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A new phospholipase C-beta from *Xenopus* oocyte: Cloning and characterization

Ma, Hai-Wen, Ph.D.

City University of New York, 1995

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A NEW PHOSPHOLIPASE C-BETA FROM *XENOPUS* OOCYTE: CLONING AND CHARACTERIZATION

by

HAI-WEN MA

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

1995

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Jan 6, 1995
Date

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ABSTRACT

A New Phospholipase C-Beta From *Xenopus* Oocyte: Cloning And Characterization

by

Hai-Wen Ma

Adviser: Professor Ravi Iyengar

Xenopus oocytes exhibit a receptor-evoked Cl^- current that is mediated through the activation of phospholipase C (PLC) and a subsequent increase in $[Ca^{2+}]_i$. A major component of the PLC response in oocytes is PTX-sensitive. However. the identity of the PLC(s) mediating this effect is unknown.

A new form of PLC (PLC-*X*β) was isolated from a *Xenopus* oocyte cDNA library. PLC-*X*β is most similar in sequence and overall structure to the β-class of mammalian PLCs. Northern blot analysis demonstrates that PLC-*X*β mRNA is expressed in *Xenopus* oocytes as 5 Kb transcripts. Immunoblot analysis demonstrates that PLC-*X*β is ubiquitously expressed as a 140 kDa protein in all the nine tissues examined.

Injection into oocytes of antisense, but not sense, oligonucleotides to a specific region of PLC-*X*β results in degradation of its mRNA and a significant reduction of Cl^- currents evoked by endogenous angiotensin receptors and expressed α_{1b}-adrenergic receptors and M_1-muscarinic

receptors. All of the receptor responses are at least partially PTX-sensitive in oocytes. Cl^- current responses to subsequent injection of IP_3 are indistinguishable in oocytes injected with antisense or sense oligonucleotides. Inhibition of the M_1-muscarinic response produced by antisense oligonucleotides was nonadditive with that produced by pertussis toxin. These results indicate that PLC-*X*β is involved in the PTX-sensitive phosphoinositide metabolism pathway in *Xenopus* oocytes.

G_o has been implicated as the signal transducer in the PTX-sensitive PLC pathway in *Xenopus* oocytes. G_o is directly involved in the α_{1b}-adrenergic receptor-mediated pathway (Blitzer et al., 1993). More recently, PLC-*X*β and α_o, but not α_q, are shown to be involved in the PTX-sensitive response evoked by $5HT_{1c}$ receptor. On the other hand, α_q, but not PLC-*X*β and α_o, is involved in the PTX-insensitive response evoked by TRH receptor (Quick et al., 1994). Taken together, these results indicate that PLC-*X*β is regulated by G_o in oocytes and represents a new effector in the PTX-sensitive PLC pathway.

PLC-*X*β has been functionally expressed in Sf9 cells by using the baculovirus expression system. In the *in vitro* reconstitution assay, the recombinant PLC-*X*β is stimulated by α_q, similar to all of the other cloned mammalian PLC-βs. However, α_o as well as βγ subunits are unable to stimulate PLC-*X*β. The regulation of PLC-*X*β by G_o in the intact oocyte but not *in vitro* indicates that the stimulatory effect is not direct. Additional as yet unknown component(s) may be required to communicate signals from G_o to PLC-*X*β.

DEDICATION

This dissertation is dedicated to my husband, Zhi-Wei, and to my parents.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1

Background

A. G proteins

1. G protein regulated signal transduction:

All cells in biological systems have the ability to receive and process information from their surroundings in order to regulate their development, control their growth, coordinate their functions, and communicate with the outside world. A variety of external signals are known. They include hormones, neurotransmitters, growth factors, light, odorants, and many other agents. Many external signals exert their functions by first binding to specific receptors on the plasma membrane of the target cells. Upon agonist binding, a large family of the cell surface receptors activate effector proteins, usually membrane-bound enzymes or ion channels. The activation of effector proteins results in either production of intracellular chemicals called second messengers or alteration in the membrane voltage potential. The second messenger initiates a cascade of molecular events leading to the change of the cell's biological activities (Iyengar, 1991).

In this class of receptors, the agonist-receptor complex does not directly stimulate the effector. G proteins, a family of guanine nucleotide-binding proteins, are required for mediating this signal flow from receptors to effectors in all eukaryotic organisms. The activated receptor stimulates G protein first, which in turn transduces the excitation signal to effector(s). G proteins are employed by many different

extracellular messengers for signal transduction, even though each signal elicits a distinct set of cellular responses (Gilman, 1987). As estimated by Dr. Lubert Stryer of Stanford University, at least a third of signal transduction processes involve G proteins. The importance of G protein-coupled signaling system has been widely appreciated and has won its researchers 1994's Nobel Prize in Physiology and Medicine.

2. GTPase cycle:

G protein is composed of three distinct subunits, designated α, β, and γ in the order of descending molecular weight. G proteins oscillate between GDP- and GTP- bound forms (Fig. 1.1). In the resting state, G proteins exist as heterotrimers with GDP bound to the α-subunits. Agonist-occupied receptors interact with heterotrimeric G proteins, promoting the dissociation of GDP from the α subunits. The GTP concentration within the cell is about 100 μM and is ten times higher than GDP concentration. Therefore, GTP rapidly binds to the empty guanine nucleotide binding site of the α-subunit. Binding of GTP induces a conformational change of α subunit and its subsequent dissociation from the $\beta\gamma$ complex. The α_{GTP}, as well as the free $\beta\gamma$ complex, are then able to interact with the downstream effectors and modulate their activities, either independently or interdependently. The α-subunit has intrinsic GTPase activity, which hydrolyzes GTP into GDP in the binding site. GDP-bound α subunits reassociate with $\beta\gamma$ subunits,

returning the system to the inactive state. Thus, the GTPase activity serves as a built-in independent deactivation device, determining how long the signaling pathway is turned on (reviewed by Gilman, 1987; Kaziro et al., 1991; Hepler and Gilman, 1992).

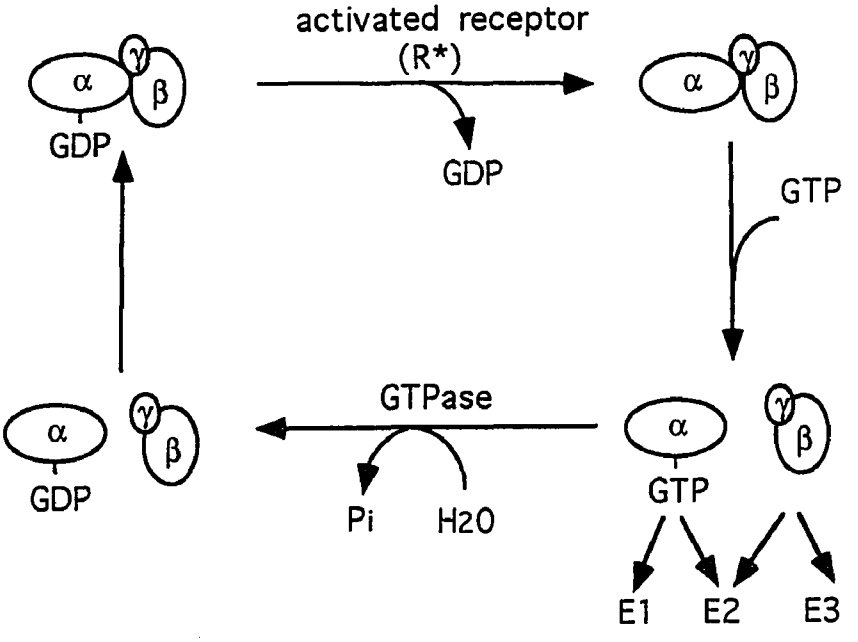

Figure 1.1. G-protein-mediated signal transduction. In the resting state, G proteins exist as heterotrimers with GDP bound to the α-subunits. Agonist-occupied receptors interact with heterotrimeric G proteins, promoting the dissociation of GDP and association of GTP to the α subunits. Binding of GTP induces a conformational change of α subunit and its subsequent dissociation from the βγ complex. The α_{GTP}, as well as the βγ complex, are then able to interact with and modulate the activities of the downstream effectors (E1, E2, or E3), either independently or

interdependently. The intrinsic GTPase activity of the α-subunit hydrolyzes GTP into GDP in the binding site. GDP-bound α subunits reassociate with βγ subunits, returning the system to the inactive state.

3. Classification of G proteins:

Multiple G proteins have been identified. Each heterotrimeric G protein that has been isolated up to now contains an unique α subunit. However, this is not necessarily true for the βγ subunits. Different α subunits may be linked to the same βγ subunits or to different pairs. In many cases, the βγ subunit complexes are functionally interchangable among different α subunits to form heterotrimers (Gilman, 1987).

G proteins are classified according to the function and sequence relationship of the α-subunits. So far, cDNAs for 21 distinct α-subunits, 4 distinct β-subunits, and 6 distinct γ-subunits have been cloned. Based on amino acid sequence similarity, these α subunits are divided into four major families represented by G_s, G_i, G_q and G_{12} (Fig. 1.2, Hepler and Gilman, 1992; Birnbaumer, 1992).

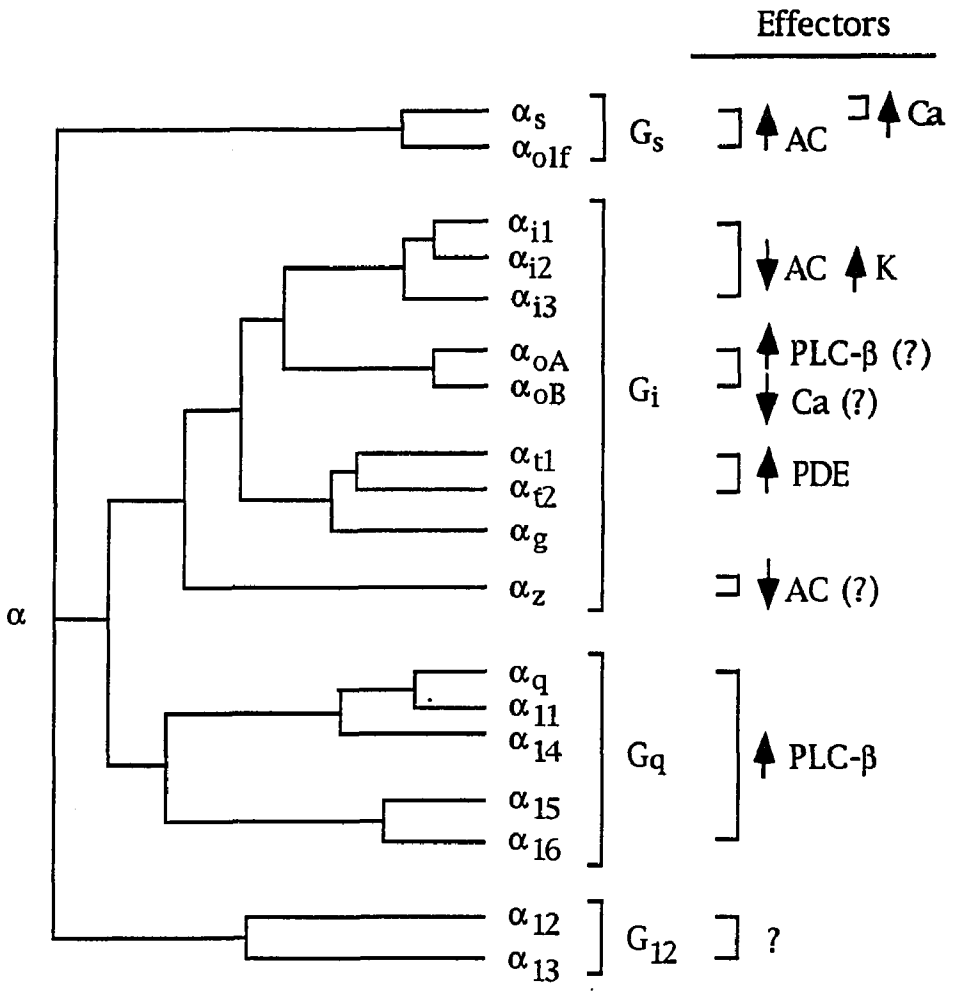

Figure 1.2. Phylogenetic tree of G protein α subunits and their effectors (adapted from Hepler and Gilman, 1992). Abbreviatons for α subunits are: s, stimultory; i, inhibitory; o, other; t, transducin; olf, olfactory; g, gustducin. Abbreviations for effectors are: AC, adenylyl cyclase; PLC, phospholipase C; PDE, phosphodiesterase; K, K^+ channel; Ca, Ca^{2+} channel.

As shown in Fig. 1.2, each G protein family can regulate one or more effectors. For example, the G_s family members stimulate adenylyl cyclases and open calcium channels. G_i family contains several subfamilies of highly similar α subunits. Not surprisingly, the function of the G_i family is also the most diversified. All G_i can inhibit adenylyl cyclases and stimulate potassium channels. G_o might be involved in stimulation of phospholipase C and inhibition of calcium channels. α_{t1} and α_{t2} stimulate the retinal phosphodiesterase. α_z has been shown to inhibit adenylyl cyclase as well. All members of G_q family stimulate phospholipase C-βs (For review, see Hepler and Gilman, 1992; Birnbaumer, 1992). The exact function of G_{12} family is not yet clear. α_{12} has been found to be a protooncogene and hence involved in proliferative processes (Xu et al., 1993). These G protein-regulated effectors generate second messengers such as cAMP, IP_3, DAG, or lead to changes in membrane potential, all of which in turn regulate numerous cellular functions.

4. Structure-function relationship of G protein α subunits:

Gα subunits belong to a superfamily of GTPases. This superfamily contains hundreds of members, including Ras. They are involved in functions as diverse as cell growth and differentiation, protein synthesis initiation and elongation, protein translocation across membranes, vesicular transport,

etc. Despite the low amino acid sequence similarity (<20%), the GTPase family members share conserved three dimensional structure and mechanism for GDP/GTP binding and GTP hydrolysis (Bourne et. al., 1990 and 1991).

The crystal structure of α_t and α_{i1} have been recently published (Noel et al., 1993; Lambright et al., 1994; Coleman et al., 1994; Spondek et al., 1994). In the crystal structure, α_t consists of two well defined domains flanking a deep guanine-nucleotide-binding cleft. One is the core GTPase domain that is structurally similar to Ras and EF-Tu. The other is a highly helical domain unique to α subunits (Noel et al., 1993).

The core GTPase domain consists of six β-sheets (β1-β6) surrounded by 5 helices (α1-α5). Several conserved sequences are clustered along one side of this domain, making interaction with the guanine ring, the ribose, the α, β, γ phosphates, and Mg^{++}. Some of these sequences are important for GTP hydrolysis. Three loops required for effector interaction are located in the other side of this domain. GTP binding or GTP hydrolysis induces conformational changes in α2, which in turn, are transmitted to the effector interaction sites for initiating or ending effector activation (Noel et al., 1993).

The second highly helical domain is formed by a stretch of 113 amino acids (residue 59-172 of α_t). This stretch is present in all Gα chains but absent in the other GTPases. This domain forms one wall of the nucleotide-binding cleft and is centrally involved in GDP/GTP binding and GTP hydrolysis. The two domains together completely bury the bound guanine

nucleotide. It is assumed that activated receptor interacts with the C-terminus of α_t, loosening the hydrogen bonds and Van der Waals contacts among residues in both domains which connect with each other and with the guanine ring, thereby promoting GDP release and GTP binding (Noel et al., 1993).

Two residues conserved in all α subunits are important for GTP hydrolysis. They are Arg (Arg^{174} in α_t, Arg^{178} in α_{i1}) and Gln (Gln^{200} in α_t, Gln^{204} in α_{i1}). Their function in GTP hydrolysis has been confirmed by biochemical studies. Mutation at either of these two sites results in decreased GTPase activity and constitutive activation of the α subunit which then continuously stimulates the effector (Graziano and Gilman, 1989; Masters et al., 1989; Landis et al., 1989; Gupta et al., 1992). The crystal structure provides detailed insight onto the mechanism of GTP hydrolysis. In the case of GTPγS-α_t, the guanidino group of Arg^{174} is found to make hydrogen bonds to the α- and γ- phosphates and the oxygen bridging the β- and γ-phosphates. In GTPγS-α_{i1}, Gln^{204} stabilizes and orients the hydrolytic water in the transition state. Arg^{178} promotes bond cleavage by stabilizing the negative charge on the equatorial oxygen atoms of the γ phosphate (Noel et al., 1993; Coleman et al., 1994).

Another line of experiments provides more information on the structure-function relationship of α subunits. The two separate domains observed in the crystal structure can be expressed separately as stable and folded recombinant proteins, as shown for α_s. The expressed core domain alone

binds GTPγS, stimulates adenylyl cyclase, but does not hydrolyze GTP. Addition of the expressed helical domain confers on the core domain the ability to hydrolyze GTP. Therefore, the helical domain serves as a built in GTPase activating protein (GAP) for Gα. The core domain is thus named as Ralph for Ras-like domain. The helical domain is named as Gail for Gα insert and GAP-like domain (Markby et al., 1993).

In addition to the inserted Gail domain, Gα subunits contain extra N and C terminal sequences compared to Ras. The N-terminus is involved in interaction with βγ-subunits. A monoclonal antibody directed against the N-terminus causes the α_t to dissociate from βγ (Mazzoni and Hamm, 1989; Mazzoni et al., 1991). A myristoylated N-terminus peptide competitively inhibits α_t binding to βγ (Kokame et al., 1992). Deletion of the N-terminus, either by mutation or protease cleavage, impairs Gα-βγ interactions (Fung and Nash, 1983; Neer et al., 1988; Journot et al., 1991; Denker et al., 1992; Graf et al., 1992).

The C-terminus of Gα subunits is the contact site for receptors. Peptide-specific antibodies against this region all uncouple G proteins from receptors (Simonds et al., 1989; Gutowski et al., 1991; Shenker et al., 1991). A synthetic 11 amino acid C-terminal peptide competes with G protein binding to receptor (Hamm et al., 1988). Work with α_i/α_q chimeras indicates that the C-terminus is involved in the specificity of interaction with receptors (Conkolin et al., 1993). In addition, a

peptide corresponding to residues 311-328 of α_t blocks activation of G_t by receptor (Hamm et al., 1988; Hamm, 1991).

Mutational analysis of α_s and studies with α_i/α_s chimeras indicates that three regions are important for effector activation (Itoh and Gilman, 1991; Berlot and Bourne, 1992). A peptide corresponding to residues 293-314 of α_t can mimic the ability of α_t to activate phosphodiesterase (Rarick et al., 1992). This region is adjacent to the C-terminus receptor activation domain and coincides with one effector activation domain mentioned above.

The proposed structural model of Gα is shown in Figure 1.3.

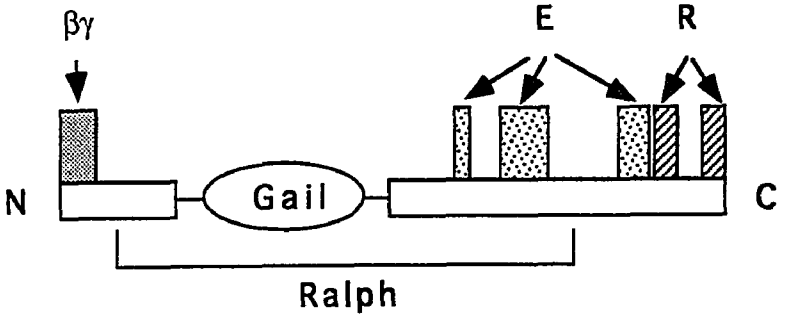

Figure 1.3. Structural model for Gα. The open bar represents the core GTPase domain (Ralph). The circle represents the inserted helical domain (Gail). The putative interaction sites of Gα with βγ, activated receptor (R), and effector (E) are also illustrated.

5. G protein β and γ subunits:

β and γ subunits are tightly associated with each other under all but denaturing conditions. Hence, the βγ dimer is believed to be the functional form (Hildebrandt et al., 1984). The sequences of all the γ subunits end with CysXXX. The cysteine residue is modified by prenylation, which is necessary for association of Gβγ with membranes (Muntz et al., 1992).

Initially, βγ subunits were thought to only play a passive role in signal transduction. They reverse the action of Gα by reforming the inactive heterotrimer and stabilize Gα in its GDP-bound form (Higashijima et al., 1987). It is clear now that βγ subunits can directly regulate effector function. βγ can activate myocardial potassium channels (Logothetis et al., 1987; Kim et al., 1989; Reuveny et al., 1994). Specific types of adenylyl cyclases are differentially regulated by βγ (Tang and Gilman, 1991; Gao and Gilman, 1991). Phospholipase-β2 and β3 are stimulated by βγ (Camps et al., 1992; Katz et al., 1992; Carozzi et al., 1993). βγ complex also increases agonist-dependent receptor phosphorylation by BARK (β-adrenergic receptor kinase), probably by facilitating BARK membrane translocation (Pitcher et al., 1992; Haga and Haga, 1992).

As mentioned above, four β and six γ subunits have been cloned from mammalian cells. Although there are many conceivable combinations, not all of them are allowed. There is some specificity in the interactions of β and γ. For example, γ1 can form a complex with β1, but not with β2 or β3. β3 does not

form complexes with γ1 or γ2 (Schmidt et al., 1992; Iniguez-Lluhi et al., 1992; Pronin and Gautam, 1992).

The structural diversity may reflect the functional specificity of the βγ complexes. β1γ1 has been found to be 10-20 times less potent in stimulating adenylyl cyclase and phospholipase C than other βγ combinations tested (Iniguez-Lluhi et al., 1992; Ueda et al., 1994). The βγ complex may also confer the specificity of receptor-G protein interaction. For example, only γ1 is able to support interaction of α_t with rhodopsin even though several different recombinant βγ complexes interact equally well with α_t (Kisselev and Gautam, 1993).

6. Pertussis toxin and G proteins:

Pertussis toxin (PTX) is produced by *Bordetella pertussis*. PTX has two components: subunit A, which contains the ADP-ribosyltransferase activity, and subunit B, which binds the toxin to cell surface receptors. After gaining entry into the cell through receptor-mediated endocytosis, PTX catalyzes the transfer of ADP-ribose from NAD to a specific cysteine residue in the α subunits of G_i family. The modified cysteine residue is located four amino acids from the carboxyl-terminus. Since the C-terminal region is involved in receptor interaction, this ADP-ribosylation results in uncoupling of the receptor from G protein and the subsequent loss of hormone action (Reviewed by Gierschik, 1992; Yamane and Fung, 1993).

PTX has its characteristic effects only on the functions of specific G proteins. The PTX substrates include α_i, α_o, and α_t. The other α subunits, which lack the cysteine in the C-terminal position, are not substrates for PTX. PTX can be used with great ease and flexibility. It can be applied to either intact cells or purified components. PTX has therefore been a very useful experimental tool for identifying G proteins and investigating their involvement in signal transduction pathways.

