CHAPTER I

INTRODUCTION

Regulation of Cellular Signaling by the Reversible Phosphorylation of Proteins

As early as the 19th century it was known that phosphates could be covalently bound to proteins; however, for a long time these phosphoproteins were considered to be mere byproducts of metabolic reactions, and nothing more (113). It wasn't until the mid 1950's that this way of thinking changed. Biochemists Edwin Kreb and Edmond Fisher discovered that glycogen phosphorylase, which is involved in glycogen metabolism, could be converted from an inactive to an active form through the transfer of a phosphate group from ATP to the protein (42). This finding made it clear that the addition and removal of a phosphate group could profoundly affect the activity and function of the target protein. In fact, the importance of protein phosphorylation as a means of regulating cellular processes is demonstrated by the finding that 30% of intracellular proteins are phosphoproteins, and it is estimated that the combined genes for protein kinases and phosphatases constitute 4% of the eukaryotic genome (10, 60). Step by step, it has become evident that the reversible phosphorylation of proteins is a fundamental mechanism that most, if not all, signaling pathways utilize to convert an external signal into the appropriate intracellular response (57, 60).

The phosphorylation state of cellular proteins is acutely regulated by the opposing actions of protein kinases and phosphatases. Protein kinases phosphorylate proteins by catalyzing the transfer of the terminal phosphate group of ATP to the hydroxyl group of

serine, threonine, or tyrosine residues within the target protein (Figure 1). Phosphorylation can also occur on histidine, aspartate, lysine, and arginine residues in prokaryotes, and to a lesser extent, on these residues in eukaryotes in some signal transduction pathways (72). This covalent addition of a negatively charged phosphate group to a target protein can alter its conformation, activity, and its interactions with other proteins, thus enabling it to modulate various biological processes. When the protein has completed its role, a protein phosphatase catalyzes the hydrolytic removal of the phosphate group, thus returning the protein to its original, unmodified state. Thus, the proper spatial and temporal regulation of protein phosphorylation within specific signal transduction pathways relies on the concerted action of both protein kinases and phosphatases (119). In fact, the aberrant regulation of protein kinases or phosphatases has been implicated in a variety of diseases such as cancer, diabetes, and Alzheimer's, thus making these enzymes major drug targets for therapeutic intervention (30, 33, 89).

Serine/Threonine Phosphatases

More than 99% of all phosphorylations in the cell occur on serine and threonine residues (58). The dephosphorylation of phospho-serine and phospho-threonine is catalyzed by serine/threonine phosphatases, which have been divided into different classes based on their substrate specificity, sensitivity to specific inhibitors, as well as their amino acid sequence homology (46, 58). Specifically, three different gene families have been described: PPM, FCP, and PPP (Figure 2). Members of these three distinct serine/threonine phosphatase families are widely distributed among eukaryotic organisms (29, 69). PPM is composed of serine/threonine phosphatases that are dependent on Mg^{2+}
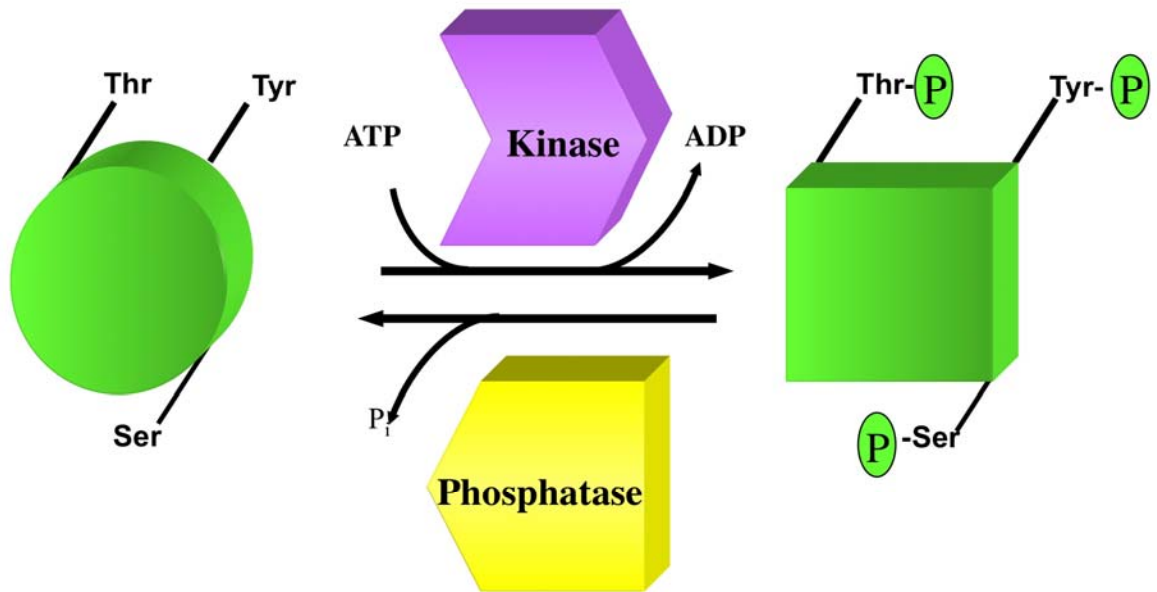
Figure 1: Reversible phosphorylation of proteins. Protein kinases transfer a phosphate group from ATP onto serine, threonine and tyrosine residues within a target protein. Protein phosphatases reverse this reaction by cleaving the phosphate from phosphorylated residues.
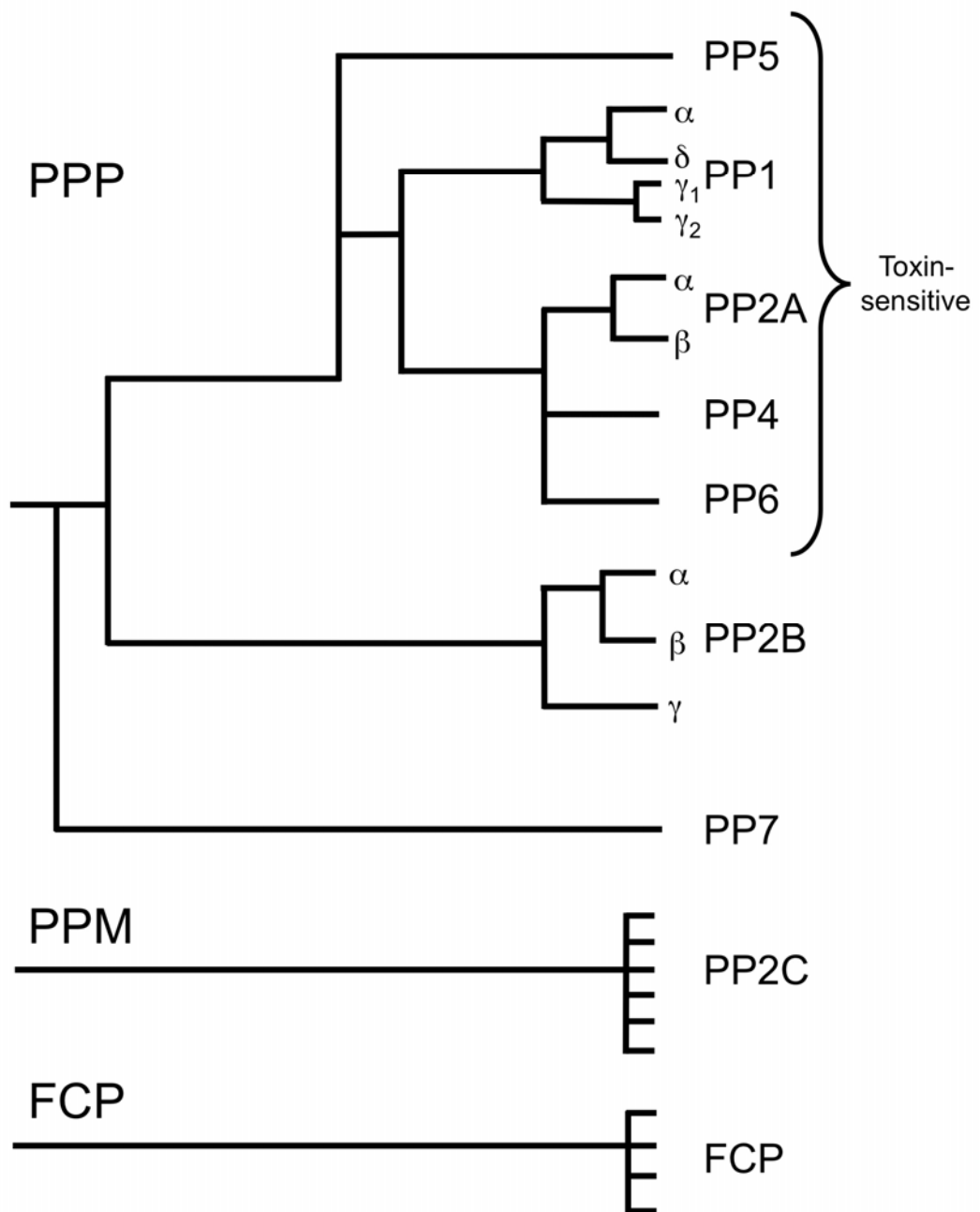
Figure 2: Phylogenetic tree of serine/threonine phosphatases. Classification of three distinct serine/threonine phosphatase gene families (PPP, PPM, FCP) based on their amino acid sequence similarity. The bracketed phosphatases are sensitive to the toxins okadaic acid, microcystin, and calyculin A. This figure is modified from (58).

or Mn^{2+} for activity; this family includes PP2C, pyruvate dehydrogenase phosphatase, and PP2C-like phosphatases (11). The FCP family is comprised of TFIIF-associating C-terminal domain phosphatases (FCP1) (7, 73) and small C-terminal domain phosphatases (SCP1, SCP2, SCP3) (144). These family members are characterized by a conserved DXDX(T/V) motif, which is essential for their activity, and their ability to dephosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II, which is required to recycle the polymerase at the end of each round of transcription (22, 66, 144). The PPP family consists of the PP1, PP5, PP7, PP2B (calcineurin), and PP2A subfamilies, all of which share significant sequence homology in their catalytic domains and are highly conserved from yeast to mammals (11, 58). In contrast to the members of the PPM and FCP families, which generally function as monomers, many protein serine/threonine phosphatases in the PPP family form multimeric holoenzyme complexes with a wide variety of regulatory, scaffolding, targeting, and inhibitory proteins. These interactions confer substrate selectivity and localization to the enzyme, thus allowing a single PPP catalytic subunit to participate in many different cellular functions (63).

