral infection. Then we determined the activity of endogenous thrombin and lysophophatidic acid (LPA) receptors in these cells by monitoring the induction of serum response factor stimulated transcription by these receptors. We found that expression of the empty G16α mutants blocked the activation of G-protein signaling by thrombin and LPA receptors. In addition, the activity of transfected m1 muscarinic receptor was also attenuated in these cells. In the control experiments, activation of serum response factor by the constitutively active mutant GqαQL was not affected by the expression of empty G16α mutants. These experiments confirmed our previous findings that empty Gα mutants can inhibit the activity of their cognate receptor; presumably by binding tightly to the receptors and preventing G-protein activation. They also proved that retroviral gene expression can be used as an effective technique to deliver empty G protein mutants into cells.

We have previously constructed xanthine nucleotide binding mutant forms of the Goα, G11α, and G16α proteins (1-3). We have demonstrated that in cells where the concentrations of XDP and XTP are negligible (4), GoαX, G11αX, and G16αX behaved like empty (nucleotide-free) G protein α subunits and formed stable complexes with their appropriate receptors. Empty mutants of Goα, G11α, and G16α retained the same receptor specificity as their wild-type counterparts. For example, GoαX interacted only with Gi-coupled receptors such as m2 muscarinic receptor (m2 MAChR), and G11αX interacts only with Gq-coupled receptors such as m1 MAChR, whereas G16αX could bind to members of both classes of G protein-coupled receptors (5,6). In addition, GoαX, G11αX, and G16αX all interacted with thrombin receptor and lysophophatidic acid (LPA) receptor which normally couple to members of both Gi and Gq families (7,8). Because of competitive binding to their cognate receptors, GoαX, G11αX, and G16αX are able to inhibit the activity of their appropriate receptors in cells. Therefore, these "empty" mutants of Goα, G11α, G16α can be used as dominant negative inhibitors against a specific class of G protein-coupled receptors.

In previous experiments, we expressed the mutant proteins in cells by transient transfection. There are several limitations to this method of introducing genes into cells including (1) the gene expression is transient; (2) transfection efficiency is normally less than 30%; (3) suspension cells can not be transfected with high efficiency. In this study, we attempted to overcome these shortcomings by constructing retroviruses encoding the empty mutant of the G16α subunit and obtaining NIH3T3 cell lines expressing empty G16α mutant proteins by retroviral infection.

Retroviral gene transfer is one of the most commonly used techniques for introducing stably expressed proteins into mammalian cells (9). The genome of retroviruses typically contain two identical RNA molecules (8-12 kilobases) which code for 3 proteins: Gag (a polyprotein processed to matrix and other core proteins), Pol (reverse transcriptase), and Env (envelope protein) (Fig.1a). Retroviruses infect cells by specific recognition by the envelope protein(s) of appropriate cell surface receptors. Infection leads to uptake of the virus nucleoprotein core (consisting mostly of gag-derived proteins, full-length genomic RNA, and the reverse transcriptase protein). The retroviral life cycle starts by making a double-stranded DNA from the RNA genome by the viral reverse transcriptase and then integrating the viral DNA into the host cell chromosome. The viral DNA can be replicated along with the host cell chromosome and thus inherited into the daughter cells. Viral DNA is also transcribed into RNA under its own long terminal repeat (LPR) promoter and gives rise to viral proteins using the host ribosomal machinery. New retroviral particles are assembled at the cell membrane and released from the host cell.

Using engineered retroviruses that carry the genes of interest the gene transfer method has several advantages. First, construction is relatively easy. Second, unlike Adenovirus-mediated gene delivery, expression from retroviruses is long-term, whereas adenoviruses do not integrate. Moreover, adeno-associated viruses have limited space for genes and regulatory units. Thus, retroviruses offer the best current compromise in terms of long-term expression, genomic flexibility, and stable integration. The main advantage for retroviruses is that their integration into the host genome which allows for their stable

transmission through cell division. This ensures that in cell types which undergo multiple independent maturation steps, such as hematopoietic cell progression, the retrovirus construct will remain resident and continue to express.