B. Phospholipase C

1. Receptor regulation of phospholipase C:

There are three inositol-containing phospholipids in eukaryotic cell membrane: phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2). Although they constitute less than 10% of the total cellular phospholipids, they play important roles in signal transduction pathways (Berridge 1993; Majerus, 1992). A cellular enzyme, inositol phospholipid-specific phospholipase C (PLC), catalyzes the hydrolysis of phosphatidylinositol-4,5,-bisphosphate to generate two products, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3).

Both DAG and IP_3 are important second messengers. DAG remains in the plasma membrane and activates protein kinase C (PKC). PKC is a serine/threonine kinase which phosphorylates

and regulates the function of a number of proteins (Nishizuka, 1992; Hug and Sarre, 1993). IP_3 is released into the cytosol and mobilizes Ca^{++} by binding to specific intracellular receptors. IP_3 receptors are homotetrameric Ca^{++} channel complexes located in the ER membranes (Furuichi et al., 1989; Maeda et al., 1991). Binding of IP_3 to the receptors induces conformational change in the receptors and leads to channel opening and release of Ca^{++} into the cytosol. Increase in intracellular Ca^{++} can affect a variety of biochemical and physiological processes either directly or indirectly through calmodulin and other related Ca^{++}-binding proteins.

The receptor-mediated stimulation of PIP_2 hydrolysis by a wide array of hormones, neurotransmitters, growth factors, and other stimuli has been well documented (Dennis et al., 1991). Based on their amino acid sequences, most receptors can be divided into two categories, growth factor receptors and G-protein-coupled receptors. Growth factor receptors, such as those for epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), possess intracellular tyrosine kinase domains (Ullrich and Schlessinger, 1991). The receptors for most hormones and neurotransmitters are G protein-linked, containing characteristic seven membrane-spanning domains (Dohlman et al., 1991). The kinetics of PLC activation initiated by growth factor receptors and G protein-coupled receptors appear to be different. The growth factor-stimulated increase in intracellular Ca^{++} concentration begins 15-30 seconds after receptor stimulation. However, there is no measurable lag

period in Ca^{++} response produced by bombesin or vasopressin (Hasegawa-Sasaki et al., 1988; Cattaneo and Vicentini, 1989). It has become clear now that differences in activation mechanisms account for differences in activation kinetics. The different activation mechanisms will be discussed below.

3. Classification and primary structure of PLCs:

PLCs are single-polypeptide enzymes. There are multiple families of PLC enzymes as deduced from protein purification and molecular cloning studies. A total of thirteen distinct PLC cDNAs have been isolated. Nine of them are from mammalian species, two from *Drosophila melanogaster*, one from *Dictyostelium discoideum*, and one from *Saccharomyces cerevisiae* (Suh et al., 1988a and 1988b; Katan et al., 1988; Mayer et al., 1988; Stahl et al., 1988; Bloomquist et al., 1988; Ohta et al., 1988; Emori et al., 1989; Meldrum et al., 1991; Shortridge et al., 1991; Drayer and Haastert, 1992; Park et al., 1992; Carozzi et al., 1992; Jhon et al., 1993; Yoko-O et al., 1993; Ferreira et al., 1993; Lee et al., 1993).

PLC isozymes have been divided into three types, β, γ, and δ, based on their primary structure. Each type contains more than one subtype (Rhee et al., 1989; Rhee and Choi, 1992). Although all three forms of PLC vary widely in size and show a low overall similarity, two regions with high similarity have been found from sequence comparisons. These two regions are designated X and Y domains, which are about 60%

and 40% identical, respectively, among the three types of PLCs (Fig. 1.4). The X domain contains about 175 amino acids. The Y domain contains about 260 amino acids. All PLCs contain a ~300 amino acid amino-terminal sequence preceding the X domain. PLC-γ differs from the others in that the region between the X and Y domains of PLC-γ is of about 400 amino acids and contains SH_2 and SH_3 src-homology domains. The regions between the X and Y domains in PLC-β and PLC-δ are only about 50-100 amino acids long and contain no SH_2 and SH_3 domains. However, β and δ-type PLCs differ in that the carboxyl-terminal following the Y domain is about 450 amino acids long in PLC-β, and the same region is only about 10 residues in PLC-δ.

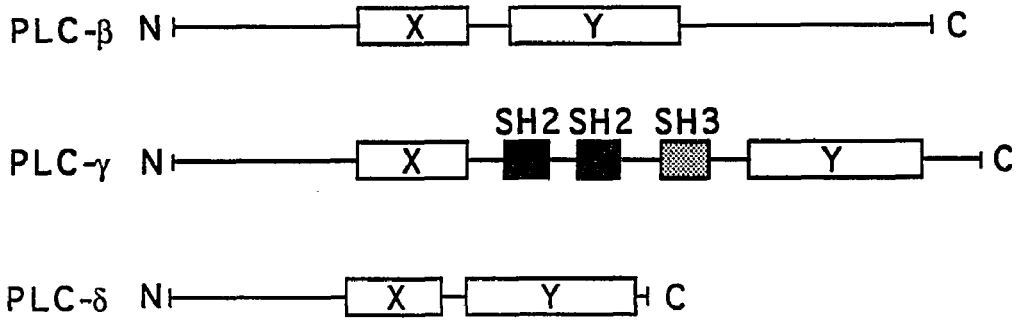

Figure 1.4. Linear display of three types of PLCs. Open boxes X and Y represent the two highly homologous regions found in three types of PLCs. SH_2 and SH_3 are the conserved *src* homology 2 and 3 domains first identified in the regulatory region of *src* family tyrosine kinases.

4. Structure-function relationship of PLCs:

The conserved X and Y domains are believed to include the catalytic domains. Deletions in either the X or Y domain, or both of them, result in complete loss of PLC activity (Emori et al., 1989; Wu et al., 1993a).

PLCs have been purified in both membrane-bound and cytosolic forms, although none of these PLC isoforms contains a membrane-spanning region according to the predicted amino acid sequence. Association with the membrane could be through lipid modification or interaction with other membrane-bound proteins, although the exact nature is not yet known. Most of the membrane-bound PLCs can be removed by high concentration of salt, indicating that ionic interactions attach the PLC to the membranes (Katan et al., 1988; Jhon et al., 1993). A series of specific deletions and truncations of PLC-β1 have been constructed. Deletion of the C-terminus does not change the intrinsic enzyme activity. However, mutants with a.a. 903-1030 deletion were only expressed in the cytosol. Therefore, the region between residues 903 to 1030 of PLC-β1 is required for particulate fraction association (Wu et al., 1993a).

As will be discussed below, G-proteins can activate PLC-β. By using truncation analysis, the region between residues 1030 to 1142 is found to be required for interaction with G-protein α subunits. Two peptides against this region (a.a. 1053-1084 and a.a. 1101-1129) can inhibit G_q-α dependent activation of PLC-

β1, presumably by competitive binding to the activated Gα protein (Wu et al., 1993a). PLC-β1 purified from bovine brain with C-terminal truncation at residue 881 by calpain has lost its ability to be activated by G_q α subunit. This truncated version of PLC-β1 still retained its catalytic activity (Park et al., 1993b). These results indicate that the C-terminal region of PLC-β1 is important for G_qα-dependent activation.

PLC-β2, but not PLC-β1, is activated by βγ subunits. A number of PLC-β2 deletion mutants and PLC-β1/β2 chimeras have been prepared in order to determine which portion of PLC-β2 is required for βγ activation. Using cotransfection assay, the authors showed that the region extending from the N terminus to the end of the Y box of PLC-β2 is required for βγ activation. The N-terminal 250 amino acids of PLC-β2 are not sufficient to confer on the chimera the ability to be activated by βγ subunits (Wu et al., 1993b; Lee et al., 1993).

PLC-γ1 is activated by polypeptide growth factor receptors which have tyrosine kinase activities. The tyrosine kinase receptor-dependent activation is mediated by the SH_2 domain of PLC-γ1, which consists of ~100 amino acids. Upon activation, the growth factor receptor first autophosphorylates itself. The SH_2 domain in PLC-γ1 binds to the specific autophosphorylated tyrosine site of the receptor with high affinity. Association of PLC-γ1 with growth factor receptors leads to phosphorylation and activation of PLC-γ1. PLC-γ can also be activated by non-receptor protein tyrosine kinases through the same mechanism (Rhee, 1991).

4. G protein dependent activation of PLC-β:

Several lines of evidence indicate that G proteins are involved in the activation of PLC by hormones and other ligands (reviewed by Fain et al., 1988). They include: (i). Introduction of GTP or its nonhydrolyzable analogs GTPγS or Gpp(NH)p into permeablized cells potentiates agonist-induced phosphoinositide breakdown. GTPγS or Gpp(NH)p alone can also stimulate the hydrolysis of phosphoinositides. (ii). Guanine nucleotides lower the affinity of the agonists but not antagonists to the PI-coupled receptors. (iii). AlF_4^-, an activator of G proteins, stimulates the hydrolysis of PIP_2. (iv). Pertussis toxin ADP-ribosylates α-subunits of most members of G_i/G_o family, causing uncoupling of G proteins from receptors. Some agonist-induced phosphoinositide pathways are PTX-sensitive, while others are not. This indicates that at least two distinct types of G proteins are involved. (v). βγ subunits of G proteins attenuate agonist-triggered PLC activity in some systems but not all.

A major step forward in developing an understanding of G protein regulation of PLCs was the demonstration that the α subunit of G_q stimulates PLC-β (Taylor et al., 1991; Smrcka et al., 1991). The G_q family contains four distinct members: G_q, G_{11}, G_{14}, and G_{16}, all of which are not susceptible to PTX modification. α_q and α_{11} subunits purified from bovine liver and brain membranes activate partially purified PLC from bovine brain (Smrcka et al., 1991; Blank et al., 1991; Shaw and

Exton, 1992). This stimulation is specific to PLC-β1 (Taylor et al., 1991). Antibodies to the carboxyl terminus of α_q block receptor-mediated stimulation of PLC (Shenker et al., 1991; Gutowski et al., 1991). When cDNAs corresponding to various α subunits of the G_q family are introduced into COS-7 cells, IP_3 formation is markedly increased after stimulation with AlF_4^-. When PLC-β1 is cotransfected, higher level of IP_3 formation is obtained (Wu et al., 1992; Lee et al., 1992). Purified α_q and α_{11} subunits from turkey erythrocytes stimulate PLC-β purified from the same system. The stimulating activity of α_q and α_{11} is inhibited by addition of βγ subunits (Waldo et al., 1991). Thus G_q family members represent the PTX-insensitive G proteins that mediate the receptor-stimulated PIP_2 hydrolysis.

In contrast to PLC-β1, PLC-β2 appears to have different properties. It is only partially activated by higher concentrations of α_q and α_{11}, and the extent of stimulation is much smaller than that for PLC-β1 (Smrcka and Sternweis, 1993). When it is cotransfected with different G_q α subunit cDNAs, it is only activated by α_{16} to a lesser extent (Lee et al., 1992). However, PLC-β2 can be specifically activated by βγ subunits of G proteins. This has been identified in both *in vitro* reconstitution assays and *in vivo* cotransfection assays (Camps et al., 1992; Katz et al., 1992). In contrast, PLC-β1 is either not affected by βγ subunits or stimulated to a much lesser extent. There is no stimulation of purified PLC-γ1 and PLC-δ1 by βγ subunits.

The third member of the mammalian β-type PLC family, PLC-β3, was isolated from a bovine brain cDNA library. Western blot analysis indicates that PLC-β3 is ubiquitously expressed (John et al., 1993). It is stimulated by not only the various α subunits of the G_q family, but also by βγ subunits. The order for the extent of activation by the α subunits of the Gq family is PLC-β1≥PLC-β3>>PLC-β2. The order for the βγ-dependent activation is PLC-β3>PLC-β2>>PLC—β1 (Carozzi et al., 1993; Park et al., 1993a; Smrcka and Sternweis, 1993). In the presence of 2 mM or higher concentration of $MgCl_2$, activation of PLC-β3 by $\alpha_{q/11}$ and βγ are additive or even synergistic (Smrcka and Sternweis, 1993).

PLC-β4 has been purified and cloned recently (Ferreira et al., 1993; Lee et al., 1993). PLC-β4 is highly homologous to *NorpA* of *Drosophila*, which is expressed predominantly in photoreceptors and involved in phototransduction. Although PLC-β4 is expressed in all nuclear layers of the retina with minor expression in the cerebellum, it is found to be expressed, among photoreceptors, in cones and not in rods (Ferreira and Pak, 1994). PLC-β4 is activated by all the α subunits of the Gq family but not the βγ subunits (Jiang et al., 1994; Lee et al., 1994). It differs from the other three mammalian PLC-βs in that it is selectively inhibited by ribonucleotides (Lee et al., 1994) It is currently unknown whether PLC-β4 plays important roles in phototransduction in the mammalian system.

C. *Xenopus* oocyte as a model system for the study of signal transduction pathway

1. General properties and electrophysiology of *Xenopus* oocyte:

The *Xenopus* oocytes used in these studies are surgically removed from female frog ovaries. Oocytes are surrounded by, sequentially from inner layer to outer layer, vitelline membrane, follicle cells, theca, and epithelial cells. There are numerous gap junctions which make connections between oocytes and the follicle cells. The gap junctions allow cell-to-cell communication by exchange of ions and small molecules and by direct electrical coupling (Browne et al., 1979). The oocytes can be detached from the surrounding follicular cells by collagenase treatment. This treatment is necessary for whole-cell voltage clamping because some responses measured in oocytes are actually occurring in the follicle cells.

Oocytes isolated from the ovary are in various stages of development, termed stage one to six (Dumont, 1972). Only the fully grown, stage V and VI oocytes are used in these experiments. They are largest in size and have an average diameter of 1.0-1.3 mm. These oocytes are growth arrested in prophase of meiosis I. After progesterone stimulation, they progress to metaphase of meiosis II, when they are again arrested until fertilization (Smith, 1989).

Xenopus oocytes are commonly used for electrophysiology studies. Two-electrode voltage clamp recordings can be easily accomplished due to its large size. Substances can also be directly injected into the cell to monitor the intracellular environment. Usually, healthy defolliculated oocytes hold a resting membrane potential of -45 to -65 mV. The approximate equilibrium potential for the major ions are: for K^+, -100 mV; for Na^+, +80 mV; and for Cl^-, -25 mV (Dascal, 1987).

2. Receptor activated IP_3 mediated-Cl^- conductance in Xenopus oocytes: General receptor--G protein--PLC signaling pathway:

In 1977, Kusano and coworkers found that bath application of acetylcholine (ACh) to voltage-clamped oocytes resulted in depolarization of the membrane (Kusano et al., 1977). This response is mediated through the acetylcholine receptor located in the oocyte membrane. Reversal potential determinations and ion substitution experiments indicated that the depolarization current is carried by Cl^- ions (Dascal and Landau, 1982; Dascal et al., 1984).

Later studies suggested that the muscarinic response was mediated by activation of PLC and generation of the second messenger IP_3. Direct injection of IP_3 into oocytes evokes a current similar to the native ACh response (Oron et al., 1985; Nadler et al., 1986). IP_3 causes the Cl^- current by mobilizing

intracellular stores of Ca^{++}. Injection of Ca^{++} into oocytes evokes a similar response, while intracellular injection of Ca^{++} chelator EGTA blocks the response to agonist or IP_3 (Miledi and Parker, 1984; Dascal et al., 1985; Gillo et al., 1987).

Several neurotransmitter receptors have been expressed in *Xenopus* oocytes after injection of appropriate mRNA, including serotonin, TRH, glutamate, M1, M2 acetylcholine receptors and many others (reviewed in Lester, 1988). These receptors activate a Cl^- conductance similar to that seen for the native muscarinic acetylcholine receptor. In all instances, these expressed foreign receptors appear to activate the Cl^- channels via the same phosphatidylinositol phosphate transduction mechanism as the native receptor (McIntosh and Catt, 1987; Takahashi et al., 1987; Oron et al., 1987; Nomura et al., 1987).

Several lines of evidences indicate that G proteins are involved in the receptor activation of Cl^- channels. GTPγS potentiates the Cl^- current response evoked by either native or expressed receptors. Direct injection of GTPγs alone can elicit Cl^- current in the absence of agonist. Injection of GDP, as well as GDPβS, attenuates the receptor evoked response (Dascal et al., 1986; Noruma et al., 1987; Kaneko et al., 1987). Further support comes from experiments utilizing PTX. PTX inhibits the Cl^- current activated by some expressed receptors but not all (Sugiyama et al., 1985; Dascal et al., 1986; Hirono et al., 1987). These data indicate that a PTX-sensitive G protein is coupling some receptors to PLC in *Xenopus* oocytes.

In summary, *Xenopus* oocytes exhibit a receptor evoked Cl^- current, which is mediated through the activation of PLC and subsequent liberation of IP_3 into cytosol. IP_3 increases intracellular Ca^{++} concentration, which in turn opens Ca^{++}-sensitive Cl^- channels in the plasma membrane. The Cl^- current in the oocytes can be measured electrophysiologically by using the voltage clamp technique. The amplitude of Cl^- current is proportional to the intracellular level of IP_3 (Gillo et al., 1987). Hence, the Cl^- current can be used as a measurement for IP_3 production and PLC activity. The Cl^- current can be evoked by various receptors. Some receptors are coupled to this pathway through PTX-sensitive G protein(s), while others use PTX-insensitive G proteins. Therefore, the *Xenopus* oocyte can be used as a model system to study hormone-stimulated, G protein-mediated phospholipase C pathway.

3. G_o as the signal transducer in the PLC pathway in *Xenopus* oocytes:

G_o was originally purified from bovine brain as the major pertussis toxin substrate (Sternweis and Robishaw, 1984). It is highly abundant in brain tissue from many different species (Neer et al., 1984; Homburger et al., 1987). G_o immuno-reactivity has also been observed in several nonneuronal tissues (Huff et al., 1985; Anholt et al., 1987; Hsu et al., 1990). G_o is present in *Xenopus* oocytes because a cDNA encoding α_o

has been cloned from a *Xenopus* oocyte cDNA library (Olate et al., 1989).

The function of G_o in mammalian cells remains unknown. In *Xenopus* oocytes, G_o has been implicated as the signal transducer in the pertussis toxin-sensitive phospholipase C pathway. Injection of purified heterotrimeric G_o enhances the native muscarinic receptor-evoked Cl^- current (Moriarty et al., 1990; Padrell et al., 1991). Injection of *E. coli* expressed α_o together with purified $\beta\gamma$ subunits can also enhance muscarinic receptor-evoked Cl^- current (Padrell et al., 1991).

The function of G_o in activating PLC seems to be attributed to the α subunit. Direct injection of GTPγS-activated α_o subunits (purified from bovine brain) into oocytes evokes the Cl^- current, whereas injection of activated α_{i1}, α_{i2}, or α_{i3} has no effect (Moriarty et al., 1990). On the other hand, injection of $\beta\gamma$ subunits inhibits the native muscarinic receptor-evoked response in a dose dependent manner. Boiled $\beta\gamma$ subunits have no inhibitory effect (Moriarty et al., 1988).

The expressed hamster α_{1b}-adrenergic receptor stimulates PLC through PTX-sensitive pathway in *Xenopus* oocytes. Injection of holo G_o but not G_{i3} enhances the norepinepherine-evoked response. When antisense oligonucleotides to the *Xenopus* α_o are injected into oocytes, the Cl^- current evoked by the expressed α_{1b}-adrenergic receptor is significantly reduced (Blitzer et al., 1993).

Taken together, G_o, probably through α_o, can activate PLC in oocytes. Since α subunits of G_q family directly activate

mammalian PLC-βs, I assume that α_o may be able to directly activate PLC in oocytes through a similar mechanism. The pathway utilized by G_o to trigger PLC-dependent Cl^- conductance in response to agonist stimulation is illustrated in figure 1.5.

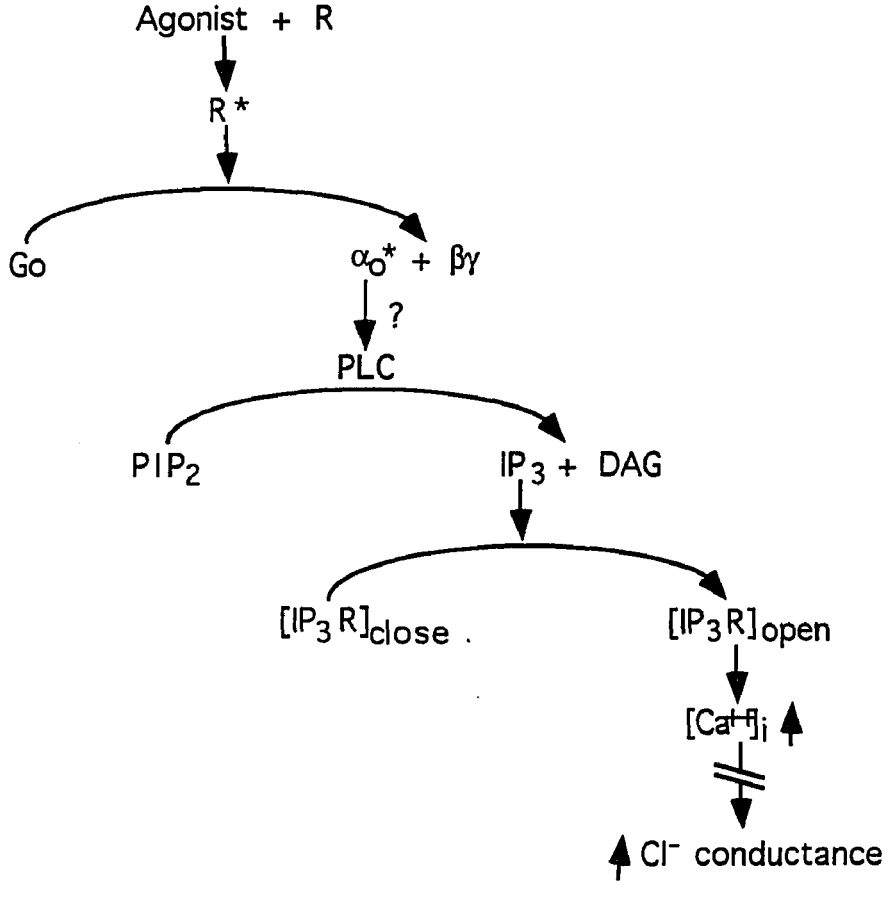

Figure 1.5. The pathway utilized by G_o to trigger PLC-dependent Cl^- conductance in response to agonist stimulation. Break in vertical arrows indicates that there is currently no direct evidence for direct linkage between the two steps.

Chapter 2

Statement of Problems and Specific Aims

The objective of this thesis project is to study how G proteins regulate PLC activity. It has been proposed that two distinct pathways operate in PLC regulation: one is PTX-sensitive, the other is PTX-insensitive. At the time this project was initiated, reports on G_q-α-dependent PLC activation were just coming out. This represents the PTX-insensitive G proteins that couple receptors to PLC. However, the PTX-sensitive PLC pathway had not been fully explored. None of the α subunits of the G_i/G_o family, which are PTX substrates, were able to stimulate PLC activity in the mammalian systems tested.