Overview of PP2A

The serine/threonine phosphatase type 2A (PP2A) makes up 1% of all cellular protein and, along with protein phosphatase 1 (PP1), accounts for over 90% of serine/threonine phosphatase activity in the cell (31, 38). PP2A plays a principal role in the regulation of numerous cellular processes including cell growth and proliferation, development, inflammation, and apoptosis (reviewed in 63, 119, 146). Its essential function in cells is reflected by the fact that deletion of the gene encoding the catalytic

subunit is lethal in yeast (71) and mice (49). In addition, the importance of PP2A in biological processes is illustrated by the existence of an astonishing array of naturally occurring PP2A inhibitors, of which okadaic acid is the best studied and most widely used (119). Okadaic acid is a polyether fatty acid that is produced by marine dinoflagellates, and is the causative agent of diarrheic shellfish poisoning. This compound is able to bind to the catalytic subunit of PP2A and efficiently block its enzymatic activity (111). Okadaic acid is also a potent tumor promoter and in fact, PP2A was first implicated as a tumor suppressor based on its tumor-promoting actions (14). Treatment of mice with okadaic acid gave rise to tumors on the skin (45), and this was later demonstrated to be caused by the activation of several cancer-promoting pathways (112). In addition, PP2A is the target of several tumor-promoting viruses, including the polyoma small and middle T antigens, as well as the simian virus SV40 small t antigen (101). The direct interactions of these viral proteins with PP2A lead to altered PP2A activity, substrate specificity, and targeting (21, 143), resulting in the deregulation of the mechanisms that control cell growth and survival (36, 116).

Unlike other phosphatases, PP2A exists in cells predominantly as a heterotrimeric complex. The core component of PP2A consists of a 36 kDa catalytic subunit (PP2Ac) and a 65 kDa A subunit that functions as the structural subunit for the enzyme. This A/C dimer interacts with a third regulatory protein, known as the B subunit, to form the heterotrimeric PP2A holoenzyme (63, 119) (Figure 3).
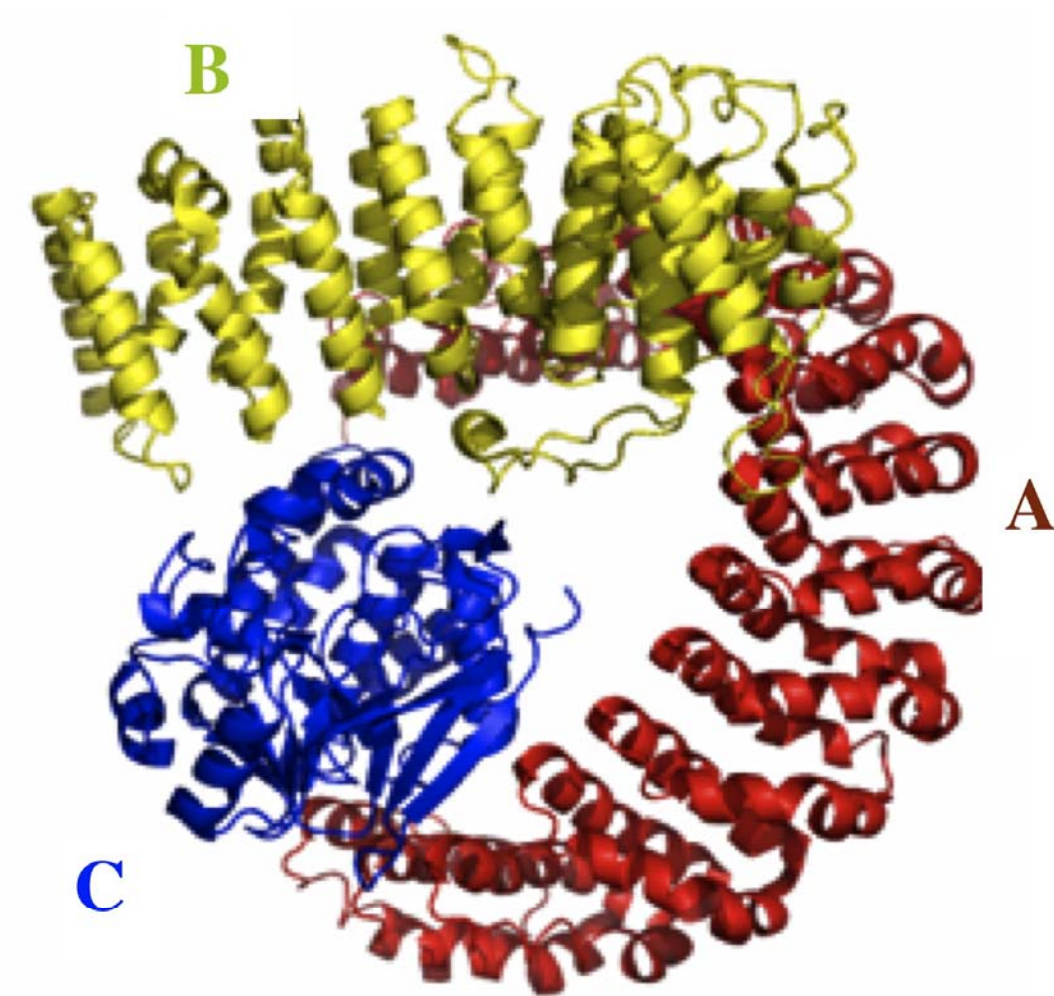
Figure 3: Ribbon diagram of the PP2A holoenzyme. The PP2A holoenzyme is comprised of a scaffolding A subunit (red), a catalytic C subunit (blue), and a regulatory B subunit (yellow). In this image, the crystal structure of the core PP2A A/C dimer is shown with a B' regulatory subunit (B'/B56). The B'/B56/PR61 subunit has a HEAT-like repeat structure, similar to that of the scaffolding A subunit. This figure is modified from (26).

The Catalytic Subunit of PP2A (C or PP2Ac)

Molecular cloning has revealed the existence of two mammalian PP2Ac isoforms, α and β, that are encoded by two distinct genes. Both isoforms are 36 kDa in size, share 98% amino acid identity, and are ubiquitously expressed (8, 50, 124). The catalytic subunit has a large conserved domain that forms a bimetallic active site for the phosphate ester hydrolysis of phosphate groups on either serine or threonine residues (41). Elucidation of the crystal structure of the PP2A holoenzyme has revealed that the C-terminal tail of the C subunit resides at a critical interface between the A and B subunits and thus stabilizes the A–B interaction (26, 141).

The Structural Subunit of PP2A (A or PR65)

The A subunit is a structural subunit that is tightly associated with PP2Ac, and forms a scaffold to which one of a number of B subunits can bind in a mutually exclusive fashion via the same or overlapping sites (106, 107). As is the case for the catalytic subunit, the A subunit is encoded by two genes, PR65α and PR65β (54). Both isoforms are ubiquitously expressed, and they share 86% amino acid identity (54). Most PP2A holoenzymes contain the PR65α isoform, while only a small fraction (10%) contain the PR65β isoform (16, 55). Interestingly, somatic alterations in the gene encoding PR65β were discovered in 15% of primary lung and colon tumor-derived cell lines (136), and mutations in the gene encoding the PR65α isoform were detected in human melanomas and breast and lung carcinomas (20).

The structure of PR65 is interesting in that it is composed entirely of 15 tandem repeats of a 39-amino acid sequence, termed a HEAT (huntingtin/elongation/A

subunit/TOR) (51). Each repeat is virtually the same, and is composed of two superimposed α-helices (Figure 3). The particular stacking of these repeats within the PR65 molecule gives rise to a stable protein with an overall asymmetrical and elongated architecture, reminiscent of a hook (C-shape) (26, 51, 141). The regulatory B subunit interacts with the PR65 scaffold protein at HEAT repeats 1-10, whereas the catalytic subunit interacts with HEAT repeats 11-15, causing the A subunit to fold in on itself to form a horseshoe-like structure. This conformation allows unimpeded access of the catalytic subunit to the PP2A substrate (26, 141),

The Regulatory B Subunits

The regulatory B subunits of PP2A are made up of four unrelated families named B, B′, B″, and B‴ (Table 1). Each of these subunits is composed of several different family members, all of which are able to bind to the A subunit in a mutually exclusive manner to form distinct ABC holoenzymes (81). Although there is significant sequence homology amongst B subunits within each family, there is little sequence similarity between families, apart from a few conserved amino acids that allow its interaction with the N-terminal HEAT domains of the PR65 scaffold subunit (81). Unlike the A and C subunits which are ubiquitously expressed, many of the B subunits have differential subcellular distributions, are expressed in a tissue-specific manner, and are developmentally-regulated. Thus it has been postulated that the B subunits confer substrate specificity and localization to the PP2A holoenzyme, thereby allowing the dephosphorylation of specific substrates in distinct cellular compartments by different holoenzyme assemblies (reviewed in 63). Given the occurrence of two A, two C, four B,