The use of recombinant retroviruses was pioneered by Richard Mulligan and David Baltimore with retrovirus packaging cell lines that could package retroviral RNA into infectious particles without the concomitant production of replication competent virus (10). In general, retrovirus packaging cells provide necessary retroviral structural gene products (Gag, Pol, and Env) *in trans* by stable transfection, but lack the retroviral packaging signal which is required for packaging retroviral RNA into virions. The gene of interest is subcloned into an engineered retroviral construct including the retroviral packaging signal but not the structural genes (11,12). In order to produce high titer (up to 10^6 unit/ml) infectious virus, which can infect target cells and transmit the gene of interest but not replicate, one needs to transfect the engineered retroviral construct into the packaging cells and then collect the viruses from the supernatant of the packaging cells.

MATERIALS AND METHODS

Materials—The retroviral packaging cells BOSC23 and the retroviral construct MSCV-GFP were kindly provided by Dr. Baltimore's laboratory.

Construction of Retroviral Constructs and Collecting of Retroviruses—The cDNAs of wild-type G16α, empty mutant G16αX (G16αD280N/Q213L) and G16αD280N were subcloned into the MSCV-GFP vector. The packaging cells BOSC23 were maintained in

DMEM containing 10% fetal bovine serum. $2x10^6$ cells were seeded in a 60-mm plate one day before transfection of the retroviral constructs. A total of 10 μg/plate of DNA was used to transfect cells with Superfect (Life Technologies, Inc.), following the manufacturer's recommendations. Two days later, the supernatant of the cells (2 ml) containing retroviruses was collected and centrifuged at 1500 x g for 5 minutes to remove cells.

NIH3T3 Cells Culture and Transfection--NIH3T3 cells were maintained in DMEM containing 10% calf serum. $1x10^5$ cells/well were seeded into 24-well plates one day before transfection. A total of 1 μg/well of DNA including 0.2 μg of pSRF-Luc reporter plasmid (Strategene, Inc.) was used to transfect cells with Superfect (Life Technologies, Inc.), following the manufacturer's recommendations. The plasmid of pCIS encoding β-galactosidase was used to maintain a constant amount of DNA for each well.

Infection of Retrovirus into NIH3T3 Cells—$4x10^5$ NIH3T3 cells/well were seeded in 12-well plates one day before the infection. At the infection, the medium was replaced by 0.5 ml of the supernatant containing retroviruses and 8 μg/ml polybrene for 5 hours. Then cells were maintained in DMEM with 10% calf serum for three more days before expression of GFP was analyzed.

Sorting of NIH3T3 cells Expressing GFP—Flow cytometric analysis of GFP and sorting of NIH3T3 cells that are GFP positive were performed using a Becton Dickinson Vantage flow cytometer/cell sorter (San Jose, CA) equipped with a 488 nm Enterprise argon laser (200 mW). Chicken red blood cells were used to calibrate for instrument drift

and quality control as described elsewhere (15). Forward angle and side scatter light gating were used to exclude dead cells and debris. Data were generated in log mode for fluorescence and in linear mode for scatter and were presented as dot-plots or histograms using the cellquest software (Becton Dickison).

Luciferase Assay--Transfected NIT3T3 cells were maintained in DMEM containing 0.05% calf serum overnight and then were stimulated with 500 nM of LPA or 1 unit/ml of thrombin in the same medium for 6 hours before cell extracts were collected to determine the activity of luciferase. The luciferase assay was performed following the protocol of Luciferase Assay System from Promega. The activity of luciferase was determined by measuring luminescence intensity using a luminometer (Monolight 2010 from Analytical Luminescence Laboratory).

RESULTS AND DISCUSSION

NIH3T3 cells express endogenous thrombin receptor and LPA receptor which couple to a variety of G proteins including members of Gi, Gq and G12/13 families (13). Activation of either receptor leads to activation of serum response factor (SRF) and SRF-mediated gene transcription through RhoA, *via* both the Gq and G12/13 pathways. The activation of SRF can be assayed by monitoring the activity of luciferase in cells that are transfected with a reporter construct in which the luciferase gene is under the regulation of SRE.L, a derivative of the c-Fos serum response element (SRE) to which SRF binds (14). We have previously shown that transient expression of empty G16α mutants

inhibited the activation of endogenous thrombin receptor and LPA receptor, as well as transfected m1 MAChR (3). In this study, we constructed NIH3T3 cell lines that stably express empty G16α mutants using recombinant retroviruses and then investigated the activation of thrombin receptor and LPA receptor in these cells.

