UNIVERSITY OF CALIFORNIA, SAN DIEGO

Mechanism of Membrane Binding of the C2 Domains of Conventional Protein Kinase C Isoforms

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Angela Michelle Scott

Committee in charge:

Professor Alexandra C. Newton, Chair
Professor Joe Adams
Professor Edward A. Dennis
Professor Jeff Esko
Professor Gourisankar Ghosh

2006

This dissertation of Angela Michelle Scott is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego
2006

DEDICATION

To my father, Michael, for his love and encouragement in everything I do and to my grandmother, Jean Reese, for always supporting and worrying about me.

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VITA

2000	B.S., Biochemistry/Molecular Biology, University of Wisconsin, Eau Claire
2006	Ph.D., Biomedical Sciences, University of California, San Diego

AWARDS AND HONORS

2004	American Society of Biochemistry/Molecular Biology Travel Award
2000-2003	Pharmacology NIH Training Grant
2000	Outstanding Student Award, College of Arts & Sciences, University of Wisconsin-Eau Claire
2000	Outstanding Senior, Chemistry Department, University of Wisconsin-Eau Claire

PUBLICATIONS

Scott, A. and Newton, A.C. The Effects of Electrostatic and Hydrophobic Interactions on the Membrane Binding Kinetics of the C2 Domain of Protein Kinase C-α. In preparation.

ABSTRACTS

Scott, A. and Newton, A.C. Membrane Binding Kinetics of the C2 Domain Mutants of Protein Kinase C-α. Presented at 2004 ASBMB Meeting, San Diego, CA.

Scott, A., Nalefski, E.A., and Newton, A.C. Membrane Binding Kinetics of the Conventional C2 Domains of Protein Kinase C. Presented at 2004 ASBMB Meeting Boston, MA.

Scott, A., Nalefski, E.A., and Newton, A.C. Stopped-Flow Analysis of Membrane Binding Kinetics of the C2 Domain Mutants of Protein Kinase C. Presented at 2003 ASBMB Meeting, San Diego, CA.

Scott, A., Nalefski, E.A., and Newton, A.C. Membrane Binding Kinetics of the C2 Domain of Protein Kinase C βII: Contribution of Electrostatic and Hydrophobic Interactions. Presented at 2003 Biophysical Society Meeting, San Antonio, TX.

ABSTRACT OF THE DISSERTATION

Mechanism of Membrane Binding of the C2 Domains of Conventional Protein Kinase C Isoforms

by

Angela Michelle Scott

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2006

Professor Alexandra C. Newton, Chair

Protein kinase C (PKC) has a central role in responding to signals that cause lipid hydrolysis leading to both short- and long-term cellular responses. Membrane binding plays a key role in the activation of PKC. During activation, the regulatory C2 domain of the conventional PKCs target the kinase to membranes in response to elevated intracellular Ca^{2+}. Once the C2 domain poises the enzyme at the membrane, it can engage a second membrane-targeting module, the C1 domain, by binding to the second messenger diacylglycerol. These interactions provide the energy for an activating conformational change in which the pseudosubstrate sequence is released from its binding cavity of PKC, allowing substrate binding and phosphorylation.

In this dissertation, the kinetics and mechanism of the critical interaction between the C2 domain and membrane were examined. Combining biophysical,

biochemical, and cellular biology techniques, the roles of electrostatic and hydrophobic interactions in the kinetics and mechanism of C2 domain membrane binding and its effects on kinase activity were elucidated. These studies show that electrostatic interactions of specific residues play a role in the retention of the C2 domain at the membrane, while hydrophobic interactions of specific residues play roles in both the membrane recruitment and retention of the C2 domain. In addition, studies with varying ionic strength and Ca^{2+} have given further insights into the role of these determinants in C2 domain membrane binding. Comparative studies of the conventional PKC isoforms and their mutants further elucidate the binding kinetics and mechanism of C2 domain binding. These studies emphasize the importance of residue 249 (numbering of PKC conventional isoforms) in both electrostatic and hydrophobic interactions that stabilize the domain-membrane complex. Taking the studies into the context of the full-length PKC, the role of these electrostatic and hydrophobic interactions in determining the kinetics, amplitude, and duration of PKC activity in cells has been monitored using live cell imaging. The imaging illustrates the importance of these electrostatic and hydrophobic interactions translates from membrane binding to kinase activity in live cells. In summary, electrostatic and hydrophobic interactions play key roles on the membrane recruitment and retention of the C2 domain.

Chapter I

Introduction: PKC, C2 domains, and the membrane

Protein Kinase C (PKC) plays an important role in a myriad of intracellular signals in response to lipid second messengers. PKC, a family of threonine/serine kinases, was first reported in the late 1970s as the 'phorbol ester receptor' (Blumberg 1980; Nishizuka 1995; Newton 2001; Parker and Parkinson 2001; Newton 2004). The cloning of PKC revealed the C1 domain, which binds phorbol esters and the natural PKC agonist, diacylglycerol (Newton 2003). Studies also showed some PKCs have an additional regulatory domain, the C2 domain, that along with the C1 domain regulate the hallmark of activation for this family of proteins—membrane binding.

Classification of protein kinase Cs

The PKC family contains 10 members that all share a highly conserved kinase domain, but differ in their regulatory domains (Newton 2003). These 10 members are divided into 3 subclasses: conventional, novel, and atypical (Figure 1-1). The two regulatory domains, C1 and C2, have different properties in each subclass. For both the conventional and novel isozymes, diacylglycerol recruits their tandem C1 domains; however, their C2 domains differ as the conventional C2 domains are Ca^{2+}-dependent and the novel C2 domains are Ca^{2+}-independent (Newton 2003). In contrast to the conventional and novel isozymes, the atypical isoforms do not have C2 domains and their C1 domains do not bind diacylglycerol (Newton 2004).

Function and biological importance of protein kinase Cs

PKC plays a central role in mediating various signals that lead to cellular responses such as differentiation, proliferation, transcription, and immunity (Newton 2003). The specific roles of PKC isoforms have proven to be elusive. Overlapping substrate specificities of PKC isozymes *in vitro* suggest redundancy of cellular functions of PKC (Mellor and Parker 1998). In mouse knockout models, the isozyme-specific knockouts of PKC do not have a severe phenotype further suggesting functional redundacy (Newton 2003). Other studies, including ones using isoform-specific inhibitors, expression of dominant negative variants, and cells with targeted isoform deletion, show roles of PKC isoforms (Larsson 2006). However, the interpretation of these studies needs to be cautious as they may be demonstrating what a PKC isoform can do instead of what the isoform actually does (Larsson 2006). In addition, the overexpression of a specific isoform of PKC leads to changes in expression of other PKC isoforms (Dempsey, Newton et al. 2000). These studies illustrate specific PKC isoforms have functions that appear to overlap and suggest an important complex interdependence and crosstalk between isozymes (Dempsey, Newton et al. 2000). One common functional theme for the isoform-specific knockouts is a lost in the adaptive responses of various functions such as reduced learning abilities and pain perception (Newton 2003).

PKC isoforms have been implicated in cancer, cardiac and lung disease, diabetes, and cerebral ischemic and reperfusion injury (Corbalan-Garcia and Gomez-Fernandez 2006) . In carcinogenesis, PKC isozymes show different expression

profiles depending on the stage and type of cancer (Koivunen 2006). However, the main role of PKC in cancer is probably indirect as few direct mutations in PKC have been linked to cancer (Koivunen 2006). The balance of PKC isoforms appears important factor in the aggressiveness of cancer as some isoforms like δ are pro-apoptotic and other isoforms, such as α and β, are pro-survival (Koivunen 2006).

Regulation of protein kinase C

With such varied roles in cellular functions and implications in disease states such as cancer, the regulation of PKC has been extensively studied. The highly controlled regulation of PKC is accomplished through three main mechanisms: phosphorylation, cofactors, and localization (Newton 2001). If any of these mechanisms are disrupted, the downstream signaling will be interrupted. The first regulatory mechanism is the use of phosphorylation to regulate the maturation and activity of the protein. For PKC to become catalytically competent, it must be phosphorylated at three sites: the activation loop, hydrophobic motif, and turn motif (Newton 2003). Without these conserved, ordered phosphorylations, the protein cannot function. In addition to the phosphorylations required for maturation, phosphorylation of serine, threonine, and tyrosine residues also play a role in regulating PKC (Newton 2003).

The second mechanism by which PKC is regulated are cofactors—diacylglycerol, Ca^{2+}, and phosphatidylserine (PS)—which help allosterically activate PKC (Newton 2001). As shown in studies, the binding of these three cofactors

increase the affinity of PKC for the membrane (Newton 2003). Specifically, the C1 domains of the conventional PKCs require diacylglycerol and PS, while the C2 domain requires Ca^{2+} and anionic phospholipids. Upon binding these cofactors, these two regulatory modules will be able to bind the membrane and undergo a conformation change allowing the protein to become active (Newton 2003).

In addition to phosphorylation and cofactors, localization also plays a key role in isoform activity and specificity (Mochly-Rosen 1995; Dempsey, Newton et al. 2000). The targeting of PKC to specific subcellular locations allows PKC to interact with specific substrates and other interacting proteins that help regulate PKC (Poole, Pula et al. 2004). The interacting proteins have four key classes: (1) proteins targeting of PKC to upstream regulators, (2) proteins directing PKC to cellular compartments, (3) proteins that are PKC substrates, and (4) proteins involved in signaling (Poole, Pula et al. 2004). Many binding partners for PKC position it near its substrates, regulators, or specific subcellular compartments (Newton 2003). In addition, binding partners can act through different binding mechanisms and some have specificity for certain isoform, while others bind multiple isoforms (Newton 2003). An example of binding partners are the C2 domain of PKCβII and its anchoring protein receptor for activated C-kinase (RACK) which bind in the presence of Ca^{2+} and PS (Rodriguez, Ron et al. 1999).

Lifecycle of the conventional protein kinase Cs

The three mechanisms of regulation—phosphorylation, conformational changes caused by cofactors, and localization initiated by protein-protein interactions—create a complex lifecycle for conventional PKCs. When newly synthesized, PKC associates with membrane in an open conformation with the pseudosubstrate outside its cavity (Figure 1-2) (Newton 2003). Phosphoinositide dependent kinase-1 (PDK-1) then phosphorylates immature PKC at its activation loop leading to two subsequent auto-phosphorylations at the turn and hydrophobic motifs. These phosphorylations lead to a conformation change in which the pseudosubstrate inserts into the active site and PKC takes on a closed conformation (Newton 2003). PKC is now catalytically competent and upon the production of diacylglycerol and Ca^{2+} through normal signaling processes, the C1 and C2 regulatory domains will be recruited to the membrane. Membrane binding of these domains releases energy that allows the pseudosubstrate to leave the active site and phosphorylation of substrates can occur. PKC quickly dephosphorylates and releases from the membrane into the cytosol. In the cytosol, PKC can bind to heat shock protein 70 (Hsp70) which stabilizes the protein, and then it can be rephosphorylated and activated again (Gao and Newton 2002). Alternatively, PKC can bind Rign Finger Interacting C Kinase protein (RINCK), an E3 ubitqitin ligase, and be shunted to the proteosome for degradation (Chen and Newton, unpublished data).

The diversity and function of C2 domains

As the translocation and binding to the membrane plays such a central role in PKC activation, understanding its regulatory modules that facilitate PKC membrane binding is vital. Regulatory modules that control binding to membranes make up an important paradigm in reversible protein activation (Newton 2003). These domains, such as C1, C2, FYVE, and pleckstrin homology domains, are controlled by a variety of signals including Ca^{2+} and lipid second messengers (Cho and Stahelin 2005)

The research of membrane binding domains began with the cloning of PKC and the discovery of two conserved regions in the regulatory N-terminus of the kinase (Hurley 2006). The Cl domain, now known to be an important regulatory module in many proteins, was the first conserved region described and determined to bind phorbol esters and diacylglycerol (Hurley 2006). The second conserved region, or C2 domain, was determined to be the Ca^{2+} binding module of PKC (Cho and Stahelin 2005). However, the C2 domain is not unique to the PKC family, but rather part of a large group of proteins that have the ability to binding a variety of ligands and substrates (Nalefski and Falke 1996). More than 250 human protein containing C2 domains have been identified (Malmberg and Falke 2005). When compared to other membrane binding domains as such the C1 domain, C2 domains show weak primary sequence homology especially in the ligand or membrane binding loops (Cho and Stahelin 2005). This greater sequence diversity allows the C2 domain to have greater variations in its functions. In general, C2 domains are classified into 5 categories

based on function: vesicular transport, lipid modification, GTPase regulation, protein phosphorylation, and unknown functions (Rizo and Sudhof 1998). Originally, all C2 domains were believed to bind Ca^{2+} and anionic phospholipids like the conventional PKCs; however, the more recent discoveries of different C2 domains have proven that these properties are not requirements (Nalefski and Falke 1996).

The structure of C2 domains

Although it now appears that C2 domains can bind different substrates and ligands, all C2 domains share a common structure (Rizo and Sudhof 1998). C2 domains are 8-stranded anti-parallel β-sandwiches with 3 ligand- and membrane-binding loops at the top of the structure and 4 loops at the bottom to connect the β-strands (Nalefski and Falke 1996). This common structure is divided into two distinct topologies based upon the connectivity of the strands (Rizo and Sudhof 1998). However, the topology of the C2 domain does not determine its function or properties such as the ability to bind Ca^{2+} (Corbalan-Garcia and Gomez-Fernandez 2006). For PKC, the topology is determined by whether the C2 domain precedes or follows the C1 domains: the conventional PKCs have topology I and the novel PKCs have topology II (Nalefski and Falke 1996; Newton 2003).

Unlike other membrane binding domains, C2 domains are unique in that they do not have a well-defined lipid binding pocket and thus show relatively weak lipid specificity (Cho and Stahelin 2005). The C1 domain of PKC selectively binds PS, while the C2 domains of the conventional PKCs do not significantly discriminate

between anionic phospholipids (Johnson, Giorgione et al. 2000). Again, this diversity allows greater divergence in the membrane targets of C2 domains.

Although not all of the C2 domains bind Ca^{2+}, it is an important regulator of membrane binding for many C2 domains. In C2 domain membrane binding, the calcium ions may play as many as three different roles: (1) inducing an electrostatic switch for membrane binding, (2) inducing conformational changes in the calcium-binding regions, and (3) directly coordinating a lipid through calcium bridging (Hurley 2006). The Ca^{2+} binding acts as an electrostatic switch by increasing the positive electrostatic potential surrounding the Ca^{2+}-binding loops, which promotes the association to the anionic phospholipids (Cho and Stahelin 2005). In general, Ca^{2+}-binding is considered the initial fast step and membrane docking is the slower, rate determining step (Nalefski and Newton 2001). In addition, Ca^{2+} binding may slow the membrane dissociation of the C2 domain by mediating the membrane penetration and coordination with anionic phospholipids (Nalefski and Newton 2001).

Comparison of archetypal C2 domains

To further understand the mechanism of Ca^{2+} binding and membrane binding for C2 domains, prominent examples—cytosolic phospholipase A_2 ($cPLA_2$), synaptotagmin I (SytIA) as well as conventional PKC isoforms, α and β, have been extensively studied (Figure 1-3). Like many C2 domains, the functions of these proteins are diverse: $cPLA_2$ liberates arachidonic acid from membranes initiating the biosynthesis of inflammatory mediators, SytIA acts as a Ca^{2+} sensor for exocytosis,

and PKC acts as a protein kinase for various downstream substrates (Xu, McDonagh et al. 1998; Frazier, Wisner et al. 2002). Despite variations in the primary sequence, the proteins have highly homologous tertiary structure (Davletov, Perisic et al. 1998).

Although the C2 domains share a similar structure, the C2 domains bind the membrane through distinct mechanisms. The C2 domains of PKC and SytIA prefer anionic phospholipids, while the C2 domain of $cPLA_2$ strongly prefers zwitterionic phospholipids such as phosphatidylcholine (PC) (Zhang, Rizo et al. 1998; Stahelin, Rafter et al. 2003). It was shown that the calcium binding loops determine the phospholipid binding specificity as exchanging the loops of SytIA-C2 and $cPLA_2$-C2 domains reverses their phospholipid binding specificities (Gerber, Rizo et al. 2001). This lipid preference may have implications for the mechanisms of C2 domain membrane binding. PKC and SytIA are believed to interact with membranes primarily through electrostatic interactions, while $cPLA_2$ may insert into the membrane through hydrophobic interactions (Davletov, Perisic et al. 1998). The different interactions are further supported by the fact that Ca^{2+}-dependent membrane binding of the PKCβ and SytIA domains, but not the $cPLA_2$ C2 domain, are inhibited by increasing ionic strength (Nalefski and Falke 1996; Nalefski and Newton 2001; Nalefski, Wisner et al. 2001).

The differential binding, in part, may be attributed to the different effects of Ca^{2+} binding to the domains. The three types of domains have different Ca^{2+} concentration requirements for binding; the C2 domain of $cPLA_2$ has the lowest Ca^{2+} threshold, while C2 domain of PKCβII and SytIA have higher Ca^{2+} thresholds

(Nalefski and Newton 2001; Nalefski, Wisner et al. 2001). These differences may have to do with their specific functions, but also suggest subtle differences in structure can change the Ca^{2+} binding of the domain.

The more charged Ca^{2+} binding loops of PKC and SytIA may be the source of the difference in Ca^{2+} binding affinity as the positive charges may lead to repulsion of like charges and destabilize the Ca^{2+}-C2 domain complex (Nalefski, Wisner et al. 2001). The Ca^{2+} binding loops of $cPLA_2$C2 domain contain two exposed clusters of hydrophobic residues and Ca^{2+} binding causes a conformational change that acts to make the Ca^{2+} binding loops more neutral in charge (Perisic, Fong et al. 1998; Bittova, Sumandea et al. 1999; Murray and Honig 2002). This neutralization of the charges allows the C2 domain of $cPLA_2$ to insert into the membrane bilayer (Davletov, Perisic et al. 1998). In contrast, the residues in the Ca^{2+} binding loops of PKC and SytIA contain negative electrostatic potential from the aspartic acid residues and Ca^{2+} binding makes the C2 domains of SytIA and PKC-C2 become very positive (Sutton, Davletov et al. 1995; Verdaguer, Corbalan-Garcia et al. 1999; Murray and Honig 2002). However, it is important to note that studies have shown that hydrophobic interactions do play a role in the membrane binding of SytIA (Gerber, Rizo et al. 2002).

Since the electrostatic interactions are believed to be dominating, the domains will not penetrate as deeply into the membrane as the $cPLA_2$C2 domain (Frazier, Wisner et al. 2002; Hurley 2006). This difference in mechanism of membrane binding is further supported by EPR studies that show that the C2 domain of $cPLA_2$ penetrates

5-7 angstroms deeper into the membrane than SytIA (Frazier, Wisner et al. 2002). For $cPLA_2$-C2 domain, the Ca^{2+} ions, promoting hydrophobic interactions, mainly slow the membrane dissociation, while Ca^{2+} binding of PKCα-C2 domain alters both membrane association and dissociation rates (Stahelin and Cho 2001).

Not only do the C2 domains have different lipid composition preference and Ca^{2+} affinities, the C2 domains translocation to different membranes within the cell. PKC-C2 and SytIA-C2 domains translocate to the plasma membrane with high affinity, while $cPLA_2$-C2 domain translocates to Golgi, endoplasmic reticulum, and nuclear envelope (Stahelin, Rafter et al. 2003; Hirabayashi, Murayama et al. 2004; Cho and Stahelin 2005; Evans, Murray et al. 2006). In vesicle binding studies, the C2 domain of $cPLA_2$ shows a clear preference for lipid vesicles mimicking nuclear membranes, while the C2 domain of PKC shows a clear preference for lipid vesicles with a similar composition of the plasma membrane (Stahelin, Rafter et al. 2003). In recent imaging studies, the lysine rich cluster, which has been shown to bind PIP_2, appears to be the main determinant for PKC-C2 domain plasma membrane binding (Evans, Murray et al. 2006). Although the C2 domains of $cPLA_2$, SytIA, and PKCs have similar structures, subtle differences cause different Ca^{2+} and membrane binding properties. Dissecting these differences, we can begin to better understand the differential mechanisms of C2 domain membrane binding.

Electrostatic and hydrophobic interactions

Electrostatic and hydrophobic interactions play critical roles in the membrane binding of regulatory modules of many proteins. Two ways electrostatic interactions are involved with modulating membrane binding are Ca^{2+} binding and interactions with basic residues. The nonspecific electrostatic interactions of Ca^{2+} binding recruit the C2 domains of both SytIA and PKC to the membrane (Zhang, Rizo et al. 1998; Nalefski and Newton 2001). It is suggested these interactions increase the domain's attraction to anionic phospholipids by increasing the electrostatic potential of the domain surface and reducing the desolvation penalty caused by membrane association and penetration (Murray and Honig 2002). Although these nonspecific electrostatic interactions along with diffusion play an important role in initiating membrane binding, these interactions are not enough to retain the binding domains at the membrane (Cho and Stahelin 2005).

The basic residues of the C2 domain also help modulate membrane binding. A recent comparison shows that membrane binding proteins have higher net charge than non-membrane binding proteins (Bhardwaj, Stahelin et al. 2006). In addition, the amino acid composition of membrane binding proteins have more basic residues, especially lysine and arginines, on their surface than non-membrane binding proteins (Bhardwaj, Stahelin et al. 2006). In part, basic protein residues can enhance the nonspecific, long-range electrostatic interactions which can accelerate the association of proteins to anionic phospholipids and help orient the protein (Murray and Honig 2002; Cho and Stahelin 2005). As the protein gets closer to the membrane, short-

range specific interactions and/or membrane penetration mainly slow the membrane dissociation (Murray and Honig 2002; Cho and Stahelin 2005)

Membrane binding proteins not only have higher proportions of basic residues, but also aliphatic and aromatic residues, in particular tryptophans, on their surface (Bhardwaj, Stahelin et al. 2006). The amino acid composition suggests the surface of membrane binding proteins involves both electrostatic and hydrophobic interactions. Additional studies show that the aromatic residues tryptophans and tyrosines are interfacial, surface exposed, and do not bury within the hydrocarbon core (Scarlata 2004). An experimental interfacial hydrophobicity scale also shows tryptophan, phenylalanine, tyrosine, and leucine are the most favorable amino acids for membrane penetration (Wimley and White 1996)).

In fact, although they are hydrophobic, the residues tryptophan and tyrosine have the highest affinity for the interfacial region (Cho and Stahelin 2005). These residues stabilize the membrane-protein complex through their contact with lipids by acting as amphipathic anchors that can hydrogen bond and can either be partially buried in the membrane or solvent exposed (Scarlata 2004). Aromatic residues, particularly tryptophans, play a unique and crucial role in binding to PC membranes by affecting both membrane association and dissociation steps (Cho and Stahelin 2005). The electrostatic and hydrophobic interactions work together to control protein association and dissociation from the membrane.

FRET association and dissociation experiments to study C2 domain interactions

To better understand the membrane association and dissociation of the C2 domains, we studied their membrane binding kinetics. In these studies, the binding kinetics of the C2 domains of conventional PKCs were studied using fluorescence resonance energy transfer (FRET) in the stopped flow analysis. These experiments allow both the on rates and off rates of the binding kinetics to be examined rather than just observing the overall lipid-binding affinity of the system. This dissection of the on and off rates allows greater detail about the mechanism of how the C2 domain is recruited to, and retained at, the membrane to be understood.

In the FRET association experiments, both the on and off rates can be determined (Figure 1-4). For the stopped flow analysis, C2 domain protein and lipid vesicles containing 35 mol % PS, 60 mol % PC, and 5 mol % dansyl-labeled phosphatidylethanolamine (dPE) are mixed in the stopped flow apparatus (Nalefski and Newton 2001; Nalefski and Newton 2003). The four intrinsic tryptophans of the protein are excited at their excitation wavelength (280nm). If the protein is bound to the dansyl-labeled vesicles, the energy at the tryptophan emission and dansyl excitation wavelengths (340nm) will transfer. This FRET interaction allows the dansyl label to emit at 465nm. This emission is recorded and then fitted to a single exponential to determine the k_{obs} of each kinetic trace. The k_{obs} is plotted against varying lipid concentrations, then fit to pseudo-first order binding reaction, and k_{on}, rate constant, k_{off} rate constant, and K_d^{calc} can be calculated.

In the FRET dissociation experiments, the off rates can be determined (Figure 5). For this stopped flow experiment, C2 domain proteins and lipid vesicles containing 35 mol % PS, 60 mol % PC, and 5 mol % dPE are pre-incubated for 30 minutes. Then C2 domain protein and labeled vesicles are mixed with 10-fold excess of unlabeled lipid vesicles in the stopped flow apparatus. Again, the four intrinsic tryptophans of the C2 domain are excited and loss of FRET, as the unlabeled vesicles compete off the labeled vesicles, will be measured as a decrease in dansyl emission at 465nm. This emission is recorded and then fitted to a single exponential to determine the k_{obs} of each kinetic trace. The k_{obs} is equal to the k_{off} rate constant in the excess of unlabeled lipid. From the k_{on} rate constant determined in the FRET association experiments and the k_{off} rate constant measured from the FRET dissociation experiment, the overall K_d^{calc} value can be determined.

Thesis overview

This dissertation describes biophysical, biochemical, and cellular biology approaches to study the kinetics and mechanism of the critical interaction between the C2 domains of conventional PKCs and the membrane. In Chapter II, stopped flow fluorimetry is used to investigate the role of electrostatic and hydrophobic interactions of specific residues using a mutational analysis of C2α domain membrane binding. These data show that electrostatic interactions of these charged residues play a role in the retention of the C2 domain at the membrane, while hydrophobic interactions of both charged and hydrophobic residues play roles in both the recruitment and retention

of the C2 domain to the membrane. In addition, studies with varying ionic strength and Ca^{2+} provide further insights into the role of these determinants in C2α domain membrane binding. The role of these electrostatic and hydrophobic interactions of specific residues will be described in the context of full-length protein by determining the kinetics, amplitude, and duration of PKC activity using live cell imaging. These studies demonstrate that electrostatic and hydrophobic interactions play a significant role in only in the membrane binding of the C2 domains, but also the activity of the full-length PKC. In Chapter III, comparative studies of the conventional isoforms, C2α, C2β, and C2γ, have helped further elucidate the kinetics and mechanism of C2 domain membrane binding. These studies showed that key residues may be responsible for differences in isoform-specific membrane binding kinetics. In Chapter IV, the role of an autophosphorylation site within the Ca^{2+} binding loops of the C2 domain is examined to determine if phosphorylation may help regulated C2 domain membrane binding. In Chapter V, a model for the membrane binding of C2 domains of the conventional PKCs is discussed.