Regulatory Subunits	Gene symbol	Known isoforms	Cell/Tissue Distribution	Subcellular Distribution
B (B55) family	PPP2R2A	Bα/B55α/PR55α	Widespread, predominant PP2A regulatory subunit	Microtubules, neurofilaments, vimentin, cytoplasm, membrane, nucleus, Golgi/reticulum
	PPP2R2B	Bβ/B55β/PR55β	Enriched in brain and testis	Cytosol
	PPP2R2C	Bγ/B55γ/PR55γ	Brain-specific	Enriched in cytoskeletal fraction
	PPP2R2D	Bδ/B55δ/PR55δ	Widely expressed, enriched in testis	Cytosol
B′ (B56) family	PPP2R5A	B′α/B6α/PR61α	Highly expressed in heart and skeletal muscle	Cytoplasm
	PPP2R5B	B′β/B56β/PR61β1	Highly expressed in brain	Cytoplasm
	PPP2R5C	B'γ1/B56γ1/PR61γ1/ B'α3	Highly expressed in heart and skeletal muscle	Cytoplasm>nucleus, focal adhesion
	PPP2R5C	B′γ2/B56γ2/PR61γ2/ B'α2	Highly expressed in heart and skeletal muscle	Nucleus>cytoplasm
	PPP2R5C	B′γ3/B56γ3/PR61γ3/ B'α1	Highly expressed in brain	Nucleus>>>cytoplasm
	PPP2R5D	B′δ/B56δ/PR61δ/ 74–kDa/B″δ	Highly expressed in brain	Nucleus, cytosol, mitochondria, microsome
	PPP2R5E	B'ε/B56ε/PR61ε	Highly expressed in brain	Cytoplasm
B″ family	PPP2R3A	B″α1/PR130	Heart,brain,lung, muscle, kidney	Centrosome and Golgi
	PPP2R3A	B″α2/PR72	Enriched in heart and skeletal muscle	Cytosol, nucleus
	PPP2R3B	B″β/PR48	Placena	Nucleus
	PPP2R3D	B″δ/PR59	Heat, kidney, lung	Nucleus
B‴ family	STRN	striatin;PR110/ SG2NA;PR93	Expressed in the brain	Cytosol, membrane-bound

Table 1: Nomenclature and distribution of the PP2A regulatory B subunits. This figure is modified from (64, 119).

at least 8 B′, four B″, and 2 B‴ isoforms, at least 75 different trimeric PP2A holoenzymes can be generated (63, 67). This extraordinary diversity of PP2A holoenzymes likely accounts for the growing list of phosphoproteins and signaling pathways regulated by PP2A.

Elucidating the Biological Functions of PP2A

Significant advances have been made in elucidating the biological roles for PP2A through the use of *in vitro* phosphatase assays, cell-permeable inhibitors, viral proteins, and molecular genetics. However, these strategies do have their limitations. For example, *in vitro,* PP2A is a very active enzyme that dephosphorylates many phosphoproteins, so although *in vitro* dephosphorylation studies have been used to implicate PP2A in the control of a particular biological process, questions often remain as to whether PP2A modulates that process *in vivo*. Potent cell-permeable inhibitors of PP21 and PP2A such as okadaic acid, calyculin A, and tautomycin are widely used on cultured cells to demonstrate an involvement of these enzymes in a particular biological process. However, these compounds exhibit overlapping specificities for PP1 and PP2A family members (40). Therefore, one must be cautious when using these inhibitors to discriminate between the activities of various protein serine/threonine phosphatases. Fostriecin is a more specific inhibitor of the PP2A enzyme, but is a difficult compound to work with as it is relatively unstable and quickly oxidized when exposed to air (131). Nevertheless, these phosphatase inhibitors have the advantage of rapidly inactivating PP2A, which allows the monitoring of signaling events that can occur within minutes of cellular stimulation. Under appropriately controlled conditions, these inhibitors can be

used in intact cell experiments to facilitate the assignment of PP1 or PP2A to specific cellular processes (40).

The role of PP2A in various biological processes has also been shown in studies of DNA tumor viruses, which induce proliferative cell growth and viral replication through the actions of their T antigens (reviewed in (47, 95). When these viral proteins, such as simian virus 40 (SV40) and polyoma virus, form complexes with host proteins, the normal functions of that host protein is altered. For example, SV40 large T antigen binds to the tumor suppressor proteins p53 and Rb, thereby inactivating their function (34, 82). PP2A is another important target for the viral antigens. In fact, PP2A is the only known target of SV40 small t in cells (101); small t inhibits PP2A activity towards most substrates by displacing the variable B subunit from the respective PP2A holoenzyme (143). Thus, the overexpression of SV40 small t in mammalian cells inhibits PP2A's role in various mitogenic signaling pathways, leading to the activation of mitogen-activated protein kinase (MAPK) (120), stress-activated protein kinase (SAPK) (123), and calcium/calmodulin-dependent protein kinase IV (CaMKIV) (137) signaling cascades. These studies also indicate that SV40 small t can be exploited as a very useful tool for dissecting PP2A's role in various biological processes.

The biological functions of PP2A have also been investigated through the use of molecular genetics. Specifically, studies in *Drosphila* have revealed a role for PP2A in developmental processes (reviewed in 63), and the genetic analyses of yeast and *Drosophila* mutants of PP2A subunits have demonstrated a role for PP2A in the control of cell cycle progression (87, 118).

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Regulation of PP2A

Due to the important role that PP2A plays in a wide variety of cellular functions, its activity is tightly controlled *in vivo* by multiple regulatory mechanisms (summarized in Figure 4). These regulatory mechanisms include a variety of post-translational modifications of the PP2A catalytic subunit (PP2Ac). Phosphorylation of tyrosine and threonine residues within PP2Ac lead to inactivation of phosphatase activity *in vitro* (24, 52). In addition, carboxymethylation of a C-terminal leucine residue of PP2Ac appears to be important for its association with the regulatory B subunits (18). The most recent post-translational modification of PP2Ac that has been reported is ubiquitination of the catalytic subunit; this modification appears to modulate PP2Ac levels, but may also control its subcellular localization (134).

The regulation of PP2A is also highly dependent upon the composition of its regulatory B subunits. Although both the A and C subunits of PP2A exist in two isoforms that are ubiquitously expressed in parallel with one another, the B subunits, in contrast, exhibit a substantial amount of variability. To date, four families of B subunits have been identified in eukaryotes; they are designated B, B′, B″, and B‴ (Table 1). Each B subunit family is encoded by multiple genes, with multiple splice variants, generating an extraordinary diversity of these subunits. The variability in the specific holoenzyme composition of PP2A increases the diversity of the PP2A enzyme and provides many possibilities for phosphatase regulation. For example, due to their specific cellular and subcellular localization, this subunit can target the phosphatase to different tissues and cellular compartments. In this way, the presence of different regulatory subunits has been shown to determine the substrate specificity of PP2A holoenzymes. Lastly, the regulation

Post-translational modifications of the PP2A catalytic subunit (C)

Association with regulatory (B) subunits

B	B'	B"	B'''
α, β, γ, δ	α, β, γ, δ, ε	PR72/130	SG2NA, striatin

Interaction with cellular proteins

Viral proteins ⟷

Inhibitor proteins

PTPA

alpha4

Figure 4: Multi-level regulation of PP2A. PP2A is regulated by post-translational modifications of the catalytic subunit, association with the variable regulatory B subunits, and its interaction with cellular proteins.

of PP2A also occurs via its interaction with numerous cellular proteins other than the cognate A and B subunits. These include i) The SV40 and polyomavirus antigens that inhibit PP2A activity by displacing the B subunit (reviewed in 63, 64), ii) the α4 protein, which directly associates with the catalytic subunit and appears to modulate substrate selectivity (25, 96), and facilitate PP2A ubiquitination (134), iii) two heat-stable protein inhibitors (I1^{PP2A} and I2^{PP2A}) (80, 88), iv) a phosphotyrosyl phosphatase activator (PTPA), which activates the phospho-tyrosine phosphatase activity of the PP2A core A/C dimer (reviewed in (63), and v) the type-2A-interacting protein (Tip) which interacts directly with the catalytic subunit and inhibits phosphatase activity (88).

Defining Cellular Substrates for PP2A

Despite the advances that have been made in our understanding of PP2A's role in various biological processes, physiologic substrates for PP2A within these pathways can be difficult to identify. This is in large part due to the fact that PP2A is such a promiscuous enzyme *in vitro*. Thus, it is often unclear whether its *in vitro* substrates also serve as substrates *in vivo*. To circumvent this issue, investigators have focused on identifying interacting proteins of PP2A that also serve as substrates for this enzyme. One approach that our laboratory has successfully utilized to identify interacting partners for PP2A is the purification of PP2A complexes from cellular extracts after crosslinking to stabilize large molecular size forms of the phosphatase (138). This approach and other biochemical strategies have revealed that PP2A associates with a variety of cellular proteins including neurofilament proteins (126), microtubules (103), biogenic amine transporters (13), nuclear factor-κB (NF-κB)/Rel transcription factors (142), and a