We subcloned the cDNAs of wild-type G16α, and the empty mutants, G16αX (G16αD280N/Q213L) and G16αD280N, into a retroviral vector MSCV-GFP which was based on the backbone of the murine stem cell virus genome. Downstream of the expression cassette of the mutant G16α is an IRES (internal ribosome entry site) sequence followed by the cDNA of GFP (green fluorescence protein) allowing us to use the expression of GFP as a marker for the production of mutant G16α (Fig. 1b). After transfecting the retroviral constructs into the packaging cells, we collected recombinant retroviruses and infected them into NIH3T3 cells. Cells were maintained in normal medium for at least three days before analysis of GFP expression. Based on the proportion of the cells that were GFP positive, we concluded that the titer of retroviruses expressing G16α mutants ranged from $0.5x10^5$ to $1x10^5$/ml. Then cells expressing GFP were isolated using a cell sorter and allowed to differentiate continuously. Analysis of GFP expression showed that cell sorting enriched the GFP-positive cells by about 10-15 fold (Table 1).

To determine whether activation of endogenous thrombin receptor and LPA receptor is inhibited in the cells expressing empty G16α mutants, we transfected these cells with the SRE.L-luciferase reporter construct and measured the activity of luciferase upon activation by thrombin or LPA. We found that cells that express G16αDN or

G16αX did not respond to thrombin or LPA whereas wild-type cells and cells expressing wild-type G16α showed increased activity of luciferase by up to 4-fold (Fig. 2 a and b). These results are consistent with our previous findings that empty G16α mutant can inhibit the activation of these receptors. To exclude the possibility that empty G16α mutants interfered with downstream components of the signaling pathway, we cotransfected the cells with the activated Gq mutant, GqαQL. We found cells expressing empty G16α mutants exhibited GqαQL-stimulated luciferase activity similar to wild-type cells (Fig. 2 a and b), indicating that the signaling pathway downstream of thrombin and LPA receptors was not affected in the cells expressing empty G16α mutants, and that the inhibition of receptor-stimulated SRF activation must come from the competitive binding of the empty G16α mutants to receptor.

Since NIH3T3 cells do not express endogenous muscarinic receptors (13), we transfected the cells with m1 MAChR and measured activation of luciferase through the endogenous Gq pathway. As expected, we found that activation of m1 MAChR in cells expressing empty G16α mutants was attenuated comparing to the wild-type cells or cells expressing wild-type G16α (Fig. 2c). However, the inhibition of transfected m1 MAChR was about 50% whereas activation of endogenous thrombin receptor or LPA receptor was almost abolished in cells expressing empty G16α. Since transiently transfected m1 MAChR was expressed at much higher level than the endogenous thrombin receptor and LPA receptor, this experiment supports the hypothesis that empty "mutants" of G16α inhibit receptor by competitive binding and an excess level of empty G16α mutants is required for effective inhibition.

In this study, we have successfully constructed recombinant retroviruses and stably expressed empty G16α mutant proteins in NIH3T3 cells by viral infection. Empty G16α mutants expressed in cells was able to inhibit the activation of G protein-coupled receptors including the thrombin receptor, LPA receptor, and m1 MAChR. These experiments agree with our previous findings that "empty" Gα mutants can act as dominant negative inhibitors against G protein-coupled receptors by binding tightly to their cognate receptor. We have shown that retroviral gene transfer can be an effective technique to deliver empty Gα mutant proteins into cells and to block the activity of G protein-coupled receptors. This technique will allow us to utilize empty Gα mutant proteins from Goα, G11α, and G16α as dominant negative inhibitors in a variety of cell types or even tissues, and help to dissect the G protein-mediated signaling network by blocking subsets of G protein-coupled receptors.

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Table 1 GFP analysis of NIH3T3 cells infected by retroviruses encoding empty G16α mutants

NIH3T3 cells	Retrovirus titer	GFP positive cells	GFP positive cells after sorting
G16α	$0.5x10^5$/ml	6%	80%
G16αDN	$0.6x10^5$/ml	7.5%	85%
G16αX	$0.8x10^5$/ml	9.5%	92%

Figure 1 Schematic drawing of retrovirus genome and recombinant retroviral construct MSV-GFP. a) Retroviral RNA encodes Gag (a polyprotein processed to matrix and other core proteins), Pol (reverse transcriptase), and Env (envelope protein), flanking by terminal direct repeat sequences (R) and unique regulatory sequence U5 and U3. b) Retroviral construct MSV-GFP consists of a multicloning site under the control of a long terminal repeat (LTR) promoter, followed by an IRES-GFP sequence.