For a decade, *Xenopus* oocyte has been a useful system for the study of receptor stimulated PLC pathway. The *Xenopus* oocyte PLC system has some unique properties: (i). Many exogenous receptors expressed in *Xenopus* oocytes are coupled to PLC through a PTX-sensitive pathway, whereas few mammalian systems use a PTX-sensitive PLC pathway. (ii). Some receptors that use a PTX-insensitive PLC pathway in their original mammalian environment use a PTX-sensitive pathway in the *Xenopus* oocytes (Moriarty et al., 1989).

Therefore, a major component of the phospholipase C response in the oocytes is PTX-sensitive. Previous studies from our laboratory indicate that α_o may be the signal transducer in PLC activation in *Xenopus* oocytes. Taken together, it seems that the *Xenopus* oocytes contain a G_o-responsive phospholipase C.

In order to better understand the PTX-sensitive PLC pathway, I intended to use *Xenopus* oocytes to:

1. Clone the gene(s) encoding PLC(s) regulated by G protein-coupled receptors.

A *Drosophila* phospholipase C gene called *plc-21* was isolated (Shortridge et al., 1991). *plc-21* is most similar to the mammalian β-type PLC and is expressed in the central nervous system. The expression pattern of *plc-21* is identical to that of the *Drosophila* α_o gene, suggesting that *plc-21* could be regulated by α_o. Since I wanted to isolate a G_o-regulated PLC, I used the *plc-21* cDNA as the probe to screen the *Xenopus* oocyte cDNA library.

2. Determine whether the new PLC is involved in the receptor-mediated PTX-sensitive PLC pathway.

Antisense oligonucleotides have been used to inhibit the expression of a number of cellular proteins. If the *Xenopus* PLC is involved in the receptor-mediated G-protein-dependent PLC pathway, inhibition of expression of the *Xenopus* PLC gene will lead to decreased receptor-evoked Cl^- current. Antisense oligonucleotides against the *Xenopus* PLC was injected into oocytes. Receptor-evoked Cl^- current was measured to see whether there was any change caused by the antisense oligonucleotides.

Several receptors are known to stimulate PLC through PTX-sensitive G proteins in the oocytes. These receptors include native angiotensin II receptor, expressed α_{1b}-adrenergic receptor, and expressed M1 acetylcholine receptor. The effect of antisense oligonucleotides was studied in the signaling

pathway of these receptors. Therefore I could know whether the new *Xenopus* PLC was involved in the PTX-sensitive pathway.

3. Determine which G-protein subunit(s) regulates the activity of the *Xenopus* PLC.

Activation of G proteins produce two functional units: α subunits and $\beta\gamma$ subunits. Since the heterotrimer is required for G protein interaction with the receptor, the antisense oligonucleotide experiments will not tell us which G protein subunit(s) regulate the activity of the new *Xenopus* PLC. It will not tell us whether the regulation by G proteins is direct either. One of the most direct and convincing experiments is the *in vitro* reconstitution assay. The *Xenopus* PLC was expressed in large quantity in sf9 cells by using the baculovirus system. The recombinant PLC was then reconstituted with α_o, α_q, and $\beta\gamma$ subunits. PLC activity was measured to see which G protein subunit could regulate its activity.

Chapter 3

Materials and Methods

Materials:

All restriction enzymes, Klenow DNA polymerase, T4 polynucleotide kinase, T3 polymerase, calf intestinal alkaline phosphatase, T4 DNA ligase were from New England Biolabs. *Taq* DNA polymerase and RNAsin ribonuclease inhibitor were from Promega. *Pfu* DNA polymerase was from Stratagene. RNAase, DNAase, proteinase K, and collagenase were from Sigma.

The Random Priming System I was from New England Biolabs. 5' RACE system kit was from Gibco BRL. The mRNA purification kit was from Pharmacia. The GeneClean II Kit was from Bio101. The Sequenase 1.0 kit was from US Biochemical. XL-1 ultracompetent cells were from Stratagene.

All radiochemicals were from ICN or NEN. Bio-Spin 6 and Bio-Spin 30 chromatography columns were from BIO-RAD. Nitrocellulose membranes were from Schleicher & Schuell. Immobilon-PVDF membranes were from Millipore.

BCA protein assay reagent was from Pierce. High molecular weight protein markers (unstained or prestained) were from Gibco BRL. Horseradish peroxidase conjugated goat-anti-rabbit IgG was from Calbiochem. ECL detection reagents were from Amersham. Hyperfilm-ECL was from Amersham. Reflection film was NEN.

Unlabeled phosphoinositides mixture was from Sigma (#P 6023). Sf-900™ II medium was from Gibco BRL. Aprotinin, leupeptin, soybean trypsin inhibitor, benzamidine,

phenylmethylsulfonyl fluoride were from Sigma. All the other chemicals were either from Sigma or the highest grade available.

Oligonucleotides were synthesized by Brookdale Center for Molecular Biology (Mount Sinai School of Medicine) or by myself using an Applied Biosystems DNA synthesizer. All the reagents used were from Applied Biosystems.

Peptides were synthesized by Research Genetics (Huntsville, AL). Antisera against KLH-conjugated peptide was raised by Pocono Rabbit Farm & Laboratory (Canadensis, PA) according to their standard protocol.

The *Xenopus* oocyte cDNA library was the gift of Dr. Douglas Melton (Harvard University). The *plc-21* cDNA clone was the gift of Dr. Randall Shortridge (Purdue University). $G_{\alpha q}$, $G_{\alpha o}$, and LacZ-transfected Cos-7 cell membranes were the gifts of Dr. Anna Aragay (California Institute of Technology). PLC-β1, β2, β3, β4, and *X*β-transfected Cos-7 cell lysates were also gifts of Dr. Anna Aragay. βγ subunits were purified bovine brain either by Dr. Donna Carty (Department of Pharmacology, Mount Sinai School of Medicine) or the gift of Dr. John Hildebrandt (Medical University of South Carolina). Partially purified PLC-110 was the gift of John Exton (Vanderbilt University).

Probes:

A. Random primer labeling of DNA probes:

The labeling reactions were performed using the Random Priming System I according to the manufacturer's instructions. 50 ng DNA was resuspended in labeling buffer (random hexadeoxyribonucleotides included) and denatured by heating in boiling water for 5 minutes. Following the denaturation, the labeling reaction was performed in 50 µl volume by adding 2 µl of 0.5 mM dNTPs (dATP, dGTP, TTP), 10 µl of α-^{32}P dCTP (3,000 Ci/mmol, 10 µCi/µl), 2 µl of 10 mg/ml BSA, and 10 units of DNA polymerase I-large (Klenow) fragment. After 1.5 hours incubation at 37°C, the probe was purified through a Bio-Spin 30 Chromatography Column. The probe was denatured in boiling water for 5 minutes and then transferred directly to the hybridization buffer.

B. 5' end labeling of oligonucleotide probes:

5' end labeling reaction was performed in 10 µl volume. The reaction mixture contained 10 pmol oligonucleotide, 1 x polynucleotide kinase buffer (70 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT), 4 µl of γ-^{32}P ATP (7000 Ci/mmol, 170 µCi/µl), and 10 units of T4 polynucleotide kinase. After 40 minute incubation at 37°C, the reaction mixture was heated at 65°C for 5 minutes to inactivate the enzyme. The probe was purified through a Bio-Spin 6 Chromatography Column.

C. cRNA probe synthesis:

Plasmid 29-1a containing nucleotide 580-1230 of PLC-*X*β was linearized by *BamH* I digestion so that the bacteriophage

T3 promoter on the vector could be used to direct the synthesis of the antisense cRNA probe. The linearized DNA was purified from an agarose gel. The riboprobe synthesis reaction was performed in 25 µl volume. The reaction mixture contained 0.5 µg template DNA, 1x transcription buffer (40 mM Tris·HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl), 10 mM DTT, 0.4 mM ATP, 0.4 mM GTP, 0.4 mM UTP, 2.4 µM CTP, 5 µl α-^{32}P CTP (800 Ci/mmol, 10 µCi/µl), 25 units RNAsin ribonuclease inhibitor, and 10 units of T3 polymerase. After incubation at 37°C for 1 hour, 0.5 unit DNAase was added to the reaction to remove the template DNA. The cRNA probe was purified by phenol/chloroform extraction and ethanol precipitation. A small aliquot of the probe was separated on a 4% acrylamide gel to check the quality of the probe.

Polymerase chain reaction (PCR):

All reactions were performed in Perkin Elmer-Cetus DNA Thermal Cycler. PCR amplification was carried out in 100 µl volume containing 1x buffer, 1-20 ng of template DNA, 0.5 µM of each primer, 0.2 mM of each dNTP, 2.5 units of heat-stable DNA polymerase. When *Taq* DNA polymerase was used, the 1x buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1% gelatin, 0.1% Triton X-100. When *Pfu* DNA polymerase was used, the 1 x buffer was 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 µg/ml nuclease free BSA.

Reactions were subjected to 30-35 cycles of the following temperature profile: denaturation (95°C, 1 minute), primer annealing (50-60°C, 1 minute), and primer extension (72°C, 3 minutes). Cycling was concluded with a final extension at 72°C for 10 minutes. Reactions were stopped by chilling to 4°C.

Screening of *Xenopus* oocyte cDNA library:

The *Xenopus* oocyte cDNA library in λgt10 was kindly provided by Dr. Douglas Melton. λ phage were grown on Y1090 strain *Escherichia coli* on LB agar plates at 50,000 phage/150 mm Plate. Phage plate replicas were prepared on duplicate nitrocellulose membranes with 2 minutes for the first set and 5 minutes for the second set. Nitrocellulose filters were denatured in 0.5 N NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris·HCl (pH 7.5), 1.5 M NaCl, and rinsed twice in 2 x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH7.0), all for 5 minutes each. Filters were baked in the vacuum oven for 2 hours at 80°C. Filters were prehybridized for 2-4 hours at desired temperature in 6x NET (1x NET is 0.15 M NaCl, 1 mM EDTA, 15 mM Tris·HCl, pH 8.3), 5x Denhardt's solution (1x Denhardt's is 0.2 mg/ml Ficoll, 0.2 mg/ml BSA, 0.2 mg/ml polyvinylpyrrolidone), 0.1% SDS, and 100 μg/ml sonicated, denatured salmon sperm DNA. Hybridization was performed by adding random primer radiolabeled probe into prehybridization solution and incubating the mixture at the same temperature overnight (usually 16-18 hours). For low

stringency hybridization, the temperature was 55°C. For high stringency hybridization, the temperature was 65°C. Hybridized filters were washed in 6x SSC three times for 5 minutes at room temperature, and in 2x SSC, 0.1% SDS three times for 5 minutes at the hybridization temperature. The positive clones were rescreened for another two rounds until pure clones were obtained.

Subcloning and characterization of cDNA clones:

Each of the purified positive phage clones was grown in 500 ml NZYM medium by the "infection at high multiplicity" method (Sambrook et al., 1989). Phage DNA was prepared by the standard method described in *Molecular Cloning* (Sambrook et al., 1989). Phage DNA was digested with *EcoR* I. The cDNA inserts were purified from agarose gel using the GeneClean II kit and ligated into pBlueScript II (pBS II) vector. The ligation mixture contained 50 ng pBS II DNA, 5-10 molar excess of restriction fragment to be cloned, 1x ligation buffer (50 mM Tris·HCl, pH7.8, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/ml BSA), and 40 units of T4 DNA ligase in 10 μl volume. The ligation mixture was incubated at 14-16°C overnight. Two μl ligation mixture was used to transform ultracompetent XL-1 blue cells by following manufacturer's instructions. Transformed bacteria were plated on LB agar plates (90 mm petri dish) containing 50 μg/ml ampicillin, 12.5 μg/ml tetracycline. The plates had been pretreated with 75 μl of 20

mg/ml Blue-gal and 50 µl of 20 mg/ml IPTG for blue/white colony selection. White colonies were selected for liquid culture in LB medium supplemented with ampicillin and tetracycline. DNA minipreps were prepared by the boiling method (Sambrook et al., 1989). DNA maxipreps were prepared by the alkaline lysis method (Sambrook et al, 1989). The correct clones were selected based on the restriction analysis of the plasmid DNA.

5'-Rapid amplification of cDNA ends (RACE):

5' RACE was performed using the 5' RACE system kit by following the manufacturer's instructions. Briefly, first strand cDNA was synthesized from 1 µg total *Xenopus* oocyte RNA by using a gene-specific primer, HW015 or HW019. This reaction was catalyzed by SUPERSCRIPT RNase H^- Reverse Transcriptase (RT). RNA template was then degraded by RNase H. cDNA was purified with GLASSMAX Spin Cartridge. Terminal deoxynucleotidyl transferase (TdT) was used to attach homopolymeric dC tails to the 3' ends of the purified cDNA. 5' of the cDNA sequence was amplified from dC-tailed cDNA by PCR using oligo dG anchor primer, and a nested gene-specific primer HW020. In order to increase the yield of the desired DNA fragment, another round of PCR was performed by using universal amplification primer (UAP) and a third nested gene-specific primer HW044. The 5' RACE product was analyzed by southern blot with HW043, an oligonucleotide corresponding to

the known 5'-most sequence, as the probe. Positive DNA fragment was purified from agarose gel and subcloned into pBS II for sequencing.

Two primers were used for first strand cDNA synthesis based on the following reasoning: 1). HW019 was used because it is close to the 5' end of the known sequence. Therefore, first strand cDNA corresponding to unknown sequence would be synthesized more efficiently. 2). However, the first strand cDNA must be purified for subsequent steps. Short cDNA (≤200 bases) can not be purified efficiently with GLASSMAX Spin Cartridge. Therefore, HW015, an oligonucleotide relatively far away from the known 5' end, was used in case the 5' unknown sequence was very short.

The sequences of the primers used in 5' RACE are listed below:

HW015 5' CATCGACATCTGGTCTCTGTCCAGGAACTGCCT 3'

HW019 5' CGGGTGTCTCTTATTACGCTGA 3'

EcoR I

HW020 5' ATCGAATTCCATATTTGGACAGGTCCAGTA 3'

HW037 5' CATCCCACTTGATGAACT 3'

HW043 5' AGAGTCTCCGGCACTTTC 3'

Pst I *Sac* I

HW044 5' CACCTGCAGAGCTCCCAGGGATTTGCTGCTGGACTCC 3'

Mlu I *Sal* I *Spe* I

Anchor Primer 5' CUACUACUACUAGGCCACGCGTCGACTAGTA CGGGIIGGGIIGGGIIG 3'

Mlu I *Sal* I *Spe* I

UAP 5' AGGCCACGCGTCGACTAGTAC 3'

DNA sequencing:

DNA sequencing was carried out by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase 1.0) and α-^{35}S dATP (1000-1500 Ci/mmol, 12.5 mCi/ml). Double-stranded plasmid DNA (5 µg per sequencing reaction) was denatured by treatment with 0.2 N NaOH, 0.2 mM EDTA for 20 minutes at 30°C. Sodium acetate (pH 4.95) was added to 300 mM, and DNA was precipitated with 2.5 volumes of ethanol. Denatured plasmid DNA was resuspended in Sequenase reaction buffer and annealed to 1 pmol primer by heating to 65°C for 2 minutes and cooling slowly to room temperature. Annealed template-primer was extended for 5 minutes at room temperature and terminated for 5 minutes at 37°C according to the manufacturer's protocol. Sequencing reactions were denatured by heating at 75°C for 2 minutes and loaded on 8% polyacrylamide sequencing gels. Gels were dried under vacuum prior to autoradiography. The cDNA fragments had been sequenced in both directions. Sequence data were assembled and analyzed by using the PC/Gene DNA analysis software (Intelligenetics).

Total RNA and poly(A)± RNA preparation :

Total RNA was isolated from stage V-VI oocytes by using the one-step guanidinium thiocyanate/phenol extraction

method described in *Current Protocols in Molecular Biology.* One gram of oocytes was homogenized on ice in 10 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M 2-mercaptoethanol, 0.5% N-lauroylsarcosine). 2 M sodium acetate (pH 4) was added to the homogenate to a final concentration of 200 mM. The resulting homogenate was extracted with equal volume of water-saturated phenol and 0.2 volume of 49:1 chloroform/isoamyl alcohol. After centrifugation, the aqueous phase was transferred to a fresh tube. RNA was precipitated twice with equal volume of isopropanol at -20°C. The RNA pellet was washed with 75% ethanol, resuspended in TE buffer (pH7.4), and stored at -70°C.

Poly(A)$^+$ RNA was purified from total RNA by using oligo(dT)-cellulose spun columns according to the manufacturer's instructions (mRNA purification kit). RNA samples were passed through spun columns twice to ensure 90% of the eluted RNA was poly(A)$^+$ RNA.

Northern blot:

RNA was separated eletrophoretically on 0.8% formaldehyde-agarose gel. Seven and half μg poly(A)$^+$ RNA was used for each sample. At the end of the run, the lane containing the RNA size marker was cut from the gel, stained in 1.5 μg/ml ethedium bromide for 30 minutes, destained in distilled water overnight, and photographed. The other RNA samples were

transferred to nitrocellulose membrane overnight in 20x SSC. Following the transfer, the filter was baked at 80°C in a vacuum oven for 2 hours. The filter was prehybridized in 5x SSPE (1x SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH7.4, 1 mM EDTA), 5x Denhardt's solution, 0.5% SDS, 100 μg/ml sonicated salmon sperm DNA, and 50% formamide for 4 hours at 42°C. Random primer labeled DNA probe was added to the prehybridization buffer. Hybridization was performed at 42°C overnight. The filter was washed three times in 2x SSC and 0.1% SDS at room temperature for 15 minutes each, three times in 2x SSC and 0.1% SDS at 65°C for 5 minutes each, and once in 0.5x SSC and 0.1% SDS at 65°C for 5 minutes.

Preparation and injection of oocytes:

Oocytes were removed surgically from mature female *Xenopus laevis* and enzymatically defolliculated with 2 mg/ml collagenase in Ca^{++} free ND96 solution for 1 hour. ND96 frog Ringer's solution is 96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 5.0 mM NaHepes, pH7.5. Stage V and VI oocytes were selected manually under dissecting microscope and additional follicular cells were removed with jeweler's forceps. Oocytes were incubated at 18°C in ND96 solution supplemented with 2.5 mM pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin. Oocytes were maintained in this solution for one day prior to microinjection.

Oocytes were injected using a Drummond microinjector. 100 pg of the α_{1b}-adrenergic receptor mRNA or 100 pg M1-muscarinic acetylcholine receptor mRNA in 50 nl were injected into each oocyte. Two days later, the oocytes were injected with 50 nl of 100 μM sense or antisense oligonucleotides to PLC-*X*β in order to achieve a final concentration of 2 μM inside the cell (assuming even distribution). Some were injected with 50 nl water as control. Norepinepherine (NE) or acetylcholine-evoked Cl^- current was recorded 20 hours after the oligonucleotide injection. Since some oocytes express endogenous angiotensin II receptor, these oocytes were only injected with oligonucleotides 20 hours before measuring the angiotensin II-evoked Cl^- response. Cl^- currents were evoked by bath application of receptor agonists. When present, the concentration of angiotensin II was 10 μg/ml, and that of norepinephrine or acetylcholine was 10 μM.

Measurement of Cl^- current:

Cl- current was measured using the standard two electrode voltage clamp technique by Dr. Robert D. Blitzer (Department of Psychiatry, Mount Sinai School of Medicine). A single oocyte was placed in a small chamber and constantly perfused with ND96 solution at room temperature. Two 3 M KCl-filled glass microelectrodes were inserted into the oocyte. One electrode was used for voltage recording. The other electrode was used for current supply. The resting potential of

the oocyte was first recorded. Only those oocytes with a resting potential more negative than -40 mV were considered healthy and used for further experiments. The membrane potential was then held at -70 mV for current recording. Agonists were applied by superfusion. Maximum amplitude of the inward current was measured as estimate of the response.

RNAase protection assay:

Total RNA was extracted from 100 oocytes 4 hours after injection .with either sense or antisense oligonucleotide. A fragment encoding the region 580-1230 was used as the probe. ^{32}P-labeled antisense RNA probe was synthesized by *in vitro* transcription as described in the previous section "cRNA probe synthesis". Hybridization of 5 µg total RNA with 5 x 10^5 cpm ^{32}P-cRNA probe was performed in a solution containing 80% formamide, 40 mM PIPES, pH 6.7, 400 mM NaCl, and 1 mM EDTA. The reaction mixture was first heated at 90°C for 10 minutes to denature the RNA and incubated at 52°C overnight. Samples were then treated with RNase A (40 µg/ml) and RNase T1 (2 µg/ml) at 30°C for 1 hour in the digestion solution containing 300 mM NaCl, 10 mM Tris·HCl, pH7.4, and 5 mM EDTA. The RNase was removed by protease K digestion (300 µg/ml) at 37°C for 30 minutes. The protected RNA-RNA duplexes were precipitated with ethanol and resolved in 5% polyacrylamide gel. The gel was dried and exposed to X-ray film at -80°C.

***Xenopus* oocyte extract preparation:**

300 stage V-VI *Xenopus* oocytes were homogenized in 2.5 ml ice-cold extraction buffer containing 50 mM NaCl, 10% sucrose, 25 mM NaHepes, pH 7.4, 1 % Lubrol-PX, 1 mM DTT, 5 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 4 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 1 mM PMSF. The homogenate was centrifuged at 100,000xg for 1 hour at 4°C. The supernatant was aliquoted and stored at -70°C. This oocyte extract was used for testing the titer of antisera raised against PLC-*X*β.

***Xenopus* tissue extract preparation:**

One gram of frog tissue was homogenized in 5 ml ice-cold homogenization buffer containing 25 mM NaHepes, pH 7.0, 300 mM NaCl, 10% sucrose 5 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). The tissue was homogenized with Polytron on ice. The homogenate was centrifuged at 100,000xg for 1 hour at 4°C. The supernatant was aliquoted and stored at -70°C.

Protein concentration determination:

Protein concentration was determined by using BCA protein assay reagent. 100 µl of protein sample was mixed with 2 ml Working reagent (50 parts of reagent A plus 1 part of reagent B). The mixture was incubated at 37°C for 30 minutes. After incubation, absorbance at 562 mm was measured *vs.* water reference. Absorbance of the blank was subtracted from the actual absorbance of each sample to yield the net absorbance. 20-120 µg of BSA were used to generate the standard absorbance *vs.* protein concentration curve. Protein concentration was determined from the standard curve.

Coupling of synthetic peptides to carrier protein and antibody production:

Three peptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH) through the C-terminus cysteine of the peptide by using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as the coupling reagent. 25 mg of KLH was resuspended in 25 ml of 50 mM sodium phosphate buffer, pH 7.4, and reacted with 10 mg of MBS (dissolved in DMSO) for 30 minutes at room temperature with constant stirring. The KLH-MBS solution was passed through a Sephadex G-25 column and washed with 10 mM sodium phosphate, pH 6.0 to remove unreacted MBS. The MBS-activated KLH recovered from the peak fractions of the column eluate was pooled together and neutralized to pH 7.2 with the addition of 10 N NaCl. 5 mg of each peptide was resuspended in 1 ml

sodium phosphate buffer, pH 7.4 and added to 5 mg of activated KLH. The reaction mixture was stirred for 3 hours at room temperature and dialyzed against 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, at 4°C overnight.