number of protein kinases. These protein kinases include calcium/calmodulin-dependent protein kinase IV (CaMKIV), the ribosomal S6 protein kinase (p70S6K), and two p21-activated protein kinases (PAK1 and PAK3) (Figure 5). The existence of these protein kinase•PP2A complexes in rat brain soluble extracts was further substantiated by several additional lines of evidence: 1) PP2Ac co-immunoprecipitated with these kinases using kinase-specific antibodies; 2) PP2Ac co-purified with GST-p70S6K, GST-PAK3 and GST-CaMKIV fusion proteins; and 3) each kinase bound to microcystin-Sepharose - an affinity resin used for isolating phosphatase holoenzymes (137, 138). To date, these approaches have been successfully utilized by our laboratory, and others, to identify several other protein kinase•PP2A complexes, including Raf1•PP2A (1), Janus-activated kinase•PP2A (44), and IκB kinase•PP2A (75). Together these studies have highlighted the importance of protein kinase•PP2A signaling modules in the control of a variety of signal transduction pathways. The realization that PP2A can regulate the activities of multiple associated protein kinases, begins to answer the question of how a single enzyme can have such a plethora of effects in the cell. Within the kinase•PP2A complex, the potential for intermolecular regulation exists, where the kinase can regulate the phosphatase and vice versa, thus allowing the fine-tuning of phosphorylation which is essential for cellular homeostasis.
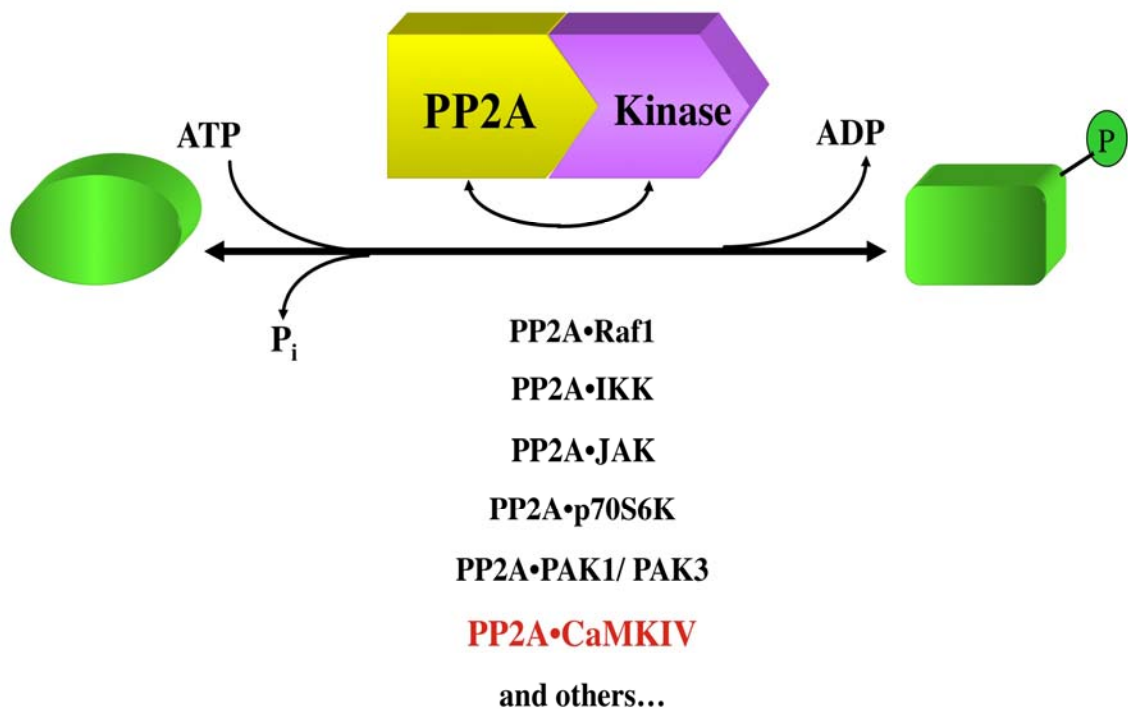
Figure 5: Protein kinase•phosphatase signaling modules. PP2A forms complexes with several protein kinases, some of which are listed above. PP2A•CaMKIV is highlighted because this is the signaling complex that our studies focus on.

Ca^{2+}/Calmodulin-Dependent Protein Kinase IV

One of the first examples of an association between PP2A and a protein kinase was the discovery from our laboratory that Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) forms a complex with PP2A (137). CaMKIV is a member of the Ca^{2+}/calmodulin-dependent family of protein kinases (CaMKs). These serine-threonine protein kinases are an important group of signaling proteins that are particularly abundant in the brain, and are activated via binding of calcium and calmodulin (CaM), the ubiquitous intracellular receptor for calcium (59, 90). Once activated, CaMKs phosphorylate Ser/Thr residues in their substrates to alter the functionality of those proteins. Some CaMKs target a specific substrate and are known as "dedicated kinases"; these include phosphorylase kinase, myosin light chain kinase, and eukaryotic elongation factor-2 kinase (also known as Ca^{2+}/CaM-dependent kinase III). Other CaMKs, known as "multifunctional kinases", have broad substrate specificities and modulate a variety of physiological processes in response to increases in intracellular Ca^{2+} (92, 93). The multifunctional kinase family is comprised of the Ca^{2+}/CaM-dependent protein kinase I (CaMKI) subfamily (α, β, γ, and δ genes), the CaMKII subfamily (α, β, γ, and δ genes), CaMKIV (one gene), and the CaMKK subfamily (α and β genes) (93). Many of the diverse actions of Ca^{2+} are mediated through these multifunctional protein kinases, which function as potent mediators of Ca^{2+}-dependent gene expression, and thus play important roles in many aspects of neuronal physiology including synaptic plasticity, learning and memory.

CaMKIV was originally identified twenty years ago. At that time, CaMKs I, II, and III had been identified, and had provided useful information about neuronal Ca^{2+}

signaling. Scientists were still interested, however, in identifying other potentially unknown CaMKs that could also play a role in mediating cellular responses to elevated neuronal Ca^{2+} levels. Therefore, a rat brain cDNA expression library was screened using both [125]calmodulin and a CaMKII monoclonal antibody (99). These cloning strategies, together with subsequent biochemical purifications, resulted in the identification of CaMKIV (43, 99). The newly discovered protein was found to be particularly abundant in the granular cells of the cerebellum, and was originally given the name 'CaM kinase Gr' (43, 99). However, this kinase was also found in other tissues, namely T-lymphocytes, and was thus later renamed to CaMKIV (91).

CaMKIV exists as two monomeric polypeptides termed CaMKIVα and CaMKIVβ, with molecular weights of 65 and 67 kDa, respectively. The β isoform of CaMKIV is identical to CaMKIVα except for the presence of an additional 28 amino acids at the amino-terminus (108). Both CaMKIV isoforms are encoded by a single 42 kb gene that is divided into 12 exons and 11 introns. Interestingly, in the rat, the last 169 amino acids of CaMKIV is identical to the entire coding sequence of another calmodulin-binding protein, calspermin, a protein of unknown function that is expressed exclusively in the testis, and is regulated by cAMP (100, 129, 130). Figure 6 depicts the organization of the rat CaMKIV gene which, as aforementioned, encodes three proteins: CaMKIVα, CaMKIVβ, and calspermin. The CaMKIVβ initiation codon is in exon I, the CaMKIVα initiation codon is in exon II, and exon XI represents the location of the testis-specific exon that encodes the calspermin transcript (130). Structurally, CaMKIV is comprised of an amino-terminal domain believed to play a critical role in enzyme activation, a central regulatory domain, and a highly acidic carboxyl-terminal domain (3) (Figure 7).Within the