Fig . 1a Genetic organization of retrovirus genome

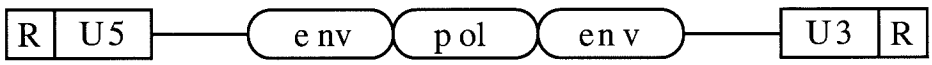


Fig. 1b Schematic drawing of recombinant retroviral construct MSV-GFP

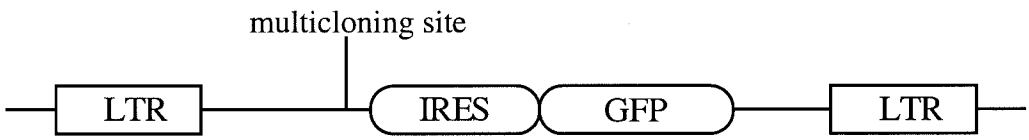


Figure 2 Endogenous thrombin receptor and LPA receptor in NIH3T3 cells expressing empty G16α mutants were inhibited. $1x10^5$ cells/well in a 24-well plate were seeded and then transfected with 0.2 μg of luciferase reporter plasmid cDNA (a, b, and c). Indicated cells were cotransfected with 0.8 μg of GqαQL cDNA (a and b) or 0.2 μg of m1 MAChR cDNA (c). The total amount of cDNA for each well was adjusted to 1.0 μg by addition of CMV-LacZ cDNA. After starvation overnight, cells were stimulated with 1 unit/ml of thrombin (a), or 0.5 μM of LPA (b), or 1 μM of carbachol (c) for 6 hours before the activity of luciferase was determined.