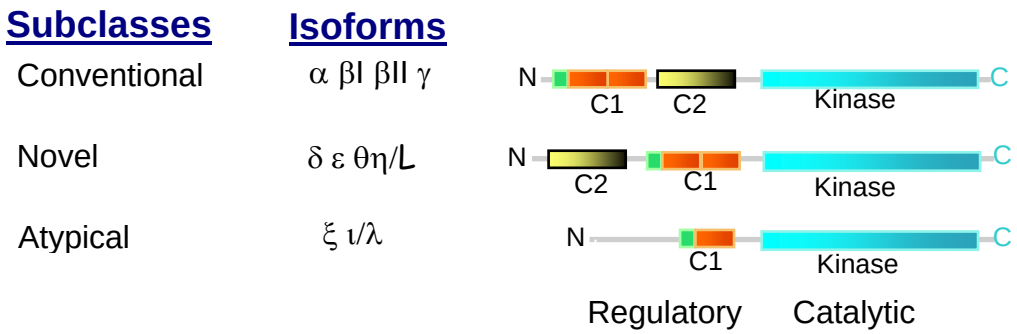


Figure 1-1. **PKC domains and classification.** The PKC family is divided into 3 subclasses: novel, conventional, and atypical. The N-terminus contains regulatory modules C1 (orange) and C2 (yellow) domains as well as the pseudosubstrate (green). The C-terminus contains the kinase (cyan) domain.

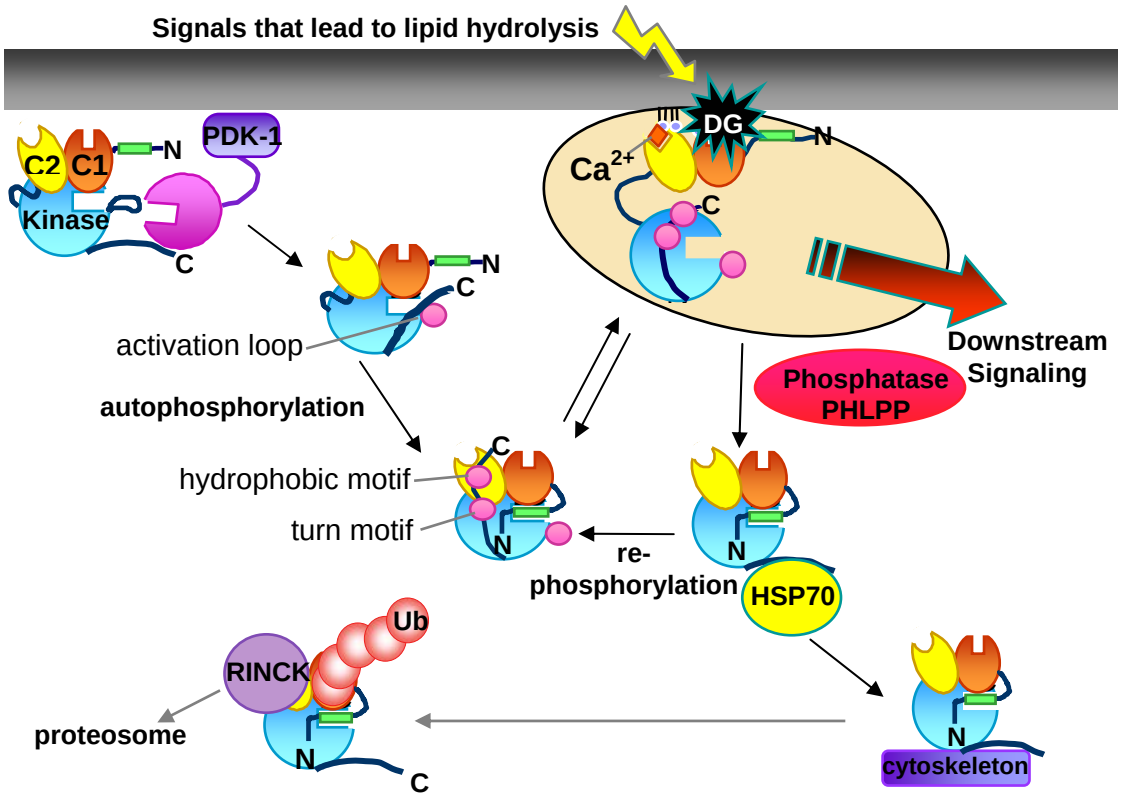


Figure 1-2. **Lifecycle of conventional PKCs: Its biosynthesis to downregulation.** Before PKC can be activated, it must go through a maturation process that includes phosphorylation by phosphoinositide dependent kinase-1(PDK-1) and two subsequent autophosphorylations. These phosphorylations allow PKC to become catalytically competent and can be activated. Upon signals that lead to lipid hydrolysis and the production of diacylglycerol and Ca^{2+}, the C1 and C2 domains bind the membrane. This membrane binding leads to a conformational change and activation of PKC. During downregulation, PKC binds either heat shock protein (HSP 70) which allows it to be rephosphorylated or binds Ring Finger Interacting C Kinase Protein (RINCK) that allows it to become ubiquitinated and degraded. Adapted from Newton, 2003.

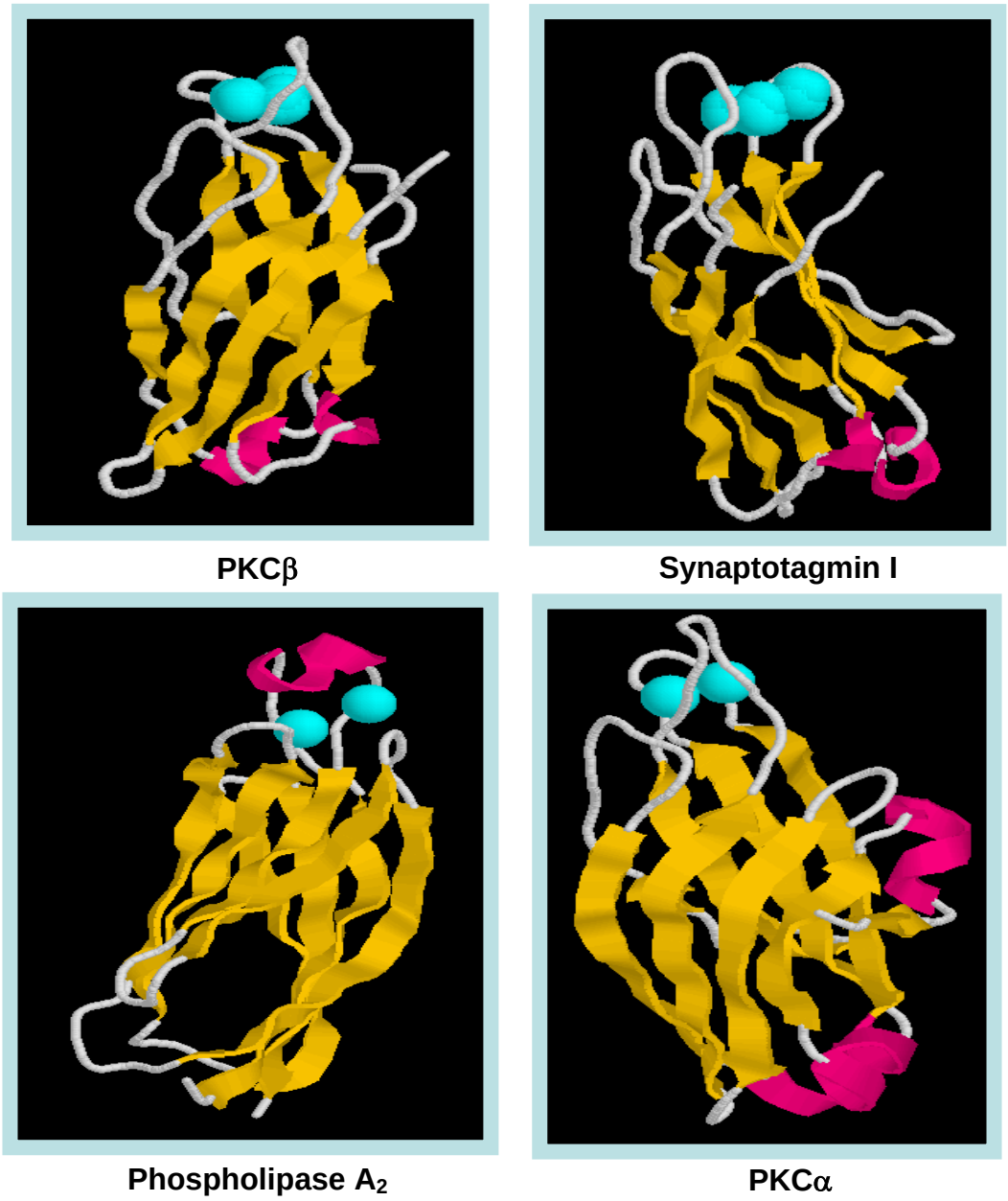


Figure 1-3. **The structures of C2 domains fo r PKCβ, synaptotagmin I, PKCα, and cytosolic phospholipase A_2 .** Ribbon diagrams of the structure of C2 domains for PKCβ, synaptotagmin I, PKCα, and $cPLA_2$ (from the left clockwise). The 4 domains have similar structure with 8-stranded anti-parallel β-sandwich and 3 Ca^{2+} binding and membrane binding loops, which coordinated 2- 3 Ca^{2+} ions, are located on the top of the structure.

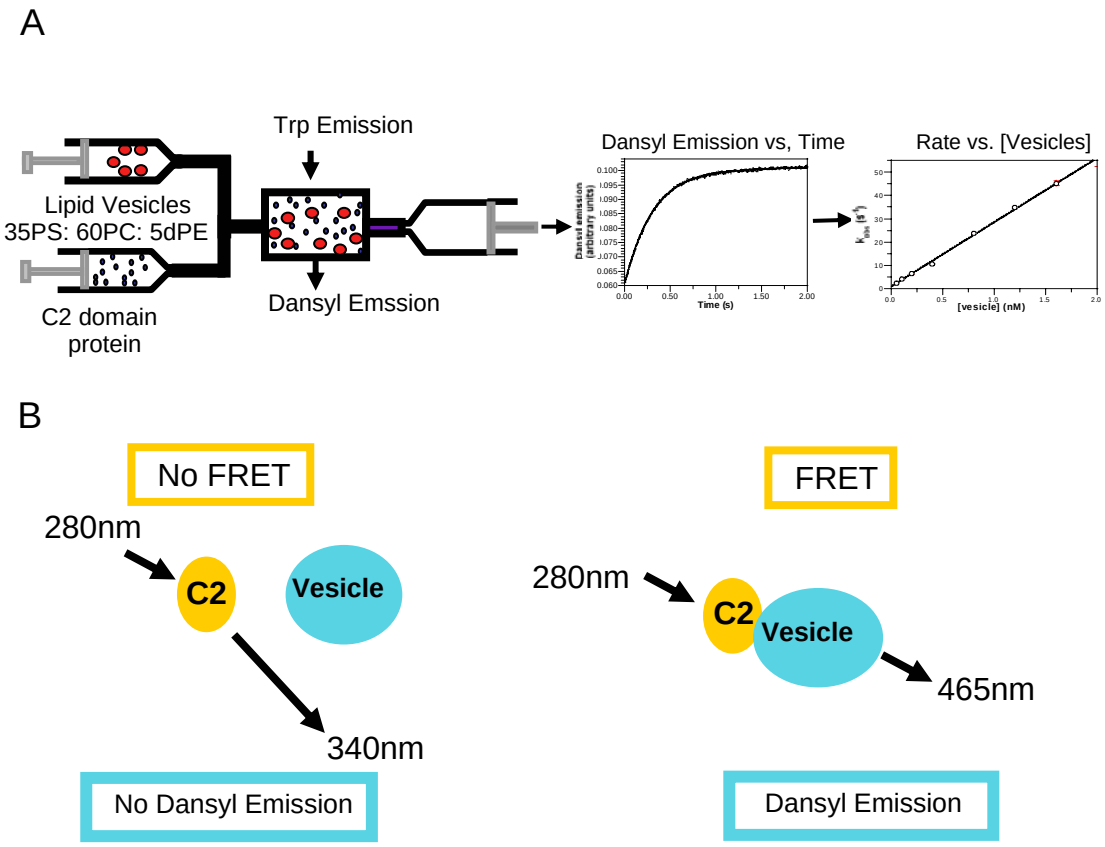


Figure 1-4. **FRET association experiment and analysis.** (A) A schematic diagram of the apparatus for the FRET association experiments. C2 domain protein and lipid vesicles are injected into chamber where the intrinsic tryptophans of the protein are excited. If the protein binds to dansyl-labeled vesicles, fluorescence resonance energy transfer (FRET) will occur and be measured. The measured dansyl emission is plotted against time and fit to a single exponential fit to calculate the k_{obs}. The k_{obs} is then plotted versus lipid concentration and fit to a pseudo-first order binding rate to determine k_{on}, k_{off}, and K_d^{calc}. (B) A schematic diagram of the FRET association experiments. When the system is excited at 280nm if C2 domain is bound to lipid vesicles, there will be a fluorescence resonance energy transfer and an emission at 465nm. If C2 domain is not bound to lipid vesicles, no FRET will occur and the emission will be at 340nm.

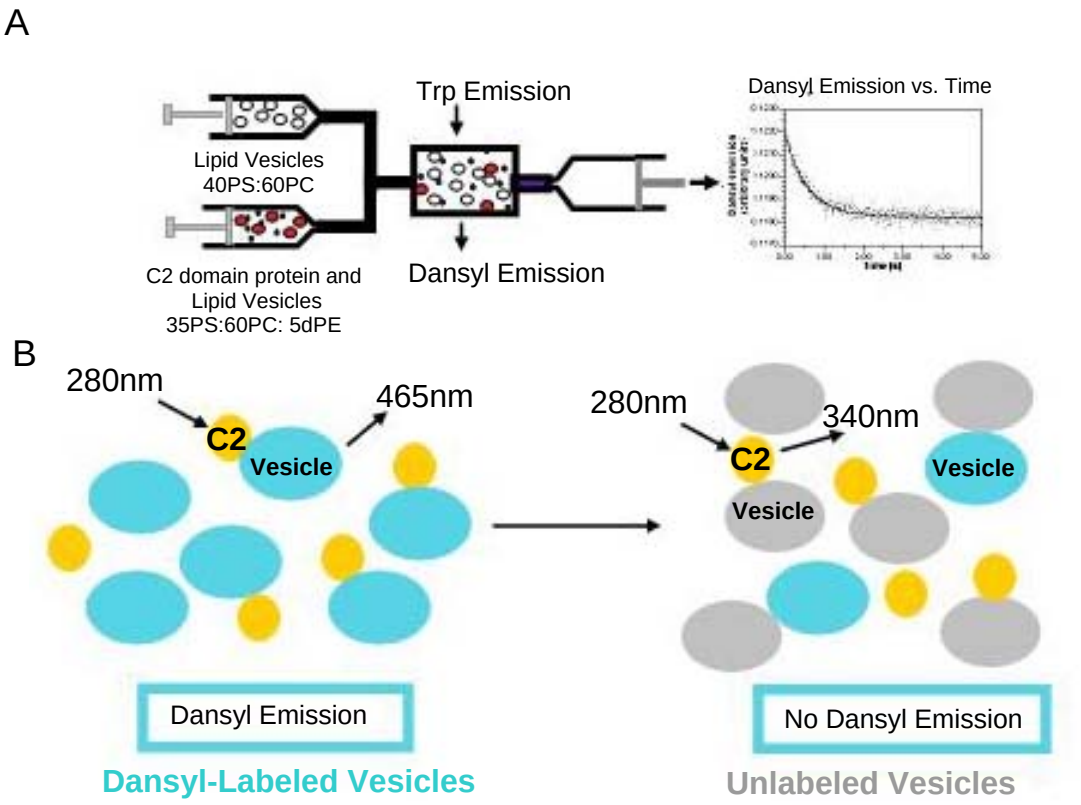


Figure 1-5. **FRET dissociation experiment and analysis.** (A) A schematic diagram of the apparatus for the FRET dissociation experiments. C2 domain protein and dansyl-labeled lipid vesicles, and 10-fold excess unlabeled lipid vesicles are injected into chamber where the intrinsic tryptophans of the protein are excited. If the unlabled lipid vesicles compete off the dansyl-labeled vesicles the C2 domain, fluorescence resonance energy transfer (FRET) will decrease and be measured. The measured dansyl emission is plotted against time and fit to a single exponential fit to calculate the k_{obs}. The k_{obs} is equal to the k_{off} in the excess of lipid. (B) A schematic diagram of the FRET dissociation experiments. When the system is excited at 280nm, if C2 domain is bound to lipid vesicles, there will be a fluorescence resonance energy transfer and an emission at 465nm. If C2 domain is not bound to lipid vesicles, no FRET will occur and the emission will be at 340nm.

Chapter II

The role of electrostatic and hydrophobic interactions in the membrane binding kinetics and mechanism of the C2 domain of protein kinase Cα

Introduction

The membrane binding of its C2 domain is critical for the activation of conventional protein kinase Cs (PKCs) that play a central role in mediating various signals that lead to cellular responses such as differentiation, proliferation, transcription, and immunity. Regulatory modules that binding membranes, such as C2, C1, FYVE, and pleckstrin homology domains, make up an important paradigm in reversible protein activation (Cho and Stahelin 2005). These regulators are controlled by a variety of signals including Ca^{2+} and lipid second messengers.

As the translocation and binding to the membrane is the hallmark of PKC activation, the mechanism by which C2α domains bind the membrane has been extensively studied. The C2 domain of the conventional PKCs was the first of this type of regulatory module to be recognized (Cho and Stahelin 2005). The crystal structure of C2α domain was first resolved in 1999 (Verdaguer, Corbalan-Garcia et al. 1999). The crystal structure reveals the typical C2 domain structure of an eight-stranded, antiparallel β-barrel with three ligand and membrane binding loops.

As the C2 domain regulation plays such an important role in activation, it is not surprising that the C2 domain has several layers of regulation. For the C2 domains of conventional PKCs, there are at least four modes of regulation: (1) Ca^{2+} binding, (2) anionic phospholipid binding, (3) PIP_2 binding, and (4) electrostatic and hydrophobic interactions of specific residues. Although the mechanisms of these regulatory cofactors are not completely understood, crystallographic, biophysical, biochemical, and cellular biology studies have given insights into the membrane binding properties

of the C2α domain. These studies have dissected these modes of regulation and given insights to the mechanism of C2 domain membrane binding.

Role of Ca^{2+} in C2 domain regulation and membrane binding

As one of the early defining characteristic of the C2 domain, the binding of Ca^{2+} and its regulation by Ca^{2+} have been extensively studied. Early studies show Ca^{2+} regulation of the C2β domain is not just a simple electrostatic switch as mutations that neutralized the Ca^{2+} binding loops do not bypass the requirement for Ca^{2+} in membrane translocation (Edwards and Newton 1997). Full-length PKC studies show the binding of Ca^{2+} to D187, D193 and D246 of PKCα is critical for the initial binding of PKC to the membrane and the other Ca^{2+} that binds to D187, D246, D193, and D254 induces the conformational change in PKCα which allows membrane penetration and activation (Medkova and Cho 1998). In lipid binding studies, Ca^{2+} binding loop 1 contains D187 and D193 and mutating these residues has a greater effect on binding than mutations of residues D248 and D254 in the Ca^{2+} binding loops3 (Medkova and Cho 1998). These results suggest that Ca1 plays a more important role in Ca^{2+} binding than Ca2 (Medkova and Cho 1998). In addition, another study confirms the importance of the three residues critical for Ca^{2+} interaction as the triple mutation of D187, D246, and D248 results in a C2 domain that could not interact with either Ca^{2+} or PS (Corbalan-Garcia, Rodriguez-Alfaro et al. 1999). In addition, these mutations lose their ability to translocate to the plasma membrane (Corbalan-Garcia, Rodriguez-Alfaro et al. 1999).

The aspartates are not the only residues within the Ca^{2+} binding loops that contribute to Ca^{2+} coordination. The crystal structure also suggests that the main chains of M186 and W247 help coordinate the Ca^{2+} ions (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). In addition, one of the phosphoryl oxygen atoms from the anionic phospholipids coordinates with Ca1 (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). The Ca1 ion does not bridging the C2 domain to an anionic phospholipid alone, N189 and R216 also help coordinate the Ca1 ion (Corbalan-Garcia and Gomez-Fernandez 2006). Ca2 is responsible for keeping Ca1 in an appropriate location and for inducing a conformational change in PKC, which partially penetrates in the phospholipid bilayer by means of Ca^{2+} binding loop 3 residues R249 and T251 (Corbalan-Garcia and Gomez-Fernandez 2006).

Together, these studies suggest that the first Ca^{2+} to bind acts as a bridge between the C2α domain and the anionic phospholipids as well as specific residues (Cho 2001). The second Ca^{2+} ions has been suggested to cause a conformational changes—either intradomain or interdomain (Cho 2001). Although the studies show there are Ca^{2+} induced conformation changes, the ability of Ca^{2+} to bridge the domain and membrane appears to be relatively more important (Cho 2001).

Role of anionic phospholipids and specific residues in C2 domain regulation and membrane binding

Ca^{2+} is not the only cofactor important for the binding of C2 domains to the membrane. Previous studies show that anionic phospholipids play an important role. These studies show that anionic phospholipids, such as PS and PG, are required for C2α domain membrane binding (Johnson, Giorgione et al. 2000; Stahelin, Rafter et al. 2003). While studies demonstrate that the C2 domains appear to have a modest preference for PS compared to other anionic phospholipids, they do not have the stereospecific requirement for sn-1,2, phosphatidyl-L-serine that the C1 domain has (Johnson, Giorgione et al. 2000)

The crystal structure also reveals possible anionic phospholipids interactions. Not only does one of the Ca^{2+}ions bridge with PS, specific residues within the Ca^{2+} binding loops may also interact with anionic phospholipids. The crystal structures show PS may interact with residues N189, T251, R216, and R249 (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). Further binding and activity studies reveal that when N189 is mutated to alanine, it did not decrease the level of membrane binding, but did prevent full enzyme activation; however, R216A, R249A, and T251A mutants of PKCα affect both overall membrane binding and enzyme activation (Conesa-Zamora, Lopez-Andreo et al. 2001). N189 seems to be specific recognition site for the serine moiety of PS, thereby, playing an important role in the activation of the enzyme.

Subcellular fractionations show that T251, R249, and R216 also play an important role in translocation (Conesa-Zamora, Lopez-Andreo et al. 2001). In vesicle binding assays of both full length PKC and isolated C2 domain, when R216 was mutated to alanine, the membrane binding of affinity of the C2 mutant decreases suggesting that this residue is important for stabilizing the domain-membrane complex at the membrane surface (Conesa-Zamora, Lopez-Andreo et al. 2001; Stahelin and Cho 2001). In addition, the double mutant R249A/R252A of PKCα as well as the single mutant R249A of PKCα show reduced vesicle binding affinity in full-length binding assays (Medkova and Cho 1998; Conesa-Zamora, Lopez-Andreo et al. 2001). Further supporting that R216 and R249 are critical for binding, the crystal structures suggest the guanidinium groups of R216 and R249 interact with the ester carbonyl group of PS(Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). However, R249 and T251 play a more crucial role in the anchorage process and consequently in the activation of the enzyme (Conesa-Zamora, Lopez-Andreo et al. 2001; Bolsover, Gomez-Fernandez et al. 2003). Taken together, R216, R249, and T251 may establish direct hydrogen bonds as well as hydrophobic interactions with acyl chains of the phospholipids (Corbalan-Garcia and Gomez-Fernandez 2006). EPR studies also add insight into the mechanism of C2 membrane binding. They suggest that R249 as well as R252 insert into the membrane (Kohout, Corbalan-Garcia et al. 2003). In these studies, R249 inserts deeper into the membrane bilayer to at or near the interface with the hydrocarbon core while R252 is near the polar group layer (Kohout, Corbalan-Garcia et al. 2003).

Moreover, R249 may have an expanded role in binding as it may also have a role in hydrophobic interactions that stabilize the C2 domain-membrane complex. In the crystal structure, the aliphatic carbons of R249 establish hydrophobic interactions with the fatty acid chains of 1,2-dicaproyl-sn-phosphatidyl-L-serine (DCPS) (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). From the crystal structure, one model, referred to as the sn-1 model, suggests the sn-1 ester oxygen atom interacts with the guanidinium groups of R249 and in the alternative model, the sn-2 model, the guanidinium groups of not only R249, but also R216A interact with the sn-2 ester carbonyl group (Verdaguer, Corbalan-Garcia et al. 1999).

The charged residues are not the only residues that participate in membrane binding. One model from the crystal structure suggests that the hydrophobic residue W247 insert into the bilayer and the W245 contacts the 1,2-diacetyl-sn-phosphatidyl-L-serine (DAPS) glycerol and acyl chains (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002). These two residues are relatively conserved in Ca^{2+} binding loop 3—completely conserved among conventional PKCs and substituted by either aromatic or hydrophobic residues in the other PKCs (Nalefski and Falke 1996). Vesicle binding studies show that the double mutant W245A/W247A dramatically reduces the binding affinity (Medkova and Cho 1998). The mutant also shows reduced activity and SPR studies further suggest that the two tryptophans penetrate into the membrane (Medkova and Cho 1998).