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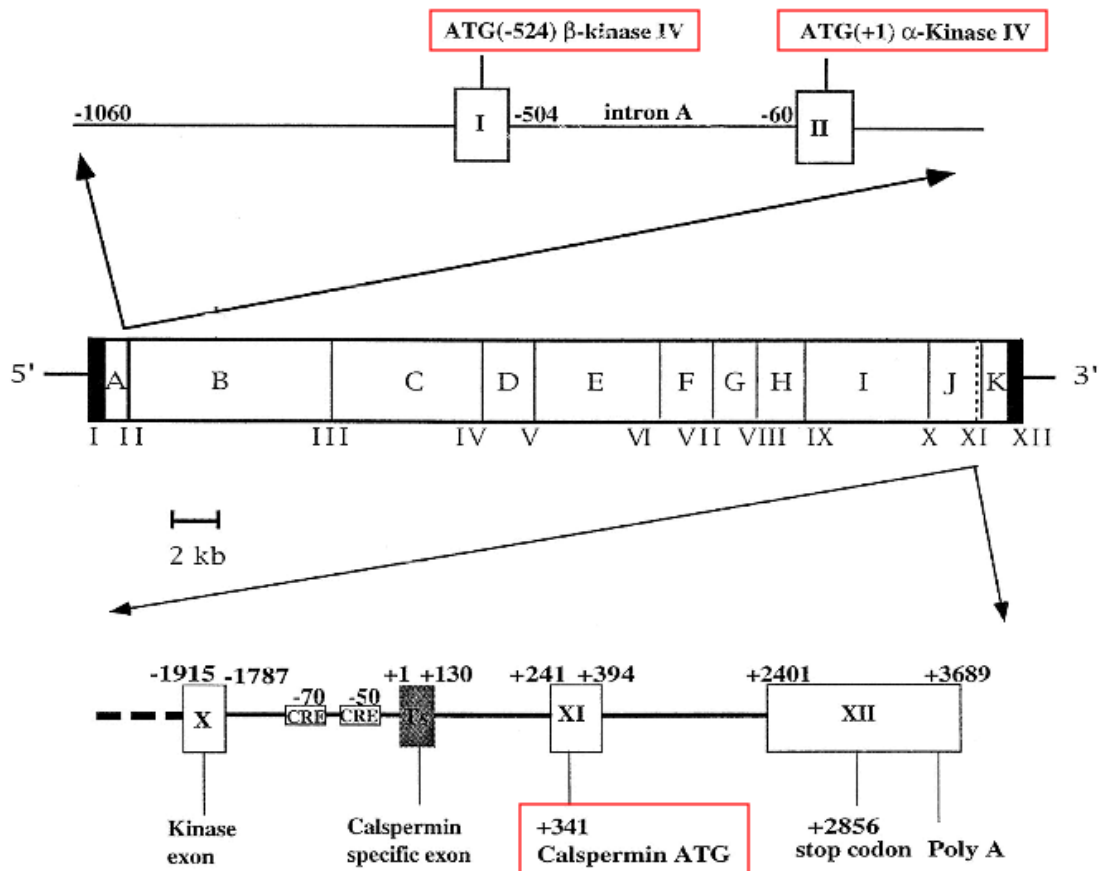
Figure 6: Schematic representation of the organization of the rat CaMKIV gene. *Top panel;* Organization of exons I and II as well as intron A. The CaMKIVα initiation codon is nucleotide +1 which is in exon II. The CaMKIVβ initiation codon is at nucleotide −524 which in exon I. *Middle panel;* Both isoforms of the kinase share exons II-XII. Exons are indicated by Roman numerals, and introns are indicated by capital letters. The dotted line between intron J and exon XI represents the location of the testis-specific exon involved in generation of the calspermin transcript. *Bottom panel;* Calspermin gene structure. Exons are indicated by boxes, and the black box is the testis-specific calspermin exon. Two CRE-like motifs are located at −50 and −70 relative to the transcriptional initiation site. This figure is modified from (130).
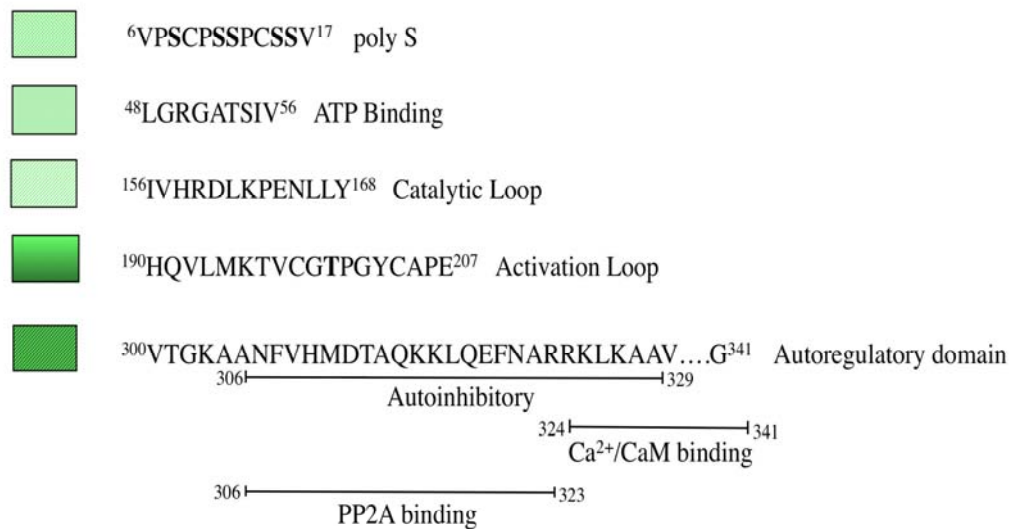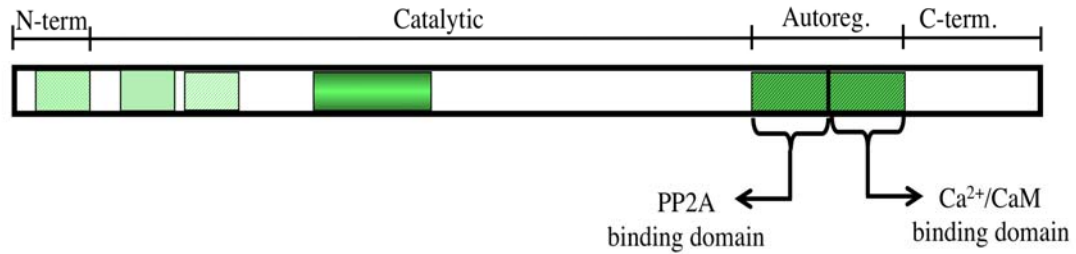
Figure 7: Schematic diagram of the domain structure of CaMKIV: The upper portion represents a schematic diagram of the CaMKIV protein. The lower portion is a key depicting the important domains within CaMKIV. This figure is modified from (3, 27).

regulatory domain, which spans residues 301 to 340, are overlapping autoinhibitory (CaMKIV residues 306 to 329) and CaM-binding (CaMKIV residues 324 to 341) domains. In addition, the autoinhibitory domain spans the region of CaMKIV that is important for its interaction with PP2A (27). In the absence of Ca^{2+}/CaM, CaMKIV is inhibited intrasterically by the autoinhibitory region, which is believed to prevent both substrate and ATP binding to the catalytic domain (3). The binding of Ca^{2+}/CaM relieves the autoinhibitory domain which leads to de-inhibition of the enzyme (3).

CaMKIV has long been regarded as a primarily nuclear protein, in part because it is responsible for Ca^{2+}-dependent gene transcription through the phosphorylation of several transcription factors (reviewed in 3). However, there is a substantial amount of immunological and biochemical data demonstrating that CaMKIV can actually be found in both the soluble and nuclear compartments of the cell (65, 102, 145). The existence of notable levels of CaMKIV in the cytoplasm indicates that CaMKIV may have physiological functions other than the phosphorylation of transcription factors. In support of this idea, one study showed that oncoprotein 18, a protein that regulates microtubule dynamics in the cell, is a major cytosolic target for activated CaMKIV (84), and another report demonstrated that CaMKIV may be involved in regulating cytoplasmic events associated with cell differentiation and survival (102).

CaMKIV Activation/Inactivation

A schematic view of the CaMKIV signaling transduction pathway is depicted in Figure 8. CaMKIV is maintained in an inactive state by its autoinhibitory domain (59). In
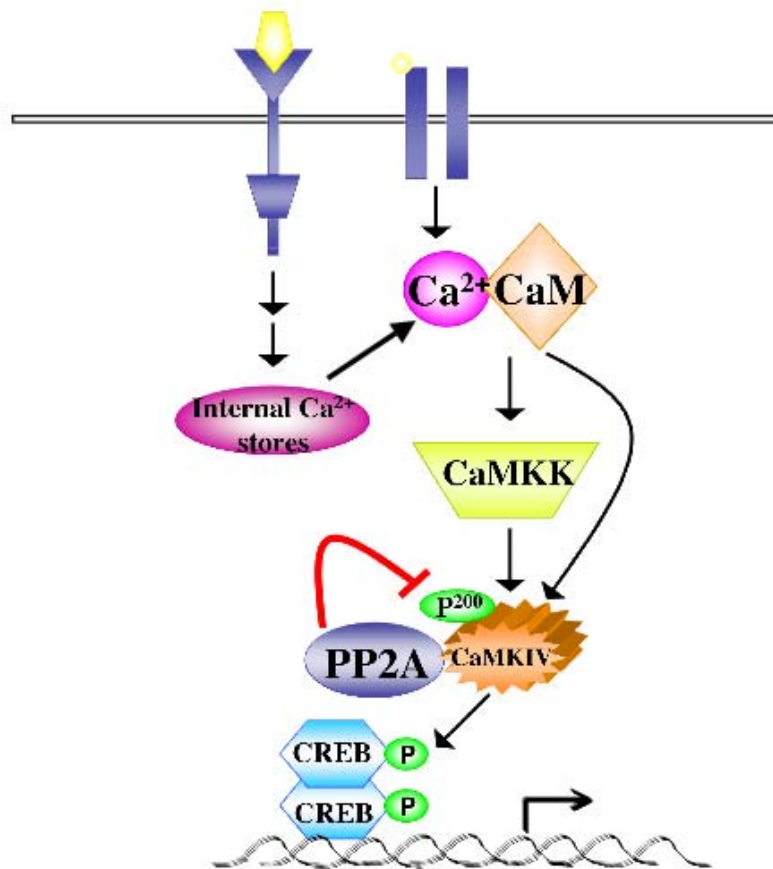
Figure 8: The CaMKIV Signaling Pathway. Upon increases in intracellular Ca^{2+}, Ca^{2+} binds to its intracellular receptor, calmodulin (CaM). The Ca^{2+}/CaM complex then binds to, and activates CaMKK. It also binds to CaMKIV, thus repositioning its autoinhibitory domain, which allows CaMKK to bind and phosphorylate CaMKIV on T200. Activated CaMKIV then phosphorylates its substates such as CREB, leading to transcription of immediate early genes. PP2A negatively regulates this pathway by dephosphorylating CaMKIV on T200.

response to increases in intracellular calcium, either from intracellular calcium stores or the opening of voltage-gated channels, calcium binds to its intracellular receptor, calmodulin. The Ca^{2+}/CaM complex subsequently binds to, and activates CaMKK, the upstream kinase in this pathway (132). The Ca^{2+}/CaM complex also binds to the calmodulin-binding domain within the autoregulatory segment of CaMKIV; this regulatory segment contains overlapping Ca^{2+}/CaM and autoinhibitory domains (Figure 6). Binding of Ca^{2+}/CaM to CaMKIV disrupts the inhibitory interactions between the autoinhibitory domain and the catalytic lobe of the kinase, thereby exposing its active site. CaMKK then binds to the activation loop and subsequently phosphorylates CaMKIV on Thr196 in the rat enzyme and Thr200 in the human enzyme (37, 115). In addition, the amino-terminus of CaMKIV has been shown to facilitate a second type of CaMKIV autoinhibition that can be relieved by Ca^{2+}/CaM-dependent autophosphorylation of Ser11 and Ser12. In the absence of active CaMKK, slow autophosphorylation occurs on those residues, which parallels the low basal activity of the enzyme. However, upon activation of CaMKIV by CaMKK, the autophosphorylation of these serine residues increases in rate and extent, thereby relieving the intrasteric autoinhibition caused by the N-terminal region of the protein (3, 23). Thus, maximal activation of CaMKIV requires 3 steps: 1) Ca^{2+}/CaM binding, 2) phosphorylation of Thr200 by Ca^{2+}/CaM-bound CaMKK, and 3) autophosphorylation of Ser 11 and 12. Fully activated CaMKIV then phosphorylates its substrates, among which the best known is the nuclear cyclic adenosine monophosphate (cAMP) response element binding protein (CREB).