The KLH-conjugated peptides were sent to Pocono Rabbit Farm and Laboratory. Antibodies to each peptide were produced by the company according to its standard protocol. Preimmune and immune serum were supplied by the company and analyzed as described below in "Immunoblot"

Immunoblot:

A 30-50 μg of protein sample was separated on 8% of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were aligned with prestained high molecular weight marker. Protein was transferred to Immobilon-PVDF membrane overnight at 4°C and 30 V in a buffer containing 25 mM Tris base, 192 mM glycine and 10% methanol.

The membrane was blocked for 1 hour at room temperature or overnight at 4°C in blocking buffer containing 1 M glycine, 5% dry milk, 5% fetal calf serum, 1% ovalbumin, and 1x PBS. After being washed three times in wash buffer (1x PBS, 1% fetal calf serum, 0.1% Tween 20, 0.1% dry milk, 0.1% ovalbumin) for 5 minutes each, the membrane was incubated in primary antibody diluted in wash buffer for 3 hours at room temperature. The membrane was then washed three times in wash buffer for 5 minutes each and once in T-PBS buffer (1 x

PBS, 0.1% Tween 20) for 15 minutes. The secondary antibody (horseradish peroxidase conjugated goat-anti-rabbit IgG) was diluted 1:3000 in wash buffer and incubated with the membrane for 1 hour at room temperature. Unbound antibodies were removed by three washes in T-PBS for 15 minutes each. The membrane was incubated in ECL detection reagents for 1 minute, and then exposed to Hyperfilm-ECL or reflection film for the desired period of time.

Construction of vector for sf9 cell transfection:

A. Assemble full length PLC-*X*β cDNA into pRC/CMV:

6 overlapping clones together span the open region frame of PLC-*X*β. They are RACE-5' spanning nucleotides 1-202, 29-1b spanning nucleotides 180-592 (another clone 33-2b spans nucleotides 279-592), 33-2a spanning nucleotides 587-1851, 27-1c spanning nucleotides 1461-2151, and 27-1a spanning nucleotides 2145-3788. All of these clones except RACE-5' have a piece of PLC-*X*β cDNA subcloned into the *EcoR* I site of pBS II. After examination of the restriction map of the PLC-*X*β cDNA, *Hind* III site at position 385, *EcoR* I site at position 587, *Cla* I site at position 1699, *EcoR* I site at position 2147, *EcoR* V site at position 2497, *EcoR* I site at position 3783, and *Xba* I site from the vector sequence after the 3783 *EcoR* I site were used to assemble the full length PLC-*X*β into the mammalian expression vector pRC/CMV. Three steps were required to accomplish this.

In the first step, the 5' end sequence of PLC-*X*β was assembled together into clone Xβ-5', which spans the region from nucleotides 60 to 1699. In the second step, the 3' end sequence of PLC-*X*β was assembled together into clone Xβ-3', which spans the region from nucleotides 1699-3788 In the third and final step, the full length sequence was subcloned into pRC/CMV.

Step 1: Since the clone RACE-5' does not contain the appropriate restriction site, the very 5' end sequence of PLC-*X*β was reamplified from the cDNA by using a different pair of primers. First strand cDNA was prepared from 10 μg total *Xenopus* oocyte RNA by using HW050 (position 1069-1088) as the primer. This first strand cDNA was used as the template to PCR amplify region nucleotide 60-789 with primers HW060 and HW061. HW060 contains *Not* I recognition sequence for subsequent subcloning. The 730 bp PCR product was named HW060/HW061. The 325 bp *Not* I-*Hind* III fragment from HW060/HW061, the 200 bp *Hind* III-*EcoR* I fragment from 33-2b, and the 1112 bp *EcoR* I-*Cla* I fragment from 33-2a were cloned into the *Not* I and *Cla* I sites of pBS II. The recombinant DNA Xβ-5' was sequenced for the whole region amplified by PCR and the regions spanning the four ligation sites. No mutation was found.

Step 2: The 447 bp *Cla* I-*EcoR* I fragment from 27-1c and the 351 bp *EcoR* I-*EcoR* V fragment from 27-1a were cloned into the *Cla* I and *EcoR* V sites of 27-1a, which already contained nucleotide sequence 2497-3788. The recombinant

DNA Xβ-3' was sequenced for the regions spanning the three ligation sites. No mutation was found.

Step 3: The 1640 bp *Not* I-*Cla* I fragment from Xβ-5' and the 2100 bp *Cla* I-*Xba* I fragment from Xβ-3' were cloned into the *Not* I and *Xba* I sites of pRC/CMV. The recombinant DNA was sequenced for the regions spanning the three ligation sites. No mutation was found.

B. Subclone PLC-*X*β into pVL1392:

PLC-*X*β was excised from pRC/CMV-*X*β and inserted into the *Not* I and *Apa* I sites of pVL1392. Recombinants were verified by restriction analysis.

Sf9 cell culture and cytosol preparation:

Sf9 cells were obtained from Dr. Ronald Magnusson (Department of Pharmacology, Mount Sinai School of Medicine). Sf9 cells were grown in sf-900 II medium at 25°C.

Sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 3-5. Two-four days post infection, cells are harvested for cytosol preparation. Cells were washed three times with 1x PBS (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 27 mM KCl, ph 7.4), and resuspended in ice-cold homogenization buffer (10 mM Hepes, pH7.5, 5 mM EDTA, 5 mM EGTA, 2.5 mM KCl, 1 mM DTT, 10 μg/ml aprotinin,10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, 1 mM PMSF). The homogenate was centrifuged at

100,000 x g for 1 hour at 4°C. The supernatant was aliquoted and stored at -70°C.

Phospholipase C assay:

The PLC assay was carried out in a total volume of 100 µl. The assay mixture consisted of the following: 25 µl of 4 x buffer (40 mM Hepes, 400 mM KCl, 40 mM NaCl, 8 mM EGTA, 4 mM $MgCl_2$, 4 mM DTT, pH 7.2), 25 µl phospholipid substrate, 10 µl of a source of PLC (either cytosol or purified protein), 25 µl $CaCl_2$ solution to yield desired free Ca^{++} concentration, and 10 µl of G protein subunits if applicable. The phospholipid substrate contains 500 pmol of unlabeled phosphoinositides mixture and 1 pmol of ^3H-PIP_2 (5.5 Ci/mmol, 0.01 mCi/ml).

The assay mixture was mixed on ice. The reactions were initiated with the addition of $CaCl_2$ solution and incubated at 30°C for 20 minutes. The reactions were stopped by the addition of 1 ml chloroform/methanol/HCl (100:100:1, by volume) followed by 250 µl 10 mM EDTA. After centrifugation, 400 µl of the aqueous phase was removed and the radioactivity was measured in a liquid scintillation counter.

Chapter 4

Cloning of a New PLC from *Xenopus* Oocyte

1. Isolation of PLC-*X*β cDNA clones from a *Xenopus* oocyte cDNA library by DNA-DNA hybridization and sequence analysis of cDNA clones:

In order to isolate a new type of phospholipase C from *Xenopus* oocyte, the *Xenopus* oocyte cDNA library in λgt10 was screened at low stringency with a 1.2Kb PCR product encompassing the X and Y domains of *Drosophila plc-21*. Positive clones were screened for another two rounds in order to obtain pure clones. Three clones with the strongest signal (from 1.2 million phage screened) were further analyzed. The cDNA inserts were excised and subcloned into pBS II and sequenced. One of the clones (clone 21.3) contained conserved X and Y domains sequences and appeared to encode a new type of PLC. The other two clones contained no PLC-homologous sequences.

The insert of clone 21.3 was only 1.2Kb in size and did not contain the complete open reading frame. In order to find the missing sequence, the insert of clone 21.3 was used as the probe to screen the cDNA library at high stringency. A total of 6×10^5 phage were screened and seven positive clones were obtained after three rounds of consecutive screening.

After restriction map analysis, four clones seemed to be different from and contained extra sequence than clone 21.3. These four clones were further analyzed by subcloning and sequencing. Three of them (clones 29.1, 33.2, 35.1) contained extra 5' end sequence, while clone 27.1 contained extra 3' end

sequence. These five overlapping clones contained most of the coding sequence of the new PLC and the entire 3' end untranslated region including the poly(A) tail.

To find the missing 5' end sequence of the new PLC, an oligonucleotide corresponding to the most 5' end sequence of clone 29.1 was used as the probe to screen the library twice. No positive clone was isolated from 1.2 million phage.

2. Use of 5'-RACE technology to isolate the 5' end of PLC-*X*β:

Since it was difficult to find the missing 5' cDNA sequence by screening the library, a 5' RACE method was used in attempt to isolate the extreme 5' end (Fig 4.1).

The 5'-RACE technique is advantageous in isolating 5' end from low-copy messages when part of the cDNA sequence is known. It employs one PCR primer specific for the gene and another hybridizing to the homopolymeric tail formed at the 3' end of the reverse transcript of mRNA. First strand cDNA is synthesized by using a gene-specific antisense oligonucleotide as the primer. This permits cDNA conversion of specific mRNA or related families of mRNAs, thus significantly increasing the target to background ratio. This also increases the potential for complete extension to the 5' end of the mRNA.

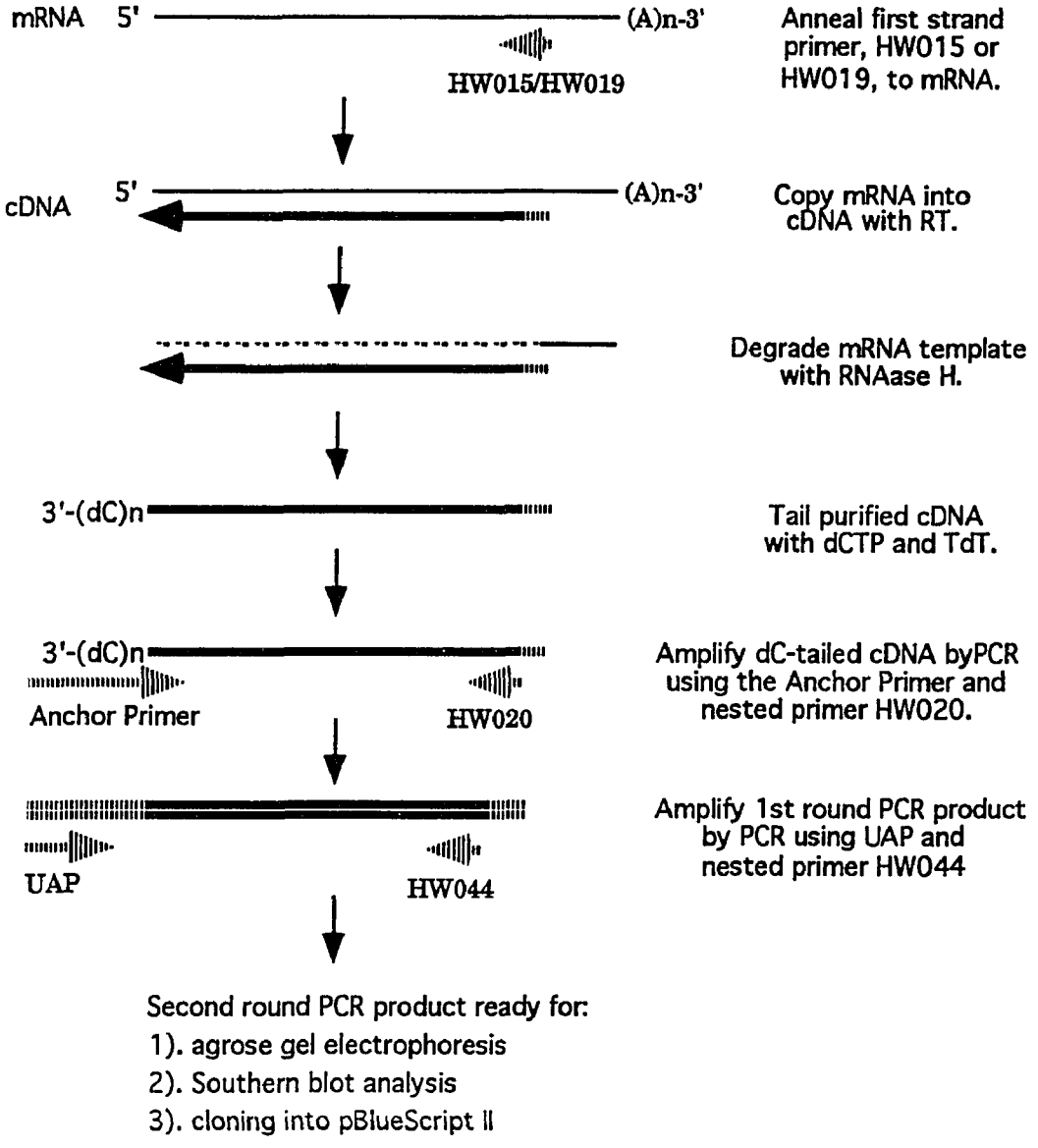

Figure 4.1. Schematic diagram of the 5' RACE procedures. Primers with leftward arrows or rightward arrows refer to their sequences complementary to the plus strand or the minus strand of the PLC-Xβ cDNA. The primer sequences are described in "Materials and Methods".

As illustrated in Fig. 4.1, First strand cDNA was synthesized from 1μg total *Xenopus* oocyte RNA by using two antisense gene-specific primers HW015 and HW019 respectively. Two primers were used for the following reasons. HW019 would lead to more efficient extension of unknown cDNA sequence because it was closer to the known 5' end. However, short cDNA (≤200 bases) can not be purified efficiently with GLASSMAX Spin Cartridges. This step of purification was required for subsequent steps. Therefore, HW015 was used in case the 5' unknown sequence was very short.

Terminal deoxynucleotidyl transferase was used to add a dC tail to the first strand cDNA. Tailed cDNA was amplified by PCR using a nested gene-specific primer, HW020, and an anchor primer provided in the kit, which anneals to the dC tail. This first round PCR product was used as the template for second round PCR using UAP, which anneals to the anchor primer, and a nested gene-specific primer HW044. This allows amplification of unknown sequences between HW044 and the 5' end of the mRNA.

Second round PCR products were subjected to Southern blot analysis with an oligonucleotide probe HW043. A 250 bp positive fragment was identified. This 250 bp DNA fragment was purified from the agarose gel and subcloned into pBS II. Even though *pfu* DNA polymerase, which was reported to have higher fidelity in DNA synthesis, was used for PCR amplification, mutation(s) could still be introduced into the

sequence by PCR. Therefore, three individual clones were selected and sequenced. They all had the same nucleotide sequence, which overlaps with clone 29.1.

The result of the southern blot analysis for the 5' RACE product is shown in Fig. 4.2.

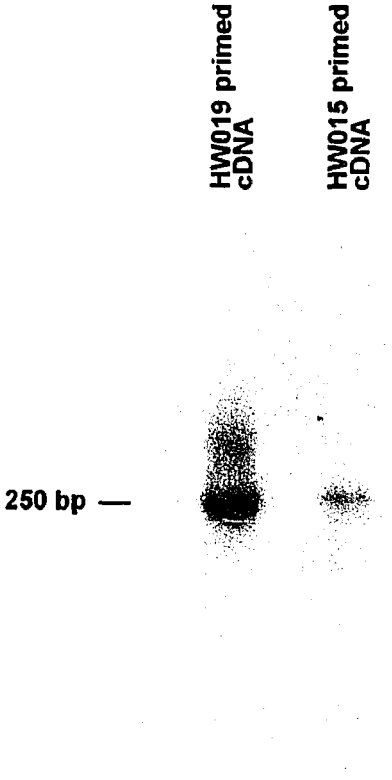

Figure 4.2. Southern blot analysis of the 5'-RACE product. First strand cDNA primed with HW015 or HW019 was used as template for two rounds PCR amplification. The second round PCR products were separated on

1.2% agarose gel and transferred to nitrocellulose membrane. The membrane was hybridized to ^{32}P-labeled probe HW043.

The 5'-RACE product and the other overlapping cDNA clones isolated from the cDNA library appeared to encode a full length PLC. Translation from the first ATG yields a putative protein 1210 amino acids long. The first ATG triplet is flanked by sequences that fulfill the Kozak criteria for initiation codons (Kozak, 1984). Twelve out of sixteen N-terminal residues of the presumed protein in this reading frame are identical to those of PLC-β1. This indicates that the presumed start site is most likely to be correct.

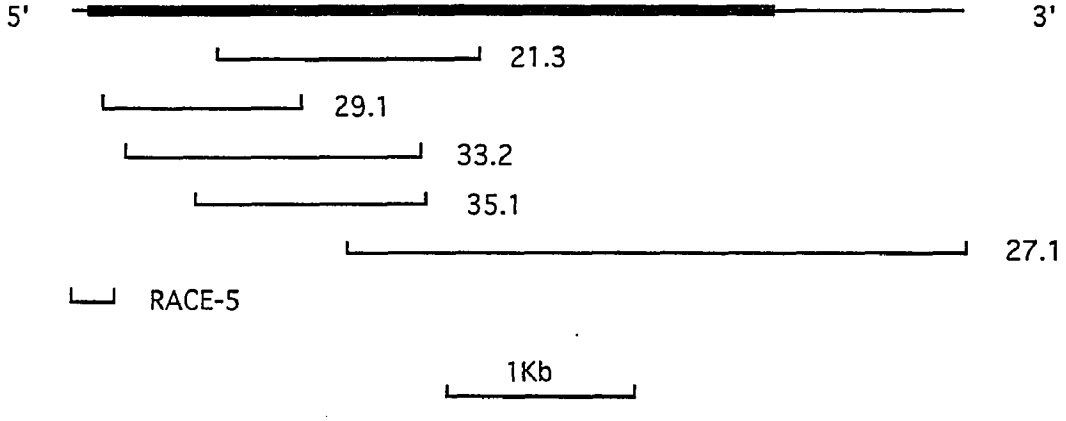

Figure 4.3. Schematic diagram of overlapping cDNA clones and cumulative full length cDNA. The thickened line represents the open reading frame.

The overlapping cDNA clones and the cumulative full length cDNA for the new *Xenopus* PLC are shown in Fig. 4.3. The relative positions of individual clones to the full length cDNA is also depicted.

3. Sequence analysis of PLC-*X*β cDNA:

The nucleotide and the deduced amino acid sequence for the new *Xenopus* PLC are shown in Fig. 4.4. The isolated cDNA is 4741 nucleotides long. It encodes a protein of 1210 amino acids with a predicted molecular weight of 138.2 kilodaltons. Two putative polyadenylation signals AATAAA are identified in the 3' untranslated region. One is just 19 nucleotides upstream of the poly(A) tail. The other is located at position 3739.

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TCTCCCTCTCTCTCTTTCGCTGCCTCCTCCTCTTCCTCGTTATTTTGCCCCCCTAGTTGC   60

CCCCTGCCCTGTTCCCCAAACATGGCGGGGGCACGGCCTGGGGTTCACTCTCTTCAACTG  120
                      M  A  G  A  R  P  G  V  H  S  L  Q  L    13

GAGCCCGTGAAAGTGCCGGAGACTCTTATCAAAGGGAGCAAGTTCATCAAGTGGGATGAG  180
 E  P  V  K  V  P  E  T  L  I  K  G  S  K  F  I  K  W  D  E    33

GAGTCCAGCAGCAAATCCCTGGTTACTCTGCGTGTGGATACAATGGGGTTTTATCTTTAC  240
 E  S  S  S  K  S  L  V  T  L  R  V  D  T  M  G  F  Y  L  Y    53

TGGACCTGTCCAAATATGGAGGTTGATATTTTGGATATCAGCGTAATAAGAGACACCCGG  300
 W  T  C  P  N  M  E  V  D  I  L  D  I  S  V  I  R  D  T  R    73

ACAGGAAAATATGCGAAAATTCCAAAGGATATAAAAATGAGGGAGATTTTGGGATTCACA  360
 T  G  K  Y  A  K  I  P  K  D  I  K  M  R  E  I  L  G  F  T    93

GGCCCAGAGCAGCGCCCTGAGGATAAGCTTGTTACAGTGGTATATGGCAATGATATTGTG  420
 G  P  E  Q  R  P  E  D  K  L  V  T  V  V  Y  G  N  D  I  V   113
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AATATCAGCTTCCTCAACTTTATGGCTGTGCAAGAAGATACAGCAAAGATTTGGACAGAA  480
 N  I  S  F  L  N  F  M  A  V  Q  E  D  T  A  K  I  W  T  E   133

GAGCTTTTTAAATTGGCACATAATATTCTGGCACAGAACTCATCTCGGAACACCTTCCTG  540
 E  L  F  K  L  A  H  N  I  L  A  Q  N  S  S  R  N  T  F  L   153

CAGAAAGCATACACCAAACTAAAGCTGCAAGTGAATCAAGATGGACGAATTCCAGTTAAG  600
 Q  K  A  Y  T  K  L  K  L  Q  V  N  Q  D  G  R  I  P  V  K   173

AATATCCTAAAGATGTTTGCAGCTGATAAGAAAAGAGTGGAAACAGCACTGGAATCTTGT  660
 N  I  L  K  M  F  A  A  D  K  K  R  V  E  T  A  L  E  S  C   193

GGCTTGAACTTTAACAGGGGGGATTCTATTAAACCAGAGGAATTTACACTAGATATTTTC  720
 G  L  N  F  N  R  G  D  S  I  K  P  E  E  F  T  L  D  I  F   213

GAACGCTTCCTGAACAAGCTGTGTCTGCGTCCAGATATTGATAAGATCCTTCTAGAACTT  780
 E  R  F  L  N  K  L  C  L  R  P  D  I  D  K  I  L  L  E  L   233

GGAGCCAAAGGGAAGCCATATCTGACTCTGGAGCAACTAATGGACTTCCTAAATCAGAAA  840
 G  A  K  G  K  P  Y  L  T  L  E  Q  L  M  D  F  L  N  Q  K   253

CAAAGGGACCCCCGGCTCAATGAGATTCTTTTCCCTCCACTAAAAAGAGACCAAGTGCGG  900
 Q  R  D  P  R  L  N  E  I  L  F  P  P  L  K  R  D  Q  V  R   273

CAGCTCATTGAGAAGTATGAGCCCAACAGGCAGTTCCTGGACAGAGACCAGATGTCGATG  960
 Q  L  I  E  K  Y  E  P  N  R  Q  F  L  D  R  D  Q  M  S  M   293

GAAGGTTTCAGCCGCTTTTTAGGTGGGCATGAGAACAGCATTGTTCCCCCGGAGATCCTT 1020
 E  G  F  S  R  F  L  G  G  H  E  N  S  I  V  P  P  E  I  L   313

GATCTAAGTGATGATATGAACCAGCCGCTCAGCAGCTACTTTATTAACTCCTCCCATAAC 1080
 D  L  S  D  D  M  N  Q  P  L  S  S  Y  F  I  N  S  S  H  N   333