Role of PIP_2 in C2 domain regulation and membrane binding

Previous studies show that the membrane binding of the C2 domain of PKC is regulated at two positions: the 3 Ca^{2+} binding loops and a lysine-rich cluster in the β3 and β4 sheets (Edwards and Newton 1997; Corbalan-Garcia, Garcia-Garcia et al. 2003; Corbalan-Garcia and Gomez-Fernandez 2006). This lysine-rich cluster appears to regulate the C2 domain membrane binding by its interaction with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (Corbalan-Garcia, Garcia-Garcia et al. 2003). In these studies, Ca^{2+} binding assays showed that the C2α domain with a K209A/K211A double mutation were deficient in their activation in the presence or absence of Ca^{2+} as well as in the presence or absence of PIP_2. More recent studies have shown that this PtdIns(4,5)P_2 binding site provides C2α with specificity for the plasma membrane (Evans, Murray et al. 2006) A $cPLA_2$-C2 and PKC-C2 hybrid mutational analysis shows the Ca^{2+} binding loops of PKC, hybridized with the rest of the $cPLA_2$-C2 domain, were sufficient for membrane binding, but it was the lysine-rich cluster that provided the specificity for the plasma membrane (Evans, Murray et al. 2006).

Interestingly, a recent study uncoupled the interaction with membranes through the C2 domain and the activation process. In addition the simultaneous mutation of K197, K199, K211 to alanine converts PKC into a constitutively active kinase, reducing its need for Ca^{2+}, PS, or PIP_2 in its activation (Rodriguez-Alfaro, Gomez-Fernandez et al. 2004). Extensive studies show the role of Ca^{2+}, anionic phospholipids, and PIP_2 in the membrane binding of the C2α domain.

Imaging studies of PKC and its C2 domains

In addition to the biochemical and biophysical techniques being used to elucidate the structure and function of the C2 domains of PKC, imaging studies have looked at the subcellular localization and translocation of PKC and its C2 domains. Studies with green fluorescent fusion protein labeled PKCα show that it translocates to the plasma membrane under various stimulus that increase intracellular Ca^{2+}, while C2 deleted mutants did not translocate to the plasma membrane with increased intracellular Ca^{2+} (Maasch, Wagner et al. 2000; Vallentin, Prevostel et al. 2000; Wagner, Harteneck et al. 2000). In addition, other imaging studies show Ca^{2+}, diacylgycerol, and phosphorylation regulate the translocation of PKCα-GFP in live cell (Tanimura, Nezu et al. 2002).

Translocation studies have also looked at specific mutations within the C2 domain in both the context of the isolated domain and full length PKC. The mutation of 3 aspartic acid residues—187, 246, and 248—within the Ca^{2+} binding loops shows that Ca^{2+} is involved in membrane translocation in COS and HeLa cells (Corbalan-Garcia, Rodriguez-Alfaro et al. 1999). Using specific mutations in the context of full length PKCα-GFP, the mutation of R249 to alanine abolishes translocation to the membrane even after stimulation with the potent diacylglycerol analog 1-2-dioctanoyl-sn-glycerol (DiC8) and ionomycin stimulation (Bolsover, Gomez-Fernandez et al. 2003). This result suggests that the R249 is so important for translocation that the C1 domain cannot compensate for the dramatically reduced affinity of the mutant C2α

domain. In contrast, another study shows that the isolated C2α domain double mutant R249A/R252A does translocate to the membrane (Stahelin, Rafter et al. 2003). Taken together, these studies suggest that the mutation of R249 may lead to the masking of an essential binding element in the context of full-length PKC, but not the C2 domain.

More recently, a C Kinase Activity Reporter (CKAR) was developed to look at the kinase activity of PKC in real time in live cells (Violin, Zhang et al. 2003). Using CKAR in conjunction with the Ca^{2+} dye Fura-2, studies demonstrate that Ca^{2+} oscillations determine the substrate phosphorylation of the conventional PKCs (Violin, Zhang et al. 2003). Additional studies reveal that an initial spike in Ca^{2+} followed by diacylglycerol generation control PKC activity (Gallegos, Kunkel et al. 2006). CKAR is a useful tool with the potential to help explore the relationship between spatial and temporal aspects of PKC activity.

Chapter overview

In this chapter, we examine the role of electrostatic and hydrophobic interactions of Ca^{2+} ions and specific charged and hydrophobic residues in the membrane binding kinetics and mechanism of the C2α domain. Specifically, we confirm that the binding of Ca^{2+} ions is the key electrostatic switch for the recruitment of the C2α domain to the membrane as well as playing an important role in the retention of the C2α domain at the membrane. We also demonstrate that both electrostatic and hydrophobic interactions of specific residues play key roles in C2α domain retention at the membrane, but that only the hydrophobic interactions of these

residues appear to play a critical role in C2α domain's recruitment to the membrane. In addition, we show the loss of these key hydrophobic interactions increases the ionic strength sensitivity of the C2α domain membrane binding. Finally, we show that by altering the electrostatic and hydrophobic interactions of specific residues, the kinase activity of protein kinase C decreases in real time in live cells.

Materials and Methods

Reagents

1-Palmitoyl-2-oleoylphosphatidyl-L-serine (POPS) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Polar Lipids. N- [5-(dimethylamino) naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dPE) was purchased from Molecular Probes. PCR primers were prepared by IDT technologies, Inc. Glutathione-Sepharose 4B was purchased from Amersham Biosciences. Electrophoresis reagents were from Calbiochem and Bio-Rad. Thrombin was purchased from Calbiochem. Antibody to PKCα was from Santa Cruz Biotechnology, Inc. All other chemicals were reagent grade.

C2α domain cloning, mutagenesis, and purification

The rat PKCα DNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). The C2 domain (residues 179-283) was PCR amplified and subcloned into a pGEX-4T3-GST plasmid using restriction sites EcoRI and XhoI. The C2α domain mutants R216A, R249A, R252A, K268A, W245A, W247A, L191A, and

W274A were created using site-directed mutagenesis. The C2 domain of rat PKCα was expressed as a glutathione-S-transferase (GST) fusion protein in BL21-pLysS cells as previously described (Johnson). The isolated C2α domain was cleaved from the glutathione-Sepharose 4B-bound fusion protein using thrombin. The concentration of C2 domain protein was determined by UV absorbance using their molar extinction coefficient (Nalefski and Falke, 2001).

Preparation of lipid vesicles

For stopped flow fluorimetry, phospholipids in chloroform were dried under nitrogen and vacuum dried for two hours. The lipids were resuspended in a buffer of varying NaCl concentration (150mM-200mM) and 20mM Hepes, pH 7.4. The resuspended lipid was freeze/thawed five times and extruded through two 0.1μM polycarbonate filters to prepare phospholipid vesicles 100nm in diameter. The phosphorus concentration was assayed as previously described to determine the phospholipid concentration (Nalefski and Newton, 2001).

Stopped flow fluorimetry

An Applied Photophysics pi*-180 stopped-flow fluorescence spectrophotometer was used to determine the kinetics. Purified C2α domain protein (0.2μM to 1.0μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol % PS: 60 mol % PC: 5 mol % dPE) at Ca^{2+} concentrations and ionic strengths as indicated. These FRET association experiments

were run at the same conditions and analyzed as previously described (Nalefski and Newton 2001; Nalefski and Newton 2003). Purified C2α domain protein (0.2μM to 1.0μM) was incubated for 30 minutes with dansyl-labeled phospholipid vesicles (35 mol % PS: 60 mol % PC: 5 mol % dPE). The protein and labeled lipid were then rapidly mixed with equal volumes of 10-fold excess unlabeled anionic phospholipid vesicles (40 mol % PS: 60 mol % PC) at Ca^{2+} concentrations and ionic strengths as indicated. These FRET trapping experiments were run at the same conditions and analyzed as previously described (Nalefski and Newton 2001). The traces were fitted to a nonlinear least-squares curve fitting using KaleidaGraph according to the exponential equation

$$F(t) = F_0 + \sum_{i=1}^{n} A_{obs(i)} e^{-k_{obs(i)} t}$$

where F(t) equals the observed fluorescence at time t, F_0 is a fluorescence offset representing the final fluorescence, and $A_{obs(i)}$ equals the amplitude, and the $k_{obs(i)}$ is the observed rate constant for *i*th of n phases. The experiments were performed under pseudo-first order conditions allowing the observed rate to be plotted as a function of lipid vesicle concentration in a linear way using the equation

$$k_{obs} = k_{on} [v] + k_{off}$$

where k_{on} represents the second-order rate constant and k_{off} represents the apparent dissociation rate constant. The calculated K_d was determined using weighted k_{on} and k_{off} rate constant averages from the FRET association and FRET trapping experiments. The S.E.M.s were also calculated.

Ca^{2+} affinity fluorimetry

Ca^{2+} titrations were performed using Jobin Yvon-SPEX FlouroMax-2 fluorescence spectrophotometer. As increasing concentrations of Ca^{2+} were added to 0.5μM C2α domain, the solution was excited at 280nm and the emission at 345mM was recorded. The data was analyzed as previously described (Nalefski and Newton 2001). The data was plotted as change in observed fluorescence versus free Ca^{2+} concentration and fitted to the following modified Hill equation

$$\Delta F_{obs} = \Delta F_{max} [x^n_H / x^n_H + ([Ca^{2+}]_{1/2}{}^n_H]$$

where ΔF_{max} equals the maximum fluorescence change, n_H represents the apparent Hill constant, and $[Ca^{2+}]_{1/2}$ represents the Ca^{2+} concentration at the midpoint in the titration, an approximation of the average Ca^{2+} dissociation constant. Since $[Ca^{2+}]_{1/2}$ was considerably greater than the concentration of total Ca^{2+} was taken as the free concentration.

PKCα cloning and mutagenesis

The rat PKCα DNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). PKCα was PCR amplified and subcloned into a pcDNA3-RFP plasmid using restriction sites EcoRI and XhoI. The mutations R249A and W247A were created using site-directed mutagenesis.

Cell transfection and imaging

HeLa 7 cells were maintained using DMEM (Cellgro) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37ºC. The constructs—pcDNA3-PKCα-WT-RFP, pcDNA3-PKCα-R249A-RFP, and pcDNA3-PKCα-W247A-RFP were transfected into HeLa cells using the FuGENE6 transfection system and allowed to grow 12-24 hours posttransfection. Cells were washed in Hanks' balanced solution (HBSS, Cellgro) and imaged in the dark at room temperature. Imaging experiments were performed on the Zeiss Axiovert microscope (Carl Zeiss MicroImaging, Inc) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software (Universal Imaging, Corp.) All optical filters were from Chroma Technologies. Data were collected as previously described (Violin, Zhang et al. 2003). Fluorescence resonance energy transfer (FRET) images were acquired through a 420/10-nm excitation filter, a 450-nm dichroic mirror, and a 535/25nm emission filter. For these experiments, cells with RFP values of approximately 3000 were selected for imaging to control for protein expression. Thapsigargin was added at the time indicated to the final concentration of 100nM. FRET ratios from several cells per

imaging plate were analyzed. The baseline for each of these ratios was subtracted; traces were normalized; and then averaged.

Results

Design of C2α mutants

The C2α domain, which shares the common structure with other C2 domains, is a 8-stranded anti-parallel β-sandwiches with 3 ligand- and membrane-binding loops at the top of the structure and 4 loops at the bottom to connect the β-strands (Verdaguer, Corbalan-Garcia et al. 1999). To study the importance of electrostatic and hydrophobic interactions of key residues in C2α membrane binding, we designed eight mutations on the domain (Figure 2-1A). The charged residues—C2α-R216, C2α-R249, and C2α-R252—were selected to examine the effect of electrostatic interactions and mutated to alanine. Residue R216 is located on the Ca^{2+} binding loop 2, while residues R249 and R252 reside on the Ca^{2+} binding loop 3 (Verdaguer, Corbalan-Garcia et al. 1999). Previous papers have shown these residues are important for membrane binding and enzyme activity (Medkova and Cho 1998; Conesa-Zamora, Lopez-Andreo et al. 2001; Stahelin and Cho 2001). In addition, EPR studies have shown R249 and R252 are involved in C2α penetration into the membrane (Kohout, Corbalan-Garcia et al. 2003). The hydrophobic residues— C2α-L191, C2α-W245, and C2α-W247—were chosen to determine the effect of hydrophobic interactions on C2 domain membrane binding and also mutated to an

alanine. Like the mutations of the charged residues, the mutated hydrophobic residues are located on Ca^{2+} binding loop 1 for C2α-L191 and Ca^{2+} binding loop3 for C2α-W245 and C2α-W247, respectively. In previous studies, it has been shown that the double mutant W245A/W247A are involved in membrane binding and important for enzymatic activity (Medkova and Cho 1998). The charged residue C2α-K268 and hydrophobic residue C2α-W274 are located on the opposite side of the C2 domain and were selected to serve as controls for these studies. The electrostatic model shows the charged surface of the C2α domain (Figure 2-1B). It shows the basic patches near the Ca^{2+} binding loops. The charged residue mutants—R216A, R249A, and R252A—will disrupt this charged area and allow us to study the importance of these basic areas within the Ca^{2+} binding loops.

Membrane binding kinetics for C2α mutants

To assess the individual contributions of the association and dissociation rate constants of the electrostatic and hydrophobic interactions of specific residues on the binding of C2α domain to membranes, we measured the changes in binding kinetics of our designed mutants. To do this, we used a FRET association experiment in which fluorescence resonance energy transfer (FRET) between dansyl-labeled lipid vesicles and the four intrinsic tryptophans in the C2α domain to measure the association rate constants (k_{on} rate constants). The k_{on} rate constants of the R216A, R249A, and R252A mutants show small changes from WT (Figure 2-2A) as the values ranged from 18.7 ± 0.7 to 19.5 ± 0.4 to 26.3 ± 0.2 $nM^{-1}s^{-1}$ compared to 22.41 ± 0.09 $nM^{-1}s^{-1}$

for wild-type C2 domain. In contrast, the k_{on} rate constants of hydrophobic residue mutants W245A and W247A decreased to 4.96 ± 0.04 $nM^{-1}s^{-1}$ and 9.0 ± 0.2 $nM^{-1}s^{-1}$ or 4-fold and 2-fold, respectively. Mutants K268A and W274A as well as L191A showed small increases in their k_{on} rate constants increasing to 34.0 ± 0.2 $nM^{-1}s^{-1}$, 27.1 ± 0.3 $nM^{-1}s^{-1}$, 31.0 ± 0.3 $nM^{-1}s^{-1}$, respectively. These results suggest that it is the hydrophobic interactions of the residues within the Ca^{2+} binding loops that are important for C2α domain recruitment, while electrostatic interactions of the charged residues play a much smaller role.

We also used fluorescence resonance energy transfer (FRET) between dansyl-labeled lipid vesicles and the four intrinsic tryptophans in the C2α domain to measure the dissociation rate constants (k_{off} rate constants) of the domain. However in these FRET dissociation experiments, we preincubated the C2α domain protein and dansyl-labeled lipid vesicles (35 mol % PS: 60 mol % PC: 5 mol % dPE) and then competed off the labeled vesicles with a 10-fold excess of unlabeled vesicles (40 mol % PS:60 mol % PC). The k_{off} rate constant for wild-type C2α domain was 0.71 ± 0.02s^{-1}. The k_{off} rate constants of charged residue mutants R216A, R249A, and R252A increased to 4.8 ± 0.2 s^{-1}, 13.1 ± 0.2 s^{-1}, and 5.03 ± 0.02 s^{-1} or 6-fold, 16-fold, and 6-fold, respectively (Figure 2-2B). In addition, the k_{off} rate constant of the mutant C2α-R249L was also determined and showed a 2-fold recovery in the retention over the mutant R249A (data not shown). This recovery suggests that not only is the basic charge of the arginine is important, but that the aliphatic carbons of the arginine contribute the C2α domain retention.

The k_{off} rate constants for mutants W245A and W247A also increased to 3.29 ± 0.02s^{-1} and 5.9 ± 0.1s^{-1} or 4-fold and 9-fold, respectively. The k_{off} rate constant of the L191A mutant remained the same at 0.864 ± 0.002 s^{-1}. For mutants K268A and W274A, the k_{off} rate constants decreased slightly to 0.556 ± 0.005 s^{-1} and 0.540 ± 0.009 s^{-1}, respectively. These results show that both electrostatic and hydrophobic interactions created by specific residues in the Ca^{2+} binding loops are critical for retaining the C2α domain at the membrane.

Overall membrane binding affinity for C2α mutants

To determine how the changes in the k_{on} rate constants and k_{off} rate constants affected the overall membrane binding, we calculated the K_d for the C2α domain mutations. The K_d^{calc} values for all C2α mutants, except the control mutants K268A and W274A and the hydrophobic residue mutant L191A, increased at least 6-fold compared to wild-type C2α domain (Table 2-1). For the R216A, R249A, and R252A mutants, the K_d^{calc} values showed an 8-, 21-, and 6-fold increase, respectively. For the hydrophobic residue mutants W245A and W247A, the differences were overall greater with K_d^{calc} values of 21-fold and 20-fold differences, respectively. The L191A mutant had no significant difference in K_d^{calc}. The control mutants K268A and W247A show slight decreases of approximately 0.5-fold in their K_d^{calc}. These results, like previous studies, show that the residues within the Ca^{2+} binding loops are critical for C2α domain membrane binding affinity.

Membrane recruitment kinetics for C2α mutants at varying ionic strengths

To examine the contribution of ionic strength on the recruitment of the C2α domain to the membrane, we measured the changes in k_{on} rate constants between wild type C2α domain and its mutants at increasing ionic strength using FRET association experiments between vesicles and the C2α mutants. The k_{on} rate constants for mutants R216A, R249A, and R252A showed little change as the ionic strength increased from 150mM NaCl to 200mM NaCl. In addition, when higher ionic strengths (175mM and 200mM NaCl) were normalized to each of their respective k_{on} rate constants at 150mM NaCl, we saw no differences between the relative change in k_{on} for wild-type C2α domain and the charged residue mutants (Figure 2-3A). In contrast, the k_{on} rate constants of hydrophobic residue mutants W245A and W247A increased as the ionic strength of the system was increased. When the k_{on} rate constants were normalized to their respective k_{on} rate constants at 150mM NaCl, both mutants W245A and W247A increased approximately 2-fold compared to wild-type C2α domain (Figure 2-3B). These results suggest the hydrophobic residues W245 and W247 have increased ionic strength sensitivity compared to the wild-type C2α domain, while the charged residues R216, R249, and R252 have similar ionic strength sensitivity as the wild-type C2α domain.

Membrane retention kinetics for C2α mutants at varying ionic strengths

To assess the contribution of ionic strength on the retention of C2α domain mutants, we measured the changes in k_{off} rate constants at increasing ionic strength

using the FRET dissociation experiments. The k_{off} rate constant of the wild-type C2α domain increased as ionic strength increased. For mutants R216A, R249A, and R252A, the k_{off} rate constants also increased as the ionic strength of the system was increased. However, when normalized to just examine the contribution of ionic strength, mutants R216A, R249A, and R252A showed no significant difference from wildtype C2α domain (Figure 2-4A). The k_{off} rate constants of mutants W245A and W247A also increased as the ionic strength of the system was increased. Like the charged residue mutants, the W245A and W247A mutants showed similar ionic strength sensitivity in their k_{off} rate constants to wild-type C2α domain (Figure 2-4B). The similar ionic strength sensitivity of the C2α mutants to wild-type C2α domain indicates that ionic strength does not play a significant role the retention of C2α domain at lipid vesicles.

Overall membrane binding affinity for C2α mutants at varying ionic strengths

To determine the effect of ionic strength on the overall binding affinity of the C2α mutants, we calculated the K_d values from the k_{on} and k_{off} measured for the FRET association and FRET trapping experiments. For mutants R216A, R249A, and R252A, the increase in their k_{off} rate constants leads to increases in their overall K_d^{calc}. However, when the K_d^{calc} values were normalized to look at just the ionic strength sensitivity, these mutants showed the same ionic strength sensitivity as wild-type C2α domain (Figure 2-5A). In contrast, mutants W245A and W247A show no ionic strength sensitivity in their binding affinity for lipid vesicles (Figure 2-5B). By

examining at the individual contributions of the k_{on} and k_{off} rate constants, the changes in the recruitment and retention cancel each other out and the overall lipid vesicle binding affinity is insensitive to increases in ionic strength. These results show that without these hydrophobic residues, the overall C2α domain lipid vesicle binding affinity is insensitive to ionic strength changes, while wild-type C2α domain and the charged residue mutants are sensitive to increasing ionic strength.

Membrane binding kinetics for double charged residue C2α mutants

To examine whether the charged residues work together to promote the binding of the C2α domain, we designed the double charged residue mutants C2α-R216A/R249A, C2α-R216A/R252A, and C2α-R249A/R252A. Previous studies have looked at the contribution of mutants R249A/R252A, R216A, or R249A, but no study has systematically looked at both their combined and individual contribution to k_{on} rate constants and k_{off} rate constants (Medkova and Cho 1998; Conesa-Zamora, Lopez-Andreo et al. 2001; Stahelin and Cho 2001). We measured the kinetics of these domains using both the FRET association and FRET dissociation experiments. The k_{on} rate constants for mutants R216A/R252A and R249A/R252A showed no change with k_{on} rate constants of 25 ± 1 $nM^{-1}s^{-1}$ and 26 ± 2 $nM^{-1}s^{-1}$, respectively, compared to wild-type C2α domain. In contrast, the k_{on} rate constant for mutant R216A/R249A showed an approximately 2-fold decrease to 9 ± 1 $nM^{-1}s^{-1}$ compared to wild-type C2α domain (Figure 2-6A). These data suggest that R216 and R249 can compensate for

each other in the recruitment of the C2α domain when only one of the residues is mutated.

In contrast, the k_{off} rate constants increase to 42 ± 4 s^{-1}, 30.3 ± 0.7 s^{-1}, 32.0 ± 0.4 s^{-1}, or 50-, 37- and 38-fold differences for mutants R216A/R249A, R216A/R252A, and R249A/R252A, respectively (Figure 2-6B). These results show the critical role that these charged residues play in retaining the C2α domain at lipid vesicles. The overall binding affinities for the R216A/R249A, R216A/R252A, and R249A/R252A mutants decreased significantly shown by 148-, 41-, and 37-fold increases in K_d^{calc}, respectively (Table 2-2).

Gibbs free energy change for single and double charged residue C2α mutants

To further look at the properties of the double charged mutations, we calculated their Gibbs free energy changes of the double charged residue C2α mutants (Table 2-3). We found that the free energy changes resulting from the single mutations of R216 and R249 to alanine were 5.2 ± 0.2 $kcalmol^{-1}$ and 7.2 ± 0.2 $kcalmol^{-1}$, while the free energy change resulting from the double mutation of R216 and R249 to alanines was as predicted at 12 ± 2 $kcalmol^{-1}$. Likewise, the free energy change resulting from the single mutations of R216 and R252 to alanine were 5.2 ± 0.2 $kcalmol^{-1}$ and 4.0 ± 0.4 $kcalmol^{-1}$, while the free energy change resulting from the double mutation of R216 and R252 to alanines was 8.7± 0.4 $kcalmol^{-1}$ as would be predicted. In contrast, we demonstrated that the free energy change resulting from the single mutations of R249 and R252 to alanine were 7.2 ± 0.2 $kcalmol^{-1}$ and 4.0 ± 0.4

kcalmol^{-1}, while the free energy change resulting from the double mutation of R249 and R252 to alanines was as less than predicted at 8.5 ± 0.7 kcalmol^{-1}. These results suggest not only that the individual residues play an important role, but also that R249 and R252 may work together to promote C2α domain membrane binding.

Effects of increasing Ca^{2+} on C2α binding kinetics

Surprisingly, the charged residue mutants R216A, R249A, and R252A showed only a modest change in their recruitment to the membrane. In previous studies, it has been suggested that electrostatic interactions play a critical role in the membrane recruitment of the C2 domains including C2 domain of PKCα (Nalefski and Newton 2001; Murray and Honig 2002). In studies looking at the C2β domain membrane binding, it is suggested that the binding of Ca^{2+} ions acts as an electrostatic switch for membrane binding (Nalefski and Newton 2001). In addition, modeling studies also show the binding of Ca^{2+} ions and its effects on electrostatic interactions are critical for C2 domain membrane binding (Murray and Honig 2002). To examine the effect of Ca^{2+} concentration of the recruitment and retention of the C2α domain at lipid vesicles, we measured the kinetics at subsaturating and saturating Ca^{2+} concentrations ((20μM and 200μM, respectively) using the FRET association and FRET dissociation experiments. For the k_{on} rate constants, there was an approximately 2-fold increase in the recruitment to the lipid vesicles from 10.12 ± 0.09 nM^{-1}s^{-1} to 22.41 ± 0.09 nM^{-1}s^{-1} (Figure 2-7A). These data support the previous studies, that Ca^{2+} binding is important for recruitment of C2α domain to the membrane. Underscoring the importance of the

Ca^{2+} binding to the C2α domain, we also looked at binding at 2 μM Ca^{2+} and could not detect any binding (data not shown).

We also looked at the effect of Ca^{2+} binding on the retention of C2α domain. For the k_{off} rate constants, increasing the Ca^{2+} concentration decreased the k_{off} rate constants for wild-type C2α domain approximately 5-fold from 3.9 ± 0.6 s^{-1} to 0.71 ± 0.02 s^{-1} (Figure 7B). These results are similar to the previous studies showing that Ca^{2+} binding is critical for not only recruitment to the membrane, but also its membrane retention (Nalefski and Newton 2001). The overall binding affinity for the lipid vesicles increased as the K_d^{calc} decreased from 0.23 ± 0.02 nM to 0.031 ± 0.007 nM. These results indicate that Ca^{2+} does play a role in membrane recruitment, but appears to play a larger role in C2α domain retention at the membrane. Interestingly, the recruitment of wild-type C2α domain to lipid vesicles is not sensitive to ionic strength as the k_{on} rate constants do not significantly vary at different ionic strengths, but the retention of the C2α domain at lipid vesicles is sensitive to increasing ionic strength. Therefore, the overall membrane binding affinity of the C2α domain is sensitive to changes in ionic strength.