CREB is a ubiquitous transcription factor that binds to the consensus cAMP-response-element (CRE) DNA sequence TGACGTCA. CREB binds DNA via its basic region and homodimerizes or heterodimerizes with closely related family members through its leucine zipper moitif (48). CaMKIV phosphorylates CREB on S133, which promotes recruitment of the transcriptional co-activator CBP (CREB-binding protein) (61), a protein that has recently been shown to display acetyltransferase activity towards both histones and other transcriptional regulatory proteins (28). The phospho-CREB/CBP complex then interacts with multiple basal transcriptional proteins to initiate the expression of immediate early genes involved in such processes as T-cell activation and neuronal long-term potentiation (3, 28). Other transcription factors phosphorylated by CaMKIV include activating transcription factor-1 (ATF-1), which is a member of the CREB family of transcription factors and is responsive to both cAMP and Ca^{2+} (83). ATF-1 can heterodimerize with CREB and may therefore affect CRE-mediated transcription by altering the equilibrium of CREB homodimers versus CREB/ATF-1 heterodimers (83). The serum response factor (SRF) is another CaMKIV substrate that is a nuclear-localized transcription factor, which has been shown to be the critical mediator of serum stimulation of the c-fos promoter (3). The SRF binds its cognate response element (SRE), which is upstream of the CRE element found in the c-fos promoter. CaMKIV stimulates CREB, ATF-1, and SRF-mediated transcription via direct phosphorylation of the activating serines on CREB (Ser 133), ATF-1 (Ser 63), and SRF (Ser 103) (39, 94, 127, 128). Thus, CaMKIV functions as a key mediator of Ca^{2+}-induced gene expression,

CaMKIV Function

Since the elevation of intracellular Ca^{2+} is necessary for the induction of long-term potentiation (LTP), several transducers of Ca^{2+} signaling have been implicated in those processes, including CaMKIV (56). CaMKIV is highly expressed in the brain, and indeed it has been shown to play an important role in LTP via phosphorylation of CREB (3, 68). LTP is an adaptive neuronal response in which long-lasting changes in neuronal cell structure and function lead to increases in synaptic strength (3). This process, which is required for long-term memory and learning, is triggered by Ca^{2+} influx via repetitive stimulation of the synapse. One study addressed the roles of Ca^{2+} and CREB in LTP by generating knockout mice in which the expression of CREB was disrupted (17). These mice exhibited deficits in long-term memory and synaptic plasticity; however, confounding effects inherent to global knockout mice made it difficult to establish a direct link between CREB function and long-term memory. Other groups also tried to disrupt CREB signaling, but found that mutant mice harbored compensatory increases in the level of several other CREB isoforms (15, 74). In recent years, scientists have moved away from studies looking at the effects of CREB disruption, and instead have turned to the disruption of CaMKIV expression as a way to implicate a role for this enzyme in LTP. For example, in one study, the authors examined the role of CaMKIV in the mammalian brain by generating mice lacking the CaMKIV gene (105). Behavioral analyses of these mice established the presence of tremors, altered gait, and moderate to severe loss of motor control, consistent with cerebellar defects. Morphological studies indicated a loss of cerebellar Purkinje neurons, and electrophysiological studies demonstrated significant defects in synaptic transmission at excitatory synapses of

Purkinje neurons in CaMKIV null mice (105). Another study examining CaMKIV knockout mice, showed that these mice exhibited impaired neuronal CREB phosphorylation and were also deficient in both LTP and long-term depression (LTD), which is proposed to be important for the formation of long-term memory (56). Transgenic mice expressing a dominant-negative form of CaMKIV (dnCaMKIV) have exhibited similar phenotypes (68). At the molecular level, these mice were impaired in activation of CaMKIV, CREB, and CRE-dependent transcription. At the level of synaptic physiology, neurons from these mice had impaired LTP, and at the behavioral level, there was impairment of the consolidation and/or retention phase of memory. Together, these data demonstrate that interference with CaMKIV signaling in the brain impairs LTP and long-term memory.

CaMKIV is also highly expressed in T cells where it appears to play an important role in the maturation of T cells in the thymus (77). T cells are critical to the mammalian immune system because they recognize antigens on the surface of invading pathogens, viruses, and tumor cells. T cells recognize an antigen through a complex mechanism that includes three different receptors: the T cell receptor (TCR), CD4 or CD8, and the IL-1 receptor. The complex mechanism by which these receptors mediate the T cell response involves calcium and many other signaling molecules, such as CaMKIV and CREB (reviewed in 117). The role of CaMKIV in the activation of T cells has been addressed in mice that express dn-CaMKIV or are null for the CaMKIV gene. Both approaches resulted in defects in CREB phosphorylation at Ser133 and T cell activation. For instance, one study showed that transgenic mice expressing a catalytically inactive form of CaMKIV were hampered in T cell activation, and exhibited a significant defect in

thymic cellularity (6). Similar results were obtained in studies with CaMKIV knockout mice (4).

Finally, CaMKIV is also expressed in the ovaries and testis, albeit to lower levels than in the brain and thymus, where it plays an important role in male and female fertility. Male mice lacking CaMKIV are impaired in their ability to exchange sperm nuclear basic proteins in male spermatids, resulting in male infertility secondary to defective spermiogenesis (140). Female fertility is also markedly reduced in CaMKIV-deficient mice due to abnormalities in follicular development and ovulation (139).

The Role of PP2A in CaMKIV Signaling

There have been two main reports addressing the role of PP2A in CaMKIV signaling; these studies were collaborative efforts between our laboratory and Dr. Anthony Means' group at Duke University. In the first study, we showed that CaMKIV and PP2A form a complex (137). This was in fact one of the first examples of a physical interaction between PP2A and a protein kinase. In this study, the authors demonstrated that PP2A coimmunoprecipitated with CaMKIV from lysates of Jurkat T cells (a human T cell line). The existence of this complex in cells was further substantiated by the copurification of CaMKIV and PP2A from rat brain soluble extracts by sequential fractionation on phenyl-Sepharose, calmodulin-Sepharose (an affinity resin for calmodulin binding proteins), Mono Q (a strong anion-exchange column), and gel filtration columns. Subsequent immunoblot analysis of the gel filtration fractions demonstrated that CaMKIV remained associated with the PP2A holoenzyme. In order to determine if PP2A exhibited any activity towards CaMKIV, the partially-purified

CaMKIV•PP2A complex was subjected to an *in vitro* kinase-phosphatse assay in the presence or absence of Ca^{2+}, calmodulin, CaMKK (the upstream kinase in the CaMKIV pathway), and okadaic acid (a potent PP2A inhibitor). The only phosphorylated protein that was detected in the presence of okadaic acid was CaMKIV, indicating that CaMKIV serves as a substrate for PP2A. Finally, to test whether PP2A could regulate CaMKIV activity in intact cells, the effect of the SV40 small t antigen on CaMKIV signaling was analyzed using a reporter assay to monitor CREB activation. The co-expression of small t with CaMKIV led to an increase in CaMKIV-mediated CREB activity, once again implicating a role for PP2A in the regulation of CaMKIV. Together, those results suggested that PP2A dephosphorylates and inactivates CaMKIV, thereby functioning as a negative regulator of CaMKIV activity.

The study of the CaMKIV•PP2A complex was further expanded upon in a subsequent report (5). In this report, the ability of PP2A to bind CaMKIV was verified, and the PP2A binding site was mapped to the autoregulatory domain of CaMKIV. Specifically, a series of FLAG-tagged CaMKIV truncation mutants containing different regions of the CaMKIV autoregulatory domain were generated and tested for their ability to co-immunoprecipitate with PP2Ac. Only the mutant that contained the full autoregulatory domain of CaMKIV (residues 1-340) was able to bind, thus indicating a requirement for the autoregulatory domain of CaMKIV for association of the kinase with PP2A. Subsequent studies from our collaborator's group narrowed down the PP2A binding site to just the autoinhibitory region of the regulatory domain (Figure 6) (27), and as expected, the coexpression of CaMKIV with PP2A binding-defective mutants, resulted in an increase in CaMKIV phosphorylation and CaMKIV-mediated CREB transcription

(5). Together, these studies demonstrated that CaMKIV and PP2A form a signaling complex via the binding of PP2A to the autoinhibitory domain of CaMKIV, and that PP2A negatively regulates the phosphorylation state and activity of the associated CaMKIV.

Summary

CaMKIV is a serine/threonine kinase that is important in the control of synaptic plasticity and T cell maturation (several key features of CaMKIV are summarized in Table 2). Activation of CaMKIV is a transient and tightly regulated event that requires Ca^{2+}/CaM binding and phosphorylation at Thr200 by CaMKK. Previous work from our laboratory showed that PP2A forms a complex with CaMKIV, and negatively regulates its phosphorylation state and activity. The goals of my research were to understand the molecular mechanisms underlying the association of CaMKIV and PP2A, as well as the inactivation of CaMKIV by this phosphatase. We began our studies by extensively characterizing a novel CaMKIV phospho-specific antibody, which allowed us to monitor the phosphorylation state of both the endogenous and exogenous enzyme. Using this antibody, we showed that the associated PP2A dephosphorylates phospho-T200 in CaMKIV both in cells (Chapter II) and *in vitro* (Chapter III). We also demonstrated that the regulation of ectopic and endogenous CaMKIV phosphorylation by PP2A are profoundly different (Chapter II). Finally, our data demonstrate that the regulatory B subunits of PP2A facilitate the interaction of CaMKIV with PP2A (Chapter III).