Fig. 2a Inhibition of thrombin receptor by empty G16α mutants in NIH3T3 cells

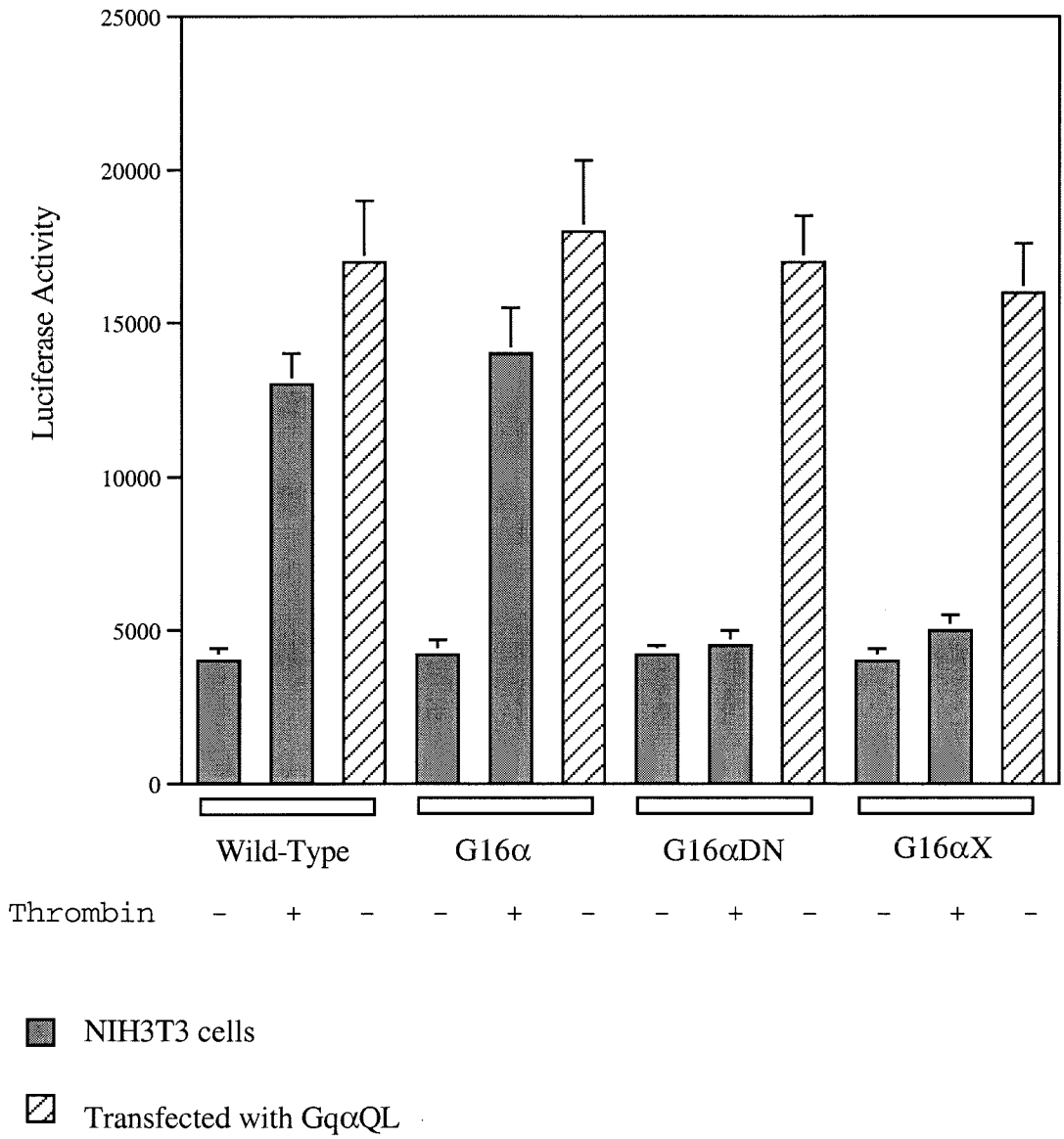


Fig. 2b Inhibition of LPA receptorby empty G16α mutants in NIH3T3 cells

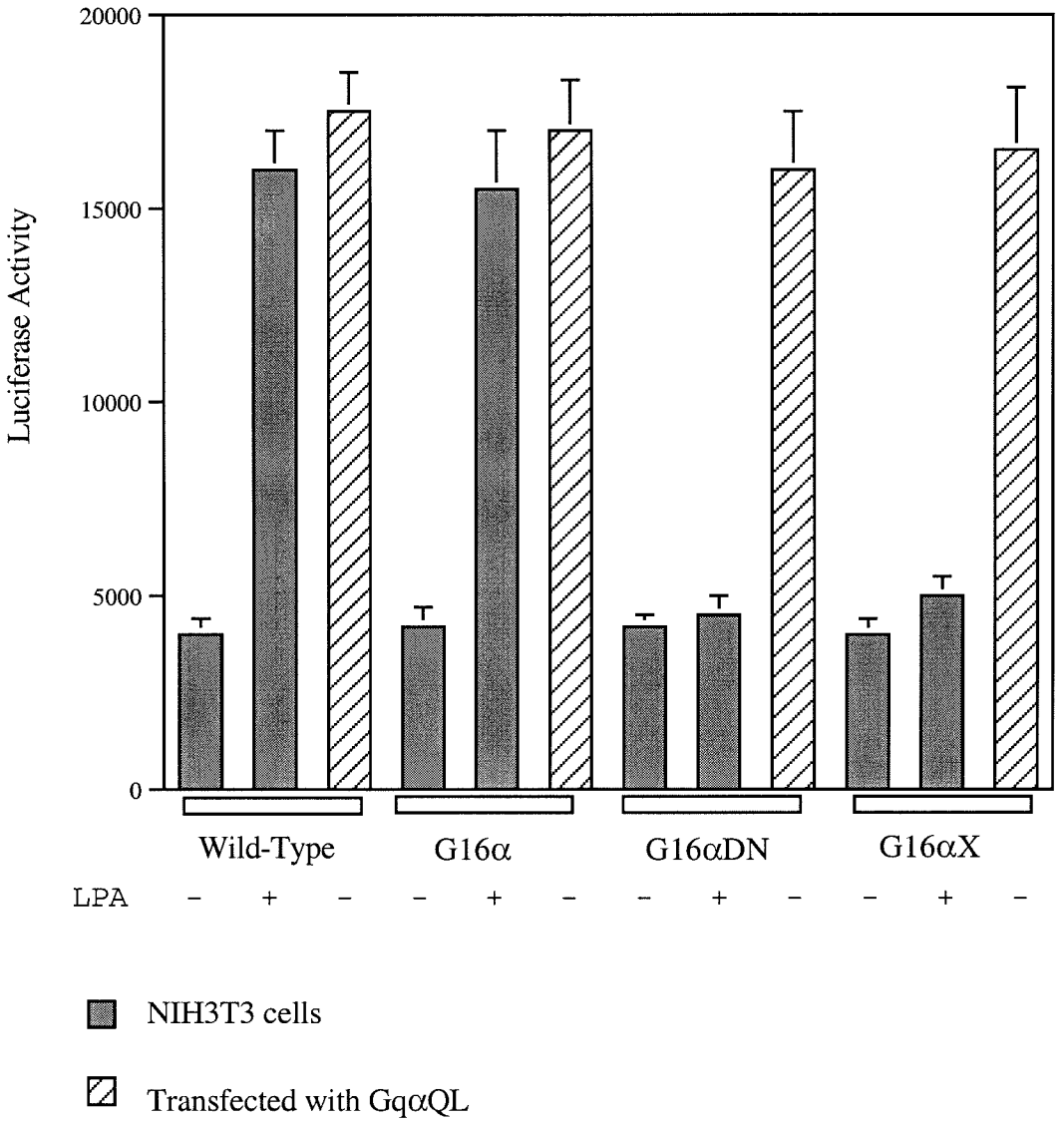
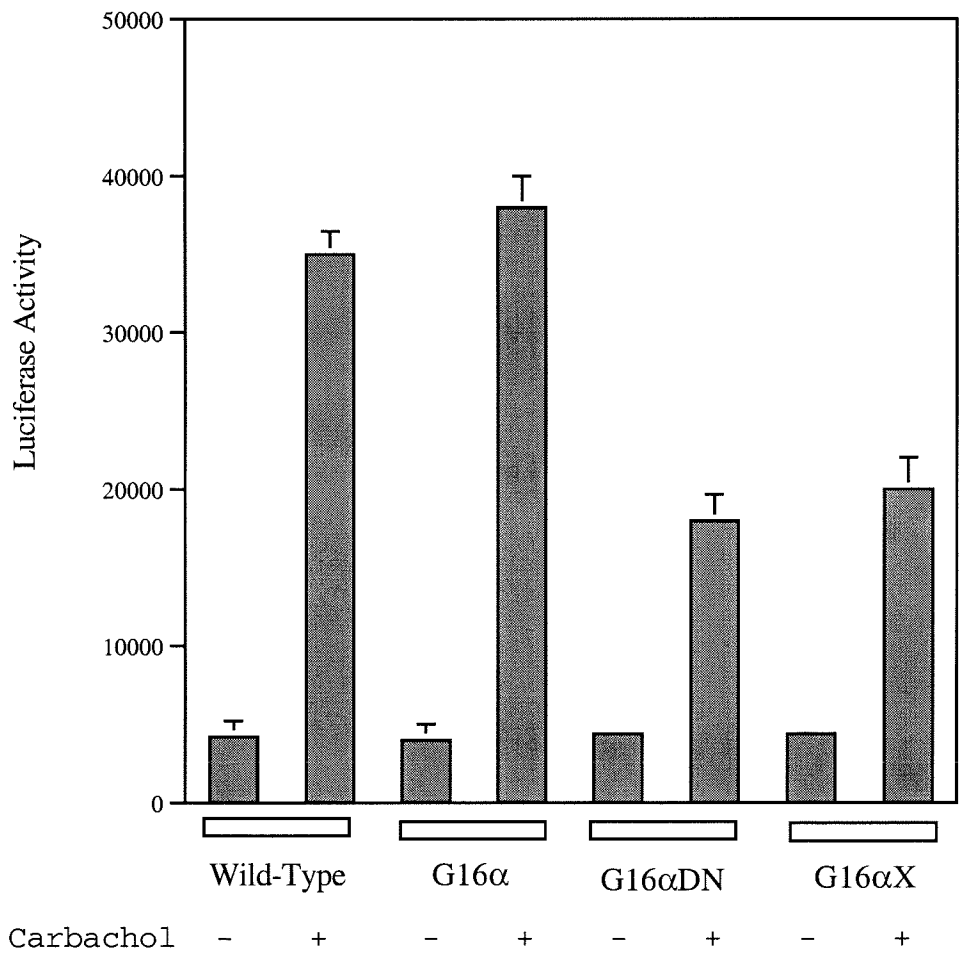


Fig. 2c Inhibition of m1 MAChR in NIH3T3 cells



Chapter Six

Summary

Heterotrimeric G proteins transduce receptor-generated signals across the plasma membrane of eukaryotic cells. The tremendous diversity of G protein-coupled receptors, G proteins, and G protein effectors makes the G protein-mediated signal transduction a complex network. In order to discern function of a specific G protein without interference from other signaling pathways, we have constructed xanthine nucleotide binding mutants from α subunits of Go, G11, and G16.