C2α mutants affect Ca^{2+} binding

Since Ca^{2+} plays such an important role in C2α domain recruitment and retention at the membrane, we wanted to assess the effect of the mutations on the C2α domain on its Ca^{2+} binding. To do this, we measured the Ca^{2+} binding affinity of the

C2α domains in the absence of lipid vesicles. The Ca^{2+} binding affinity of the C2α domain was 32 ± 4 μM, similar to previous studies (Table 2-4) (Medvoka, Kohout). The mutant R216A appears to not play a role in Ca^{2+} binding as its affinity is similar to the wildtype C2α domain at 38 ± 2 μM. However, the mutants R249A and R252A show a significant decrease in Ca^{2+} binding affinity to 1409 ± 69 μM and 395 ± 27 μM, respectively. However, in the presence of saturating lipid vesicles, most of the Ca^{2+} binding affinity for the R249A and R252A mutants is recovered as their Ca^{2+} binding affinities are 71 ± 3 μM and 27.6 ± 0.3 μM, respectively (data not shown). For the mutants W245A and W247A, their Ca^{2+} binding affinities were reduced to 74 ± 4 μM and 263 ± 12 μM, respectively. The mutations within the Ca^{2+} binding loops affect the overall Ca^{2+} binding affinities of the mutant C2α domains.

Mutations in C2α domain affect PKCα activity in live cells

To determine whether the mutation of R249 and W247 to alanine have an effect on PKCα activity in live cells, we introduced these mutations into full-length PKC and measured PKCα activity. In previous studies, mutations in the Ca^{2+} binding loops including the mutants R249A and W245A/W247A reduce PKCα activity *in vitro* (Medkova and Cho 1998; Conesa-Zamora, Lopez-Andreo et al. 2001). Previously in our lab, a C kinase activity reporter (CKAR) was developed to monitor PKC activity in live cells (Violin, Zhang et al. 2003; Gallegos, Kunkel et al. 2006). To determine if the decrease in membrane binding affinity affected the kinase activity

of these mutants, HeLa cells were transfected with CKAR and PKCα-WT-RFP, PKCα-R249A-RFP, or PKCα-W247A-RFP, stimulated with thapsgargin to release intercellular Ca^{2+} stores, and then imaged to measure the CKAR phosphorylation within the cells.

Compared to the activity of cells transfected with PKCα-WT-RFP, the cells with transfected PKCα-R249A-RFP and PKCα-W247A-RFP showed a slower response time to thapsgargin as well as decreased rate and amount of overall phosphorylation of CKAR and an increased percent decay of substrate phosphorylation (Figure 2-8). For cells with PKCα-WT, the time for it to reach one-half maximal activity was approximately 120sec, while cells with PKCα-R249A-RFP and PKCα-W247A-RFP took longer, 160sec and 210sec, respectively. In addition, the overall amplitude, or total activity, for cells with PKCα-WT was greater than for cells with PKCα-R249A-RFP and PKCα-W247A-RFP. The percent decay for cells (defined as the decrease from the highest FRET ratio to the FRET ratio at 1200sec) transfected with PKCα-WT-RFP was less at 23 ± 6 % compared to 53 ± 9 % and 68 ± 12 % for cells with PKCα-R249A-RFP and PKCα-W247A-RFP, respectively. These results show that the mutants R249A and W247A not only affect the binding of PKCα, but also slow its activation, decrease its overall activity, and increase the rate of decay of the signal.

Discussion

We have examined the role of electrostatic and hydrophobic interactions in PKCα membrane binding and activation using stopped flow kinetics and activity assays in live cells. Using the stopped flow kinetics, we have been able to look at the individual contributions of the on and off rate constants as well as the overall effect of these interactions on membrane binding. We show that interactions of charged residues play a key role in retaining the C2 domain at the membrane, while interactions of hydrophobic residues play a role in both the recruiting and retaining of the C2 domain at the membrane. In addition, we showed that the key electrostatic component in C2 domain recruitment is Ca^{2+} binding to the domain. Live cell imaging of the CKAR showed a slowed rate of activation, reduced overall response, and an increased rate of decay in response to increased intracellular Ca^{2+}.

The role of charged residues in C2α domain binding to membranes

To examine of role of the charged residues within the Ca^{2+} binding loops, we mutated three arginine residues—R216, R249, and R252—located within the Ca^{2+} binding loops of the C2α domain. These mutants only have a modest effect on the recruitment of the C2α domain to the membrane, but a significant effect on the retention of the domain at the membrane and overall membrane binding affinity. These results are consistent with previous studies that have implicated mutants R216A, R249A, and R249A/R252A in decreased membrane binding affinity, translocation to the membrane, and enzymatic activity (Medkova and Cho 1998;

Conesa-Zamora, Lopez-Andreo et al. 2001; Stahelin and Cho 2001; Bolsover, Gomez-Fernandez et al. 2003). The differences in membrane association and dissociation rates for mutants R216A and R249A/R252A in the study by Stahelin et al. from our study might be due in part to difference in the use of monolayer versus bilayer in the different systems and differences in ionic strength. However, in both studies it is clear that reduction of the residency time is the dominant effect of the R216A mutant, but at the lower ionic strength of their studies, it showed that the R249A/R252A double mutant has a greater effect on the recruitment of C2α to the membrane.

Our studies found that the mutation of R249 to an alanine had the greatest impact on the off rate constants of membrane binding. Given its proposed location and key role, R249 may be playing a role in both electrostatic and hydrophobic interactions. The drastic decrease in residency time (increased k_{off} rate constants) at the membrane suggests that R249 provides stability for the C2 domain-phospholipid complex. R249 may provide this stability in three ways: first, the guanidinium group of R249 has been suggested to interact with the sn-2 ester carbonyl group of PS; second, the main chain nitrogen of R249 forms hydrogen bonds with the sn-1 ester oxygen atom of the fatty acyl chains of PS; and third, that the aliphatic carbons of the R249 interact with the acyl chains of PS (Verdaguer, Corbalan-Garcia et al. 1999; Ochoa, Corbalan-Garcia et al. 2002) . The importance of the aliphatic carbons in providing more stability is supported by the partial recovery of C2α domain retention at the membrane as shown by an increase in residency time between the mutants R249L and R249A. Our studies also found that the mutation of R216 and R252 to an

alanine significantly decreased the retention of the C2α domain to the membrane. Like R249, the guanidinium group of R216 may also interact with the sn-2 ester carbonyl of the fatty acyl chain of PS (Verdaguer, Corbalan-Garcia et al. 1999). Taken together, these data suggest that R216, R252, and most importantly R249 play an important role in the stabilization of the domain-lipid complex.

To further understand the role of electrostatic interactions, the effect of mutating R216, R249, or R252 to alanine was studied at varying ionic strength. The on rate constants of the R216A, R249A, and R252A mutants were insensitive to changes in ionic strength similar to wildtype C2α domain. This insensitivity suggests that at physiological and higher ionic strengths, the recruitment of the C2α domain to the membrane is not affected by changes in ionic strength.

In contrast, the off rate constants were sensitive to changes in ionic strength. The off rate constants of the R216A, R249A, and R252A mutants show the same ionic strength sensitivity as wild-type C2α domain. This similar ionic strength sensitivity supports the model that R249 and R252 are inserted into the membrane rather than be solvent exposed. This model is supported by EPR studies that have shown both R249 and R252 may penetrate into the membrane bilayer interacting with polar and anionic groups within the headgroup layer (Verdaguer, Corbalan-Garcia et al. 1999; Kohout, Corbalan-Garcia et al. 2003). These studies indicate that R249 penetrates deeper into the bilayer than R252 suggesting that R249 may interact at or near the interface with the hydrocarbon core, while R252 is located closer to the bilayer surface. Likewise, the off rate of mutant R216A shows similar ionic strength insensitivity to the R249A

and R252A mutants suggesting that R216 is not solvent exposed. Regardless, the similar ionic strength sensitivities between the on and off rate constants of wild-type C2α domain and these mutants suggest that the ionic strength sensitivity of the C2α region is located outside these residues in the Ca^{2+} binding loops.

In addition, the effect of making double mutation of arginine to alanine at positions R216, R249, and R252 were studied. In previous studies, the double mutant R249A/R252A had reduced membrane binding affinity and enzymatic activity (Medkova and Cho 1998; Stahelin and Cho 2001; Stahelin, Rafter et al. 2003). In our studies, looking at both the single and double mutations, we showed that individually R216 and R249 may compensate for each other in the recruitment of the C2α domain to the membrane as the single mutants show only a modest decrease in on rate constant. However, when both R216 and R249 are lost, a significant decrease in the recruitment is observed, suggesting that interaction of only one of these residues with the sn-2 ester carbonyl of the fatty acyl chain of a phospholipid is sufficient for C2α recruitment.

All three double charged mutants, in particular R216A/R249A, show a large decrease in both residency time at the membrane and overall membrane binding affinity. This result suggests that the role of the guanidinium groups and their interactions with the sn-2 ester carbonyl group of anionic phospholipids is essential to C2α domain membrane retention and binding. The overall binding of the double mutants R216A/R252A and R249A/R252A was significantly decreased; however, the change was less than expected suggesting that R252 may be able to somehow

compensate for the loss of the guanidinium group of R216 and R249. The overall free energy change in the stability of the mutant relative to the wild-type C2α domain suggests that the R249A/R252A mutant is non-additive, while mutants are R216/R249A and R216A/R252A are additive. These results suggest that the replacement of the two arginines at positions 249 and 252 with alanine may lead to more favorable interactions than expected. The loss of the two bulky arginines groups may get rid of some steric hindrance and create a hydrophobic interaction with the acyl chains of a phospholipid.

As previously reported, the Ca^{2+} binding was significantly affected by mutants R249A and R252A suggesting that these residues play a significant role in the coordination of the Ca^{2+} ions, while R216 does not play an important role in Ca^{2+} binding (Medkova and Cho 1998). However, the addition of phospholipids appears to help stabilize the Ca^{2+} binding of these mutants as most of their Ca^{2+} affinity is recovered in the presence of saturating lipid vesicles. Interestingly, lower Ca^{2+} binding affinity of the W247A mutant maybe caused, in part, by the role of the tryptophan's main chain nitrogen in the coordination of Ca^{2+}.(Ochoa, Corbalan-Garcia et al. 2002).

To further examine the importance of electrostatic interactions in C2α membrane binding, we also looked at membrane binding in the presence of low and high Ca^{2+} concentrations. Previous studies have shown that Ca^{2+} is critical for overall binding affinity (Medkova and Cho 1998; Nalefski and Newton 2001; Stahelin and Cho 2001; Murray and Honig 2002). In our studies, we have shown that Ca^{2+} binding

is critical in both the recruitment and retention of the C2α domain at the membrane. The on rate constant of the C2α domain increases while the off rate constant decreases as Ca^{2+} is increased from a subsaturating level to a saturating level. However, again the recruitment of the C2 domain at varying Ca^{2+} concentrations appeared to be insensitive to ionic strength, but the retention of the C2 domain at the membrane was sensitive to changes in ionic strength. These results, along with the same ionic strength sensitivity of the charged residue mutants, further suggest that the ionic strength sensitive region is located outside the Ca^{2+} binding loops.

The role of hydrophobic residues in C2α domain binding to membranes

To examine of role of hydrophobic interactions, we mutated three residues – L191, W245, and W247—located within the Ca^{2+} binding loops of the C2α domain. The mutation of L191 located on Ca^{2+} binding loop 2 had no significant effect on either the on rate constant or off rate constant suggesting that this residue does not have a significant role in C2α domain membrane binding. However, the other two hydrophobic residue mutants W245A and W247A appear to play important roles in both the recruitment to and retention of the C2α domain on the membrane. For both mutants W245A and W247A, the on rate constants show a significant decrease and the off rate constants show a significant increase leading to a drastic reduction in overall membrane binding affinity. These results suggest that both W245 and W247 are involved in both the recruitment and retention of C2α domain. These data are consistent with previous studies that have suggested that these two residues penetrate

into the membrane (Medkova and Cho 1998; Verdaguer, Corbalan-Garcia et al. 1999; Stahelin and Cho 2001).

To further understand the role of hydrophobic interactions, the effect of mutating W245 and W247 to alanine were studied are varying ionic strength. The on rate constants of mutants W245A and W247A were sensitive to changes in ionic strength while the on rate constants of wild-type C2α domain were insensitive to changes in ionic strength. This sensitivity suggests that hydrophobic interactions dominate the recruitment of the C2α domain to the membrane, but when either tryptophan is lost, weaker electrostatic interactions become important for membrane recruitment. In contrast, like the charged residue mutants, off rate constants for the mutants W245A and W247A show the same ionic strength sensitivity as wild-type C2α domain. These data further support the model in which the component that makes the membrane retention of the C2α domain sensitive to ionic strength is located outside the Ca^{2+} binding loops.

The effect of charged and hydrophobic residue mutants on PKCα activity in live cells

After showing the importance of electrostatic and hydrophobic interactions in the binding of the isolated domain, we addressed if the differences in on and off rate constants of a charged and hydrophobic residue mutants affected the kinase activity of PKCα. It had been shown previously that mutants in the Ca^{2+} binding loops had significant effects on enzymatic activity *in vitro* (Medkova and Cho 1998; Conesa-

Zamora, Lopez-Andreo et al. 2001). Taking advantage of CKAR, we measured the activity of mutants R249A and W247A using the activity reporter in HeLa cells. The results of the imaging correlate with the *in vitro* binding assays suggesting these mutation not only affect membrane binding, but also the activation of PKCα. In these experiments, the cells with transfected PKCα-R249A-RFP and PKCα-W247A-RFP show decreased response time to thapsigargin as well as a decreased rate of overall phosphorylation of CKAR, and an increased decay compared to cells transfected with PKCα-WT-RFP. These results show that the changes in membrane recruitment and retention of the C2α domain mutants correlate with the differences in the activity of PKCα in these mutants in live cells.

Conclusions

Taken together, our data suggests a model where electrostatic interactions created by the binding of Ca^{2+} ions and hydrophobic interactions of specific residues with membrane phospholipids are the critical factors in the recruitment of the C2α domain. The retention of the C2α domain involves not only the electrostatic interactions of Ca^{2+} binding and hydrophobic interactions created by the hydrophobic residues, but also electrostatic and hydrophobic interactions of the charged residues within the Ca^{2+} binding loops. Our data also suggest that R249 plays a dual role in electrostatic and hydrophobic interactions that is critical for the retention of the C2α domain.

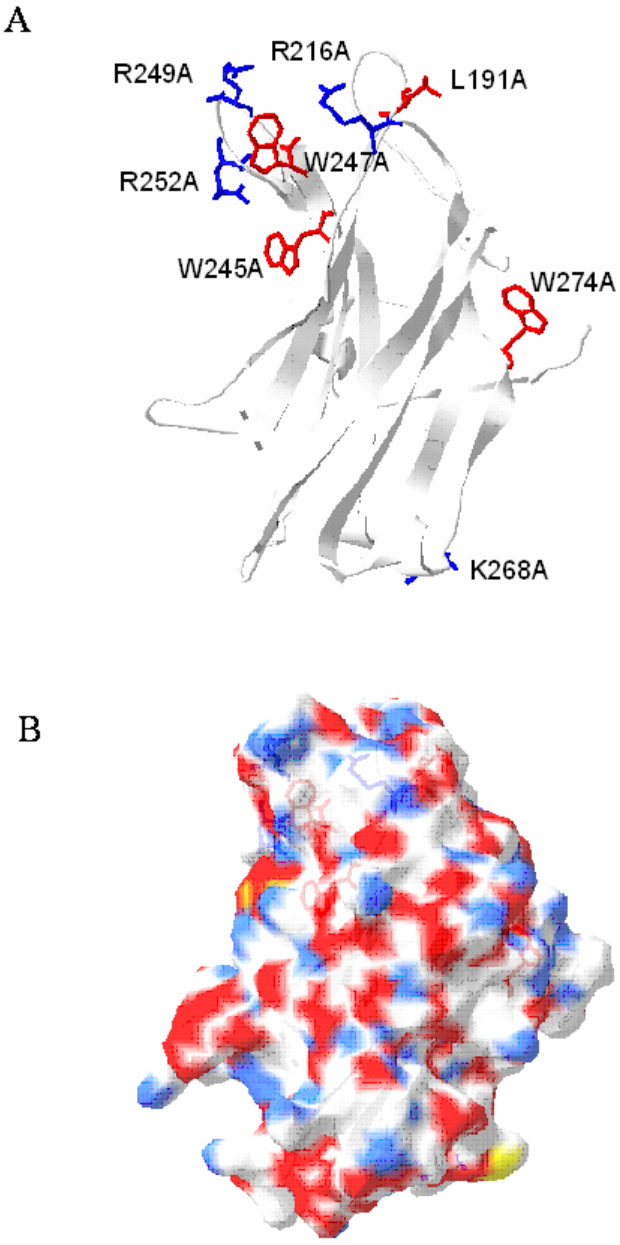
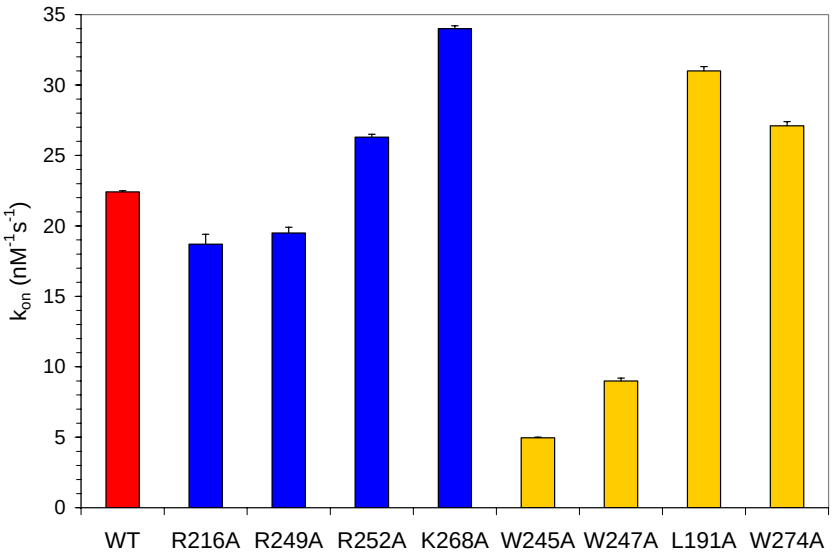


Figure 2-1. **Structure of C2α domain and mutations**. (A) Ribbon diagram includes the C2α mutants used in this study. Mutants C2α-R216A, C2α-R249A, and C2α-R252A are utilized to study the interactions of these specific residues in C2α domain membrane binding and C2α-W245A, C2α-W247A, and C2α-L191A are hydrophobic residue mutants used to examine the interactions of these specific residues in C2α domain membrane binding. C2α-K268A and C2α-W274A served as control mutants. (B) Electrostatic potential model of the C2α domain shows the charge of the domain's surface. Blue indicates basic surfaces, red shows the acidic surfaces, and yellow shows the non-polar surfaces.

Figure 2-2. **Charged and hydrophobic residue mutations on the C2α domain affect its recruitment to and retention at lipid vesicles**. (A) The binding of purified C2α-WT, C2α-R216A, C2α-R249A, C2α-R252A, C2α-K268A, C2α-W245A, C2α-W247A, C2α-L191A, and C2α-W274A and anionic phospholipid vesicles was measured by FRET association stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled phospholipid vesicles (35 mol %PS:60 mol % PC:5 mol % dPE) at 200μM Ca^{2+} and 150mM NaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{obs} was determined using a monophasic fit. The k_{on} is calculated from the slope of the linear plot of k_{obs} versus vesicles concentration. The weighted averages are calculated from three to seven independent experiments. (B) The binding of purified C2α-WT, C2α-R216A, C2α-R249A, C2α-R252A, C2α-K268A, C2α-W245A, C2α-W247A, C2α-L191A, and C2α-W274A and anionic phospholipid vesicles was measured by FRET dissociation stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was incubated for 30 minutes with dansyl-labeled phospholipid vesicles (35 mol %PS: 60 mol % PC:5 mol % dPE). The protein and labeled lipid were then rapidly mixed with equal volume of 10-fold higher concentration of unlabeled phospholipid vesicles (40 mol % PS:60 mol % PC) at 200μM Ca^{2+} and 150mMNaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{off} was determined using a monophasic fit. The weighted averages are calculated from three to seven independent experiments.

A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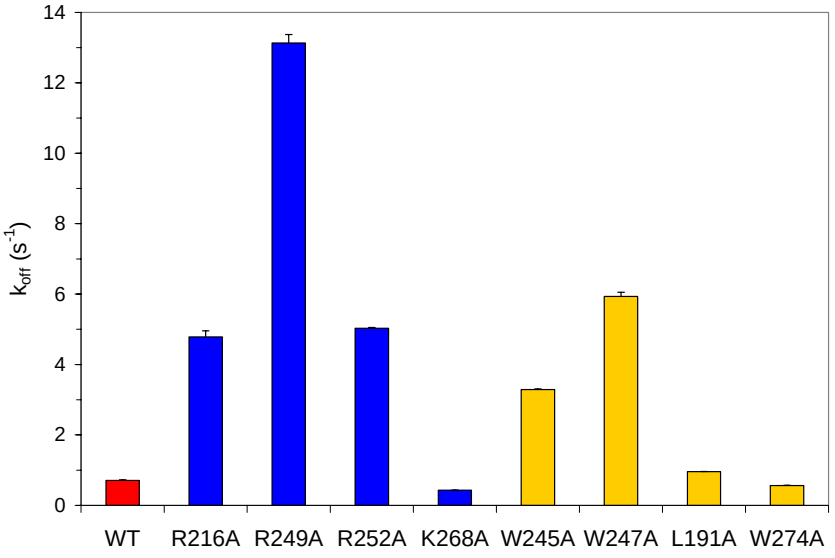
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W247A
L191A
W274A

Table 2-1. **Effect of charged and hydrophobic residue mutations on the C2α domain on its overall binding affinity to lipid vesicles.** Weighted averages of between three and seven independent experiments carried out as described in the figures 2-2A and 2-2B. The K_d^{calc} values indicate the calculated K_d from the weighted averages ± S.E.M. The fold difference values were calculated from comparing the K_d^{calc} of wildtype C2α domain to the K_d^{calc} of the mutants and represented as fold difference ± S.E.M.

	K_d^{calc} (nM)	Fold Difference from WT
WT	0.0379 ± 0.0009	1.00 ± 0.04
R216A	0.31 ± 0.01	8.3 ± 0.4
R249A	0.69 ± 0.01	18.2 ± 0.7
R252A	0.19 ± 0.02	5.1 ± 0.1
K268A	0.0164 ± 0.0002	0.4 ± 0.01
W245A	0.790 ± 0.009	20.8 ± 0.6
W247A	0.88 ± 0.02	23.2 ± 0.8
L191A	0.0279 ± 0.0003	0.7 ± 0.02
W274A	0.0199 ± 0.0004	0.5 ± 0.02

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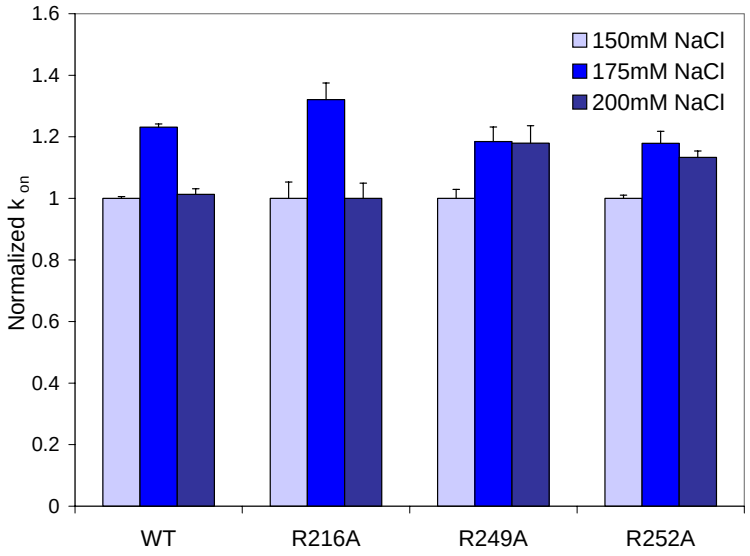

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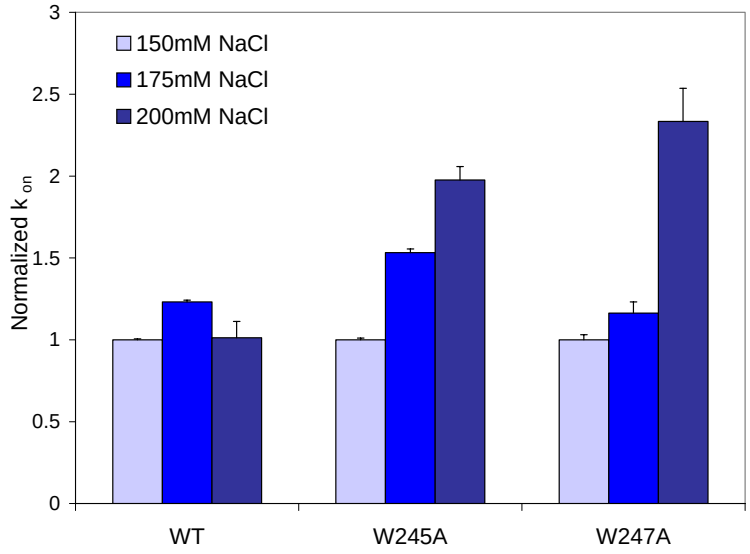

Figure 2-3. **Hydrophobic residue mutations on the C2α domain affect recruitment to lipid vesicles at varying ionic strength, but charged residue mutations do not.** (A) The binding of purified C2α-WT, C2α-R216A, C2α-R249A, and C2α-R252A and anionic phospholipid vesicles was measured by FRET association stopped flow analysis as described in figure 2-2A. The conditions were the same except for increasing concentrations of NaCl as indicated. The weighted averages are calculated from three to seven independent experiments. (B) The binding of purified C2α-WT, C2α-W245A, and C2α-W247A and anionic phospholipid vesicles was measured by FRET association stopped flow analysis as described in figure 2-2A. The conditions were the same except for increasing concentrations of NaCl as indicated. The weighted averages are calculated from three to seven independent experiments.