ACCTACCTCACAGCTGGCCAGCTAACTGGAAATTCATCAGTGGAGATGTACAGGCAAGTG 1140
 T  Y  L  T  A  G  Q  L  T  G  N  S  S  V  E  M  Y  R  Q  V   353

CTGTTGACTGGCTGCCGCTGTATTGAGCTAGATTGCTGGAAGGGTCGTCCGCAGGATGAA 1200
 L  L  T  G  C  R  C  I  E  L  D  C  W  K  G  R  P  Q  D  E   373

GAACCATTCATAACCCATGGATTCACTATGACCACCGAAATTCCTTTTAAGGAAGTAATT 1260
 E  P  F  I  T  H  G  F  T  M  T  T  E  I  P  F  K  E  V  I   393

GAAGCAATAGCAGAAAGTGCCTTTAAAACATCGCCGTTCCCAGTCATTTTGTCATTTGAA 1320
 E  A  I  A  E  S  A  F  K  T  S  P  F  P  V  I  L  S  F  E   413

AATCATGTTGACTCTTCAAAGCAGCAAGCAAAGATGGCCGAGTACTGCAGGAACATCTTC 1380
 N  H  V  D  S  S  K  Q  Q  A  K  M  A  E  Y  C  R  N  I  F   433

GGAGATGCGTTGTTAATAGACCCGCTGGAGAAGTATCCTCTCCAGCCTGGAGTTGCACTT 1440
 G  D  A  L  L  I  D  P  L  E  K  Y  P  L  Q  P  G  V  A  L   453

CCTAGTCCCCAGGAACTTATGGGTAAGATCCTAGTGAAGAATAAAAAAAGACACAAAGTG 1500
 P  S  P  Q  E  L  M  G  K  I  L  V  K  N  K  K  R  H  K  V   473

GCAGTAAAAGGCCCAGAAGGCAGTGTAAAAAGAAGGGTCAGCGAACAAACCTCCAATACT 1560
 A  V  K  G  P  E  G  S  V  K  R  R  V  S  E  Q  T  S  N  T   493
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TACAGTGACTCATCCAGTGTTTGCGAGTCTTCTGCTACAGGTCTACCACCTTCAGAATCA 1620
 Y  S  D  S  S  S  V  C  E  S  S  A  T  G  L  P  P  S  E  S   513

GCTGATGTTTCATTGACACTTTCCAATGGGGATGAAAAAATCGAGGAGAAACCCCCTAAA 1680
 A  D  V  S  L  T  L  S  N  G  D  E  K  I  E  E  K  P  P  K   533

TATACAAAGCCACGGAAATCGATTGATCATGATGCTTATAGTGAGGAGGAAGAAGAAGAA 1740
 Y  T  K  P  R  K  S  I  D  H  D  A  Y  S  E  E  E  E  E  E   553

GAGCCGTCAGATCCCAAGAAATCTGATGAGGGTACAGCAAGTAGTGAGGTGAATGCCACA 1800
 E  P  S  D  P  K  K  S  D  E  G  T  A  S  S  E  V  N  A  T   573

GAGGAGATGTCCACACTTGTGAATTATGTAGAACCTGTTAAGTTCAAGTCATTTGATGCG 1860
 E  E  M  S  T  L  V  N  Y  V  E  P  V  K  F  K  S  F  D  A   593

GCCAAAAAAAGAAATAAGTATTATGAGATGTCCTCATTTGTTGAAACCAAGGCATTGGAG 1920
 A  K  K  R  N  K  Y  Y  E  M  S  S  F  V  E  T  K  A  L  E   613

CAGCTCACTAAATCCCCAATGGAATTTGTGGAATATAACAAGAAGCAGCTCAGCCGTATT 1980
 Q  L  T  K  S  P  M  E  F  V  E  Y  N  K  K  Q  L  S  R  I   633

TACCCAAAAGGAACTCGGGTAGATTCCTCAAATTACATGCCCCAGCTCTTCTGGAATGCA 2040
 Y  P  K  G  T  R  V  D  S  S  N  Y  M  P  Q  L  F  W  N  A   653

GGATGTCAAATGGTGGCTTTGAACTTTCAAACGCTGGATCTTCCAATGCAGCTGAATGTG 2100
 G  C  Q  M  V  A  L  N  F  Q  T  L  D  L  P  M  Q  L  N  V   673

GGCATTTTTGAGTATAATAGACGCAGTGGTTACCTCCTCAAACCGGAATTCATGTGCAGG 2160
 G  I  F  E  Y  N  R  R  S  G  Y  L  L  K  P  E  F  M  C  R   693

ACAGACAAGCATTTTGATCCTTTTACTGAAAATATCGTCGATGGAATTGTCGCAAATACG 2220
 T  D  K  H  F  D  P  F  T  E  N  I  V  D  G  I  V  A  N  T   713

GTTAAAATCAAGATTATATCAGGGCAGTTCCTATCTGAAAAGCGAGTAGGCATATTCGTA 2280
 V  K  I  K  I  I  S  G  Q  F  L  S  E  K  R  V  G  I  F  V   733

GAGGTCGATATGTTTGGTCTGCCAGTTGATACCAAGAGGAAATTTCGCACAAAAACATCC 2340
 E  V  D  M  F  G  L  P  V  D  T  K  R  K  F  R  T  K  T  S   753

CAAGGAAACTCATTTAATCCTGTCTGGGATGAAGAGCCATTTATTTTGCCTAAGGTTGTA 2400
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 L  P  T  L  A  T  L  R  I  A  V  F  E  E  G  G  K  F  V  G   793

CATCGAATCCTGCCTGTCTCAGCTATCCGACCAGGATATCACTACATTTGCCTTAGAAAT 2520
 H  R  I  L  P  V  S  A  I  R  P  G  Y  H  Y  I  C  L  R  N   813

GAGCTTAACCAGCCTGTCTGTCTCCCAGCGCTGCTTGTTTACACTGAAGTAAATGATTAT 2580
 E  L  N  Q  P  V  C  L  P  A  L  L  V  Y  T  E  V  N  D  Y   833

ATTCCTGATGACCACCAGGGCTATGCGAATGCCTTAACTAACCCCATCAAGCACATCAGC 2640
 I  P  D  D  H  Q  G  Y  A  N  A  L  T  N  P  I  K  H  I  S   853

CTCATGGACCAAAGGGCAAAGCAACTGGCAGCTCTAATGGGAGAGAATGAGCAACCATTC 2700
 L  M  D  Q  R  A  K  Q  L  A  A  L  M  G  E  N  E  Q  P  F   873
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AACAAAGACAAGATTGATTGGGATCCAATATCAAAGCCCCCAATAAGATCCCGCGATGAT 2760
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 T  P  E  K  I  K  I  P  I  I  P  V  P  S  P  P  A  Q  R  D   913

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AAGCACCTGCGCAAGGTGTCGTCTCTGTGCAAGGAGCAGAGCACTTGGGTGACACCTCTG 3000
 K  H  L  R  K  V  S  S  L  C  K  E  Q  S  T  W  V  T  P  L   973

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 L  R  E  I  Q  H  E  Q  E  R  K  L  K  H  S  H  L  L  E  A  1033

GTTCAGAAGCTCCAGGAAGTGGCCATTTCTTATCATTCCACTCAACTCAAGAAGTTAAAG 3240
 V  Q  K  L  Q  E  V  A  I  S  Y  H  S  T  Q  L  K  K  L  K  1053

GAAATCAATGAAAAGGAGAAGAAAGAGCTGCAGAAGATCCTGGACAGAAAAAGACATAAC 3300
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 S  S  S  H  S  S  P  P  S  I  T  R  S  W  G  S  E  S  G  E  1193

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 K  D  S  L  H  S  P  G  D  S  S  D  E  A  T  R  L           1210