Based on the crystal structures of transducin and Gi α subunits, we replaced an aspartic acid in the conserved NKXD motif of Goα by an asparagine that was expected to coordinate with xanthine instead of guanine. This mutation was introduced into both the wild-type Goα subunit and the GTPase-deficient Goα mutant (Q205L). As expected, the double mutant GoαD273N/Q205L, GoαX, switched nucleotide specificity; it bound xanthine nucleotides instead of guanine nucleotide. Interestingly, the single mutant D273N was not able to bind either nucleotide. We found that GoαX retained the characteristic properties of wild-type Goα in the presence of XDP or XTP; GoαX bound βγ in the XDP form and dissociated from βγ in the XTP form because of a conformational change similar to that in the activated wild-type Goα. This is the first example of an engineered heterotrimeric G protein α subunit that switched nucleotide specificity from guanine nucleotides to xanthine nucleotides.

The empty state (nucleotide-free) of G proteins is an important intermediate during the GTP-GDP exchange catalyzed by the activated receptor. Since the concentration of xanthine nucleotides in cells is negligible, GoαX provides an excellent

model to study the properties of the empty form of Gα. We found that GoαX formed a stable complex with receptor and inhibited the activity of cognate receptor both *in vitro* and in cells. In addition, GoαX retained the same receptor binding specificity as the wild-type Goα and interacted with only Gi-coupled receptors.

To test whether the same strategy can apply to other G proteins, we introduced similar DN mutations in two other Gα subunits, G11α and G16α. Both G11α and G16α belong to the Gq family. However, G16α is considered a promiscuous G protein which interacts with all types of G protein-coupled receptors, whereas G11α only interacts with Gq-coupled receptors. Mutant proteins of both G11αX (G11αD277N/Q209L) and G16αX (G16αD280N/Q213L) were found to bind xanthine nucleotides instead of guanine nucleotides. Similar to GoαX, G11αX and G16αX also interacted with their appropriate receptors. In transfected COS-7 cells, empty G11α mutant proteins bound to the Gq-coupled receptors and inhibited their activity, but had no effect on the Gi-coupled receptor. On the other hand, empty G16α mutant proteins interacted with and inhibited both Gi- and Gq-coupled receptors. These experiments indicate that, along with empty Goα mutants, empty mutants of G11α and G16α can also be used as dominant negative inhibitors against appropriate G protein-coupled receptors.

Finally, in order to express empty G proteins in a variety of cell types, we constructed retroviruses encoding empty G16α mutants. We obtained NIH3T3 cell lines stably expressing empty G16α mutants by viral infection. We found that empty G16α mutants blocked the activation of both thrombin and LPA receptors in NIH3T3 cells, consistent with previous experiments in COS-7 cells. These experiments suggest that

retroviral gene transfer can be an effective technique to deliver empty Gα mutant proteins into cells and to block activation of G protein-coupled receptors.

In summary, we successfully engineered G protein mutants that can be regulated by xanthine nucleotides, not by guanine nucleotides. In cells GoαX, G11αX and G16αX are in the empty conformation and can only be activated by addition of exogenous XDP or XTP. These G protein mutants provide us with a unique way to activate a particular G protein α subunit, or to block βγ subunits from their natural effectors by introducing XTP or XDP *in vivo* without interfering with other cellular activities. These mutant proteins also make it possible to study the empty form of G proteins. The experiments on receptor interaction of these empty Go, G11 and G16 α subunits showed that they all exhibited dominant negative activities toward their respective receptors. Therefore, these "empty" Gα mutants can be used to specifically block one class of G protein-coupled receptors *in vivo*, and to differentiate between receptor-mediated signal pathways in a complex physiological system. Retroviral gene transfer makes it possible to introduce empty Gα mutants in an efficient fashion in a variety of cells and even in animals, and to study the functions of G protein-coupled receptors in different developmental processes.