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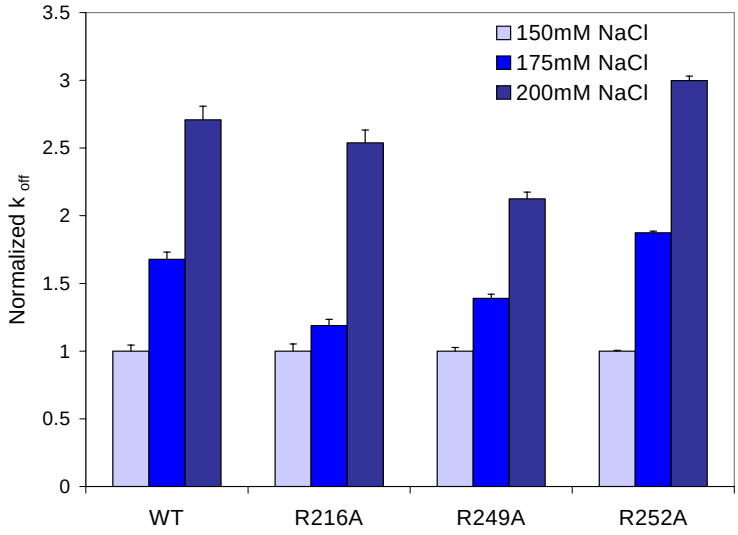

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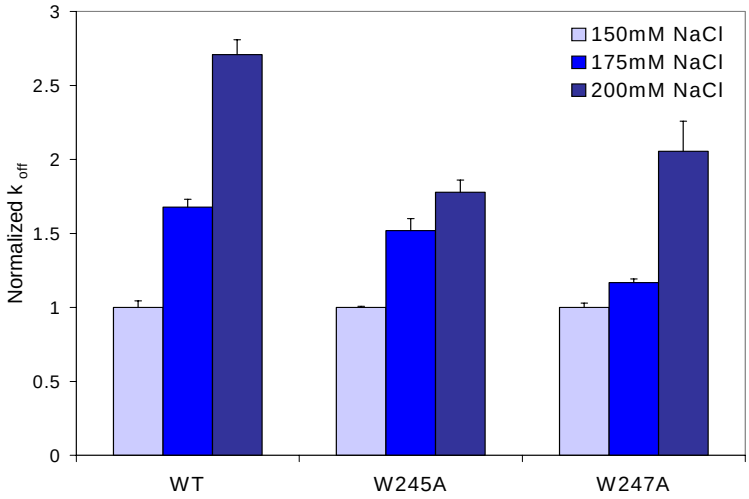

Figure 2-4. **Charged and hydrophobic residue mutations on the C2α domain show the same ionic sensitivity as wildtype C2α domain in their retention at lipid vesicles.** (A) The binding of purified C2α-WT, C2α-R216A, C2α-R249A, and C2α-R252A and anionic phospholipid vesicles was measured by FRET dissociation stopped flow analysis as described in figure 2-2B. The conditions were the same except for increasing concentrations of NaCl as indicated. The weighted averages are calculated from three to seven independent experiments. (B) The binding of purified C2α-WT, C2α-W245A, and C2α-W247A and anionic phospholipid vesicles was measured by FRET stopped flow analysis as described in figure 2-2B. The conditions were the same except for increasing concentrations of NaCl as indicated. The weighted averages are calculated from three to seven independent experiments.

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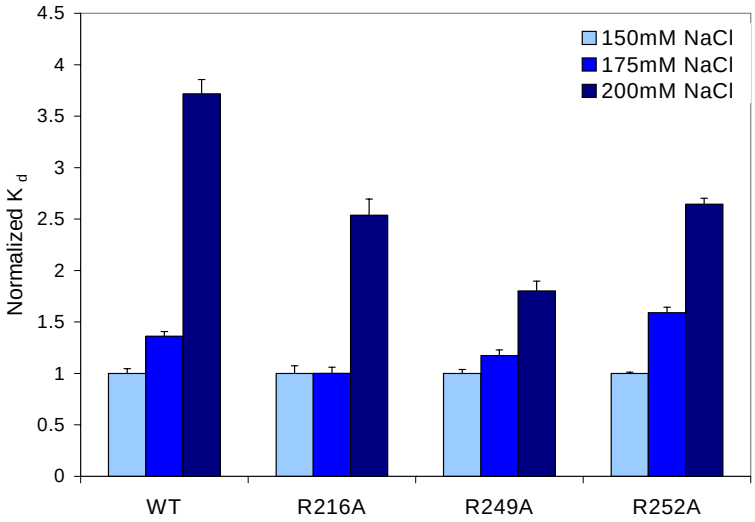

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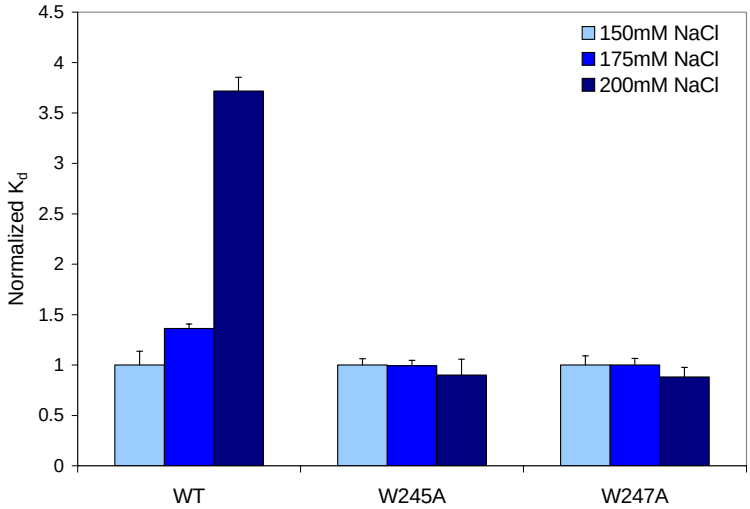
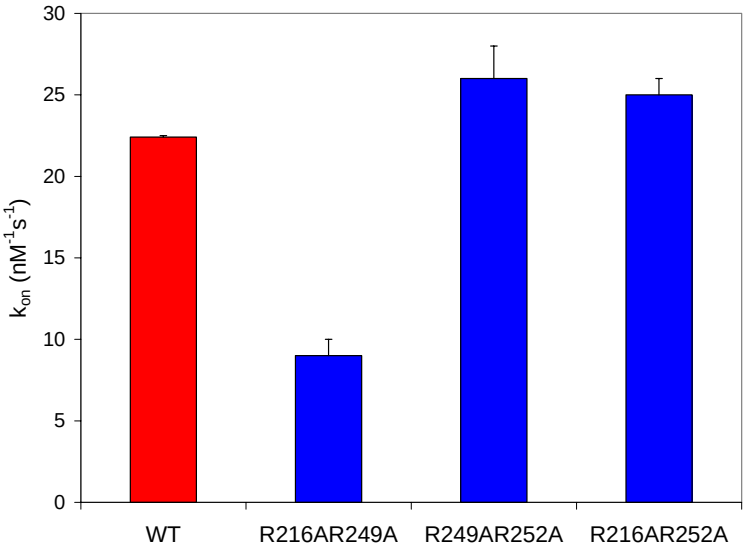


Figure 2-5. **Charged residue mutations on the C2α domain show the same ionic sensitivity as wildtype C2α domain to their overall lipid vesicle binding affinity, while hydrophobic residue mutations on the C2α domain do not.** (A) The K_d^{calc} values were calculated from the k_{on} and k_{off} rate constants of the FRET association and dissociation experiment at varying ionic strength for wild-type C2α domain and mutants R216A, R249A, and R252A. Weighted averages of between three and seven independent experiments carried out as described in the figures 2-3A and 2-4A. (B) The K_d^{calc} values were calculated from the k_{on} and k_{off} rate constants of the FRET association and dissociation experiment at varying ionic strength for wild-type C2α domain and the mutants W245A and W247A. Weighted averages of between three and seven independent experiments carried out as described in the figures 2-3B and 2-4B.

A



B

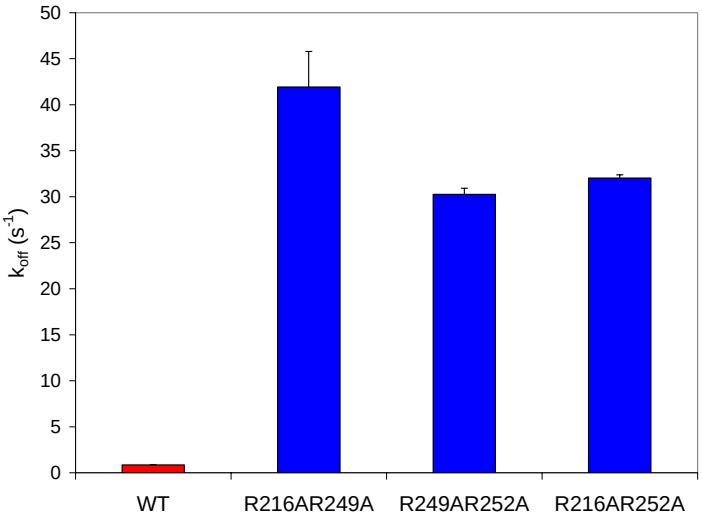


Figure 2-6. **Double charged mutations on the C2α domain affect recruitment to, and retention at, lipid vesicles.** (A) The binding of purified C2α-WT, C2α-R216A/R249A, C2α-R216A/R252A, and C2α-R249A/R252A and anionic phospholipid vesicles was measured by FRET association stopped flow analysis as described in Figure 2-2A. The weighted averages are calculated from three to five independent experiments. (B) The binding of purified C2α-WT, C2α-R216A/R249A, C2α-R216A/R252A, and C2α-R249A/R252A and anionic phospholipid vesicles was measured by FRET dissociation stopped flow analysis as described in Figure 2-2B. The weighted averages are calculated from four to seven independent experiments.

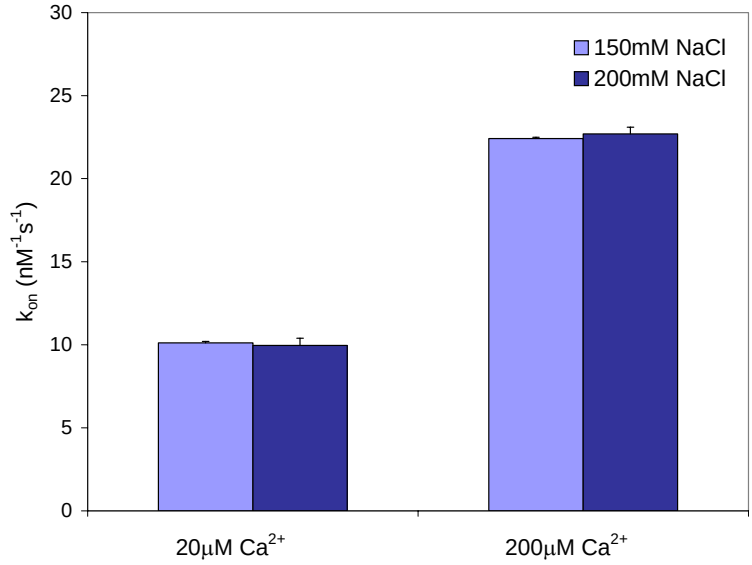
Table 2-2. **Effect of double charged mutants on the overall binding affinity to lipid vesicles of the C2α domain.** Weighted averages of between three and seven independent experiments carried out as described in the figures 2-6A and 2-6B.The K_d^{calc} values indicate the calculated K_d from the weighted averages ± S.E.M. The fold difference values were calculated from comparing the K_d^{calc} of the wildtype C2α domain to the K_d^{calc} of the mutants and represented as fold difference ± S.E.M.

	K_d^{calc} (nM)	Fold Difference from WT
WT	0.032 ± 0.001	1.00 ± 0.04
R216A/R249A	4.7 ± 0.7	148 ± 22
R216A/R252A	1.28 ± 0.05	41 ± 2
R249A/R252A	1.16 ± 0.09	37 ± 3

Table 2-3. **Effect of single and double charged mutants on free energy of lipid vesicle binding of the C2α domain.** Weighted averages of between three and seven independent experiments carried out as described in the figures 2-6A and 2-6B. The Δ(Δ G) values indicate the change in free energy of the C2α mutant compared to the wildtype C2α domain calculated from the K_d^{calc} represented as the weighted averages ± S.E.M.

Compared to C2α-WT	Δ(ΔG) (kcalmol^{-1})
R216A	5.2 ± 0.2
R249A	7.2 ± 0.2
R252A	4.0 ± 0.4
R216AR249A	12 ± 2
R216AR252A	8.7 ± 0.4
R249AR252A	8.5 ± 0.7

A



B

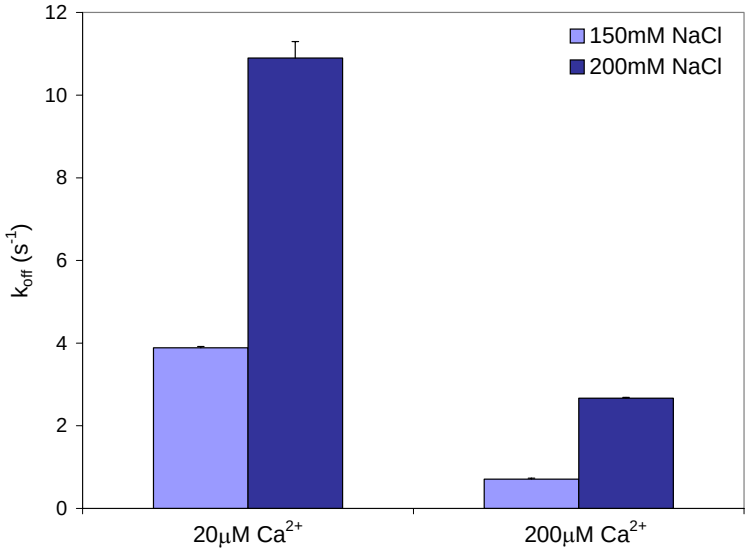


Figure 2-7. **Increasing Ca^{2+} affects the C2α domain recruitment to, and retention at, lipid vesicles.** (A) The binding of purified C2α-WT and anionic phospholipid vesicles at 20μM and 200μM Ca^{2+} was measured by FRET association stopped flow analysis as described in Figure 2-2A. The weighted averages are calculated from three to seven independent experiments. (B) The binding of purified C2α-WT and anionic phospholipid vesicles at 20μM and 200μM Ca^{2+} was measured by FRET dissociation stopped flow analysis as described in Figure 2-2B. The weighted averages are calculated from three to four independent experiments.

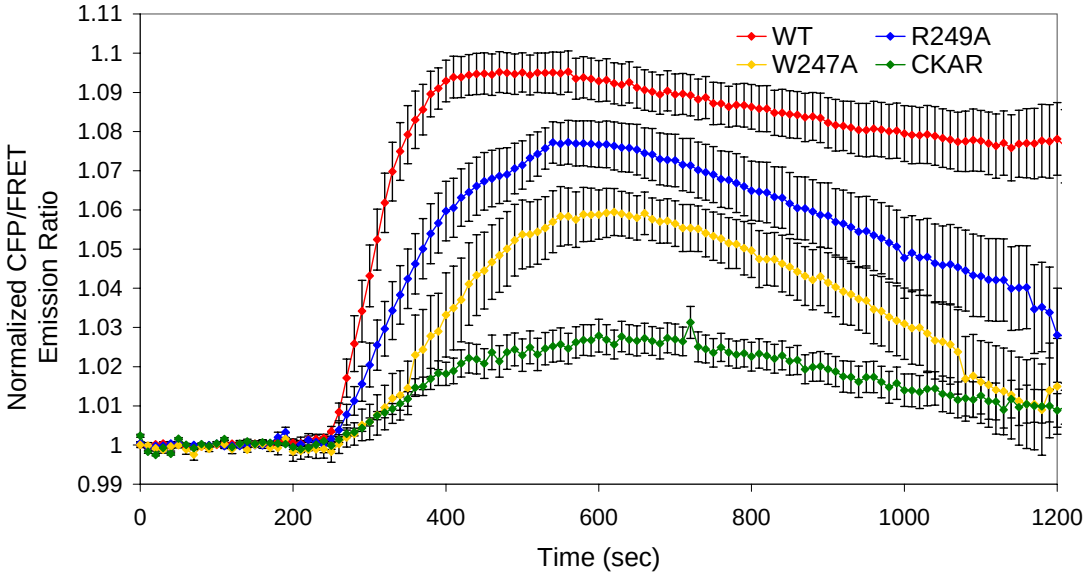


Figure 2-8. **Effects of charged and hydrophobic residue mutants on PKCα kinase activity in live cells.** CKAR phosphorylation in HeLa cells expressing PKCα-WT-RFP, PKCα-R249A-RFP, and PKCα-W247A-RFP were imaged after the addition of thapsgargin. The traces were analyzed as described in the materials and methods and averages ± S.E.M are calculated from at least three independent experiments. The RFP values used to control for PKCα expression were 2868 ± 166 for PKCα-WT-RFP, 2847 ± 125 for PKCα-R249A-RFP, and 2814 ± 245 for PKCα-W247A-RFP.

Table 2-4. **Effect of charged and hydrophobic residue mutants on Ca^{2+}- binding affinity.** The binding of purified C2α-WT, C2α-R216A, C2α-R249A, C2α-R252A, C2α-W245A, and C2α-W247A and Ca^{2+} was measured. As increasing concentrations of Ca^{2+} was added to 0.5μM C2α domain, the solution was excited at 280nm and the emission at 340mM was recorded. The data was plotted as change in observed fluorescence versus free Ca concentration and fitted to a modified Hill equation. Weighted averages of between three and seven independent experiments carried out as described. The $[Ca2+]_{1/2}$ values indicate the weighted averages ± S.E.M.

C2α	$[Ca2+]_{1/2}$ (μM)
WT	32 ± 4
R216A	38 ± 2
R249A	1409 ± 69
R252A	395 ± 27
W245A	74 ± 4
W247A	263 ± 12

Chapter III

A comparison of the membrane binding kinetics and mechanism of the C2 domains of the conventional protein kinase Cs

Introduction

In this chapter, the membrane binding kinetics and mechanism of membrane binding of the conventional C2 domains—C2α, C2βII, and C2γ—will be compared to gain insights into the mechanism of C2 domain membrane binding of the conventional PKCs. In general, the conventional isoforms show similar structure and enzyme activity, but have different cellular distribution and different levels of expression (Dempsey, Newton et al. 2000). PKCα is found in all cell types, PKCβI and PKCβII are found in several tissues, but especially the brain, and PKCγ is found only in the central nervous system (Hannun, 1992). The conventional isoforms shares the same cofactor requirements: their C1 domains require diacylglycerol and PS, while their C2 domains require Ca^{2+} and anionic phospholipids (Newton 2003).

Structure and sequence homology of the C2 domains of conventional PKCs

As true with most C2 domains, the conventional C2 domains of PKC have 8-stranded anti-parallel β-sandwiches with 3 ligand and membrane binding loops at the top of the structure and 4 loops at the bottom to connect the β-strands isoforms (Rizo and Sudhof 1998). The conventional C2 domains are also similar not only in structure, but in sequence (Kohout, Corbalan-Garcia et al. 2002). The isoforms, C2α, C2β, and C2γ, have 64 percent identical sequences (Kohout, Corbalan-Garcia et al. 2002; Torrecillas, Corbalan-Garcia et al. 2003). Providing further details, the structures of C2α and C2β domains have been solved using X-ray crystallography

(Figure 3-1) (Sutton and Sprang 1998; Verdaguer, Corbalan-Garcia et al. 1999). The structures are very similar; C2α and C2β domains only have a 0.43 angstroms difference between C_α atoms (Torrecillas, Corbalan-Garcia et al. 2003). Although the structure for C2γ domain has not been resolved, but with 74 percent sequence homology to C2α domain, the structure is expected to very similar to C2α domain (Torrecillas, Corbalan-Garcia et al. 2003).

In general for the conventional isoforms of PKC, the Ca^{2+} binding loops 1 and 3 are highly conserved (Corbalan-Garcia and Gomez-Fernandez 2006). However, there are a few interesting sequence differences between the isoforms (Figure 3-2). In Ca^{2+} binding loop 1, the residue 216 is an arginine for C2α, while it is a lysine for both C2β and C2γ (Nalefski and Falke 1996). Also in Ca^{2+} binding loop 3, the residue 249 is an arginine for C2α and C2γ, while it is a leucine for C2β. These subtle differences in sequence at the critical tips of the Ca^{2+} binding loops may help explain differences in the conventional isoforms.

Differences in the Ca^{2+} binding of the C2 domains of conventional PKCs

Although these structures have high homology in structure and sequence, the subtle differences between the conventional isoforms offer clues to difference in Ca^{2+} binding affinity. An interesting difference between the two crystal structures C2α and C2β is that C2α coordinates two Ca^{2+} ions, while C2β can coordinate up to three Ca^{+2} ions (Verdaguer, Corbalan-Garcia et al. 1999). Instead a H_2O molecule occupied the position of the third Ca^{2+} ion in the structure of C2α (Verdaguer, Corbalan-Garcia et

al. 1999). The number of Ca^{2+} ions that actually bind the conventional C2 domains has been debated in the literature. Another crystal structure shows that the C2α domain can, in fact, bind three Ca^{2+}; however, this result may be due to the high Ca^{2+} concentration and not physiologically relevant (Kohout, Corbalan-Garcia et al. 2002). Most Ca^{2+} binding studies agree that C2β and C2γ bind three and two Ca^{2+} ions, respectively (Kohout, Corbalan-Garcia et al. 2002). The idea that C2α may bind a third Ca^{2+} weakly and it dissociates so quickly that current methods do account for this binding cannot be excluded (Nalefski, Wisner et al. 2001). There is also even evidence of possible fourth Ca^{2+} binding site when the C2 domains of PKCβ and SytIA are bound to the membrane (Nalefski, Wisner et al. 2001).

Regardless of the number of Ca^{2+} bound, it is clear that the C2α and C2γ domain have higher binding affinity for Ca^{2+} than the C2β domain in the presence of lipid (Kohout, Corbalan-Garcia et al. 2002). In addition, C2α and C2γ domains do not bind Ca^{2+} cooperatively in the absence of lipid, but the C2β domain does (Kohout, Corbalan-Garcia et al. 2002). Although the domains contain the same tryptophans, the fluorescence increases for the C2α and C2γ domains as Ca^{2+} binds, but decreases as Ca^{2+} binds for the C2β domain (Kohout, Corbalan-Garcia et al. 2002). These results suggest subtle differences in the environment of those tryptophans. Although all three conventional C2 domains bind Ca^{2+}, there are variations in the number of ions bound and the degree of cooperativity of the binding.

Differences in the membrane binding of the C2 domains of conventional PKCs

In addition to similarities in binding Ca^{2+}, the C2 domains of the conventional PKC isoforms also show similarities in overall membrane binding. C2α, C2β, and C2γ domains have similar rates of membrane association, but different dissociation rates: the C2γ domain has the slowest dissociation rate, followed by the C2α domain, and then significantly higher than the C2β domain (Kohout, Corbalan-Garcia et al. 2002). All three isoforms are sensitive to ionic strength and not sodium sulfate suggesting that electrostatic interactions are important and dominate over hydrophobic interactions in membrane binding (Kohout, Corbalan-Garcia et al. 2002) .

Although the C2 domains of the conventional PKCs have similar structure and properties, there are subtle differences. Could these variations lead to different subcellular localization or the different Ca^{2+} sensitivities serve to help with their diverse functions? In this chapter, the membrane binding kinetics were determined using the FRET association experiments only. The difference in the k_{off} rate constants determined by the FRET association experiments and the FRET dissociation experiments show a difference in the absolute numbers; however, they do not show a difference in the relative effects of the C2 mutants as compared to wildtype C2 domain.

Chapter overview

In this chapter, we examine the role of electrostatic and hydrophobic interactions in the membrane binding kinetics and mechanism of the conventional C2

domains. Specifically, we show that the recruitment of the conventional C2 domains—C2α, C2β, and C2γ—are similar, but the membrane retention of these domains and overall membrane binding affinity varied. In addition, we show the loss of the key basic residue R249 plays an important role in the isoform-specific differences in membrane binding kinetics. We also show that the C2β domain has greater sensitivity to mutations in its Ca^{2+} binding loops than the C2α domain.

Materials and Methods

Reagents

1-Palmitoyl-2-oleoylphosphatidyl-L-serine (POPS) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Polar Lipids. N- [5-(dimethylamino) naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dPE) was purchased from Molecular Probes. PCR primers were prepared by IDT technologies, Inc. Glutathione-Sepharose 4B was purchased from Amersham Biosciences. Electrophoresis reagents were from Calbiochem and Bio-Rad. Thrombin was purchased from Calbiochem. Antibody to PKCα was from Santa Cruz Biotechnology, Inc. All other chemicals were reagent-grade.

C2α, C2β, and C2γ domain cloning, mutagenesis, and purification

The rat PKCα DNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). The C2α domain (residues 179-283) was PCR amplified

and subcloned into a pGEX-4T3-GST plasmid using restriction sites EcoRI and XhoI. The C2α domain mutants R216A, R249A, R252A, R249L, and W247A were created using site-directed mutagenesis. The rat pGEX-KG-PKC-C2β was made as previously described (Johnson, Giorgione et al. 2000). The C2β domain mutants K216A, R252A, and W247A were created using site-directed mutagenesis.
The rat PKC-C2γ was PCR amplified and subcloned into a pGEX-4T3-GST plasmid using restriction sites EcoRI and XhoI. The C2 domain of rat PKCs were expressed as a glutathione-S-transferase (GST) fusion protein in BL21-pLysS cells as previously described (Johnson, Giorgione et al. 2000). The isolated C2 domain was cleaved from the glutathione-Sepharose 4B-bound fusion protein using thrombin. The concentration of C2 domains were determined by UV absorbance using their molar extinction coefficient as previously described (Nalefski, Wisner et al. 2001).

Preparation of Lipid Vesicles

For stopped flow fluorimetry, phospholipids in chloroform were dried under nitrogen and vacuumed dried for two hours. The lipids were resuspended in a buffer of 150mM NaCl, 20mM Hepes, pH 7.4. The resuspended lipid was freeze/thawed five times and extruded through two 0.1μM polycarbonate filters to prepare phospholipids vesicles 100nm in diameter. The phosphorus concentration was assayed as previously described to determine the phospholipid concentration (Nalefski and Newton 2001).