ACCCCCTAAAAATATAGAAATAAACTTTTTTACTTTCAGAAGCTAATCTTGGAATCCAAT 3780

CGGAATTCCGGAAGCACCCAAGTCTGTGGACTGGCGTAAGAAAGGCTATGTAACACCAGT 3840

TAAAGACCAGGGACAATGTGGTTCATGCTGGGCACCCAGTACAACAGGTGCATTAGAAGG 3900

TCAGCACTACAGGAAGACTAGTAAACTGATTTCTCTAAGTGAGCAAAATCTTGTAGACTG 3960
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TCAATCTGGATGTGAGAAAGATCTCATGAAGGCAGTAGCTTCAGTGGGACCAGTCTCTGT 4200
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CGACAATGGATACATCAACATTGCCAAGGATAGACACAACCACTGTGGAATAGCAACTGC 4440
TGCCAGTTATCCTCTGGTTTAATATAAACATTGTATATGGACCTCATTAGAACTATTATG 4500
TGCACATGTTGTAATCCTCCACTTTTAGCAAGTACTGGACTGTTTCGTTTTTTTTTTTTT 4560
ACCTGATGGAATTCTGCTCAAGGCTGTCTTTTAAACTTGTGTTGAAAGTTGACGTCCCAC 4620
ATTGAAGCTGGGTTGTTTTAGTATTGTTTACATGTTGATTTTAATGGTGGTTTTATGTCA 4680
TCTTAAGCTTTTATAATGTAAAATAAAAACTATAATTGTGAAAAAAAAAAAAAAAAAAAA 4740
A                                                               4741
```

Figure 4.4. Nucleotide and deduced amino acid sequence of the new *Xenopus* PLC. Two putative polyadenylation signals AATAAA are underlined. The nucleotide sequence of the new *Xenopus* PLC has been deposited in the GenBank^{TM}/EMBL Data Bank with accession number L20816.

4. Sequence comparison of all known PLC-βs:

The predicted protein contains the X and Y box sequences conserved in all PLCs, which encompass the presumed catalytic domain. The X and Y boxes are separated by a short stretch that does not contain any SH2 or SH3 domains. There is a long

tail after the Y box, a feature characteristic of PLC-βs. Sequence comparison reveals that the deduced *Xenopus* PLC protein is more similar in primary sequence and overall structure to the β-type mammalian PLCs than to the other types. It shares substantial (32-64%) homology with the mammalian PLC–β1 to PLC–β4 as well as the two *Drosophila* β-type PLCs. Among the known PLC-βs, it is closest to PLC–β3 with 64% identity and ~80% similarity. Therefore, the newly cloned *Xenopus* PLC most likely belongs to the β-type and is named PLC–*X*β.

All known β-type PLCs are compared in pairwise fashion by using the PALIGN program in PC/GENE. The percent identity is shown in Table 4.1.

Table 4.1

Comparisons of phospholipase C-βs

	β1	β2	β3	β4	*X*β	*norpA*	*plc-21*
β1	-	47	53	39	58	35	36
β2	-	-	41	32	44	32	34
β3	-	-	-	32	64	32	36
β4	-	-	-	-	33	54	35
*X*β	-	-	-	-	-	32	38
norpA	-	-	-	-	-	-	34

Indicated PLCs were compared in a pairwise fashion using PALIGN program in PC/GENE on an IBM PS2 computer. Every two PLC-β sequences are compared respectively. Numbers are percent amino acid identity. 100% identities and repeats are shown by "-".

The familial relationship between the various PLC-βs from different species was explored by multilayered comparison by the method of Hein (Hein, 1990). Multilayered analysis was performed on a Sun workstation by Dr. Lutz Birnbaumer (University of California, Los Angeles). The resultant phylogenetic tree is shown in Fig. 4.5. Such comparison indicates that the PLC-*X*β is closest to the mammalian PLC-β3 and fits well into the mammalian PLC family as compared to the *Drosophila* PLC-βs (*plc-21* and *norpA*).

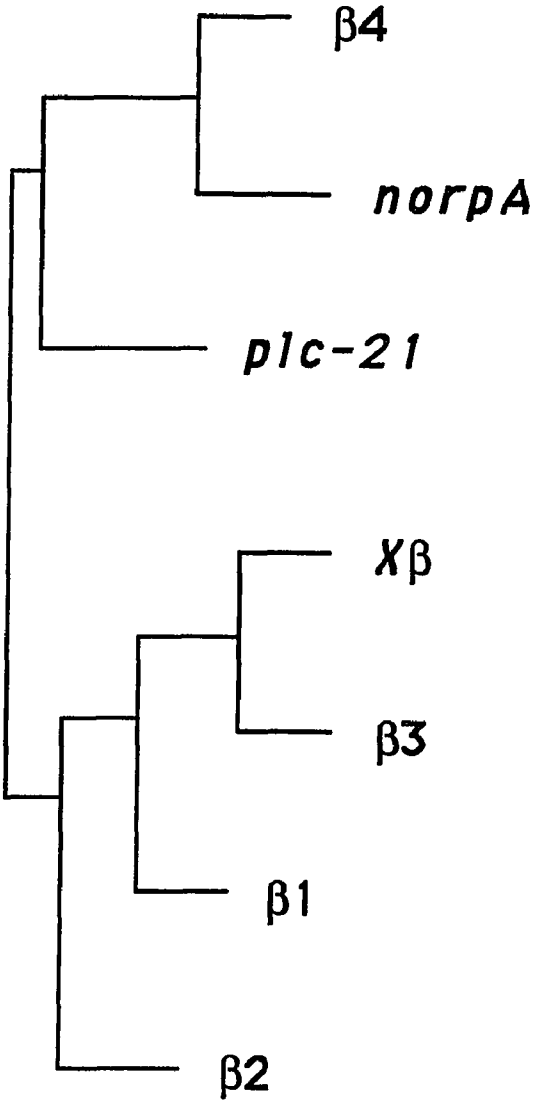


Figure 4.5. Phylogenetic Tree of PLC-βs. Multilayered comparison was performed by the method of Hein. Comparison is restricted to 1200 amino acids.

Chapter 5

Characterization of PLC-*X*β in intact *Xenopus* oocytes

1. The size of PLC-*X*β mRNA:

After the full length cDNA for PLC-*X*β was isolated, PLC-Xβ was then studied in the intact *Xenopus* oocyte system. Its expression pattern in oocytes was studied first.

Poly(A)+ RNA was isolated from stage V and VI oocytes. The size of the mRNA encoding PLC–*X*β was determined by Northern blot analysis with two different probes. 7.5 μg *Xenopus* oocyte poly(A)+ RNA was used for each hybridization. Probe A encoded nucleotides 757-2151, which contained conserved X and Y domains and the region between. Probe B encoded nucleotide 2145-3788, which contained the coding region after the Y domain.

Two distinct probes were used in an attempt to identify the mRNA encoding PLC-Xβ, as well as to determine if there were additional PLC-βs in the oocyte. Different PLC–βs share high homology in the two conserved X and Y domains, while the sequences other than the X and Y boxes are rather divergent. Therefore, probe A might be able to identify more transcripts than probe B. The two probes gave essentially identical hybridization patterns (Fig. 5.1). This did not necessarily mean that there were no other PLC-βs expressed in the oocytes, because the hybridization stringency was not lowered when probe A was used. The degree of conservation at the nucleotide level is less than that at the amino acid level. Therefore, hybridizaton of members of a related gene family to

one DNA probe would probably only occur at low stringency, even though the protein sequences are highly conserved.

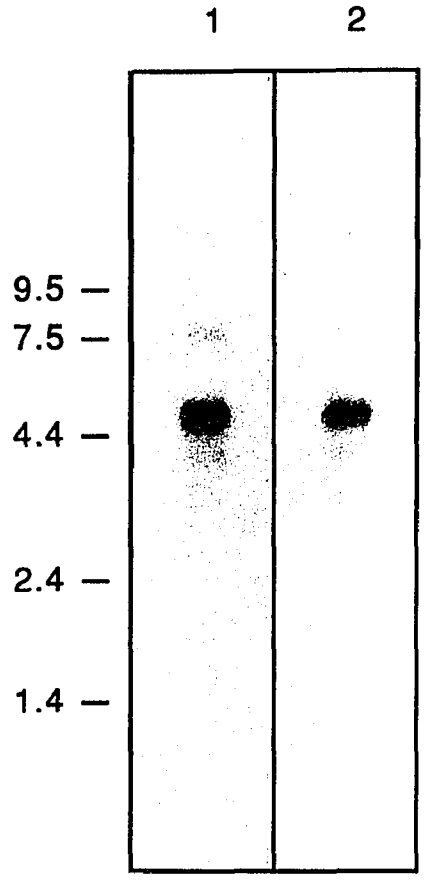


Figure 5.1. Northern blot analysis for PLC-*X*β mRNA. *Xenopus* oocyte poly(A)$^+$ RNA (7.5 μg) was probed with cDNA fragments encoding

nucleotides 757-2151 (lane 1) or nucleotides 2145-3788 (lane 2). The size of the molecular weight markers in kilobases is given. Picture is from a 20 hour autoradiogram.

Northern analysis demonstrated the expression of 7.5 Kb, 5.0 Kb, and 4.2 Kb transcripts in the oocytes, with the 5.0 Kb transcript being the most prominent one. The 5.0 Kb transcript was close to the ~4.8 Kb size of the combined cDNAs. There was a AATAAA polyadenylation signal 19 nucleotides upstream the poly(A) tract. This polyadenylation signal could be used to generate the 5.0 Kb transcript.

Multiple transcripts hybridizing to the PLC-Xβ cDNA probe may be the result of alternative processing of the same pre-mRNA from a single gene, or represent transcripts from a family of related genes. Another AATAAA polyadenylation signal was found at position 3739. The less abundant 4.2 Kb message could be generated from alternative polyadenylation by using this polyadenylation signal. The origin of the other less abundant 7.5 Kb message is currently unknown.

2. Inhibition of receptor-evoked Cl^- current by antisense oligonuleotide against PLC-*X*β:

The *Xenopus* oocyte has been a useful model system for the study of receptor stimulated-phospholipase C pathway for a decade. It exhibits a receptor-evoked Cl^- current, which is mediated through the activation of PLC and subsequent

liberation of intracellular Ca^{++}. This Ca^{++}-dependent Cl^- current can be evoked by native receptors in the oocyte as well as many mammalian G protein-coupled receptors when expressed in the oocyte. The native angiotensin and muscarinic receptors couple to the phospholipase C pathway through pertussis toxin-sensitive G proteins. Some cloned receptors expressed in oocytes also show at least partial pertussis toxin sensitivity while others do not.

The function of the newly cloned PLC-Xβ in the receptor-evoked Cl^- current in the *Xenopus* oocyte was studied by antisense oligonucleotide inhibition experiments. Antisense oligonucleotides have been used to inhibit the expression of a number of cellular proteins. Once inside the cell, the antisense oligonucleotide would bind to the complementary mRNA, leading to degradation of specific mRNA via an RNAase H dependent mechanism or other mechanisms. Degradation of specific mRNA would in turn result in inhibition of the expression of the corresponding protein. Therefore, if PLC-Xβ is involved in the receptor-mediated G protein-dependent PLC pathway, inhibition of expression of PLC-Xβ by antisense oligonucleotide will lead to decreased receptor-evoked Cl^- current.

A specific antisense oligonucleotide was designed against a region (nucleotide 938-958) that was distinct for PLC-Xβ in comparison to the mammalian PLC-β1, β2, and β3. The corresponding sense oligonucleotide was also synthesized. Injection of the sense oligonucleotides did not affect either the

native or the expressed receptor-evoked Cl- current as compared to the water-injected controls (data not shown). Hence, in some of the studies described below, the response of the oocytes injected with antisense oligonucleotides were compared with those of the sense oligonucleotide-injected oocytes.

First I examined the effect of antisense oligonucleotide on the angiotensin II receptor-evoked Cl^- current response. Some oocytes express native angiotensin II receptors on their plasma membrane. The endogenous angiotensin II receptors activate phospholipase C via PTX-sensitive G protein(s) (Sandberg et al., 1990; Sakuta et al., 1991). Oligonucleotides were injected into oocytes in a 50 nl volume to yield a final concentration of 2 μM assuming uniform distribution. 20-24 hours later, the Cl^- current response of individual oocytes was measured in the presence of 10 μg/ml angiotensin II. Injection of antisense oligonucleotide significantly reduced Cl^- current evoked by angiotensin II receptor. Injection of water or sense oligonucleotides had no effect. On the average, the antisense oligonucleotides inhibited angiotensin II response by 60%. Result of one representative experiment is shown in Fig. 5.2.

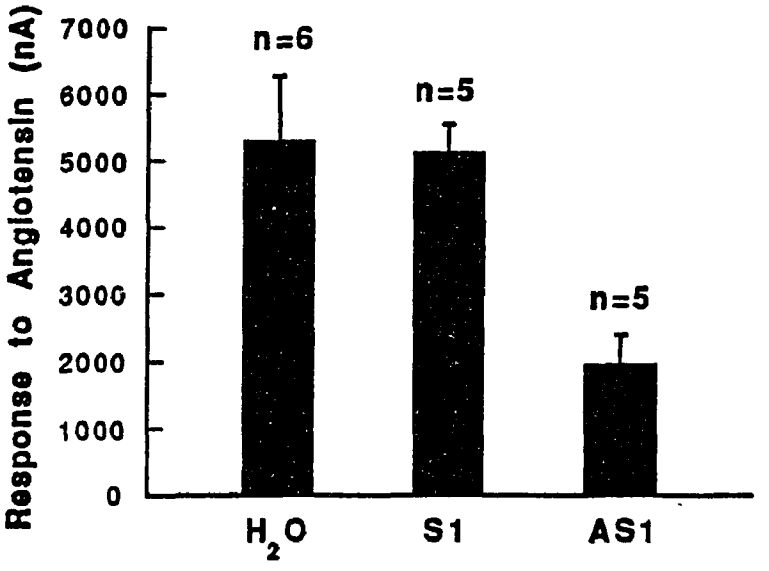


Figure 5.2. Comparison of the angiotensin II receptor-evoked Cl^- current response in oocytes injected with water (H_2O), sense (S) or antisense (AS) oligonucleotides. Oligonucleotides to PLC-Xβ were injected into an oocyte in a 50 nl volume to yield a final concentration of 2 μM assuming uniform distribution inside the oocyte. 20-24 hours later, Cl^- currents were evoked by bath application of 10 μg/ml angiotensin II. Currents were recorded at peak depolarization. Number of oocytes in each group is indicated. Cl^- current values are mean ± S.E.

Stimulation of α_1-adrenergic receptor in liver activates phospholipase C via pertussis toxin-insensitive G protein(s) (Lynch et al., 1986). Expression of α_1-adrenergic receptors in COS-7 cells also leads to a pertussis toxin-insensitive response (Cotecchia et al., 1990).

Xenopus oocytes do not possess native α-adrenergic receptors. Cloned hamster α_{1b}-adrenergic receptor (α_{1b}-AR) has been expressed on the oocyte plasma membrane by injection of

in vitro transcribed α_{1b}-AR mRNA into the oocytes. However, in the oocyte the coupling of expressed α_{1B}-adrenergic receptor to phospholipase C is largely pertussis toxin-sensitive. The use of antisense oligonucleotides indicates that α_o is involved in transducing a significant component of the α_1-adrenergic response (Blitzer et al., 1993).

The effect of PLC-*X*β antisense oligonucleotides on expressed α_{1b}-adrenergic receptor-evoked Cl^- current response was also examined. 100 pg of α_{1b}-adrenergic receptor mRNA was injected into each oocyte. Two days later, oocytes were injected with either sense or antisense oligonucleotides. Norepinephrine-evoked response was recorded 20-24 hours after the oligonucleotide injection. On the average, the norepinephrine response was inhibited by the antisense oligonucleotides by 50%. Representative result is shown in Fig. 5.3.

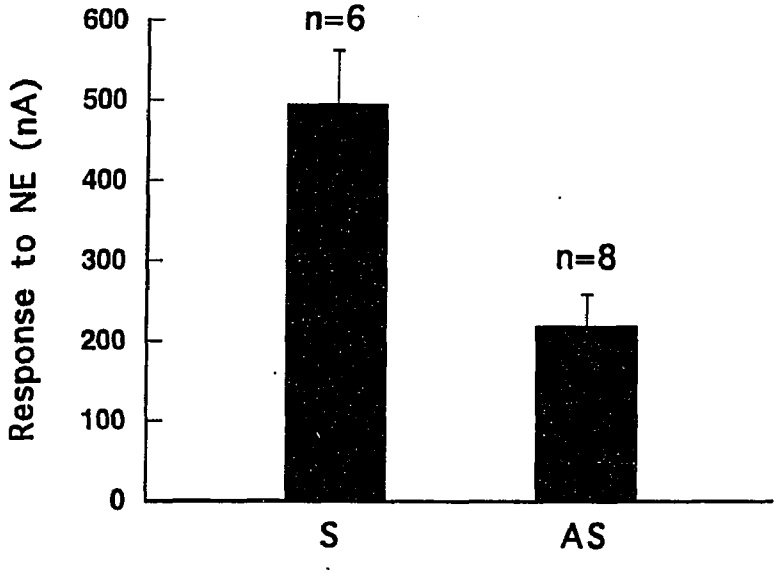


Figure 5.3. Comparison of the α_{1b}-adrenergic receptor-evoked Cl^- current response in oocytes injected with sense (S) or antisense (AS) oligonucleotides. 100 pg of α_{1b}-adrenergic receptor mRNA was injected into each oocyte. Two days later, oligonucleotides to PLC-*X*β were injected into oocyte in a 50 nl volume to yield a final concentration of 2 μM assuming uniform distribution inside the oocyte. Another 20-24 hours later, Cl^- currents were evoked by bath application of 10 μM norepinephrine (NE). Currents were recorded at peak depolarization. Number of oocytes in each group is indicated. Cl^- current values are mean ± S.E.

Similar to α_{1b}-adrenergic receptor, the M_1-muscarinic receptor stimulates phospholipase C via pertussis toxin-insensitive G proteins in their native mammalian environment. When expressed in *Xenopus* oocyte, it stimulates phospholipase C at least partially through pertussis toxin-sensitive G proteins (Blitzer et al., 1993).

The effect of PLC-*X*β antisense oligonucleotides on expressed M_1-muscarinic receptor-evoked Cl^- current response was also examined. 100 pg of *in vitro* transcribed rat M_1-muscarinic receptor mRNA was injected into each oocyte. Two days later, oocytes were injected with either sense or antisense oligonucleotides. Acetylcholine-evoked Cl^- current response was recorded another 20-24 hours later. On the average, the acetylcholine response was inhibited by the antisense oligonucleotides by 50%. Representative result is shown in Fig. 5.4.

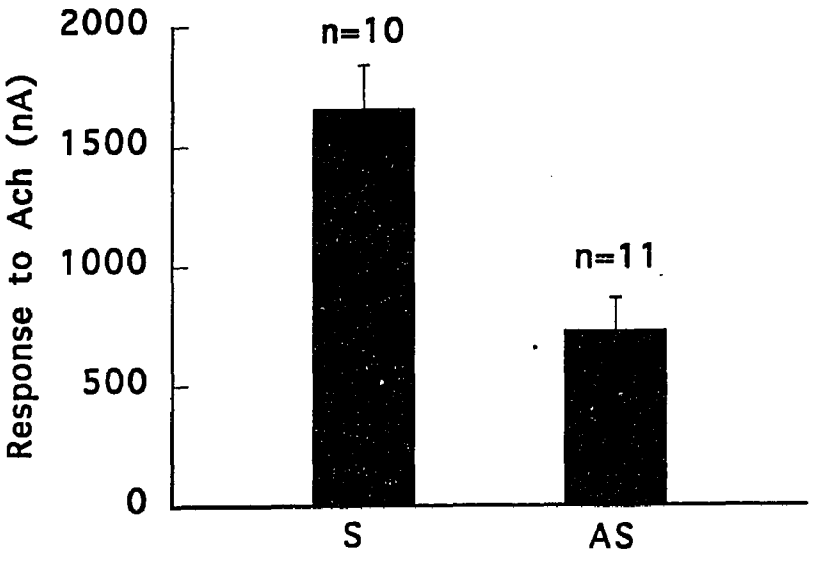


Figure 5.4. Comparison of the M_1-muscarinic receptor-evoked Cl^- current response in oocytes injected with sense (S) or antisense (AS) oligonucleotides. 100 pg of rat M_1-muscarinic receptor mRNA was injected into each oocyte. Two days later, 50 nl oligonucleotides to PLC-$X\beta$ were injected into oocyte to yield a final concentration of 2 μM assuming uniform distribution inside the oocyte. Another 20-24 hours later, Cl^- currents were evoked by bath application of 10 μM acetylcholine (Ach). Currents were recorded at peak depolarization. Number of oocytes in each group is indicated. Cl^- current values are mean $\pm$ S.E.

Injection of IP_3 evoked a similar Cl^- current response in oocytes injected with either sense or antisense oligonucleotides (Fig. 5.5). This indicates that the antisense oligonucleotide treatment affects a step before the IP_3 function. Release of Ca^{++} into the cytosol and subsequent steps to open of Cl^- channels are not affected.

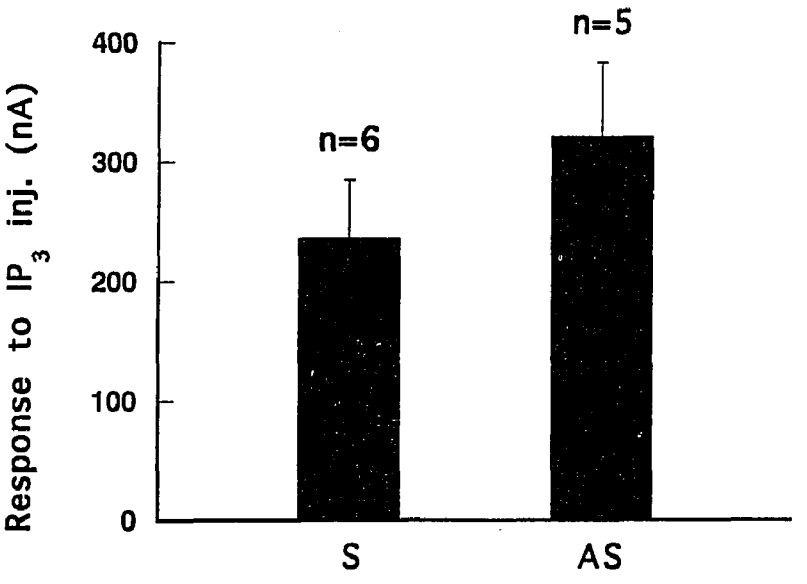


Figure 5.5. Comparison of IP_3-evoked Cl^- current response in oocytes injected with sense (S) or antisense (AS) oligonucleotides. 50 nl oligonucleotides to PLC-*X*β were injected into oocyte to yield a final concentration of 2 μM assuming uniform distribution inside the oocyte. 20-24 hours later, Cl^- currents were evoked by intracellular injection of IP_3 with a picospritzer. Currents were recorded at peak depolarization. Number of oocytes in each group is indicated. Cl^- current values are mean ± S.E.

Taken together, the antisense experiments indicate that PLC-*X*β mediates a significant proportion of the receptor-evoked Cl^- current in *Xenopus* oocytes. For the three receptors tested here, coupling of these receptors to PLC are not fully attenuated by pertussis toxin (50-80%). This implies that these receptors may or may not exclusively couple to PTX-sensitive G proteins in the oocytes. Thus it is possible that these receptors may activate phospholipase C through PTX-sensitive as well as

PTX-insensitive pathways at the same time. Since inhibition of receptor response by antisense oligonucleotide to PLC-*X*β was only partial, the experiments in Fig. 5.2-5.5 could not determine whether the observed effects were due to the partial inhibition of PLC-*X*β expression or due to the involvement of PLC-*X*β in only one pathway. Partial inhibition of PLC-*X*β expression would lead to only partial inhibition of the receptor response, because there would be some residual protein functioning in response to agonist stimulation. On the other hand, if the receptor is coupled to both PTX-sensitive and insensitive PLC pathways and PLC-*X*β only mediates one of the two components, even complete inhibition of PLC-*X*β expression would only lead to partial inhibition of the receptor response. In order to address this question, more experiments were performed to test whether inhibition by antisense oligonucleotide to PLC-*X*β was additive with the pertussis toxin blockade of the receptor signal.

Muscarinic receptors were chosen for this experiment. Muscarinic receptors were the least PTX-sensitive among the three receptors tested. This allowed me to determine whether PLC-*X*β functioned in the PTX-sensitive or insensitive component of the muscarinic response. Oocytes expressing M_1-muscarinic receptors were injected with sense or antisense oligonucleotides. Each group was treated with or without 10 μg/ml pertussis toxin overnight. Individual treatment with pertussis toxin or injection of antisense oligonucleotides inhibited the M_1-muscarinic response 40-50% respectively.

However, the inhibition by pertussis toxin and antisense oligonucleotides was not additive (Fig. 5.6). This indicates that PLC-$X\beta$ is involved in the PTX-sensitive pathway.

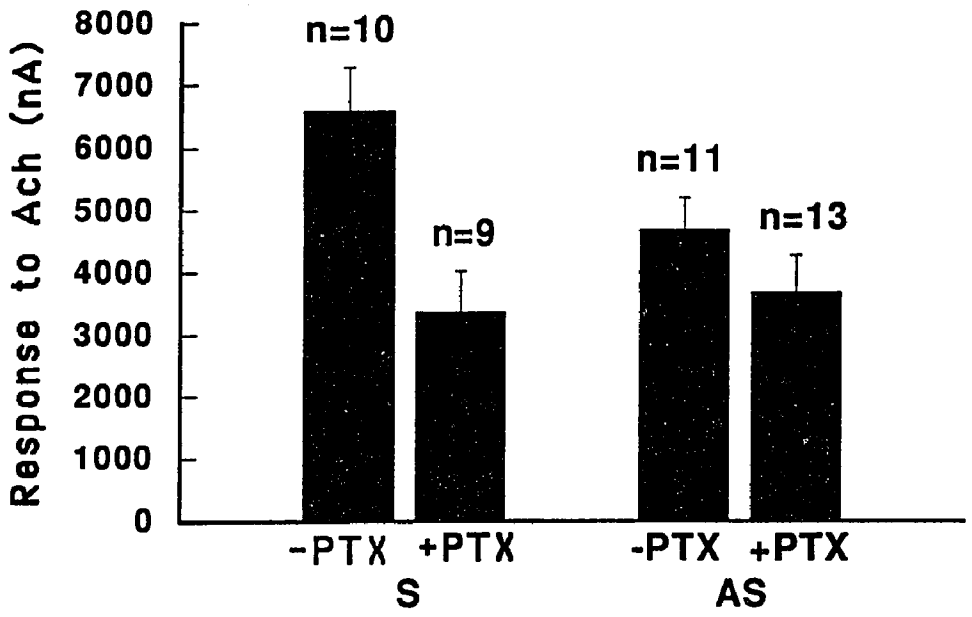


Figure 5.6. Effect of pertussis toxin treatment on the M_1-muscarinic receptor-evoked Cl^- current response in oocytes injected with sense (S) or antisense (AS) oligonucleotides. 100 pg of rat M_1-muscarinic receptor mRNA was injected into each oocyte. Two days later, 50 nl oligonucleotides to PLC-$X\beta$ were injected into oocyte to yield a final concentration of 2 μM assuming uniform distribution. Another 20-24 hours later, Cl^- currents were evoked by bath application of 10 μM acetylcholine (Ach). Where indicated, oocytes were also pretreated with 10 μg/ml pertussis toxin overnight. Currents were recorded at peak depolarization. Number of oocytes in each group is indicated. Cl^- current values are mean ± S.E.

3. Degradation of PLC-*X*β mRNA by antisense oligonucleotide:

I investigated whether the inhibition of Cl- current by antisense oligonucleotides was due to degradation of PLC-*X*β mRNA. Solution hybridization/RNase protection assay, a quantitative method for estimation of the amount of RNA, was used to determine the effect of injection of sense or antisense oligonucleotides on the stability of PLC-*X*β mRNA.