Summary of the features of CaMKIV	
Structure	Monomeric (59)
Tissue Distribution	Limited – most abundant in brain and thymus (76)
Subcellular Distribution	Nucleus and cytosol (145)
Substrates	CREB, SRF, MEF2, ATF1/2 and others (3, 59, 104)
Physiological roles	Transcription, spermatogenesis, LTP, neuronal memory, osteogenesis, T cell maturation and others (3, 104)
Diseases in which it may be involved	Learning disorder, delayed neuronal death, osteoporosis, cardiac hypertrophy (62)
Inhibitors	KN-62, KN-93 (59)
Activation mechanism	Activated upon phosphorylation by CaMKK on Thr200 in human CaMKIV and Thr196 on mouse CaMKIV (23, 132)

Table 2: Key features of CaMKIV. Modified from (62).

CHAPTER II

DIFFERENTIAL REGULATION OF ENDOGENOUS AND ECTOPIC CAMKIV PHOSPHORYLATION BY PP2A

Introduction

CaMKIV is a multifunctional serine/threonine kinase that is activated by three main events: 1) the binding of Ca^{2+}/CaM , which relieves intramolecular autoinhibition of the enzyme; 2) phosphorylation of CaMKIV on the threonine residue in its activation loop (Thr200 in human CaMKIV) by the upstream kinase CaMKK; and 3) autophosphorylation of Ser11 and Ser12, which relieves the intrasteric autohinibition caused by the N-terminal region of the protein. A key feature of CaMKIV is that, once activated, it is transformed from a Ca^{2+}/CaM-dependent enzyme to a fully Ca^{2+}/CaM-independent or autonomous enzyme (27). Importantly, CaMKIV achieves autonomous activity and transcriptional competence only after Ca^{2+}/CaM binding to CaMKIV and subsequent phosphorylation on Thr200 (27). So, in essence, the main role of Thr200 phosphorylation by CaMKK is to generate autonomous activity, which subsequently allows CaMKIV to drive transcription. In this way, brief elevations in intracellular Ca^{2+} can lead to an activation of CaMKIV that outlasts the duration of the Ca^{2+} signal, allowing CaMKIV to mediate transcription even after the intracellular Ca^{2+} returns to basal levels .

The activation of CaMKIV is very transient. In T cells, CaMKIV activity peaks by 1 min and returns to basal activity by 5 min (23, 53), and in HEK293 cells, CaMKIV

activity peaks at 5 min and returns to near baseline levels by 15 min (5). *In vitro* studies, as well as cellular studies using the SV40 small t antigen (described in Chapter I), both suggested that the rapid deactivation of CaMKIV is due to the action of PP2A, which inactivates the kinase, thereby extinguishing the autonomous kinase activity of CaMKIV and abrogating its ability to drive transcription (5, 137). However, because CaMKIV can be phosphorylated on multiple residues, the precise site of PP2A dephosphorylation remained unknown. In the studies outlined in this chapter, we describe our extensive characterization of a novel CaMKIV phospho-specific antibody that allowed us to track the kinetics of CaMKIV phosphorylation and dephosphorylation in cells. Most importantly, this antibody allowed us to determine that the inactivation of CaMKIV is due to dephosphorylation by PP2A on its Thr200 residue.

During the course of these studies with the CaMKIV phospho-specific antibody, we made the unexpected finding that the phosphorylation of endogenous CaMKIV was regulated by PP2A, whereas the regulation of ectopic CaMKIV phosphorylation was mediated by an okadaic acid-insensitive phosphatase. We demonstrate that this differential regulation of ectopic versus endogenous CaMKIV was not due to differences in the subcellular localization of the two proteins. Rather, endogenous CaMKIV was associated with PP2Ac, thus allowing it to be tightly regulated by the phosphatase, whereas ectopic CaMKIV had very little to no PP2Ac associated with it. In addition, in this chapter we show data from our immunofluorescence studies demonstrating that CaMKIV can be localized in both the cytoplasm and the nucleus. This is an interesting finding because there has been a lot of controversy in the field regarding the precise localization of CaMKIV regulation. This issue has been further complicated as several

reports demonstrate that CaMKIV is primarily nuclear (35, 79) and CaMKK is cytosolic (97, 109), thus raising the question of how CaMKIV can get phosphorylated given the apparent spatial discrepancy from its upstream activator. Our data confirm more recent reports suggesting that CaMKIV can be localized in both cellular compartments (145), thereby allowing it to get phosphorylated in the cytoplasm by CaMKK, followed by its translocation to the nucleus where it can mediate CREB-dependent transcription.

Materials and Methods

Antibodies and Reagents

Anti-FLAG M2-agarose, FLAG peptide (DYKDDDDK), and rabbit and mouse anti-FLAG antibodies were obtained from Sigma (St. Louis, MO). Mouse monoclonal antibodies recognizing CaMKIV and PP2A catalytic subunit were from BD Biosciences Pharmingen (San Diego, CA). The affinity-purified CaMKIV phospho-specific antibodies described in this chapter were obtained from New England Peptide, Inc. (Gardner, MA) and Bethyl Laboratories, Inc. (Montgomery, TX); these antibodies are designated as p-T200 (NEP) and p-T200 (BL), respectively. The GAPDH monoclonal antibody was purchased from Abcam Inc. (Cambridge, MA). Secondary antibodies for fluorescence detection were obtained from Rockland (Gilbertsville, PA) or Molecular Probes (Eugene, OR). Normal rabbit IgG was obtained from The Jackson Laboratory (Bar Harbor, ME), and protein A-Sepharose was from Zymed Laboratories Inc. (San Francisco, CA). Lipofectamine 2000 and *Trans*-IT Expression transfection reagents were purchased from Invitrogen (Carlsbad, CA) and Mirus (Madison, WI), respectively.

Hanks' balanced salt solution without $CaCl_2$ (HBSS) was from Invitrogen. Plasmid purification kits were obtained from Qiagen (Valencia, CA). Okadaic acid and ionomycin were purchased from Alexis Biochemicals (San Diego, CA) and Sigma (St. Louis, MO), respectively. The CaMKK inhibitor, STO-609, was from Tocris Bioscience (Ellisville, MO). The Odyssey blocking buffer was from LI-COR (Lincoln, NE). Mammalian expression plasmids for FLAG-CaMKIV and FLAG-CaMKIV T200A were kindly provided by Dr. Tony Means (Duke University).

Cell Culture and Transfection

The human embryonic kidney cell line QBI-293A (HEK293A) was a gift from Dr. Tony Means (Duke University). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The human embryonic kidney cell line QBI-293FT (HEK293FT; from Quantum Biotechnologies) was maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Jurkat T cells were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK293A cells grown to ~90-95% confluency in a 100 mm dish were transfected with the appropriate expression vectors using Lipofectamine 2000 according to the manufacturer's directions. HEK293FT cells were grown to 45% confluency in 60 mm dishes and transfected with the appropriate

expression vectors using the *Trans*-IT Express transfection reagent according to the manufacturer's directions.

Subcellular Fractionation

Native HEK293A cells (five 10-cm plates) and HEK293A cells transfected with FLAG-CaMKIV (one 10-cm plate) were grown to near confluency, washed twice with PBS and harvested by scraping, followed by centrifugation at 13,000 x g for 1 min. Pelleted cells were lysed in 500 μl of buffer containing 100 mM Tris, pH 8.0, and 0.5% Igepal. Cell lysates were incubated on ice for 5 min and centrifuged at 13,000 x g at 4°C for 5 min. The supernatant was used as the cytosolic fraction. The pellet was resuspended in 75 μl of a nuclear extraction buffer containing 20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM Na_3VO_4, 1 μM microcystin, and 1 mM PMSF. After incubating on ice for 20 min (with vortexing every 5 min), the extracts were centrifuged at 13,000 x g at 4°C for 5 min. The resulting supernatant was used as the nuclear fraction. Aliquots of both fractions (10 μl) were resolved on a 12% polyacrylamide gel followed by immunoblot analysis.

Immunofluorescence and Confocal Microscopy

HEK293A cells were plated at a density of 2×10^5 cells/6-well dish and transfected with mammalian expression plasmids encoding CFP-nuc (Clontech, Palo Alto) alone or CFP-nuc and FLAG-CaMKIV using Lipofectamine 2000. At 40 h post-transfection, cells were washed with PBS and fixed in 4% formaldehyde for 20 min. Subsequently, cells were washed with PBS containing 10 mM glycine (PBS-gly) and

permeabilized with 0.5% Triton X-100 (Sigma) for 4 min. After another PBS-gly wash, cells were blocked for 1 hour in 0.25% (w/v) ovalbumin diluted in PBS-gly, and then incubated with a 1:100 dilution (in PBS-gly) of the monoclonal FLAG antibody or a 1:100 dilution (in PBS-gly) of a polyclonal CaMKIV antibody for 1 h. Excess antibody was removed with three washes of PBS-gly, and the cells were incubated for 1 h with a 1:600 dilution (in PBS-gly) of the Alexa 594 anti-mouse antibody or a 1:600 dilution (in PBS-gly) of the Alexa 488 anti-rabbit antibody for 1 h. The cells were washed three times with PBS-gly, mounted with Aqua Poly/Mount (Polysciences, Inc., PA), and examined by confocal microscopy using the 60x objective.