Stopped Flow Fluorimetry

An Applied Photophysics pi*-180 stopped-flow fluorescence spectrophotometer was used to determine the kinetics. Purified C2 domain protein (0.2 μM to 1.0 μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol % PS: 60 mol % PC:5 mol % dPE) at Ca^{2+} concentrations and ionic strengths as indicated. These FRET association experiments were run at the same conditions and analyzed as previously described (Nalefski and Newton 2001). Purified C2 domain protein (0.2μM to 1.0μM) was incubated for 30 minutes with dansyl-labeled phospholipid vesicles (35 mol % PS: 60 mol % PC:5 mol % dPE). These traces were fitted to a nonlinear least-squares curve fitting using KaleidaGraph according to the exponential equation

$$F(t)= F_0 + \sum_{i=1}^{n} A_{obs(i)} e^{-k_{obs(i)}t}$$

where F(t) equals the observed fluorescence at time t, F_0 is a fluorescence offset representing the final fluorescence, and $A_{obs(i)}$ equals the amplitude, and the $k_{obs(i)}$ is the observed rate constant for *i*th of n phases. The experiments were performed under pseudo-first order conditions allowing the observed rate to be plotted as a function of lipid vesicle concentration in a linear way using the equation

$$k_{obs} = k_{on} [v] + k_{off}$$

where k_{on} represents the second-order rate constant and k_{off} represents the apparent dissociation rate constant. The calculated K_d was determined using weighted k_{on} and k_{off} averages from the FRET association experiments and the S.E.M were also calculated.

Results

Comparison of C2α, C2β, and C2γ

To further understand the membrane binding kinetics and mechanism of C2 domain, the membrane binding kinetics of the C2 domains of the three members of the conventional class of PKCs were compared. The on rate constants of C2α, C2β, and C2γ showed only modest differences with C2γ having the highest k_{on} rate constant at 25.0 ± 0.4 $nM^{-1}s^{-1}$ followed by C2α with a k_{on} rate constant of 22.40 ± 0.09 $nM^{-1}s^{-1}$ and then C2βII with a k_{on} rate constant of 21.3 ± 0.8 $nM^{-1}s^{-1}$ (Figure 3-3A). However, the k_{off} rate constants of the isoforms showed a 3-fold increase between C2γ and C2β from 1.5 ± 0.06 s^{-1} to 4.89 ± 0.06 s^{-1} and a 2-fold increase in k_{off} rate constant between C2γ and C2α from $1.5 \pm 0.06s^{-1}$ to 2.81 ± 0.05 s^{-1} (Figure 3-3B). The overall lipid vesicle binding affinity of C2γ at 0.0600 ± 0.0008nM was 2-fold and 4-fold greater than C2α and C2β with K_d^{calc} values of 0.126 ± 0.002 nM and 0.226 ± 0.004 nM, respectively (Table 3-1).

Membrane binding kinetics of C2α-R249L

Looking closer at the differences between the kinetics of the conventional C2 domains, the sequence alignment shows high homology especially in the Ca^{2+} binding loops. However, in the highly conserved Ca^{2+} binding loop 3, there is one mutation in which the charge is not conserved. In both C2α and C2γ, residue 249 is an arginine, while in C2β residue 249 is a leucine. The loss of this basic residue coincides with an increase in the off rate constant as the mutational analysis of C2α in the previous chapter showed that electrostatic interactions of charged residues play an important role in retention of the C2 domain at the membrane.

To confirm this observation, residue 249 was mutated from an arginine to a leucine in C2α. Using the FRET association experiment, the k_{on} rate constants of C2α and C2β were similar at 22.40 ± 0.09 $nM^{-1}s^{-1}$ and 21.3 ± 0.8 $nM^{-1}s^{-1}$, respectively, and mutants R249A and R249L showed slightly slower recruitment to the lipid vesicles at 19.4 ± 0.4 $nM^{-1}s^{-1}$ and 15.5 ± 0.7 $nM^{-1}s^{-1}$, respectively (Figure 3-4A). The k_{off} rate constant of the R249L mutant was approximately 2-fold higher than C2α at 6.5 ± 0.4 s^{-1} compared to 2.81 ± 0.05 s^{-1}, respectively (Figure 3-4B). Interestingly, the k_{off} rate constant of the R249L mutant was only approximately 1.3-fold higher than C2β which has a k_{off} rate constant of 4.89 ± 0.09 s^{-1}. However, strikingly, the R249L mutant recovered the membrane retention of the C2α domain by 2-fold compared to the k_{off} rate constant of the R249A mutant with a k_{off} rate constant of 13.4 ± 0.42s^{-1}. Overall

lipid vesicle binding affinities of C2α, C2β, C2α-R249A, and C2α-R249L showed similar trends as the k_{off} rate constants with affinities of 0.126 ± 0.002 nM, 0.226 ± 0..004 nM, 0.69 ± 0.02 nM, and 0.42 ± 0.03 nM, respectively. These results suggest the residue at position 249 plays a role in the differential kinetics of the conventional isoforms.

Design of C2α and C2β Mutants

The C2α domain shares the common structure with other C2 domain of 8-stranded anti-parallel β-sandwiches with 3 ligand and membrane binding loops at the top of the structure and 4 loops at the bottom to connect the β-strands (Verdaguer, Corbalan-Garcia et al. 1999). As shown by previous studies, there are subtle difference in the binding kinetics of the conventional PKC isoforms. In these studies, we look at the membrane binding kinetics of two residues that are conserved and one homologous residue between C2α and C2β to further dissect the mechanism of conventional C2 domain membrane binding. To do this, we designed three mutations in the C2α and C2β domains: R252A and W247A in both domains and R216A and K216A in C2α and C2βII, respectively (Figure 3-5). Previous papers have shown residues 216 and 249/252 in a double alanine mutation in the context of full-length PKC are important for membrane binding and enzyme activity (Medkova and Cho 1998; Conesa-Zamora, Lopez-Andreo et al. 2001; Stahelin and Cho 2001) . In addition, EPR studies have shown R252 is involved in C2α penetration into the

membrane (Kohout, Corbalan-Garcia et al. 2003). The hydrophobic residue mutant W247A (in a double alanine mutation with W245) is involved in membrane binding and important for enzymatic activity as well as suggested to be involved with membrane penetration (Medkova and Cho 1998).

Comparison of C2α and C2β mutants

As stated above, the structural and sequence homology between the conventional isoforms is quite high. In these studies, we examined the membrane binding kinetics of mutations in C2α and C2β to determine if there are any isoform-specific differences between the mutations. FRET association experiments were performed to determine both the on and off rate constants of mutants in residues 216 (R216A for C2α and K216A for C2β), 252 (R252A for both C2α and C2β), and 247 (W247A for C2α and C2β). Consistent with the previous kinetic isoform results, the k_{on} rate constants were similar in the C2α and C2β mutants (Figure 3-6A). The k_{on} rate constants of the mutants R216A and R252A in C2α were similar at 18.7 ± 0.7 $nM^{-1}s^{-1}$ and 26.3 ± 0.2 $nM^{-1}s^{-1}$ compared to the k_{on} rate constant of 22.41 ± 0.09 $nM^{-1}s^{-1}$ for wild-type C2α domain. Likewise, the k_{on} rate constants of the mutants K216A and R252A of C2β were similar at 14.5 ± 0.3 $nM^{-1}s^{-1}$ and 22 ± 2 $nM^{-1}s^{-1}$ compared to the k_{on} rate constant of 21.3 ± 0.8 $nM^{-1}s^{-1}$ for the wild-type C2β domain. These results indicate that the residues 216 and 252 may have small roles in the membrane recruitment of the C2 domain.

In contrast, the mutant W247A resulted in at least a 2-fold decrease in the recruitment of the C2 domain to the lipid vesicles. The k_{on} rate constants of C2α were reduced from 22.41 ± 0.09$nM^{-1}s^{-1}$ to 9 ± 0.2$nM^{-1}s^{-1}$and the k_{on} rate constants reduced from 21.3 ± 0.8$nM^{-1}s^{-1}$ to 10.7 ± 0.5$nM^{-1}s^{-1}$ for C2β. These results suggest that this tryptophan is critical for membrane recruitment in both C2 domains.

Overall, the k_{off} rate constants of C2α appeared less sensitive to the mutations than the k_{off} rate constants of C2β (Figure 3-6B). Between the two C2 domains, there was a 2-fold difference in the k_{off} rate constants as the k_{off} rate constant of C2α was 2.81 ± 0.05s^{-1} and the k_{off} rate constant of C2β was 4.89± 0.09s^{-1}. The k_{off} rate constants of mutants R/K216A, R252A, and W247A were 8.1 ± 0.1s^{-1}, 5.41 ± 0.08s^{-1}, and 7.18 ± 0.07s^{-1} for the C2α domain, respectively, and were 14.5 ± 0.3s^{-1}, 19 ± 2 s^{-1}, and 29.7 ± 0.6s^{-1} for C2β domain, respectively. These results show that the k_{off} rate constants for mutants 216, 252, and 247 of C2β were 3-fold, 4-fold, and 6-fold greater than their wildtype C2β domain, while the k_{off} rate constants for mutants 216, 252, and 247 were 3-fold, 2-fold, and 2.5-fold greater their wildtype C2α domain. A possible explanation for the increased sensitivity of the C2β mutants is without an arginine at position 249, the other residues, especially R252 and W247, play a more central role in the retention of C2β at the membrane.

Likewise, the overall lipid vesicle binding affinities of C2α mutants 216, 252, and 247 were approximately 2-fold less than the difference in lipid vesicle binding affinities of C2α mutants 216, 252, and 247, respectively (Table 3-2). Overall, these

results suggest the membrane binding kinetics of C2β is more sensitive to the loss of electrostatic and hydrophobic interactions of specific residues than the membrane binding kinetics of C2α.

Comparison of PKCα and PKCβII activity in live cells

After comparing the membrane binding kinetic differences in the isolated C2 domains of the conventional C2 domains, we determined whether there are differences in activity between PKCα and PKCβII in live cells. A C kinase activity reporter (CKAR) was developed to monitor PKC activity in live cells (Violin, Zhang et al. 2003; Gallegos, Kunkel et al. 2006). To determine if the difference in isoform-specific kinetics affect the kinase activity of PKCα-RFP and PKCβII-RFP, HeLa cells were transfected with CKAR and PKCα-RFP or PKCβII-RFP and stimulated with thapsgargin which releases intracellular Ca^{2+} stores, and imaged to measure the CKAR phosphorylation within the cells. Compared to cells monitored for transfected PKCα-RFP, the cells with transfected PKCβII-RFP showed a slower response time to thapsgargin, but an increased amount of overall phosphorylation. In addition, the PKC signal in the cells with transfected PKCβII-RFP was sustained longer. However, it is important to note that the overall expression of protein was not controlled. The intensity of the RFP signal is different between these two constructs and Western blotting is needed to control for PKC expression.

Discussion

Comparison of conventional C2 domain membrane binding kinetics

We have compared the membrane binding kinetics of the C2 domains of conventional PKCs. As shown in previous studies, the on rate constants of the three isoforms are quite similar (Kohout, Corbalan-Garcia et al. 2002). As shown in Chapter 2, the electrostatic interactions of Ca^{2+} binding and the hydrophobic interactions of the tryptophans play a major role in membrane recruitment. The similar on rate constants suggest that although there are differences in the Ca^{2+} binding properties between the three isoforms, they are not significant enough to change the overall recruitment of the C2 domain to the membrane. In contrast, the off rate constants of the three conventional isoforms show significant differences. The C2β domain shows the fastest dissociation rate constant, followed by the C2α domain, and the C2γ domain has the slowest dissociation rate. These off rate constants suggest the C2γ domain has the most stable domain-lipid complex and the C2β domain has the least.

Importance of residue R249 in membrane binding kinetics

To further explore the differences in membrane binding between the conventional isoforms, we examine specific residue differences. One residue that appears to offer an explanation to the isoform specific kinetics is at position 249. In both C2α and C2γ, residue 249 is an arginine and in C2β residue 249 is a leucine. To test this hypothesis, R249 in C2α was mutated to leucine. As expected, this mutant

does not affect the on rate constants. The data did show that loss of the charged residue dramatically affects C2α membrane retention as the off rate constant increased. However, the increase in the off rate constants was less than in the mutant C2α-R249A suggesting the aliphatic chain of R249 allows favorable hydrophobic interactions to occur promotes membrane binding. This idea agrees with some models of C2 membrane binding that R249 plays a role not only in electrostatic interactions, but also plays a role with hydrophobic interactions. The kinetics of mutant R249L suggests that this residue plays a role in the stability of the domain-membrane complex and in the differences between conventional C2 domains.

Comparison of conventional C2α and C2β domain membrane binding kinetics

To further look at difference between the C2 domain isoforms, we compared mutations of residues 216, 252, and 247 to alanine in both the C2α and C2β domains. These results show that the C2α mutants for residues 216, 252, and 247 had 3-fold, 4-fold, and 4-fold greater membrane binding affinities than of C2β mutants, respectively. However, when comparing the fold differences from their respective wildtype C2 domains, mutants R252A and W247A of C2β show a greater sensitivity in their kinetics to these mutants than the mutants in C2α. Interestingly, the mutation of residue R216 to alanine shows the same sensitivity in its on and off rate constants in both the C2α and C2β domains. These results suggest that C2β is more sensitive to mutations within Ca^{2+} binding loop 3 than C2α, but not mutations within Ca^{2+} binding loop 1.

One possible explanation is residues R252 and W247 play a more critical role in membrane retention for C2β than C2α as C2β does not have an arginine at position 249. Without this residue, which we showed in last chapter to not only be involved with electrostatic interactions but also hydrophobic interactions, the domain-membrane complex is not as stable. Without the arginine at position 249, the role of W247 and R252 in membrane penetration, as shown in EPR studies, become more important (Kohout, Corbalan-Garcia et al. 2003). For C2β, W247 and R252 may be required to be the anchors in the membrane; therefore, their mutations have a greater impact on the binding kinetics of C2β. Overall, these results suggest the membrane binding kinetics of C2β is more sensitive to the loss of electrostatic and hydrophobic interactions of specific residues than the membrane binding kinetics of C2α.

Comparison of conventional PKCα and PKCβ activity in live cells

To evaluate if the isoform-specific binding kinetic differences translate into different cellular activity of the isoforms, we imaged PKCα and PKCβ in live cells. These results show that PKCα activates faster than PKCβ under conditions of increased intracellular Ca^{2+}. However, the signaling PKCβ is prolonged as compared to PKCα. These data suggest that while it is possible to look at isoform differences, more controlled studies are needed. The role of the different C1 domains were not accounted for in these studies and may explain why the activity of PKCα and PKCβ do not correlate with our binding studies.

Conclusions

Taken together, our data suggest there are isoform-specific kinetic differences between the conventional PKC isoforms—C2α, C2βII, and C2γ. Although the conventional C2 domains appear to recruit to the membrane in a similar manner, the retention at membrane and overall stability of the domain-membrane complexes are different. The C2γ domain has the longest residency time at the membrane followed by C2α, and then C2βII. Our data suggest that R249, which plays a dual role in electrostatic and hydrophobic interactions, may be the key residue for the membrane retention of the C2α domain. In fact, when residue 249 is missing, as it is in C2β, the overall retention at the membrane decreases and the domain is more sensitive to the mutation of other key residues, such as R252 and W247. The isoform-specific differences in conventional C2 domain binding may offer insights into the differences in the specific functions and regulation of the conventional PKCs.

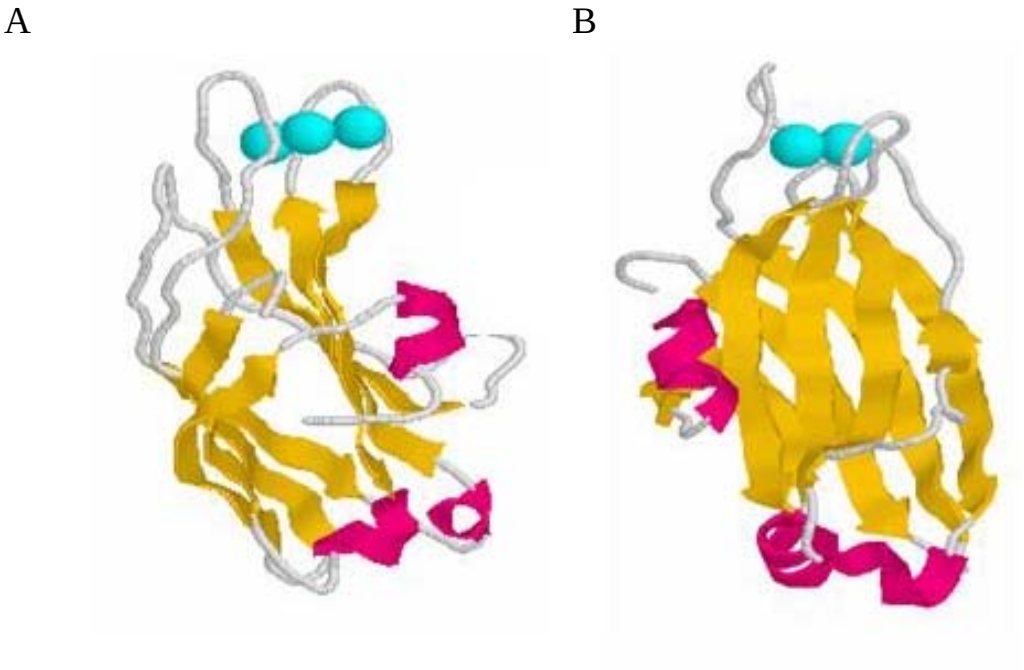


Figure 3-1. **Structure of conventional C2 domains—α and β.**
The structures of C2 domains for PKCβ (left) and PKCα (right). The C2 domains have similar structure with 8-stranded anti-parallel β-sandwich and 3 Ca^{2+} binding loops. The key difference between the two domains is that C2β coordinates 3 Ca^{2+} ions while C2α coordinates only 2 Ca^{2+} ions.

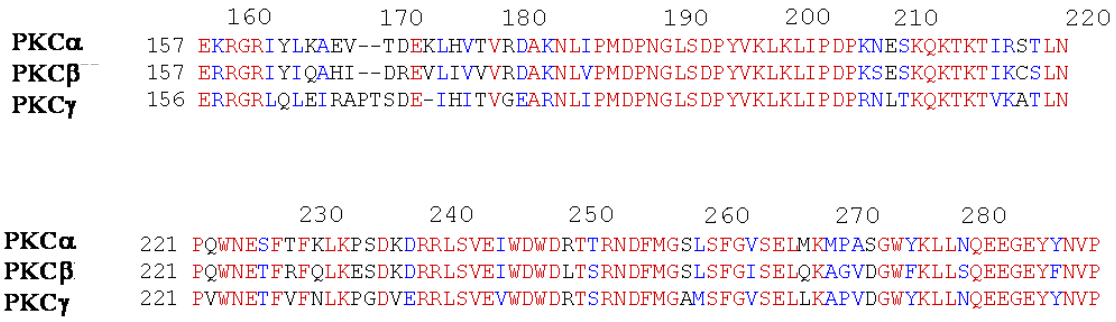


Figure 3-2. **Sequence Alignment of conventional PKCs.** In this primary sequence alignment for the C2 domains of conventional PKC, the amino acids denoted in red share identical residues between the isoforms, while the amino acid denoted in blue indicate homologous residues.

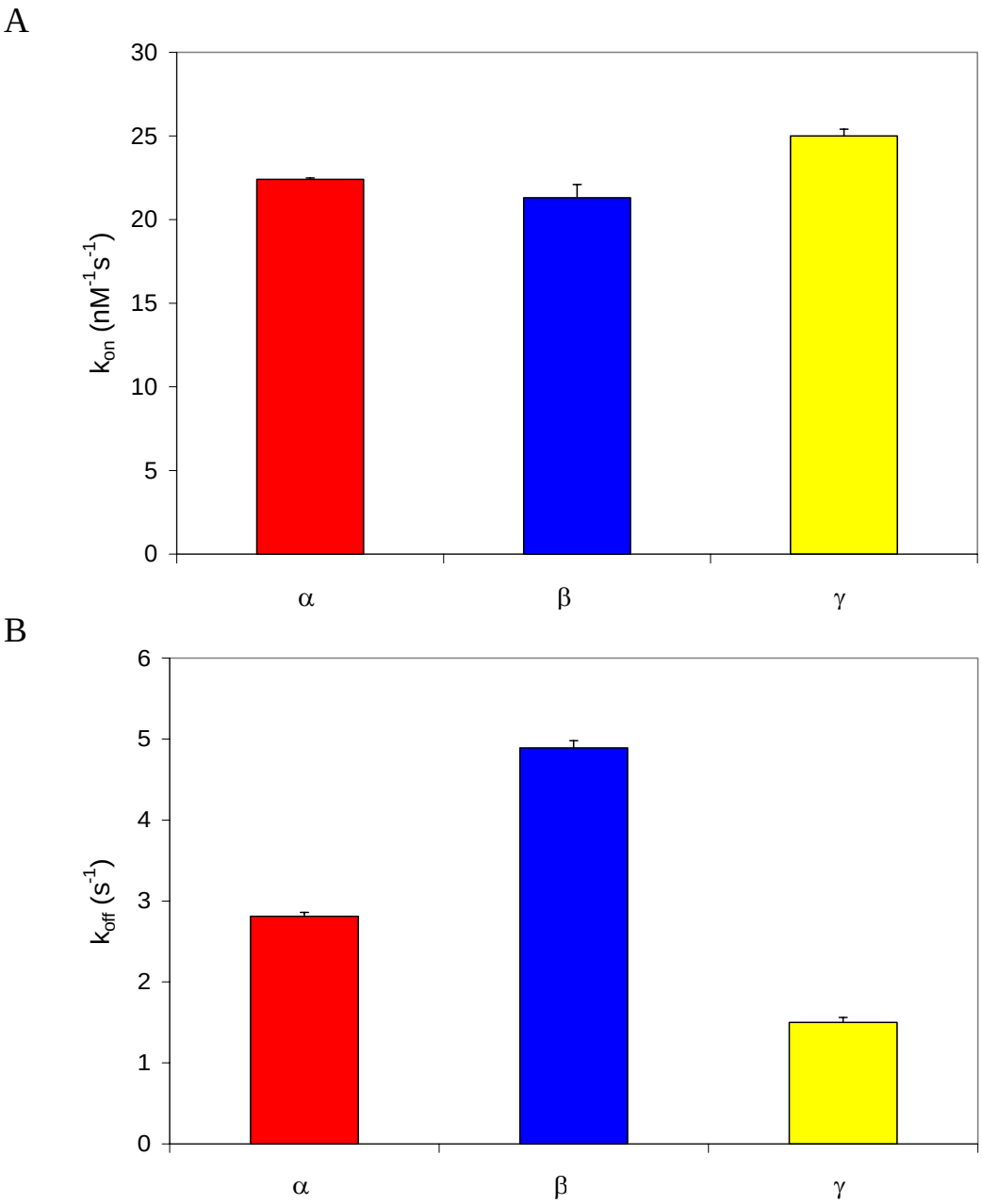
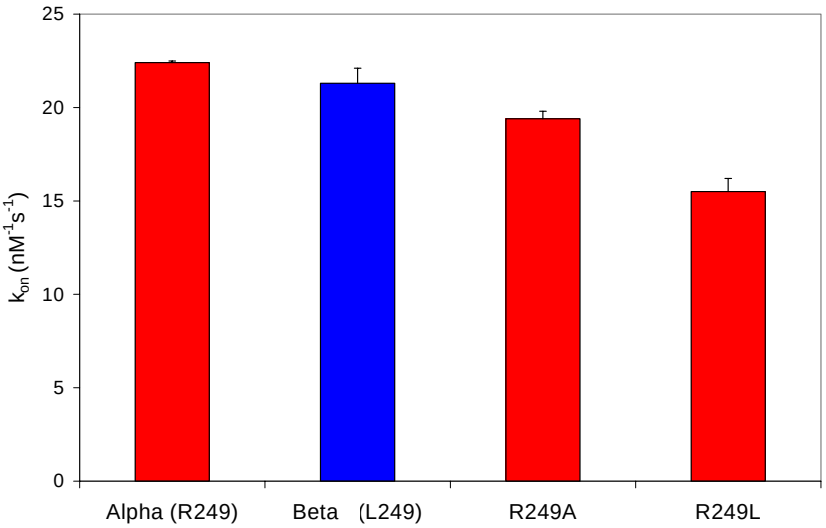


Figure 3-3. **Differences in the membrane binding kinetics of conventional C2 domains--α, β, and γ.** (A) The k_{on} rate constants and (B) the k_{off} rate constants of purified C2α, C2β, and C2γ binding to anionic phospholipid vesicles was measured by FRET association stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol % PS:60 mol % PC:5 mol % dPE) at 200μM Ca^{2+} and 150mM NaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{obs} was determined using a monophasic fit. The k_{on} is calculated from the slope and the k_{off} is calculated from the y-intercept of the linear plot of k_{obs} versus lipid concentration. The weighted averages are calculated from three to seven independent experiments.

Table 3-1. **Differences in the overall binding affinity to lipid vesicles of the conventional C2 domains.** Weighted averages of between three and seven independent experiments carried out as described in the figure 3-3A and 3-3B. The K_d^{calc} values indicate the calculated K_d from the weighted averages ± S.E.M. The fold difference values were calculated from comparing the K_d^{calc} of C2γ domain to the K_d^{calc} of C2α and C2β and represented as fold difference ± S.E.M.