Total RNA was extracted from 100 oocytes 4h after injection with either sense or antisense oligonucleotides. The 4h point was chosen because Melton and co-workers have shown that significant degradation of specific message is seen by this time after injection of corresponding antisense oligonucleotides (Woolf et al., 1990). A fragment encoding the region 580-1230 of PLC-*X*β was used as the probe. Five μg of total RNA was hybridized with ^{32}P-labeled cRNA probe and digested with RNase A and RNase T1. The protected RNA-RNA duplexes were then resolved on a polyacrylamide gel and visualized by autoradiography. Most of the PLC-*X*β mRNA (~85% as estimated by densitometry) appears to be degraded in oocytes injected with antisense oligonucleotides (Fig. 5.7).

Figure 5.7. RNase protection assay of PLC-Xβ mRNA in oocytes injected with either sense (S) or antisense (AS) oligonucleotides. 100 oocytes/group were injected with oligonucleotides. Total RNA was extracted 4 h later. Five μg of total RNA for each group was hybridized

with ^{32}P-labeled cRNA probe complementary to nucleotides 580-1230. RNase protected fragments were resolved on 5% polyacrylamide gel. Picture of a 48-h autoradiogram is shown in the upper panel. Lower panel shows ethedium bromide staining of 5 μg of total RNA separated on 1% agarose/formaldehyde gel.

Chapter 6

In vitro studies of PLC-Xβ

1. Development of antibody for PLC-*X*β:

Antibodies were raised against PLC-*X*β in order to facilitate further functional studies. There is a family of closely related PLC-βs that share conserved amino acid sequences. Isolation of cDNA clones yield information on primary structure of various proteins. This allows us to generate sequence-specific antibodies which would only recognize one PLC-β.

Three peptides were selected in order to develop PLC-Xβ specific antisera. Two criteria were used in selecting the peptides. First and more importantly, their sequences are distinct for PLC-Xβ while the sequences at the corresponding regions are quite divergent for other known PLC-βs. Second, they contain several hydrophilic residues which make them more antigenic. Sequences of the three peptides and corresponding sequences in the other known mammalian PLC-βs are shown in Fig. 6.1.

For peptide #1:

PLC-Xβ	SPGDSSDEATRL	1199-1210
PLC-β1	GENPGKEFDTPL	
PLC-β2	AKADAQE--SRL	
PLC-β3	ADSESQEENTQL	
PLC-β4	----DRRPATVV	

For peptide #2:

PLC-Xβ	KIEEKPPKYTKPR	526-538
PLC-β1	----------	
PLC-β2	GTE----------	
PLC-β3	VGLEKTS--LEPQ	
PLC-β4	QEEEAHPEY-KFG	

For peptide #3:

PLC-Xβ	QPFNKD----------KIDWD	871-881
PLC-β1	EEVKKEADPGETPSEAPSEARPT	
PLC-β2	E----------KPF	
PLC-β3	AQASTEMCQETPSQQQGSQLSSN	
PLC-β4	D----------IADVPSDTSKN	

Figure 6.1. Selection of peptides for anti-PLC-*X*β antibody development. Sequences are compared for PLC-*X*β and all known mammalian PLC-βs at the corresponding regions. Positions of the three peptides for PLC-*X*β are shown at the right of the sequence.

All three peptides were synthesized with a cysteine residue at the C-terminus. The peptides were coupled to the carrier protein KLH through the C-terminal cysteine using MBS as the coupling reagent. One rabbit was immunized for each peptide by Pocono Rabbit Farm & Laboratory according to their standard protocol. Test bleeding was performed 70 days after initial peptide injection. The quality and titer of the antisera were tested by immunoblotting.

Xenopus oocyte extracts were prepared as described in "Materials and Methods" and used as the source of PLC-*X*β protein. *Xenopus* oocyte extracts were separated on 8% SDS-PAGE and transferred to Immobilon-P membranes. The membranes were blotted with preimmune serum, different dilution of antipeptide immune serum, or immune serum that had been preincubated with the corresponding peptides. The anti-peptide#3 immune serum recognized a 140 kDa protein in

the oocyte extracts. This protein was not recognized by either the preimmune serum or immune serum that had been blocked by the antigen peptide (Fig. 6.2). The size of this protein was in accordance with the predicted size of PLC-*X*β. This suggests that the protein recognized by the immune serum represents the native PLC-*X*β expressed in the oocytes.

This immune serum also specifically recognized two other 100 kDa and 65 kDa protein, which can be blocked by preincubation with the antigen peptide. These two proteins may represent the proteolytic fragments of intact PLC-*X*β protein. Both the preimmune and immune serum recognized a protein of ~55 kDa, which could not be blocked by preincubation with the antigen peptide. Therefore, this recognition was not specific for the antipeptide antibodies.

Only one rabbit was immunized for each peptide. Unfortunately, the antisera against peptide #1 and #2 were not immunoreactive against PLC-*X*β, even when 1:100 dilution of antiserum was used for immunoblot (data not shown).

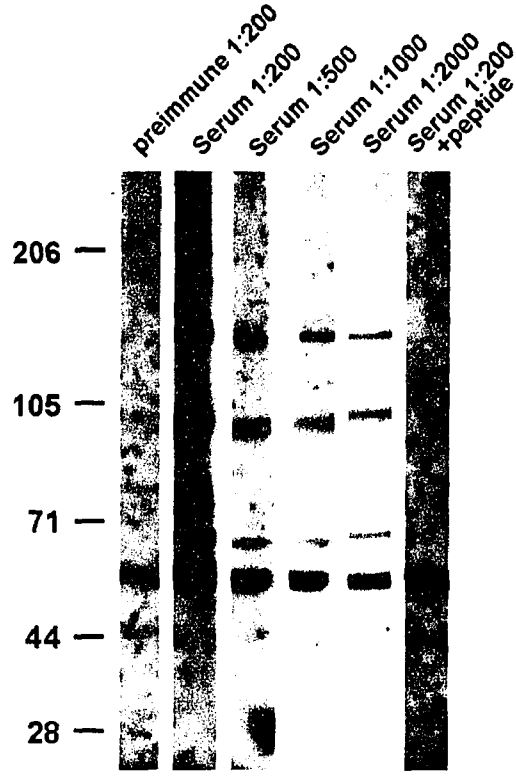


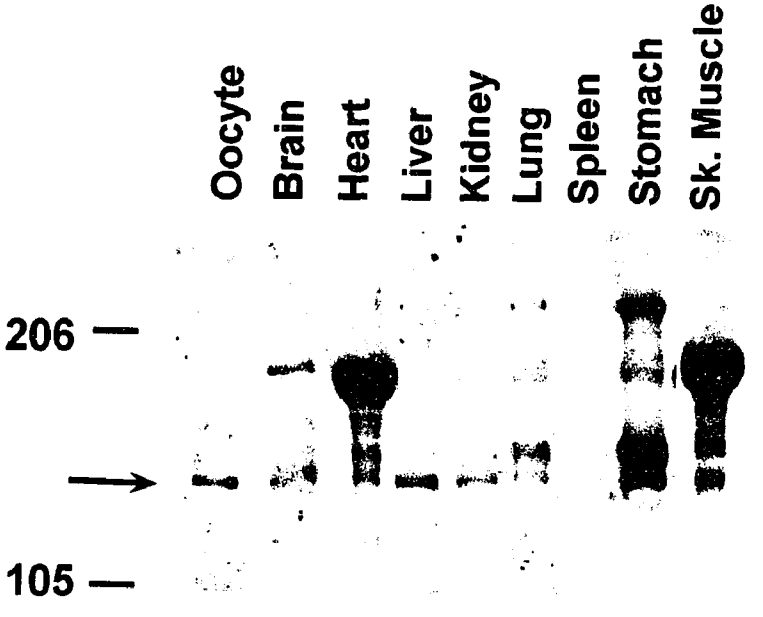
Figure 6.2. Immunoreactivity of anti-peptide#3 preimmune or immune serum against *Xenopus* oocyte extract. 100 μg of Xenopus oocyte extract/lane were separated on 8% SDS-PAGE and transferred to Immobilon-P membrane. The membranes were blocked and exposed to the different primary sera. When indicated, the 1:200 serum was preincubated with 100 μM antigen peptide overnight at 4°C.

The tissue distribution of PLC-*X*β was studied by immunoblot. A protein of 140 kDa was recognized in all tissues examined (Fig. 6.3A). The immunoreactivity was blocked by

preincubation of the serum with peptide (Fig. 6.3B). Therefore, this 140 kDa protein represents the PLC-*X*β and PLC-*X*β is ubiquitously expressed.

The serum also recognized several other proteins with different sizes in several tissues. They might represent nonspecific binding, or alternatively processed PLC-*X*β, or proteins related to PLC-*X*β. To address this question, the same tissue extracts were tested with preimmune serum and immune serum that had been preblocked with peptide. Except the 140 kDa protein mentioned above, all other proteins can be recognized by the preimmune serum as well as the preblocked immune serum. Therefore, the recognized proteins other than PLC-*X*β represent nonspecific binding.

6.3 A



6.3 B

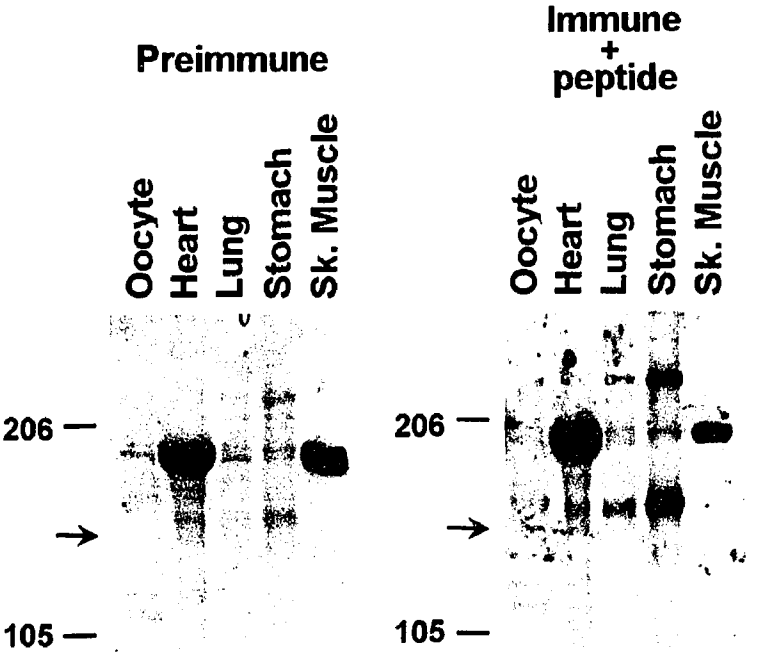


Figure 6.3. Tissue distribution of PLC-*X*β. 50 μg tissue extracts were separated on 8% SDS-PAGE and transferred to Immobilon-P membrane. The membrane was incubated with immune serum raised against peptide #3 (A), preimmune serum, or immune serum preblocked with antigen peptide (B). All primary sera were diluted 1:1000 for blotting. Arrow indicates the position of 140 kDa PLC-*X*β. When indicated, the serum was preincubated with 250 μM antigen peptide overnight at 4°C.

The specificity and crossreactivity of the antiserum against peptide #3 was studied. Cos-7 cells were transfected with PLC-β1, β2, β3, β4 and Xβ cDNA respectively by Dr. Anna

Aragay. Total cell extracts were prepared and immunoblotted with anti-PLC-Xβ antidody. The antibody only recognized PLC-Xβ. It did not recognize the other PLC-βs (Fig. 6.4).

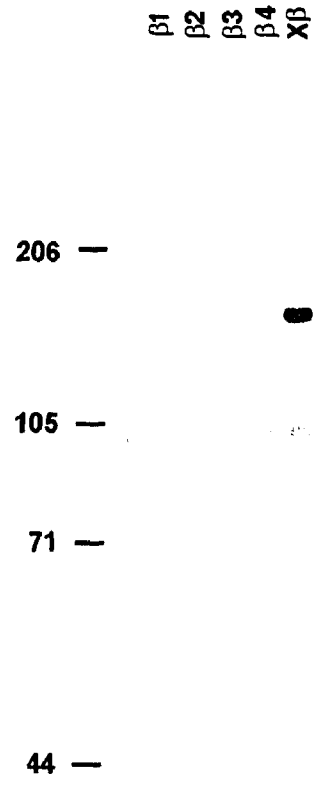


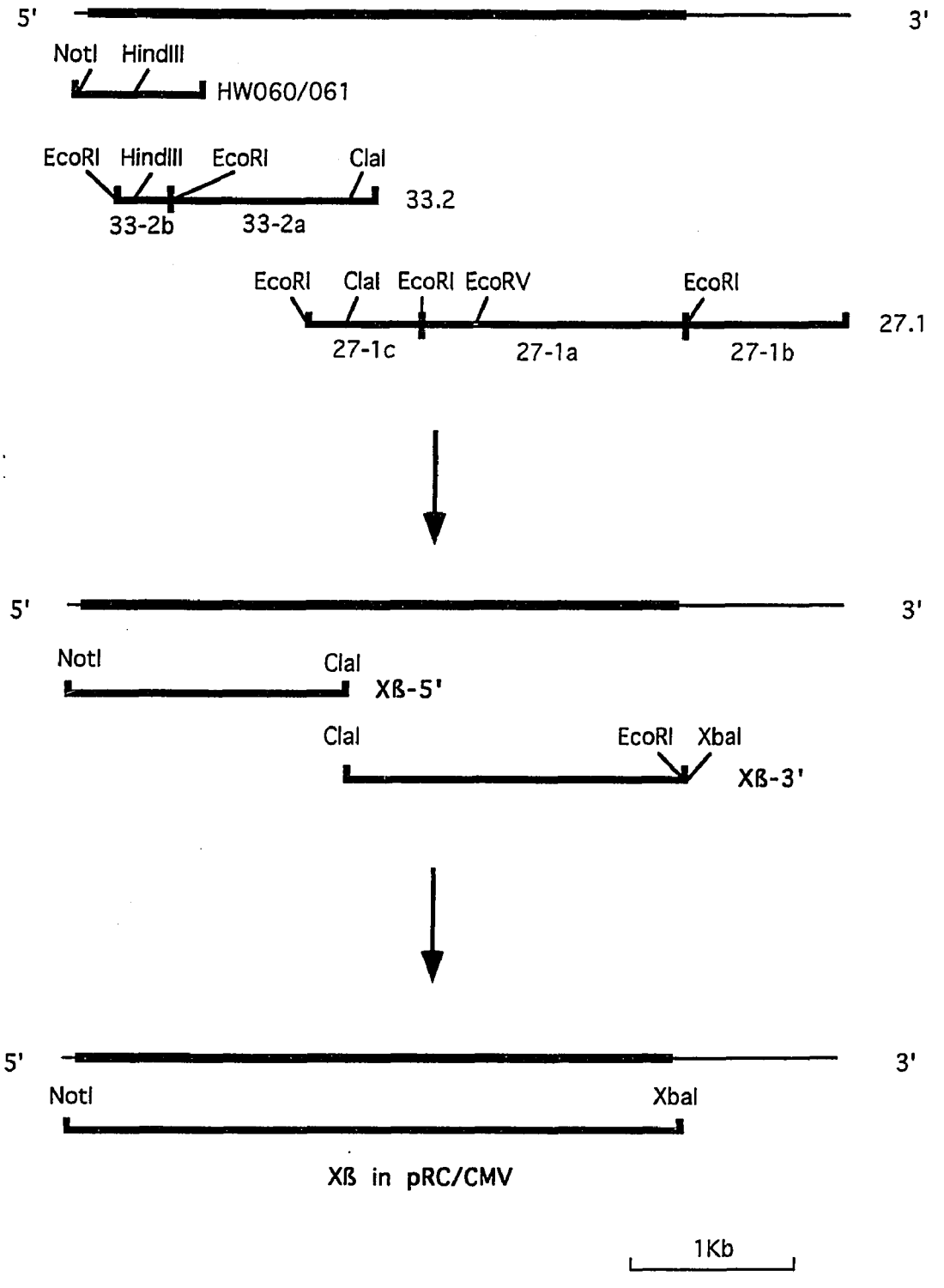
Figure 6.4. Specificity of anti-PLC-Xβ antibody against Cos-7 expressed PLC-βs. Cos-7 cells were transiently transfected with PLC-β1, β2, β3, β4 and Xβ cDNA respectively by lipofection. Cells were directly homogenized in 1x SDS sample buffer. 20 μl cell extracts/lane were separated on 8% SDS-PAGE and immunoblotted with anti-PLC-Xβ antidody.

2. Expression of PLC-*X*β in Sf9 cells:

Detailed functional studies require large amount of PLC-*X*β protein. However, it is very time-consuming and technically demanding to purify sufficient amounts of PLC-*X*β from tissues. Furthermore, several closely related PLC-βs may be present in one cell type. Their close structural and functional similarities may make their biochemical resolution difficult. A variety of prokaryotic and eukaryotic host/vector systems have been developed for the production of large quantities of proteins from cloned genes. Among them, the baculovirus expression system has been used for the expression of a wide variety of heterologous genes (Luckow and Summers, 1988). Under the transcriptional control of the polyhedrin promoter in the recombinant baculovirus, large quantities of proteins can be produced in the cultured insect cells with proper modifications. Therefore, PLC-*X*β was expressed in Sf9 insect cells using the baculovirus expression system.

First, the full length PLC-*X*β cDNA was introduced into the transfer vector pVL1392. The unique restriction sites *Not* I, *Hind* III, *Cla* I, *EcoR* V, and *Xba* I were used to assemble and subclone the full length PLC-*X*β cDNA into pRC/CMV. PLC-*X*β cDNA was then removed from pRC/CMV and inserted into pVL1392. The strategy for constructing the baculovirus expression vector for PLC-*X*β is shown in Fig. 6.5 and explained in detail in "Materials and Methods".

6.5 A


5'
3'
NotI
HindIII
HW060/061
EcoRI
HindIII
EcoRI
ClaI
33.2
33-2b
33-2a
EcoRI
ClaI
EcoRI
EcoRV
EcoRI
27.1
27-1c
27-1a
27-1b
5'
3'
NotI
ClaI
Xß-5'
ClaI
EcoRI
XbaI
Xß-3'
5'
3'
NotI
XbaI
Xß in pRC/CMV
1Kb

6.5 B

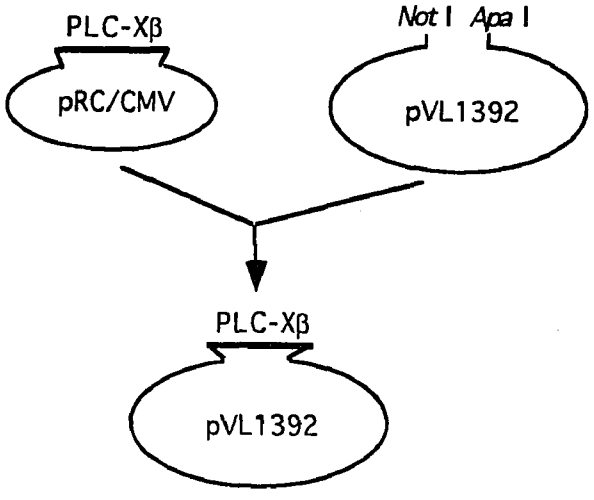


Figure 6.5. Strategy for constructing baculovirus expression vector for PLC-*X*β. (A). Strategy for assembling and subcloning of full length PLC-*X*β cDNA into vector pRC/CMV. Top line indicates the full length cDNA with the thick portion representing the open reading frame. Individual clones and restriction sites used for assembly are illustrated. (B). PLC-*X*β cDNA was excised from pRC/CMV and ligated into pVL1392.

The recombinant baculovirus was generated by Dr. Ronald Magnusson. Recombinant virus was plaque-purified and confirmed by Southern analysis with PLC-*X*β cDNA as the probe.

Sf9 cells were infected with baculovirus encoding PLC-*X*β. Cell extracts were prepared 48 hr post infection. As shown in Fig. 6.6, PLC-Xβ was expressed as a single protein with an apparent molecular mass of ~145 kDa. The immunoreactive

band was not detected in noninfected cells or cells infected with adenylyl cyclase 2.

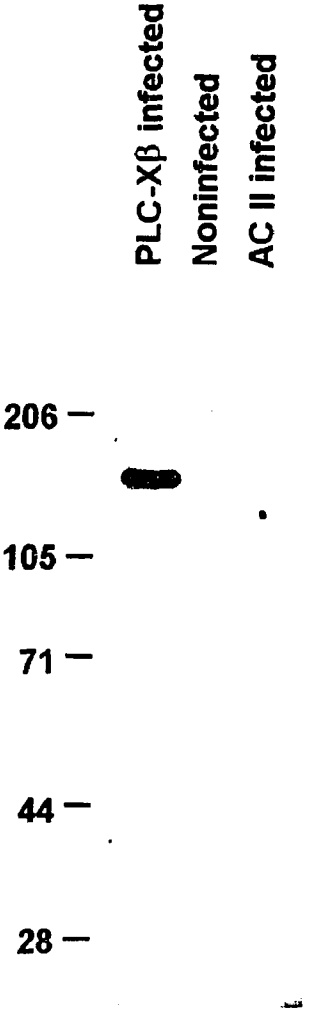


Figure 6.6. Expression of PLC-*X*β in Sf9 cells. Sf9 cells were infected with baculovirus encoding PLC-*X*β or adenylyl cyclase 2 (AC II). Cell extract was prepared 48 hr post infection. 30 μg cell extract/lane were separated on 8% SDS-PAGE and immunoblotted with anti-PLC-Xβ antiboby.

The subcellular distribution of PLC-Xβ in Sf9 cells was investigated. Two days post infection, the Sf9 cell homogenate was prepared either in the absence or in the presence of 300 mM NaCl. The homogenate was then separated into cytosolic (S) and particulate (P) fractions. As shown in Fig. 6.7, PLC-Xβ was detected in both the cytosolic and the particulate fractions. More PLC-Xβ was associated with the membrane function than with the cytosolic function. A large part of the membrane-associated enzyme can be solubilized by 300 mM NaCl extraction. The determination of the subcellular distribution of PLC-Xβ would aid in the selection of subcellular fractions for the future purification of the enzyme.

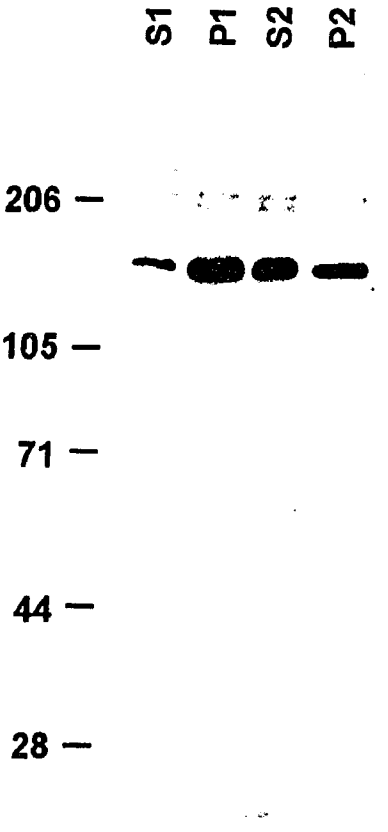


Figure 6.7. Subcellular distribution of PLC-Xb in Sf9 cells. Two days post infection, the Sf9 cells were homogenized in buffer either with or without 300 mM NaCl. The homogenate was then clarified by centrifugation at 100,000xg for 1 hour at 4°C. The resulting membrane pellet was resuspended in buffer containing 1% cholate. S1 and P1 denote the cytosolic (S) and particulate (P) fractions prepared in the absence of NaCl. S2 and P2 denote the cytosolic and particulate fractions prepared in the presence of 300 mM NaCl. 30 µg protein/lane were separated on 8% SDS-PAGE and immunobloted with anti-PLC-Xβ antibody.

In order to determine whether there is increased activity along with the expression of PLC-*X*β protein, the PLC activity of the Sf9 cell cytosol extraction was measured in the presence of 1 mM free Ca^{++}, which activates PLC directly. As shown in Fig. 6.8, there was considerable amount of PIP_2 hydrolyzing activity in the PLC-*X*β infected Sf9 cell cytosol. In contrast, the cytosol from non-infected or adenylyl cyclase 2-infected Sf9 cells did not show any measurable PLC activity.

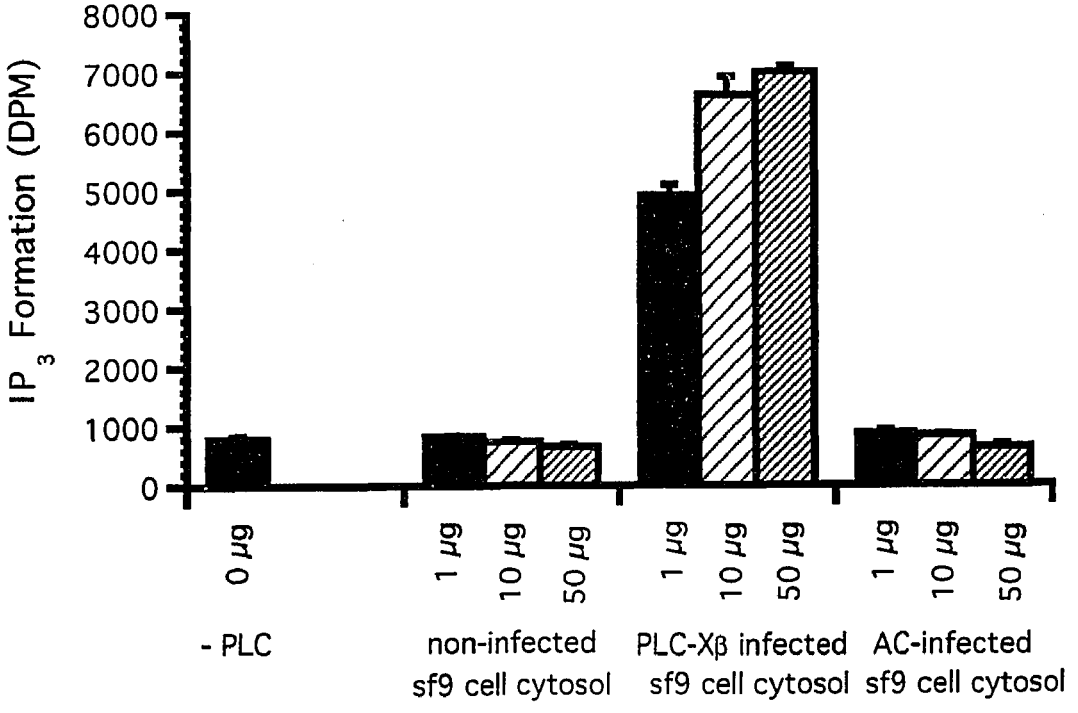


Figure 6.8. PLC activity in Sf9 cell extract. Four days post infection, Sf9 cell cytololic fractions were prepared as described under "Materials and Methods". Varying amount of cytosol was incubated with phospholipid vesicles in the presence of 1 mM free Ca^{++}. Assays were performed in 100 µl volume.

3. G protein-dependent regulation of PLC-*X*β:

The antisense oligonucleotide experiments indicate that PLC-*X*β, together with G_o, are involved in the receptor-mediated PLC pathway. However, it does not necessarily mean that PLC-*X*β is directly stimulated by α_o. Alternatively, our current data do not rule out the possibility that G proteins other than G_o, and PLCs other than PLC-*X*β, are also involved in this pathway. Therefore, PLC-*X*β could be stimulated by α_o or βγ or other G-protein α subunits. In vitro reconstitution assays were performed in order to understand how PLC-*X*β is regulated by G proteins.

It is clear now that both α and βγ subunits are capable of activating phospholipase Cs. Therefore, I tested α_o, α_q, and βγ-dependent regulation of PLC-*X*β. The Sf9 cell expressed PLC-*X*β was used for this study.

Membrane preparations containing the α subunits of G_o and G_q proteins were prepared from Cos-7 cells transfected with corresponding Gα cDNA and were used as the source of α_o and α_q. These membrane preparations were enriched for specific Gα proteins and had been washed with 1 M KCl to deplete endogenous PI-PLC. α_q and α_{11}-transfected Cos-7 membranes have been used to study activation of PLC-β1, β2, and β3 (Wu et al., 1992; Lee et al., 1992; John et al., 1993). The activation by membranes can be blocked by preincubation with corresponding anti-Gα antibodies (Wu et al., 1992).

$G\alpha_q$, $G\alpha_o$, and LacZ-transfected Cos-7 membranes were incubated with PLC-Xβ-infected Sf9 cell extract. GTPγs-dependent activation of PIP_2 hydrolysis was measured. As shown in Fig. 6.9, α_q significantly stimulated PLC-*X*β activity. However, α_o did not mediate any increase in PLC activity as compared to lacZ-transfected membrane.

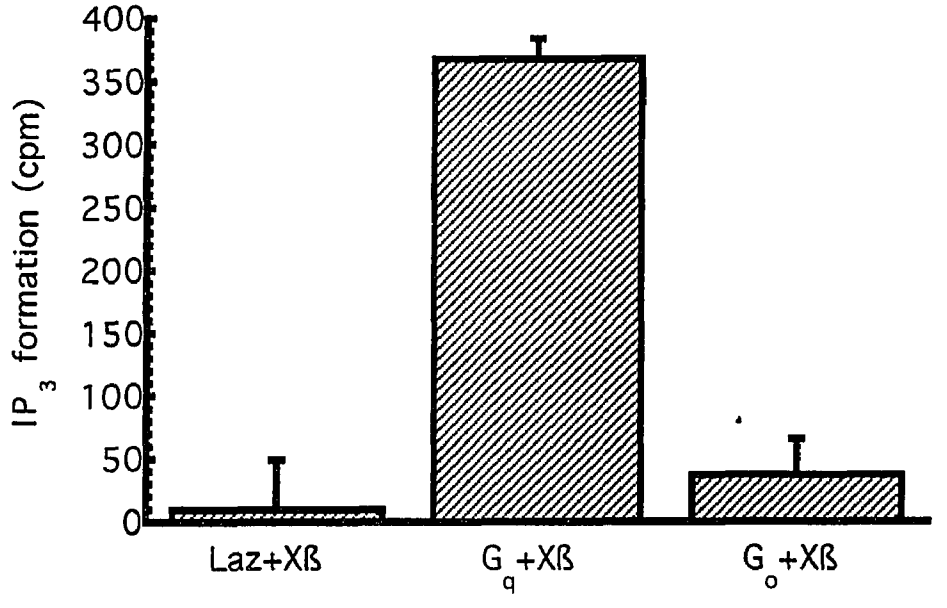


Figure 6.9. Activation of PLC-*X*β by Gα subunit-transfected membranes. $G\alpha_q$, $G\alpha_o$, and LacZ-transfected Cos-7 membranes (4.5 μg of protein) were incubated with PLC-*X*β-infected Sf9 cell extract (o.5 μg of protein). GTPγS-dependent activation of PIP_2 hydrolysis was measured as described under "Materials and Methods". Final free Ca^{++} concentration was 100 nM. Values are mean±S.E of triplicate determinations.

The effect of βγ subunits on PLC-*X*β activity was also studied. Purified bovine brain βγ subunits was incubated with varying amount of PLC-*X*β protein. In all circumstances, there was no significant increase in PLC-*X*β activity mediated by βγ subunits (Fig. 6.10).

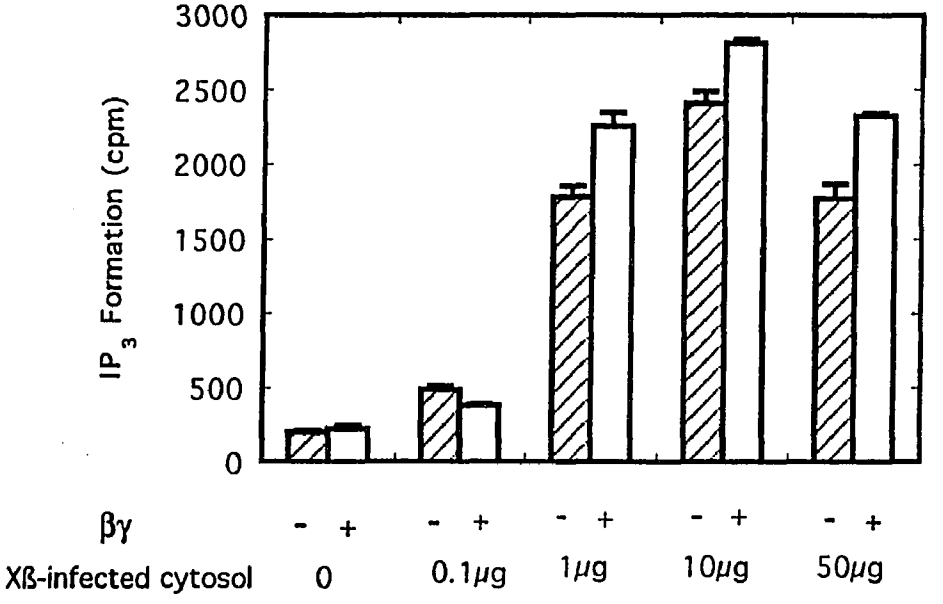


Figure 6.10. Effect of βγ subunits on the activity of PLC-Xβ. Indicated amount of cytosolic extract protein from PLC-*X*β-infected Sf9 cells were assayed for PLC activity in the presence or absence of 250 nM purified bovine brain βγ subunits. Final free Ca^{++} concentration was 100 nM. Values are mean±S.E.

PLC-110, the C-terminal truncated form of PLC-β3, was purified from bovine brain cytosol and was generously

provided by Dr. John Exton. PLC-110 has been demonstrated to be greatly stimulated by βγ subunits (Blank et al., 1993). I also tested the effect of purified βγ subunits on the activity of PLC-110 as the positive control. As shown in Fig. 6.11, PLC-110 was stimulated by βγ subunits by more than 10 fold, while no stimulation was observed for PLC-*X*β.

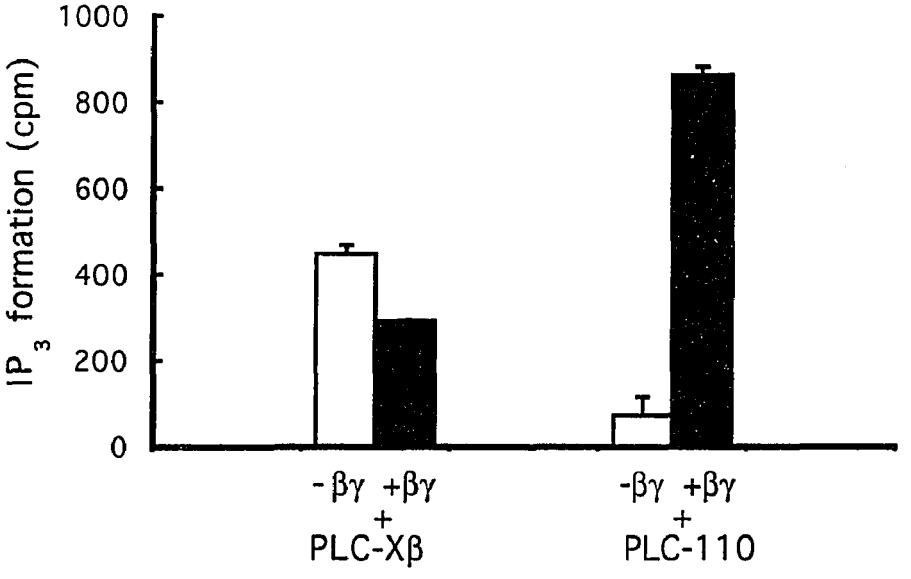


Figure 6.11. Effect of βγ subunits on PLC-*X*β and PLC-110. 10 μl of partially purified PLC110 and o.5 μg of PLC-*X*β-infected Sf9 cells extract were assayed for PLC activity in the presence or absence of 500 nM purified bovine brain βγ subunits. Final free Ca^{++} concentration was 100 nM. Values are mean±S.E.

Chapter 7

Discussion

Phospholipase C plays an important role in transmembrane signaling. This enzyme catalyzes the hydrolysis of plasma membrane phospholipid, PIP_2, generating two second messengers (IP_3 and diacylglycerol) in response to the binding of various ligands to their cell surface receptors. A number of distinct PLC enzymes have been molecularly cloned, sequenced, and purified. PLC isozymes can be divided into three types, namely β, γ, and δ, on the basis of their primary structure. Current studies indicate that receptor-mediated activation of PLC can be achieved by at least two distinct pathways. One pathway involves the activation of PLC-γs by growth factor and immune system receptors through tyrosine phosphorylation. The other pathway involves the activation of PLC-βs by G protein-coupled receptors through certain members of the large family of heterotrimeric G proteins (reviewed in Rhee and Choi, 1992).

There are two general pathways of activation of PLC mediated through G proteins as distinguished by their susceptibility or insensitivity to pertussis toxin inactivation. It is clear now that the pertussis toxin-insensitive mechanism is mediated by the α subunits of the G_q family. The G_q class includes four different α subunit gene products: α_q, α_{11}, α_{14}, and α_{16}. *In vitro* reconstitution experiments performed with purified proteins have demonstrated that the α subunits of G_q, G_{11}, and G_{16} can stimulate phospholipase C-βs (Hepler et al., 1993; Kozasa et al., 1993). Similar conclusions have been reached by transient transfection experiments where all α