	K_d^{calc} (nM)	Fold Difference from C2γ-WT
C2α	0.126 ± 0.002	2.10 ± 0.03
C2β	0.226 ± 0.004	3.76 ± 0.05
C2γ	0.06003± 0.0008	1.00 ± 0.01

A



B

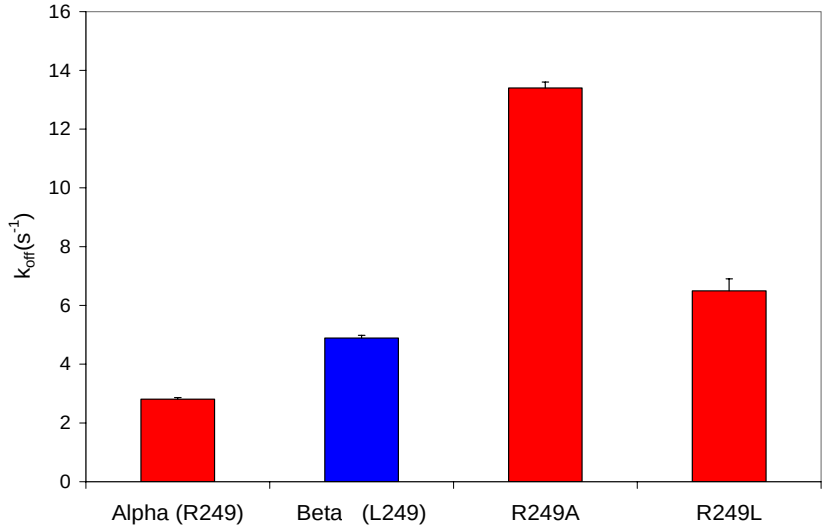


Figure 3-4. **Differences in the membrane binding kinetics of key residue 249 of the conventional C2α domains.** (A) The k_{on} rate constants and (B) the k_{off} rate constants of purified C2α-WT, C2β-WT, and C2α-R249A, and C2α-R249L binding to anionic phospholipid vesicles was measured by FRET association stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol % PS:60 mol % PC:5 mol % dPE) at 200μM Ca^{2+} and 150mM NaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{obs} was determined using a monophasic fit. The k_{on} is calculated from the slope and the k_{off} is calculated from the y-intercept of the linear plot of k_{obs} versus lipid concentration. The weighted averages are calculated from three to seven independent experiments.

Table 3-2. **Differences in the overall binding affinity to lipid vesicles of changed and hydrophobic mutations on the conventional C2 domains—α and β.**
Weighted averages of between three and seven independent experiments carried out as described in the figure 15. The K_d^{calc} values indicate the calculated K_d from the weighted averages ± S.E.M. The fold difference values were calculated from comparing the K_d^{calc} of wild-type C2α or C2β domain to the K_d^{calc} of C2α or C2β mutants, respectively, and represented as fold difference ± S.E.M.

	K_d^{calc} (nM)	Fold Difference from WT
C2α-WT	0.126 ± 0.002	1.00 ± 0.02
C2α-R216A	0.432 ± 0.009	3.43 ± 0.09
C2α-R252A	0.206 ± 0.004	1.63 ± 0.04
C2α-W247A	0.79 ± 0.02	6.3 ± 0.2
C2β-WT	0.226 ± 0.004	1.00 ± 0.02
C2β-R216A	1.4 ± 0.2	6.2 ± 0.9
C2β-R252A	0.9 ± 0.2	4.0 ± 0.9
C2β-W247A	3.1 ± 0.2	13.7 ± 0.9

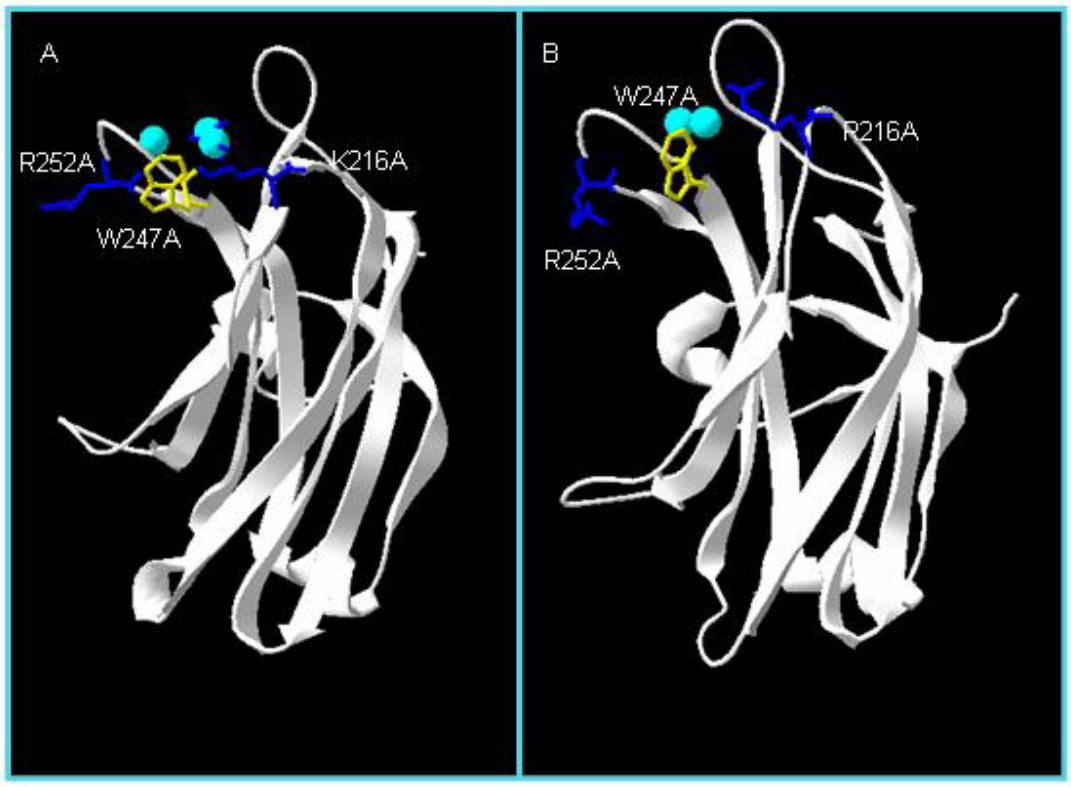
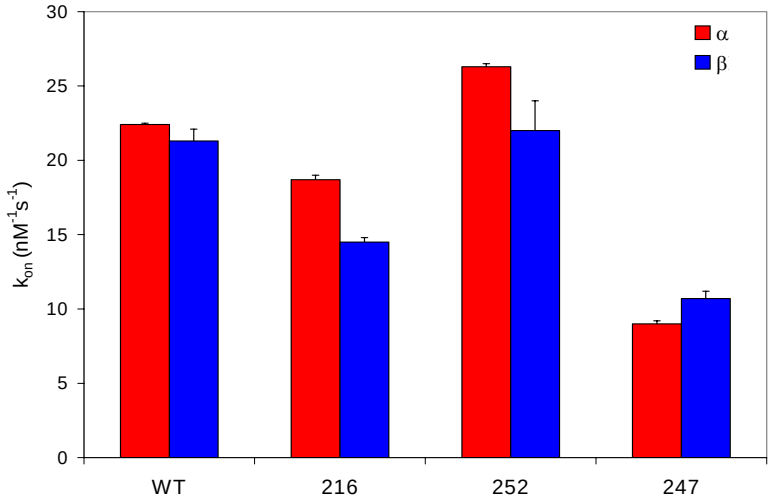


Figure 3-5. **Structure of C2α and C2β domain with mutated residues indicated.**
Ribbon diagrams include the mutants studied on both (A) C2β and (B) C2α domains. C2-R/K216A and C2-R252A are charged residue mutants and C2-W247A is hydrophobic residue mutant studied to examine the differences in electrostatic and hydrophobic interactions involved with conventional C2 domain membrane binding.

A



B

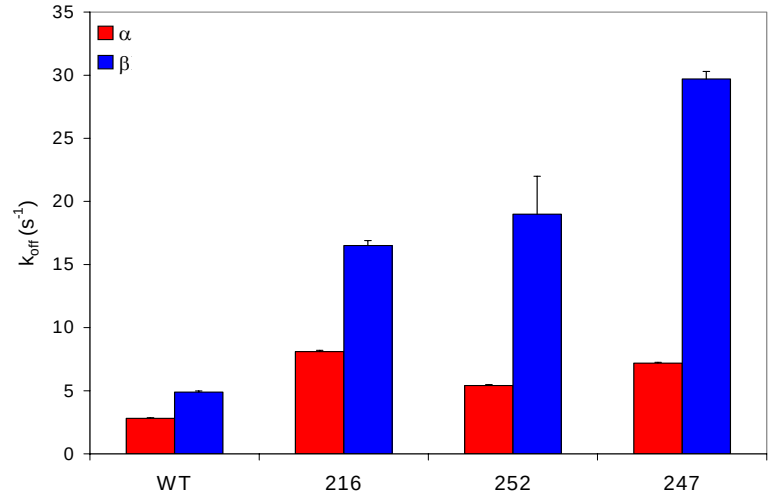


Figure 3-6. **Differences in the membrane binding kinetics of C2α and C2β domains.** (A) The k_{on} rate constants and (B) the k_{off} rate constants of purified C2α-WT, C2α-R216A, C2α-R252A, C2α-W247A, C2β-WT, C2β-R216A, C2β-R252A, and C2β-W247A binding to anionic phospholipid vesicles was measured by FRET association stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol % PS:60 mol % PC:5 mol % dPE) at 200μM Ca^{2+} and 150mM NaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{obs} was determined using a monophasic fit. The k_{on} is calculated from the slope and the k_{off} is calculated from the y-intercept of the linear plot of k_{obs} versus lipid concentration. The weighted averages are calculated from three to seven independent experiments.

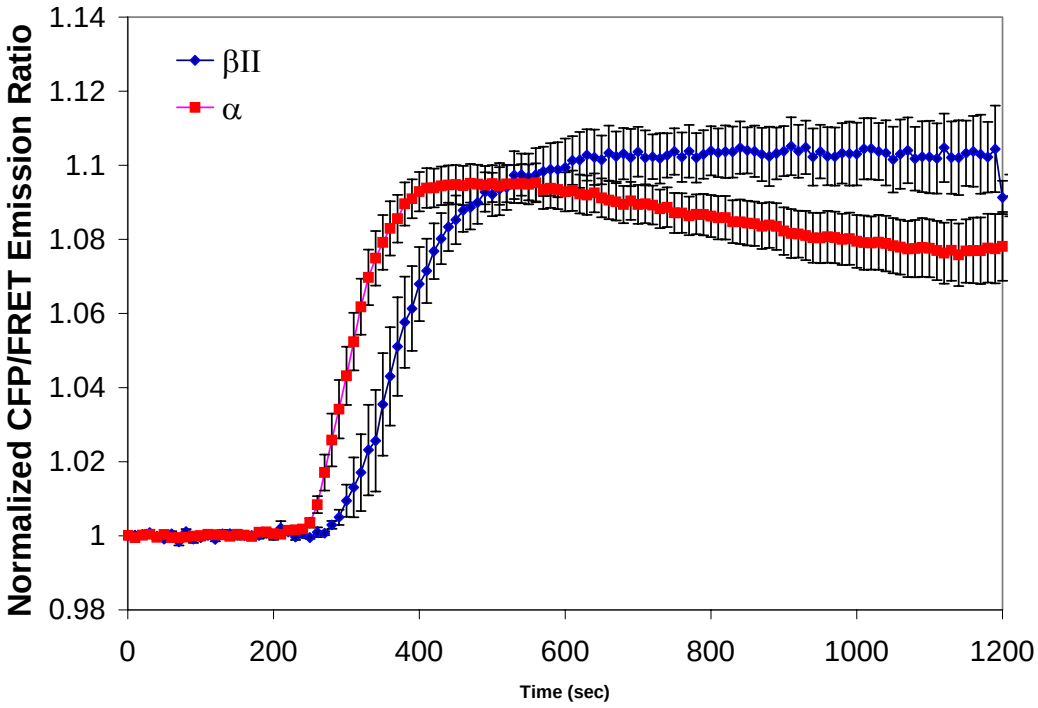


Figure 3-7. **Differences in kinase activity between PKCα and PKCβII in live cells.** CKAR phosphorylation in HeLa cells expressing PKCα-WT- RFP and PKCβII-WT-RFP were imaged after the addition of thapsgargin at 3 minutes. The traces were analyzed as described in the materials and methods and averages ± S.E.M are calculated from at least two independent experiments.

Chapter IV

The possible role of autophosphorylation in the membrane binding kinetics of the C2α domain of protein kinase C

Introduction

As C2 domain membrane binding plays such as important in activation, it is not surprising that the C2 domain binding has several layers of regulation. In the previous chapters, we have examined the importance of electrostatic and hydrophobic interactions of both Ca^{2+} binding and specific residues located within the Ca^{2+} binding loops on the membrane binding kinetics and mechanism of C2 domain. In addition, anionic phospholipids and PIP_2 have been shown to help provide specificity for C2 domain localization at the plasma membrane (Newton 2003; Corbalan-Garcia and Gomez-Fernandez 2006). However with the important role in regulation of C2 domain membrane binding, are these four factors the keys to C2 domain membrane binding or could there be other factors?

Autophosphorylation of residue 250 on the C2 domains of conventional PKCs

A study shows that there is an autophosphorylation site located at residue 250 in the C2 domain of PKCα (Ng, Squire et al. 1999). Residue 250, a threonine, is located on the Ca^{2+} binding loop 3 in close proximity to W247, R249, and R252 that we studied in the previous chapters. The study shows after stimulation with the potent PKC agonist tetradecanoyl phorbol acetate (TPA), the T250 site becomes phosphorylated after 30 minutes and that this phosphorylation is an autophosphorylation (Ng, Squire et al. 1999). In C2β, residue 250, a serine, also serves as an autophosphorylation site. This observation raises the possibility that

autophosphorylation plays a role in regulating the Ca^{2+} and membrane binding of the C2 domains.

Phosphorylation as a regulatory mechanism in other conventional PKCs

Phosphorylation is one of the three key mechanisms that tightly regulate PKC (Newton 2003). PKC requires three ordered phosphorylations at the activation loop, turn motif, and hydrophobic motif to mature and become catalytic competent. Recent studies demonstrate that phosphorylation sites exist in the C2 domains of novel PKCs. In the C2δ domain, tyrosine 64 can be phosphorylated (Joseloff, Cataisson et al. 2002). In fact, the study suggests this phosphorylated tyrosine can interact electrostatically with Arg 67 and, thereby, prevent the binding of phosphopeptides and inactivate the enzyme (Joseloff, Cataisson et al. 2002) Furthermore, the C2 domain of Aplysia nPKC AplII contains two autophosphorylation sites. When one of these sites, Ser 36, is phosphorylated, the domain's affinity for anionic phospholipids increases *in vitro* (Pepio and Sossin 2001). In addition, phosphorylation of this site in cells increase the rate of membrane translocation rate and the mutation of this site dramatically decreases membrane translocation (Pepio and Sossin 2001).

Chapter overview

These studies set a precedent for regulation of the C2 domain by phosphorylation. To address whether this autophosphorylation site affects C2α domain membrane binding, we determined the binding kinetics of an alanine and a phosphomimic mutant of the residue 250. In addition, we looked at how this

phosphomimic mutant affected translocation in live cells and its *in vitro* kinase activity.

Methods

Reagents

1-Palmitoyl-2-oleoylphosphatidyl-L-serine (POPS) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were purchased from Avanti Polar Lipids. N- [5-(dimethylamino) naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dPE) was purchased from Molecular Probes. [γ-32]ATP and [^{3}H]dipalmitoylphosphatidylcholine were from PerkinElmer Life Sciences. PCR primers were prepared by IDT technologies, Inc. Glutathione-Sepharose 4B was purchased from Amersham Biosciences. Electrophoresis reagents were from Calbiochem and Bio-Rad. Thrombin was purchased from Calbiochem. Antibody to PKCα was from Santa Cruz Biotechnology, Inc. All other chemicals were reagent-grade.

C2α domain cloning, mutagenesis, and purification

The rat PKCα DNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). The C2 domain (residues 179-283) was PCR amplified and subcloned into a pGEX-4T3-GST plasmid using restriction sites EcoRI and XhoI. The C2α domain mutants T250A and T250D were created using site-directed mutagenesis. The C2 domain of rat PKCα was expressed as a glutathione-S-transferase (GST)

fusion protein in BL21-pLysS cells as previously described (Johnson, Giorgione et al. 2000). The isolated C2α domain was cleaved from the glutathione-Sepharose 4B-bound fusion protein using thrombin. The concentration of C2 domains were determined by UV absorbance using their molar extinction coefficient (Nalefski, Wisner et al. 2001).

Preparation of lipid vesicles

For stopped flow fluorimetry, phospholipids in chloroform were dried under nitrogen and vacuumed dried for two hours. The lipids were resuspended in a buffer of varying NaCl concentration (150 mM-200 mM) and 20 mM Hepes, pH 7.4. The resuspended lipid was freeze/thawed five times and extruded through two 0.1μM polycarbonate filters to prepare phospholipids vesicles 100 nm in diameter. The phosphorus concentration was assayed as previously described to determine the phospholipid concentration (Nalefski and Newton 2001). For protein kinase C activity assay, the phospholipids prepared as described above except they were sonnicated for 1 min instead of extruded.

Stopped flow fluorimetry

An Applied Photophysics pi*-180 stopped-flow fluorescence spectrophotometer was used to determine the kinetics. Purified C2 domain protein (0.2 μM to 1.0 μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol %PS:60mol

%PC:5mol %dPE) at calcium concentrations and ionic strengths as indicated. This FRET association experiments were run at the same conditions and analyzed as previously described (Nalefski and Newton 2001; Nalefski and Newton 2003). These traces were fitted to a nonlinear least-squares curve fitting using KaleidaGraph according to the exponential equation

$$F(t)= F_0 + \sum_{i=1}^{n} A_{obs(i)} e^{-k_{obs(i)} t}$$

where F(t) equals the observed fluorescence at time t, F_0 is a fluorescence offset representing the final fluorescence, and $A_{obs(i)}$ equals the amplitude, and the $k_{obs(i)}$ is the observed rate constant for $_i$th of n phases. The experiments were performed under pseudo-first order conditions allowing the observed rate to be plotted as a function of lipid vesicle concentration in a linear way using the equation

$$k_{obs} = k_{on} [v] + k_{off}$$

where k_{on} represents the second-order rate constant and k_{off} represents the apparent dissociation rate constant. The calculated K_d was determined using weighted k_{on} and k_{off} rate constants averages from the FRET association and FRET trapping experiments and the S.E.M were also calculated.

PKCα cloning and mutagenesis

The rat PKCα DNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). PKCα was PCR amplified and subcloned into a pcDNA3-

HA or pcDNA3-YFP plasmid using restriction sites EcoRI and XhoI. The mutations T250A and T250D were created using site directed mutagenesis.

Cell transfection and western blotting

Cos 7 were maintained using DMEM (Cellgro) with 10% fetal bovine serum and 1% penicillin/streptomycin at 37ºC. The constructs were transfected into cos 7 cells using the FuGENE6 transfection system. For Western blotting, the cos 7 cells were lysed in 0.1% trition, 200 μM benzamindine, 40 μgmL^{-1} leupeptin, 1mM PMSF, and 1mM DTT after 24 hour transfection**.** The detergent souble cell lysates were obtained by centrifuging the whole cell lysates at maximum speed in a microcentrifuge for 2 min. These lysates were separated by SDS-Page electrophoresis and Western blotted using the appropriate antibody.

Kinase assay

Protein kinase C assays were done at varying lipid concentrations to determine the lipid dependent activation. Protein kinase C activity was measured by assaying the rate of phosphorylation of a protein kinase C-selective peptide as previously described (Edwards et al 1997). The cos 7 cells were lysed in 0.1% trition, 200μM benzamindine, 40μgmL^{-1} leupeptin, 1mM PMSF, and 1mM DTT after 24 hour transfection. The detergent souble cell lysates were obtained by centrifuging the whole cell lysates at maximum speed in a microcentrifuge for 2 min. The amount of protein was determined using a Bradford assay. The kinase assay was performed in

triplicate with increasing lipid concentrations and normalized to PKC expression determined by Western blotting.

Imaging

The constructs were transfected into HeLa cells using the FuGENE6 transfection system and allowed to grow 12-24hours posttransfection. Cells were washed in Hanks' balanced solution (HBSS, Cellgro) and imaged in the dark at room temperature. Imaging experiments were performed on the Zeiss Axiovert microscope (Carl Zeiss MicroImaging, Inc) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor software (Universal Imaging, Corp.) All optical filters were from Chroma Technologies. Data were collected as previously described (Violin, Zhang et al. 2003). Flourescence resonance energy transfer (FRET) images were acquired through a 420/10-nm excitation filter, a 450-nm dichroic mirror, and a 535/25nm emission filter. For these experiments, UTP and thapsgargin was added at the time indicated to the final concentration of 100nM. FRET ratios from several cells per imaging plate were normalized and analyzed.

Results

The structure of T250

To look at the possible role of autophosphorylation, we mutated the threonine at position 250 to alanine or to glutamic acid to serve as a phosphomimic. T250 is located at the top of calcium binding loop 3 (Figure 4-1A). In addition, the sequences

of the calcium binding loops of the PKCs were aligned and revealed that the threonine is conserved in the conventional isoforms of PKC, but not the novel isoforms (Figure 4-1B). This brings up the possibility that the autophosphorylation may play a part of the differentially roles, regulation, and membrane binding of these subclasses of C2 domains.

Phosphomimic mutant of T250 reduces the binding and translocation of C2α to the membrane

To examine the effects of the autophosphorylation of T250 on membrane binding, we used the FRET association experiment as described in chapter 1. The k_{on} rate constants for C2α-WT and C2α-T250A were similar at $22.40 \pm 0.09 nM^{-1}s^{-1}$ and $23.8 \pm 0.3 nM^{-1}s^{-1}$, respectively (Table 4-1). However, there was no detectable binding to determine the k_{on} or k_{off} rate constants for C2α-T250D. In addition, the k_{off} rate constant increased 2-fold between C2α-WT and C2α-T250A from $2.81 \pm 0.05 s^{-1}$ and $5.13 \pm 0.2 nM^{-1}s^{-1}$, respectively. Since we could not detect any binding using our FRET association stopped flow analysis, we examined the lipid binding of C2α-T250D using a sucrose-loaded vesicle binding assay. At a high lipid concentration (2.0mM), C2α-WT is located in the pellet showing a high binding affinity between it and the lipid vesicles (Figure 4-2A). In contrast, C2α-T250D is primarily still the supernatant with only a small percentage in the pellet indicating that the protein has a significantly lower affinity for the lipid compared to C2α-WT.

In addition, we compared the translocation of YFP-C2α-WT and YFP-C2α-T250D. In these studies, YFP-C2α-WT or YFP-C2α-T250D was cotransfected with a myrisoylated-palmitoylated CFP into COS 7 cells. Then, the cells were imaged and upon stimulation with the natural agonist UTP and then the SERCA pump inhibitor thapsgargin, the changes in FRET were measured. In these preliminary studies, we see that YFP-C2α-WT translocates to the membrane upon both UTP and thapsgargin stimulation, but YFP-C2α-T250D appears to translocation only after thapsgargin stimulation (Figure 4-2B).

We also determined the PKCα kinase activity of the T250A and phosphomimic mutants *in vitro*. In these experiments, a kinase assay in the presence and absence of calcium were performed on lysates that were transfected with PKCα-WT, PKCα-T250A, or PKCα-T250D. Both mutations, T250A and T250D show a significant decrease in kinase activity both in the presence or absence of calcium (Figure 4-2C).

Discussion

The existence of an autophosphorylation site within the Ca^{2+} binding loops may be another level of regulation for the C2 domain. The addition of the negatively charged phosphate group may disrupt the electrostatic switch created by the binding of Ca^{2+} to the C2 domain. Alternatively, the phosphate group may physically disrupt the binding of the Ca^{2+} ions or may have a more subtle effect and neutralize part of the positive charge (created not only by the Ca^{2+}, but also the basic residues in this region)

of this region that promotes interaction with the PS and, thereby, membrane binding. In addition, in early chapters stopped flow analyses revealed that this area, the tip of Ca^{2+} binding loop 3, is critical for the stabilization of the C2 domain – membrane complex. Could this autophosphorylation act as a means for downregulation?

When comparing the binding of C2α-T250A to wild-type C2α domain, the k_{on} rate constants were similar, while the k_{off} rate constants show a 2-fold difference. This result suggests that the hydrophobic interactions of T250 may play a role in the retention of the C2α domain at the membrane. As suggested by earlier EPR studies and supported by the membrane binding kinetics of R249A and R252A mutants, residue T250, along with a significant portion of the Ca^{2+} binding loop 3, is likely located in the headgroup region of the bilayer (Kohout, Corbalan-Garcia et al. 2003). Therefore, the aliphatic portion of threonine may be interacting with the acyl chains within this region.

Furthermore in the FRET association experiments, the phosphomimic mutant C2α-T250D had no detectable membrane association and so we could not measure k_{on} or k_{off} rate constant. By adding the negative charge, the binding was greatly reduced as also shown by the sucrose-loaded binding assays. This data suggest that the negative charge disrupts lipid vesicle binding possibly through neutralizing the positive charge of the Ca^{2+} binding loop 3. However, the effects of Ca^{2+} binding have not been studied and may give a greater insight into the disruption of binding.

The mutation of C2α-T250 not only affects the binding of the C2 domain to the membrane, but also disrupts the translocation to the membrane in live cells and

reduces the *in vitro* kinase activity of PKC. The loss of translocation further suggests that the negative charge added by autophosphorylation would disrupt membrane binding. And in turn, this loss of binding and translocation translate into decreased kinase activity. It is interesting to note, the mutation of the T250 to alanine also showed a decrease in kinase activity again suggesting the importance of hydrophobic interactions of the threonine with the membrane.

In preliminary data looking at autophosphorylation of the C2α domain, we demonstrate that the addition of a negative charge disrupts C2α domain binding. Since the autophosphorylation occurs after stimulation of PKC and disrupts membrane binding, the question becomes could the autophosphorylation of the C2 domain be involved in the downregulation of PKC. This question is especially interesting since most previous studies have looked at and for cofactors that promote membrane binding. These results may shift the perspective of C2 domain binding and begin to explore the possibility that the C2 domain is involved not in PKC activation, but also its downregulation. The autophosphorylation of the C2α domain reduces its membrane binding affinity and may be a new mechanism of regulation for the C2 domain and the conventional PKCs.

A

B

```
beta     KCSLNPEWNETFRFQLKESDKDRRLSVEIWDWDLTSRNDFMGSLSFGISELQKAGVDGWF 104
alpha    RSTLNPQWNESFTFKLKPSDKDRRLSVEIWDWDRTTRNDFMGSLSFGVSELMKMPASGWY 104
gamma    KATLNPVWNETFVFNLKPGDVERRLSVEVWDWDRTSRNDFMGAMSFGVSELLKAPVDGWY 104
delta    KPTMYPEWKSTFDAHIYEG---RVIQIVLMRAAEDPMSEVTVGVSVLAERCKKNNG---- 90
eplison  ----SPAWHDEFVTDVCNG---RKIELAVFHDAPIGYDDFVANCTIQFEELLQNGSRHFE 108
eta      ----KPTYNEEFCTNVSDG---GHLELAVFHETPLGYDHFVANCTLQFQELLRTAGTSDT 107
theta    KPTMYPPWDSTFDAHINKG---RVMQIIVKGKNVDLISETTVELYSLAERCRKNNGKTEI 105
```

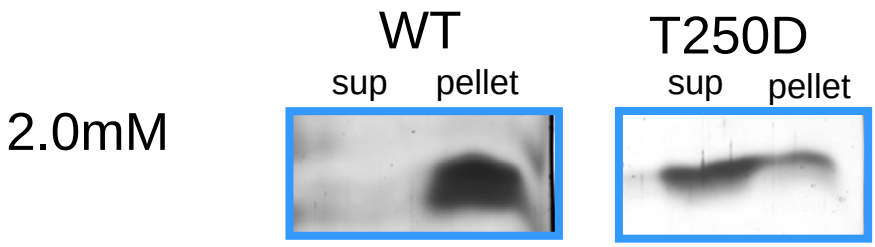
Figure 4-1. **Location of T250 on C2α domain and a sequence alignment of the third Ca^{2+} binding loop of the conventional and novel PKCs.** (A) Ribbon diagram includes the residue T250 which has been suggested to be an auto-phosphorylation site. {B) In this primary sequence alignment for the C2 domains of conventional PKC, the amino acids previously mutated in the C2α domain are denoted in red and the amino acids denoted in blue indicate the possible auto-phosphorylation sites that are conserved in the conventional PKCs, but not the novel or atypical PKCs.

Table 4-1. **The autophosphorylation mimic C2α-T250D showed decreased recruitment to lipid vesicles compared to C2α-T250A and wildtype C2α domain.** Weighted averages of two independent experiments carried out as described in the figure 14.The k_{on} and k_{off} rate constants indicate the weighted averages ± S.E.M. The binding of purified C2α-WT, C2α-T250A, and C2α-T250D and anionic phospholipid vesicles was measured by FRET association stopped flow analysis. The C2α domain protein (0.2μM to 0.5μM) was rapidly mixed with equal volumes of increasing concentrations of dansyl-labeled anionic phospholipid vesicles (35 mol %PS: 60 mol % PC:5 mol % dPE) at 200μM Ca^{2+} and 150mM NaCl, 20mM Hepes, pH 7.4. The dansyl emission was measured over a time course and the k_{obs} was determined using a monophasic fit. The k_{on} is calculated from the slope and k_{off} from the y-intercept of the linear plot of k_{obs} versus vesicles concentration. The weighted averages are calculated from two independent experiments.

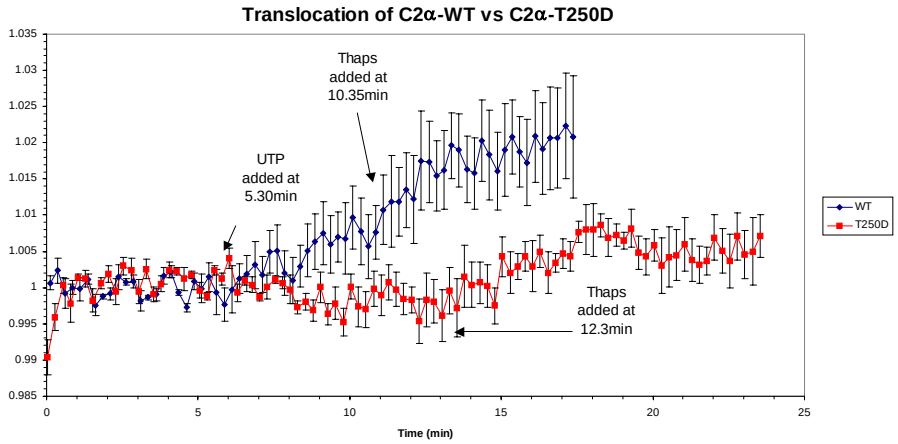
	k_{on} ($nM^{-1}s^{-1}$)	k_{off} (s^{-1})
WT	23.8 ± 0.3	2.81 ± 0.05
T250A	22.41 ± 0.08	5.31 ± 0.2
T250D	n.d.	n.d

Figure 4-2. **Mutating the auto-phosphorylation site T250 decreases C2α membrane binding and translocation as well as PKCα enzymatic activity.** (A)Sucrose-loaded vesicle binding assay show a significant decrease in membrane binding for the auto-phosphorylation mimic T250D as compared to wildtype C2α domain at 2.0mM lipid vesicles (40 mol % PS:60 mol % PC)(supernantant, pellet, respectively). (B) The translocation of C2α-T250D shows decreased membrane translocation compared to wildtype C2α domain in cos 7 cells. The traces were analyzed as described in the materials and methods and averages ± S.E.M are calculated from one independent experiment. (C) The protein kinase C activities of detergent-souble lysates from cos 7 cells expressing PKCα-WT, PKCα-T250A, and PKCα-T250D were measured in the presence of Ca2+ and multilamellar vesicles containing 140μM PS and 3.8μM diacylgycerol using the protein kinase C selective peptide as described. The results were normalized to protein expression determined by Western blot. The averages ± S.E.M are calculated from one independent experiment.

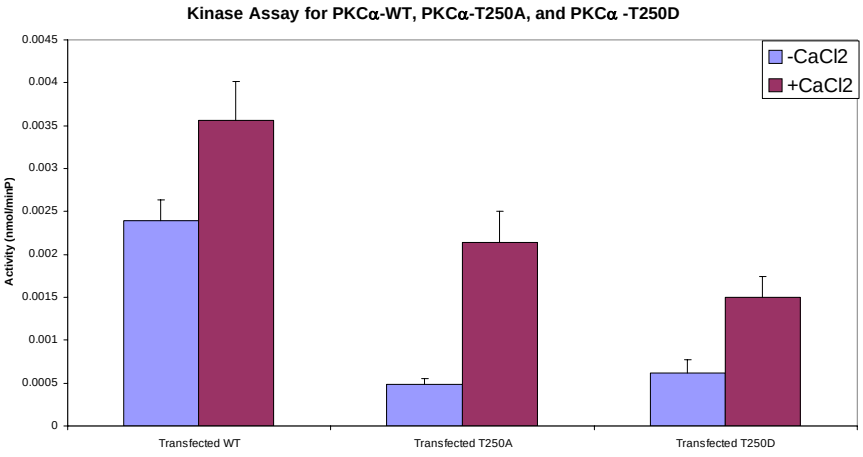
A


WT
sup
pellet
T250D
sup
pellet
2.0mM

B


Translocation of C2α-WT vs C2α-T250D
Thaps added at 10.35min
UTP added at 5.30min
Thaps added at 12.3min
WT
T250D
Time (min)

C


Kinase Assay for PKCα-WT, PKCα-T250A, and PKCα -T250D
-CaCl2
+CaCl2
Activity (nmol/minP)
Transfected WT
Transfected T250A
Transfected T250D

Chapter V

Conclusions: A model of membrane binding of C2 domain of conventional PKCs

Introduction

The translocation and binding to membranes of the regulatory modules of PKC are considered the hallmarks of activation. Since the cloning of PKC, we have known of a region that is responsible for its Ca^{2+} binding dependency (Hurley 2006). This region, the C2 domain, is not only found in PKC, but a variety of other proteins (Nalefski and Falke 1996). Extensive studies provide clues to the mechanism of how the C2 domain recruits to and stays at the membrane, but the overall mechanism of C2 membrane binding is still unclear.

In this dissertation, we dissected the binding kinetics of this critical interaction between the C2 domains of conventional PKCs and the membrane using stopped flow fluorimetry. Using this method, we examined the individual contributions of the on and off rate constants of C2 domain binding to membranes. These measurements allow us to specifically look at the recruitment to, and retention at, the membrane rather than the overall membrane binding affinity that previous studies have examined. These greater insights into which residues and cofactors are involved in membrane recruitment and which residues and cofactors are involved in membrane retention will allow a greater understanding of the mechanism of C2 domain membrane binding.

Model

The C2 domains of the conventional PKCs are thought to follow a two step mechanism for membrane-protein binding: (1) first, diffusion and electrostatic forces lead to the formation of the nonspecific collisional complexes; and (2) once the initial

complex is formed, specific interactions with phospholipid head groups and membrane penetration allow a tightly bound complex to form (Cho and Stahelin 2005). Using the previous studies and the study presented in this thesis, a new model of the mechanism of C2 domain membrane binding has been elucidated. The relationship between specific residues of C2 domain, Ca^{2+}, anionic phospholipids, and the membrane are more clearly defined. The model discussed below incorporates information from the literature and from the experiments described in this thesis.

In developing a model based on our studies, we considered studies that demonstrate the lipid stoichiometry of the conventional C2 domains: 3 ± 1 of PS phospholipid groups for C2α, 4 ± 1 for C2β, and 5 ± 1for C2γ are required for the binding of the C2 domains (Kohout, Corbalan-Garcia et al. 2002). Taken as a whole, these data suggest 3 to 5 PS group are necessary for C2 domain binding. Given that each phospholipid is approximately 1nm in diameter and the C2 domain is approximately 6nm in diameter, the amount of lipid that a C2 domain may encounter is 30 phospholipids; however, the boundary lipid and PS selectively are not known (Verdaguer, Corbalan-Garcia et al. 1999). According to these assumption and calculations, a C2 domain, given the composition used in this study of 35 mol % PS, 60 mol % PC: 5 mol % dPE, may contact approximately 10PS, 17 PC, and 1dPE groups as the C2 domain binds the lipid vesicles.

Orientation

The results of stopped flow confirm that the Ca^{2+} binding loops are critical for C2 domain membrane binding (Edwards and Newton 1997). In particular, residues R216, R249, R252, W245, and W247 play important roles in the membrane binding. The mutating the control residues K268 and W274 to alanine show no effect on the membrane binding kinetics indicating that this part of the C2 domain is not involved with membrane binding. In addition to the Ca^{2+} binding loops, a basic cluster (residues K209 and K211) on the β3 and β4 strands turn are required for specific C2 domain plasma membrane binding and PIP_2 binding (Corbalan-Garcia, Garcia-Garcia et al. 2003) EPR studies provide further information on the orientation of the C2 domain. Both Ca^{2+} binding loop 1 and 3 insert into the membrane; on Ca^{2+} binding loop 1 residue N189 and on Ca^{2+} binding loop 3 residues R249 and R252 insert into the membrane (Kohout, Corbalan-Garcia et al. 2003). The requirement of the basic cluster and the insertion of Ca^{2+} binding loops 1 and 3 suggest a nearly parallel model for the orientation of the C2 domain relative to the membrane (Figure 5-1) (Kohout, Corbalan-Garcia et al. 2003). In this model, residues N189, R249, and R252A reside in the phospholipid head group layer.

Recruitment to the Membrane

The C2 domains of the conventional PKCs follow a two step mechanism for membrane binding: (1) first, diffusion and electrostatic forces lead to the formatiom of the nonspecific collisional complexes; and (2) once the initial complex is formed,

specific interactions and membrane penetration allow a tightly bound complex to form (Figure 5-2)(Cho and Stahelin 2005). In general, protein residues and other factors such as Ca^{2+} binding help create nonspecific electrostatic interactions that enhance recruitment of the C2 domain to anionic membranes (Cho and Stahelin 2005). In contrast to this standard idea, results of this thesis revealed that the major determinants of recruitment for C2 domains of conventional PKCs onto the membrane were Ca^{2+} binding and hydrophobic interactions of specific hydrophobic residues in our studies. The binding of Ca^{2+} may allow three major events: (1) Ca^{2+} may bridges a PS to the domain, and (2) Ca^{2+} may cause subtle changes in the confirmation of the C2 domain, and (3) Ca^{2+} may serve as an "electrostatic switch" (Cho and Stahelin 2005; Hurley 2006). In the case of the C2 domains of the conventional PKCs, experimental evidence supports that Ca^{2+} may have all three effects. These events prepare the C2 domain for membrane binding. The electrostatic switch and bridging to Ca^{2+} may act to bring the domain closer to the membrane. Then, the subtle conformational change may help exposed the tryptophans in Ca^{2+} binding loop 3 and help the domain penetration in to the membrane.

Although electrostatic interactions are supposed to play a key role in membrane association, the mutation of residues R216, R249, and R252 to alanine did not cause significant changes in the recruitment of the C2 domain to the membrane. These data are surprising as we predicted these basic residues should promote recruitment; however, double charged mutant R216A/R249A does show a significant

decrease in membrane recruitment suggesting R216 and R249 can compensate for each other.

Although the classical model for C2 domain membrane binding does not suggest the importance of hydrophobic interactions in membrane recruitment, our data show that hydrophobic interactions are critical for C2 domain membrane binding. In fact, the literature does suggest that aromatic residues, particularly tryptophans, are critical for both the association and dissociation of C2 domains from PC membranes (Cho and Stahelin 2005). In fact, one of the crystal structures suggests the oxygen from the main chain of W247 coordinates one of the Ca^{2+} (Verdaguer, Corbalan-Garcia et al. 1999). These data correlate with the fact that the mutant W247A affects Ca^{2+} binding affinity. The coordination of this Ca^{2+} ion may help stabilize the Ca^{2+} binding and the conformational change needed for membrane binding. In addition, the crystal structures reveal that W245 contacts the glycerol and acyl chains of DAPS (Ochoa, Corbalan-Garcia et al. 2002). Although it interacts to PS in the crystal structure, the residue W245 may not necessarily interact with a PS group and could interact with a PC group. The crystal structures only observed the interaction with PS and did not look at the potential interactions with PC (Ochoa, Corbalan-Garcia et al. 2002). While the electrostatic switch of Ca^{2+} binding has been thought to dominate membrane association, our studies reveal that the hydrophobic interactions of W245 and W247 play into important in membrane recruitment. Our data suggest interactions of Ca^{2+} binding help get the domain close to the membrane, but the hydrophobic interactions may play a major role in the insertion into the membrane.

Retention at the Membrane

Once the domain binds the membrane, the literature suggests protein residues and other factors enhance short-range specific interactions and/or membrane penetration that slow the membrane dissociation (Cho and Stahelin 2005). The bridging of Ca^{2+} ions to PS is important for stabilizing the interaction as well as recruiting the domain to the membrane. Our studies, along with others, show that the membrane retention of the C2 domain is reduced at low Ca^{2+} levels (Nalefski and Falke 1996).

Our studies also showed that both electrostatic and hydrophobic interactions have major roles in retention of the C2 domain at the membrane. The penetration of W245 and W247 leads to the penetration of the Ca^{2+} binding loop 3. The Ca^{2+} binding loop 3 also includes residues R249 and R252 that we found are critical for retaining the domain at the membrane. In our studies, residue R249 was the greatest determinant of the stabilization of C2 domain-membrane complex.

In the C2α domain, R249 acts through both electrostatic and hydrophobic interactions to stabilize C2 domain membrane binding. The crystal structures of C2α domain suggest R249 accomplishes this stabilization in three ways: (1) the aliphatic carbons of R249 interact with the fatty acyl chains of a PS group, and (2) the main chain nitrogen of R249 hydrogen bonds with the sn-1 ester oxygen of PS, and (3) the guanidinium groups of R249 interact with the fatty acyl sn-2 ester carbonyl group of PS (Verdaguer, Corbalan-Garcia et al. 1999). With multiple ways to stabilize the

domain-membrane interaction, R249 appears to be very important for the retention of C2α domain. Our studies also confirmed residue R249 is involved in hydrophobic interactions as the aliphatic chain in the mutant R249L partially recovers the residency time lost in the mutant R249A. Furthermore, analysis of constructs mutated at residue 249 highlights a mechanistic difference between the membrane binding of C2α and C2γ domains and the C2β domain. The C2α and C2γ domains with an arginine at position 249 have a longer residency and are part of a more stable domain-membrane complex than the C2β domain. These data suggest that R249 in C2α and C2γ domains play a central role in anchoring the C2 domain in the membrane.

In addition to confirming many of the interactions described by the crystal structure, it is interesting that the various described interactions suggest the R249 may be 'snorkeling' (Killian and von Heijne 2000). In this mechanism, the aliphatic chain of the arginines prefers to localization in the hydrophobic part of the bilayer, while the positively charged guanidinium group prefer that more polar environment (Killian and von Heijne 2000).

In addition to the charged residue R249, two other charged residues—R216 and R252—appear to be involved in the stabilization of C2 domain membrane binding. Crystal structures suggest that the guanidinium groups of R216, like R249, interact with the fatty acyl sn-2 ester carbonyl groups of PS or with the fatty acyl sn-2 ester carbonyl group (Verdaguer, Corbalan-Garcia et al. 1999). The interactions of R252 are less apparent as the residue is poorly defined in the crystal structure (Verdaguer, Corbalan-Garcia et al. 1999). As both of the other arginines within the

Ca^{2+} binding loops interact with a PS group, one possibility is that the guanidinium groups of R252 also interact with a PS headgroup. It is clear that the basic residues R216, R249, and R252 stabilize the C2 domain-membrane complex through multiple interactions with phospholipids.

The arginines in the Ca^{2+} binding loops are not the only residues that act to stabilize C2 domain membrane binding, W245 and W247 also increase the residency time of the C2 domain. Tryptophans have been shown to have a high affinity for the interfacial layer of the membrane (Cho and Stahelin 2005). The aromaticity of the tryptophans promote partitioning into the electrostatically complex interface environment and unfavorable interactions of the flat, rigid side chain with the hydrocarbon core lead tryptophans to prefer the membrane interface (Braun and von Heijne 1999). The Ca^{2+} binding loop 3 inserts into the membrane and it seems likely that W245 and W247 will insert into the membrane interface and interact with the lipid carbonyls (Killian and von Heijne 2000).

Future Directions

In this dissertation, the recruitment and retention of the C2 domains of the conventional PKC have been dissected to better understand the mechanism of C2 domain membrane binding. As the C2 domain regulation plays such as important in activation, it is not surprising that the C2 domain has several levels of regulation. For the conventional C2 domains of PKC, there are four mechanism of regulation: (1) Ca^{2+}, (2) anionic phospholipids, (3) PIP_2, and (4) electrostatic and hydrophobic

interactions of specific residues. However, there may be more levels of C2 domain regulation. One area of regulation that is not fully resolved in PKC is causes the kinase to leave the membrane. In general the metabolism of diacylglycerol is predicted to be the main mechanism of PKC downregulation, but are there other mechanisms? One possible source of additional regulation may be the autophosphorylation site at residue 250 located within calcium binding loop 3 of the conventional C2 domain. In preliminary studies, we showed that a phosphomimic mutation, T250D, significantly reduces binding of the domain to membranes. To confirm these results, further studies examining the binding, translocation, and activity of the phosphomimic mutant are needed.

In addition, these studies examined the consequences of loss of electrostatic and hydrophobic interactions mostly in the context of the isolated C2 domain *in vitro*. In imaging studies, we showed the loss of specific charged and hydrophobic residues affect kinase activity in live cells. These studies should be expanded looking at different stimuli and subcellular localization. In addition, we showed isoform-specific differences between the activities of different conventional PKC isoforms in live cells. Using the insights we have gained in these experiments *in vitro*, we can pursue imaging in live cells in real time to better understand the importance of the electrostatic and hydrophobic interactions in PKC activity in the context of the whole cell.

Conclusions

In summary, this thesis addressed the roles of electrostatic and hydrophobic interactions in C2 domain membrane binding. We found that electrostatic interactions of charged residues within the Ca^{2+} binding loops with membrane phospholipids play a role in the retention of the C2 domain at the membrane. In addition, hydrophobic interactions of both charged and hydrophobic residues within the Ca^{2+} binding loops with membrane phospholipids play roles in both the recruitment and retention of the C2 domain to the membrane. The electrostatic interactions from Ca^{2+} binding also play significant role in the recruitment and retention of the C2 domain membrane binding. Taken together, these insights provide a greater understanding of the mechanism of C2 domain membrane binding.

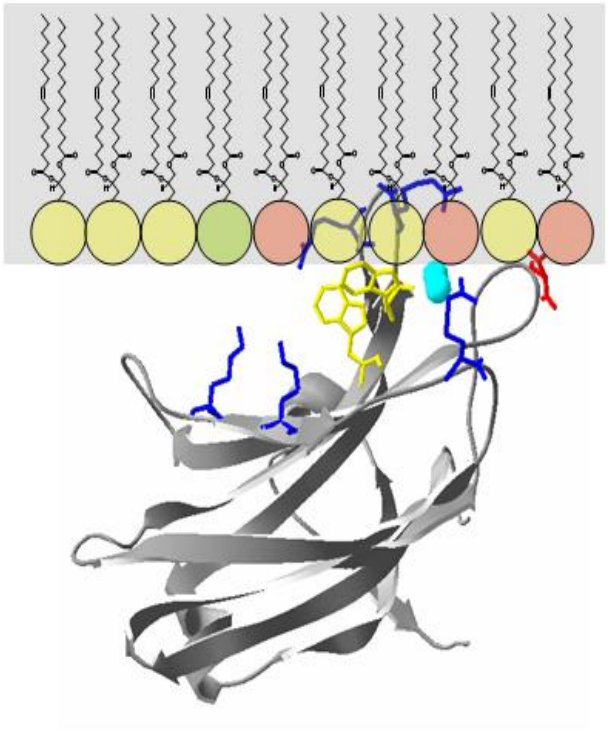


Figure 5-1. **The orientation of the conventional C2α domain.** Residues R216, R249, R252, W245, and W247, which are found within the Ca^{2+} binding loops help orient the C2 domain within the membrane. Both Ca^{2+} binding loop 1 and 3 insert into the membrane; on Ca^{2+} binding loop 1 residue N189 and on Ca^{2+} binding loop 3 residues R249 and R252 insert into the membrane. In addition to the residues located on the Ca^{2+} binding loops, a basic cluster on the β3 and β4 strands turn is required for specific C2 domain plasma membrane and PIP_2 binding. The basic cluster and the insertion of Ca^{2+} binding loops 1 and 3 suggest a nearly parallel model for the orientation of the C2 domain relative to the membrane. The red phospholipid head groups represent an anionic phospholipid, the yellow phospholipid head groups represent a neutral phospholipid, and green phospholipid head group represents $PIP_{\backslash\backslash}$

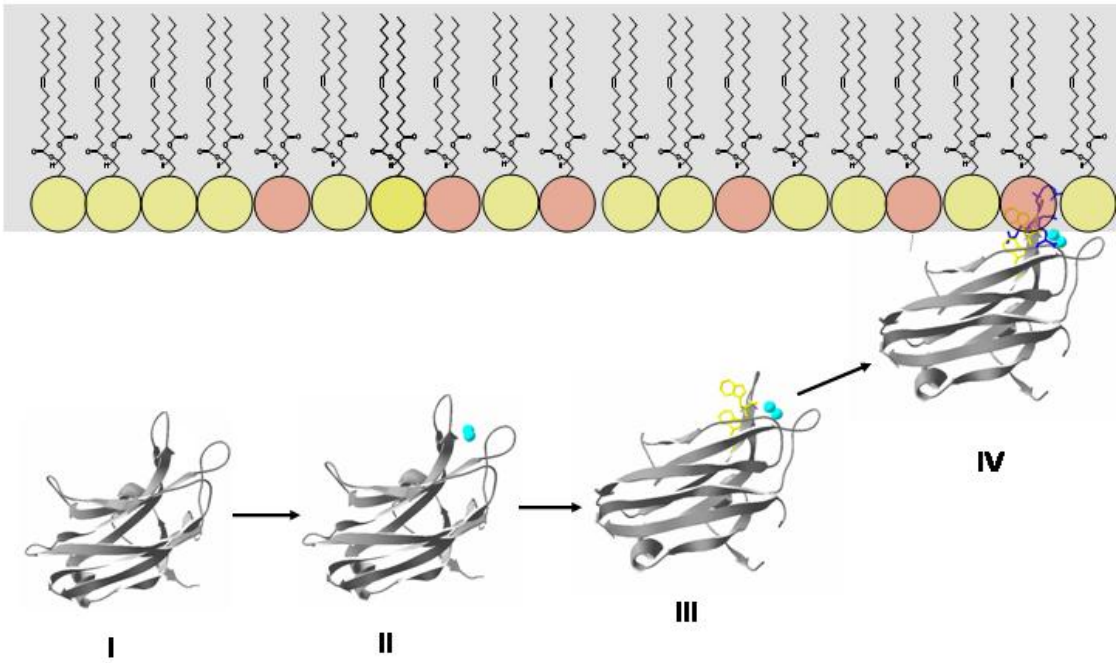


Figure 5-2. **The role of electrostatic and hydrophobic interactions in the recruitment and retention of the conventional C2α domain to the membrane.** The electrostatic interactions of Ca^{2+} binding to the C2α domain promote membrane binding by three mechanisms: bridging an anionic phospholipid to the C2 domain, causing subtle changes in the confirmation of the C2 domain, and serving as an electrostatic switch (Structure I and II). The subtle conformational change may help expose the tryptophans in Ca^{2+} binding loop 3 and facilitate penetration of the C2α domain into the membrane (Structures II and III). Our data suggest interactions of Ca^{2+} binding help get the domain close to the membrane, but the hydrophobic interactions may play a major role in the insertion into the membrane. Although the classical model for C2 domain membrane binding does not suggest the importance of hydrophobic interactions in membrane recruitment for the C2 domains of the conventional PKCs, we show that hydrophobic interactions are critical. The bridging of Ca^{2+} ions to anionic phospholipids is important for stabilizing the domain-membrane interaction (Structure IV). The penetration of W245 and W247 allows the Ca^{2+} binding loop 3, including residues R249 and R252 that we found are critical for retaining the domain at the membrane, to penetrate the membrane bilayer. In fact, residue R249 was the greatest determinant of the stabilization of C2 domain-membrane complex and acts through both electrostatic and hydrophobic interactions to stabilize C2 domain membrane binding. The guanidinium groups of R216 and R252 may also interact with anionic phospholipids. Since the Ca^{2+} binding loop 3 inserts into the membrane, it seems likely that W245 and W247 will insert into the membrane interface and interact with the lipid carbonyls. The red phospholipid head group represents an anionic phospholipid and the yellow phospholipid head group represents a neutral phospholipid.

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