subunits of the G_q family have been individually expressed (Lee et al., 1992; Jiang et al., 1994). All mammalian PLC-βs identified so far can be stimulated by α_q, which is similar to another well studied adenylyl cyclase system in which all adenylyl cyclases identified so far can be stimulated by α_s. The mechanism by which G_q activates phospholipase C-β appears to follow the well established model that agonist-activated receptors promote release of an activated α subunit, which then directly stimulates the effector protein.

Some receptor-mediated PIP_2 hydrolysis can be blocked or partially blocked by prior treatment with pertussis toxin. The PTX-sensitive PLC signaling pathway occurs notably in cells of hematopoietic origin such as neutrophils, macrophages, basophils, platelets, and HL-60 cells granulocytes, as well as in cells of other origins such as neuronal cells, renal cells, vascular smooth muscle cells, fibroblasts, endothelial cells and fat cells (Cockcroft and Thomas, 1992). The pertussis toxin sensitive mechanism for activation of phospholipase C is less well understood. Members of the G_i family, including $G\alpha_i$, $G\alpha_o$, and $G\alpha_t$ are sensitive to PTX modification. However, there is no evidence that phospholipase C can be directly activated by α subunits of G_i or G_o *in vitro*. An alternative mechanism has been postulated based on the finding that βγ subunits can activate PLC-β2 and β3 directly (Camps et al., 1992; Katz et al., 1992; Carozzi et al., 1993). Thus, agonist-occupied receptors activate heterotrimeric G_i or G_o and promote release of βγ subunits, and the βγ subunits can stimulate the effector.

The objective of this research project was to study how pertussis toxin-sensitive G proteins regulate PLC activity. At the time this project was initiated, reports on $G\alpha_q$-dependent PLC activation were just coming out. However, little was known about components involved in the PTX-sensitive PLC pathway. For a decade, the *Xenopus* oocyte has been a useful system for the study of receptor stimulated PLC pathway. A major component of the phospholipase C response in the oocytes is PTX-sensitive, whereas very few mammalian systems use PTX-sensitive PLC pathway. Several lines of evidence indicate that G_o may be the signal transducer in PLC activation in *Xenopus* oocytes (Moriaty et al., 1990; Padrell et al., 1991; Blitzer et al., 1993). Therefore, it seemed likely that the *Xenopus* oocytes contain a G_o-responsive phospholipase C.

In order to better understand the PTX-sensitive PLC pathway, I decided to determine the identity of the PLC involved in *Xenopus* oocyte and study its G protein-dependent regulation.

A new β-type phospholipase C was isolated from a *Xenopus* oocyte cDNA library. In phylogenetic analysis, the newly cloned *Xenopus* PLC-β appears to fit well in the mammalian branch and is substantially divergent from the two *Drosophila* β-type PLCs. Within the mammalian branch, PLC-*X*β is closest to PLC-β3. However, it does not appear that PLC-*X*β is the *Xenopus* homologue of PLC-β3. Generally, identity is very high between mammalian and *Xenopus* forms for the same signaling component. *Xenopus* α_q is 95% identical to the

mammalian α_q (Quick et al., 1994). *Xenopus* α_o as well as *Xenopus* IP_3 receptor are 90% identical to their mammalian forms, respectively (Olate et al., 1989; Kume et al., 1993). In contrast, PLC-*X*β is only 64% identical to PLC-β3 and 58% identical to PLC-β1. These numbers support the notion of a distinct identity for PLC-*X*β. The functional properties of PLC-*X*β also indicate that it is more likely to be a distinct form.

The data presented here indicate that PLC-*X*β is a component of the G protein-receptor regulated phospholipase C pathway in *Xenopus* oocytes. I tested the effect of antisense oligonucleotide to PLC-*X*β on three receptor-mediated PLC responses: angiotensin II receptor, α_{1b}-adrenergic receptor, and M_1-muscarinic receptor. Receptor-evoked Cl^- current response was inhibited 40-60% by injection of antisense oligonucleotides, but not sense oligonucleotides or water. The partial ablation of the receptor response may be due to the slow turnover of the PLC-*X*β protein, or the involvement of additional PLC-β(s) in the same pathway, or both.

Injection of IP_3 evoked similar Cl^- current responses in oocytes injected with either sense or antisense oligonucleotides. This indicates that the antisense oligonucleotide treatment affects a step prior to the IP_3 generation. Release of Ca^{++} into cytosol and following steps to open of Cl^- channels are not affected. Solution hybridization/RNase protection assay demonstrated that ~85% of the PLC-*X*β mRNA was degraded in oocytes injected with antisense oligonucleotides. These data indicate that the inhibitory effect of the antisense

oligonucleotides was most probably due to the inhibition of PLC-*X*β expression. However, it should be noted that sometimes oligonucleotides can bind to targets other than one desired, and hence cause "nonspecific" effects. I think it is unlikely to be the case here, since the sense oligonucleotides do not have any effect compared to water-injected controls (Fig. 5.2), and the antisense oligonucleotides to PLC-*X*β have differential effects on the Cl^- current responses evoked by $5HT_{1c}$ receptor and TRH receptor (Quick et al., 1994).

The PLC response mediated by angiotensin II receptor, α_{1b}-adrenergic receptor, and M_1-muscarinic receptor are all pertussis toxin-sensitive in *Xenopus* oocyte. G_o has been implicated as the signal transducer in the pertussis toxin-sensitive phospholipase C pathway in *Xenopus* oocyte based on the following observations. (1). Injection of purified heterotrimeric G_o enhances the native muscarinic receptor-evoked Cl^- current, as does the injection of *E. coli*-expressed α_o together with purified βγ subunits (Moriarty et al., 1990; Padrell et al., 1991). (2). Direct injecton of GTPγS-activated α_o subunits evokes the Cl^- current, whereas injection of activated α_{i1}, α_{i2}, or α_{i3} has no effect (Moriarty et al., 1990). On the other hand, acute injection of functionally active βγ subunits do not evoke a Cl^- current in several attempts (Blitzer, Landau, and Iyengar, unpublished data). Injection of βγ subunits inhibits the native muscarinic receptor evoked response in a dose dependent manner (Moriarty et al., 1988). (3). Antisense oligonucleotides to the *Xenopus* α_o significantly reduce the Cl^-

current response evoked by α_{1b}-adrenergic receptor (Blitzer et al., 1993). (4). A dominant negative mutant of α_o, which lost its GTPγS binding but retained its capacity to interact with $\beta\gamma$ subunits, significantly decreased the TRH-induced Cl^- current response in a dose-dependent manner (Slepak et al., 1993). (5). GAP-43, a neuronal protein enriched in the axonal growth cones, is thought to play a role in determining the growth cone motility by modulating signal transduction cascades in the growth cones. GAP-43 can activate purified G_o by increasing guanine nucleotide exchange and steady state GTP hydrolysis. It is found that GAP-43, when injected into *Xenopus* oocytes, dramatically increases Cl^- current in response to the native muscarinic receptor and expressed $5HT_{1c}$ serotonin receptor stimulation. Higher levels of GAP-43 alone cause a transient Cl^- current even in the absence of receptor stimulation (Strittmatter et al., 1993). All these findings support the notion that G_o, most likely through the α subunit of the native protein, can activate phospholipase C in oocytes.

The relationship between G_o and the newly cloned PLC-*X*β was investigated. The α_{1b}-adrenergic receptor-evoked response was inhibited by both antisense oligonucleotides to α_o and PLC-*X*β, suggesting that α_o and PLC-*X*β are probably involved in the same signaling pathway. Inhibition of M_1-muscarinic receptor-evoked Cl^- current by pertussis toxin and PLC-*X*β are not additive, indicating that PLC-*X*β is responsible for transducing the pertussis toxin-sensitive component of the M_1-muscarinic signal. Further evidence supporting the notion

that PLC-*X*β is downstream of $G_{\alpha o}$ comes from the study performed by Quick and his co-workers (Quick et al., 1994). In their study, the serotonin 1c ($5HT_{1c}$) and thyrotropin-releasing hormone (TRH) receptors are expressed in the same *Xenopus* oocyte by coinjection of the corresponding mRNAs. 5HT-induced Cl^- currents were reduced 80% by PTX pretreatment. TRH-induced Cl^- currents were reduced only 20% by PTX pretreatment. The specificity for a given receptor-G protein-effector coupling was examined. 5HT-induced Cl^- currents were significantly inhibited by antisense oligonucleotides to α_o as well as to PLC-*X*β, but not by antisense oligonucleotide to α_q. In contrast, TRH-induced Cl^- currents were significantly inhibited by antisense oligonucleotides to α_q, but not by antisense oligonucleotide to α_o and PLC-*X*β. Taken together, these results indicate that PLC-*X*β is regulated by G_o in the native oocytes and represents a new effector in the pertussis toxin-sensitive PLC pathway. It seems that G_q does not act through PLC-*X*β, at least in the TRH receptor signaling pathway expressed in oocytes.

G protein-dependent regulation of PLC-*X*β activity was studied by another approach, i.e. *in vitro* reconstitution assay, in order to confirm the results obtained from *in vivo* studies. In this line of experiments, PLC-*X*β was shown to be stimulated by α_q, similar to all of the other cloned mammalian PLC-βs. This result seems to be in contrast to the finding of Quick et al. mentioned above. It should be remembered, however, that the conditions for the *in vitro* assay do not necessarily reflect the

actual conditions inside the living cell. Changes in the relative concentration of receptors, G proteins, and effectors or co-localization of individual receptors with G proteins and effectors in a given cell type may influence the particular signaling pathway for a given receptor.

α_o as well as $\beta\gamma$ subunits were unable to stimulate PLC-*X*β in the *in vitro* reconstitution experiments. The inability of $\beta\gamma$ subunits to stimulate PLC-*X*β is somewhat expected because acute injection of functionally active $\beta\gamma$ subunits do not evoke a Cl^- current in *Xenopus* oocyte (Blitzer, Landau, and Iyengar, unpublished observation). This observation provides evidence that PLC-*X*β is functionally distinct from PLC-β3 despite their close structural similarity, since $\beta\gamma$ subunits are able to stimulate PLC-β3. Close examination of their amino acid sequences and "domain-swap" experiment may help to elucidate the region(s) responsible for $\beta\gamma$ interaction.

Current data indicate that PLC-*X*β may be regulated by G_o in the intact oocyte but not *in vitro* . One explanation for this is that the regulation of PLC-*X*β by G_o is not direct. An additional, as yet unknown, component(s) may be required to communicate signals from G_o to PLC-*X*β. This intermediate component(s) may be missing in the *in vitro* assay, and hence the stimulation by α_o was not observable. Further studies are needed to determine whether this hypothesis is correct.

With the progress of modern molecular biology techniques, it is becoming apparent that there is high molecular diversity for receptors, G proteins, and various effectors. A

large number of distinct signal transduction pathway combinations can be formed given the fact that many different receptors, G proteins, and effectors are often present in the same cell. This allows a particular cell to make distinct adjustments in response to different stimuli. Different modes of action may operate in the receptor-mediated enzyme activation. For example, while most effectors are regulated by G proteins directly, it is also possible that some effectors are regulated by G proteins indirectly. The indirect regulation of an effector by G protein, as proposed for regulation of PLC-*X*β by α_o, is not unprecedented. Two examples are given below.

Eight distinct adenylyl cyclases have been isolated from mammalian cells. All of them can be stimulated by α_s (Iyengar, 1993). Type 2 adenylyl cyclase is unique in that its basal activity is prominently stimulated by PKC activation (Jacobowitz et al., 1993; Yoshimura and Cooper, 1993; Lustig et al., 1993). Therefore, even though type 2 adenylyl cyclase cannot be stimulated by α_q directly, it can be stimulated by α_q indirectly through the activation of PKC within an intact cellular context.

Phospholipase A_2 (PLA_2) hydrolyzes phosphatidylcholine and phosphatidylethanolamine, releasing arachidonic acid. For many years, it has been known that PLA_2 is stimulated by G proteins and this effect is pertussis toxin sensitive. However, the nature of the G proteins that regulate PLA_2 remains uncertain and direct coupling between G protein and PLA_2 has not been demonstrated (Exton, 1994). Recently PLA_2 was

shown to be a substrate of MAP kinase. Phosphorylation of a specific serine residue by MAP kinase leads to activation of PLA_2 (Lin et al., 1993; Nemenoff et al., 1993). There is also evidence that activation of certain G proteins, including α_i, and $\beta\gamma$ subunits, leads to activation of MAP kinase (Winitz et al., 1993; Alblas et al., 1993; Crespo et al., 1994). Therefore, it is possible that G protein regulation of PLA_2 may be mainly indirect and the effect is mediated through MAP kinase.

Similar to the examples described above, PLC-*X*β appears to be regulated by α_o indirectly. The exact mechanism is not clear right now. It could be a protein kinase that is activated by α_o, which in turn phosphorylates and activates PLC-*X*β. It could also be another kind of protein that is activated by α_o, which in turn helps to recruit PLC-*X*β to the plasma membrane. Regardless of the nature of the intermediate component(s), discovery of the protein which lies immediately downstream of α_o will undoubtedly be very helpful for a fully understanding of this unique pertussis toxin-sensitive phospholipase C pathway. The yeast two-hybrid system, which is based upon direct protein-protein interaction, may be useful in such characterizations.

The new model for the pathway utilized by G_o to trigger PLC-dependent Cl^- conductance in response to agonist stimulation is illustrated in Figure 7.1.

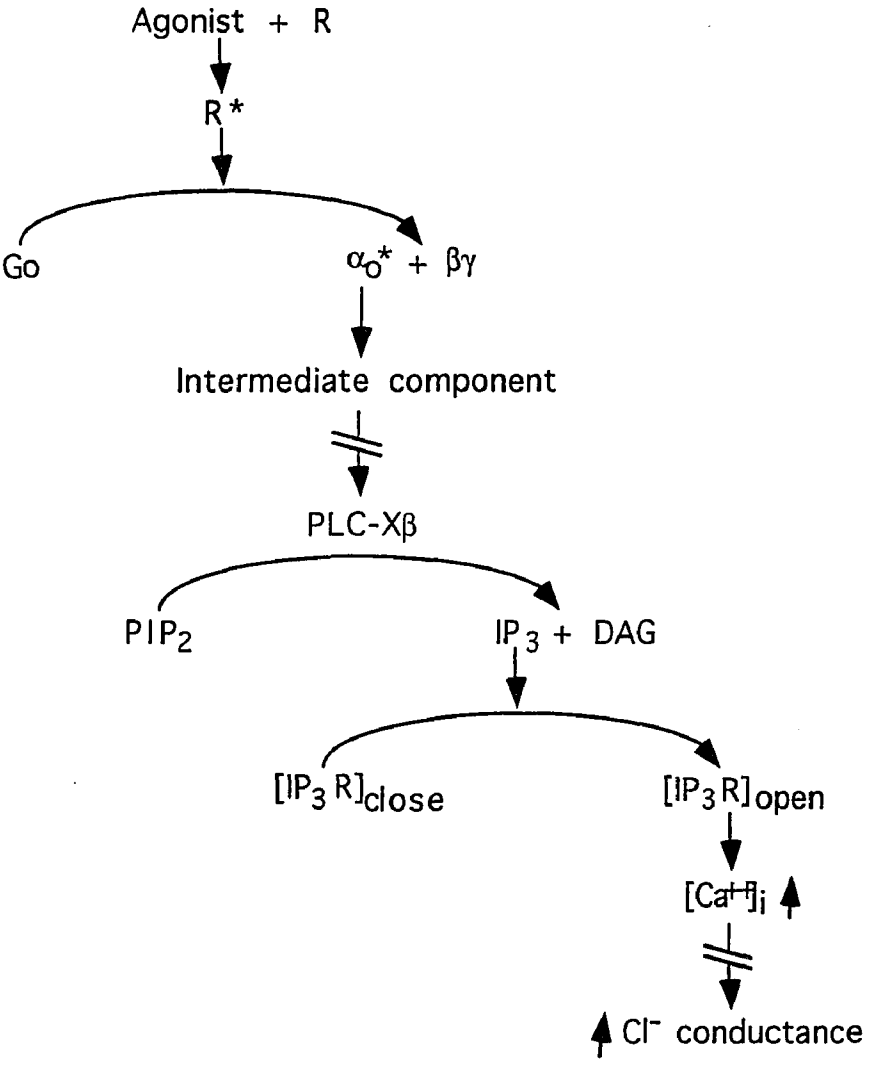


Figure 7.1. The pathway utilized by G_o to trigger PLC-dependent Cl^- conductance in response to agonist stimulation. Some as yet unknown component(s) may communicate the signal flow from α_o to PLC-Xβ. Break in vertical arrows indicates that there is currently no direct evidence for direct linkage between the two steps.

G_o was originally isolated from bovine brain as the major PTX-substrate from brain. It may account for 1.5% of total membrane protein of brain (Sternweis and Robishaw, 1984; Neer et al., 1984). The prominence of G_o suggests a role in brain function. Many neurotransmitters are coupled to pertussis toxin-sensitive G proteins through their cell surface receptors. However, the specific cellular function of G_o is still unknown. The results presented in this research dissertation indicate that G_o, through intermediate component(s), activates a β-type phospholipase C in *Xenopus* oocytes. The interactions occur in the oocytes may also occur in the brain. If they do, my identification of the PLC-*X*β regulated by G_o and subsequent deciphering the specific pathway will add more information to our understanding of the mechanisms that underlie the complex events that happen in brain. Study of the specific function of G_o may of potential clinical use. For example:

G_o is one of the most prominent proteins in neuronal growth cone membranes (Strittmatter et al., 1990). The growth cone is the motile structure at the tip of elongating axons and dendrites, which is responsible for transduction of extracellular signals and directing neurite growth in the target area (Lockerbie, 1987). G_o has been suggested to play an important role in neurite development. A group of membrane-bound target-derived factors have been described to cause growth cone collapse. Growth cone collapse induced by these factors is blocked by pertussis toxin (Igarashi et al., 1993). In other studies, a large increase in intracellular Ca^{++} concentration is

shown to be necessary for neurite growth inhibitor-induced growth cone collapse (Cohan et al., 1987; Bandtlow et al., 1993). Currently, we do not know whether the increase in $[Ca^{++}]_i$ is due to activation of phospholipase C. In future studies, it will be interesting to see whether neurite growth inhibitor-induced growth cone collapse is accompanied by changes in phosphoinositide metabolism. It will also be interesting to see whether PLC-*X*β or a mammalian homologue is localized in the growth cone.

Axons regenerating within the adult mammalian central nervous system is generally limited to 1 mm (Patterson et al., 1988). This makes it difficult for the victims of neurological trauma or stroke to recover full function. Since G_o is important for neurite development, understanding of the specific pathway and mechanism of G_o function will be helpful in developing modifying agents which may have therapeutic potential on nerve regeneration.

Chapter 8

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