Coimmunoprecipitations

For immunoprecipitations using antibody-conjugated agarose beads, cells were washed once with Hanks' balanced salt solution lacking $CaCl_2$ and then harvested in 500 μl of ice-cold lysis buffer containing 25 mM NaH_2PO_4, 2 mM EDTA, 2 mM EGTA, 10 μg/ml leupeptin, 100 μg/ml Pefabloc, and 100 nM okadaic acid. Cell lysates were transferred to a 1.5-ml Eppendorf tube, incubated on ice for 30 min, and centrifuged at 14,000 x g for 10 min at 4°C. The clarified lysates were then incubated with a 20 μl of 50% slurry of anti-FLAG agarose beads for 4-16 h at 4˚C. After two washes with lysis buffer (1 ml), and one wash with Tris-buffered saline (TBS; 25 mM Tris, pH 7.4, 137 mM NaCl, and 3 mM KCl), bound proteins were eluted from the anti-FLAG agarose beads by incubation in 40 μl of TBS containing 300 ng/μl FLAG peptide at 4˚C for either 1 h or overnight. The eluted protein was clarified by centrifugation at 14,000 x g for 1 min, and the supernatant was removed and filtered through a disk (Kontes/Kimble

#420162-0000) to remove residual FLAG resin. The filtrate was then subjected to SDS-PAGE and immunoblot analysis. In some experiments, bound proteins were directly eluted from the anti-FLAG agarose beads in SDS sample buffer. For immunoprecipitations using unconjugated antibodies, cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% Igepal, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 17 μg/ml aprotinin, and 5 μg/ml leupeptin, followed by centrifugation for 10 min at 14,000 x g. Clarified lysates were incubated overnight at 4˚C with 2 μg of rabbit polyclonal CaMKIV or 2 μg of rabbit polyclonal control IgG, and subsequently incubated for 1 h with 20 μl of a 50% slurry of Protein A-Sepharose beads. Bound proteins were washed three times in lysis buffer, eluted in SDS sample buffer, and subjected to western analysis.

Characterization of CaMKIV Phospho-Specific Antibodies

For characterization of the CaMKIV phospho-specific antibody from New England Peptide, Inc., p-T200 (NEP), recombinant wildtype CaMKIV (CaMKIV WT) or a T200A CaMKIV mutant were utilized as substrates for recombinant CaMKK in an *in vitro* kinase reaction that was performed by Dr. Means group (Duke University). Proteins were resolved by SDS-PAGE and immunoblot analysis was carried out with antibodies recognizing phosphorylated and total CaMKIV. To determine if this p-T200 (NEP) antibody could recognize phosphorylated CaMKIV in cells, HEK293A cells were transfected with FLAG-CaMKIV WT or a T200A CaMKIV mutant. Eighteen hours post-transfection, cells were either untreated or stimulated with 2 μM ionomycin for 5, 15, and 30 min. The cells were then washed once with Hanks' balanced salt solution without

CaCl$_2$ (HBSS) and then scraped from the plates in 1 ml of ice-cold lysis buffer containing 25 mM NaH$_2$PO$_4$, 2 mM EDTA, 2 mM EGTA, 10 µg/ml leupeptin, 100 µg/ml Pefabloc, and 100 nM okadaic acid. The lysed cells were transferred to a 1.5-ml Eppendorf tube, incubated on ice for 30 min, and centrifuged at 14,000 x g for 30 min at 4°C. For immunoprecipitation experiments, clarified cell lysates were incubated for 4 h at 4˚C on a rotator with 20 µl of a 50% slurry of anti-FLAG agarose beads. After two washes with TBS (25 mM Tris base, pH 7.4, 0.14 M NaCl, and 2.7 mM KCl), FLAG-CaMKIV was eluted from the resin by incubation with 300 ng/µl FLAG peptide overnight. The eluted protein was recovered by centrifugation at 14,000 x g for 10s. Aliquots of the cell lysates and FLAG peptide eluates were subjected to immunoblot analysis with the p-T200 (NEP) antibody.

For characterization of the CaMKIV phospho-specific antibody from Bethyl Laboratories, p-T200 (BL), HEK293FT cells were transfected with pcDNA3 (empty vector), FLAG-CaMKIV/pSG5 (FLAG-KIV), or a FLAG-CaMKIV/pSG5 construct containing a single threonine to alanine mutation (FLAG-T200A-KIV) using *Trans*-IT Express transfection reagent. Twenty-four h post-transfection, the cell culture media was removed and replaced with serum-free DMEM containing 5 µM STO-609 (a CaMKK inhibitor) or an equivalent volume of DMSO (vehicle). Following overnight incubation, the cells were treated for 5 min by replacing the media with serum-free DMEM containing 2 µM ionomycin or an equivalent volume of DMSO. The cells were then washed once with phosphate-buffered saline (PBS) and harvested from the dish in 200 µl of ice-cold lysis buffer (20 mM Tris, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 0.5% Igepal, 20 mM Na$_4$P$_2$O$_7$, 40 mM β-glycerol-phosphate, 1 mM, Na$_3$VO$_4$, 1 mM

PMSF, 3 mM benzamidine, 5 mM pepstatin, and 5 μg/ml leupeptin). The lysed cells were transferred to a 1.5-ml Eppendorf tube, incubated on ice for 20 min, and centrifuged at 14,000 x g for 10 min at 4°C. For immunoprecipitation experiments, clarified cell lysates were incubated for 4-16 h at 4˚C on a rotator with 20 μl of a 50% slurry of anti-FLAG agarose beads. After three washes with lysis buffer, bound proteins were eluted from the resin with SDS sample buffer. Aliquots of the cell lysates (30 μg of protein) and FLAG immune complexes were subjected to immunoblot analysis.

Analysis of CaMKIV T200 Phosphorylation

To analyze endogenous CaMKIV phosphorylation, Jurkat T cells were serum-starved in serum-free DMEM for 1 h prior to treatment with 2 μM ionomycin, 1 μM okadaic acid, or a combination of both reagents for the indicated time points; for the combination treatments, okadaic acid was added 10 min before stimulation with ionomycin. The treated Jurkat T cells were washed once with PBS and lysed in lysis buffer. Aliquots of the clarified cell lysates (45 μg of protein) were immunoblotted with the indicated antibodies. For analysis of ectopic CaMKIV phosphorylation, HEK293A cells grown to ~90-95% confluency in 6-well plates were transfected with empty vector or FLAG-CaMKIV using Lipofectamine 2000. Fourty-eight h post-transfection, the cells were stimulated for the indicated time points either 2 μM ionomycin alone, or a combination of okadaic acid and ionomycin (pre-treatment with 1 μM okadaic acid for 10 min, followed by treatment with 2 μM ionomycin). The cells were washed once with PBS and then scraped from the dish in 200 μl of ice-cold lysis buffer. The lysed cells were centrifuged at 14,000 x g for 10 min at 4°C, and clarified lysates were incubated for

4-16 h at 4˚C on a rotator with 20 µl of a 50% slurry of anti-FLAG agarose beads. After two washes in 1 ml lysis buffer, and one wash in 1 ml of TBS, bound proteins were eluted from the resin by incubation in 40 µl of TBS containing 300 ng/µl FLAG peptide at 4˚C for either 1 h or overnight. The eluted protein was recovered by centrifugation at 14,000 x g for 1 minute, and the supernatant was removed and filtered through a disk, as described above. The lysates and FLAG peptide eluted material were subjected to western analysis using the indicated antibodies.

Western Analysis

Protein samples were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to 0.45 µm nylon-supported nitrocellulose membranes in 10 mM CAPS, pH 11, containing 10% methanol (1 h at 150 V). Proteins on the membrane were visualized with Ponceau S, followed by washing in TTBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, and 0.2% Tween 20). The membranes were blocked in Odyssey buffer for detection with the Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska), incubated for 1 h with the appropriate primary antibody (1/1000 dilution), washed 4 times (5 min each), followed by incubation for 1 h with the corresponding secondary fluorophore-conjugated secondary antibody (1:20,000 dilution). All incubations and washes were done at room temperature, and all antibodies were diluted in Odyssey blocking buffer. After washing 3 times (10 min each), visualization and quantification of the immunolabeled proteins were accomplished using the Odyssey^{TM} infrared imaging system and Odyssey software, which measures integrated pixel intensity. For analysis of the New England Peptide, Inc. CaMKIV phospho-specific

antibody, p-T200 (NEP), a slightly different protocol was used. Namely, membranes were block in 0.5% BSA in TTBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, and 0.2% Tween 20), followed by incubation with the indicated primary antibody. Membranes were then incubated with alkaline phosphatase secondary antibodies and visualized by colorimetric detection.

Results

Characterization of Two CaMKIV Phospho-T200-Specific Antibodies

Previous work from our laboratory demonstrated that PP2A dephosphorylates CaMKIV *in vitro* (137). As an extension of these studies, we were interested in determining the precise site of CaMKIV dephosphorylation by PP2A. Therefore we spent a significant amount of time working with a company (New England Peptide, Inc.) to develop and characterize a CaMKIV phospho-specific antibody, p-T200 (NEP), that would allow us to monitor regulated changes in CaMKIV phosphorylation on its Thr200 residue. We chose this specific residue because CaMKIV requires phosphorylation of T200 by CaMKK in order to stimulate CREB-mediated transcription (23). Upon obtaining the p-T200 (NEP) antibody, which was generated and affinity-purified by New England Peptide, Inc., we first examined its ability to recognize phosphorylated CaMKIV. To this end, recombinant CaMKIV (CaMKIV WT) or a phosphorylation-deficient mutant of CaMKIV (CaMKIV T200A) (115) that had been incubated in the presence or absence of CaMKK in an *in vitro* kinase assay, were resolved by SDS-PAGE followed by immunoblot analysis. Figure 9 shows a Ponceau stain of the purified proteins

42
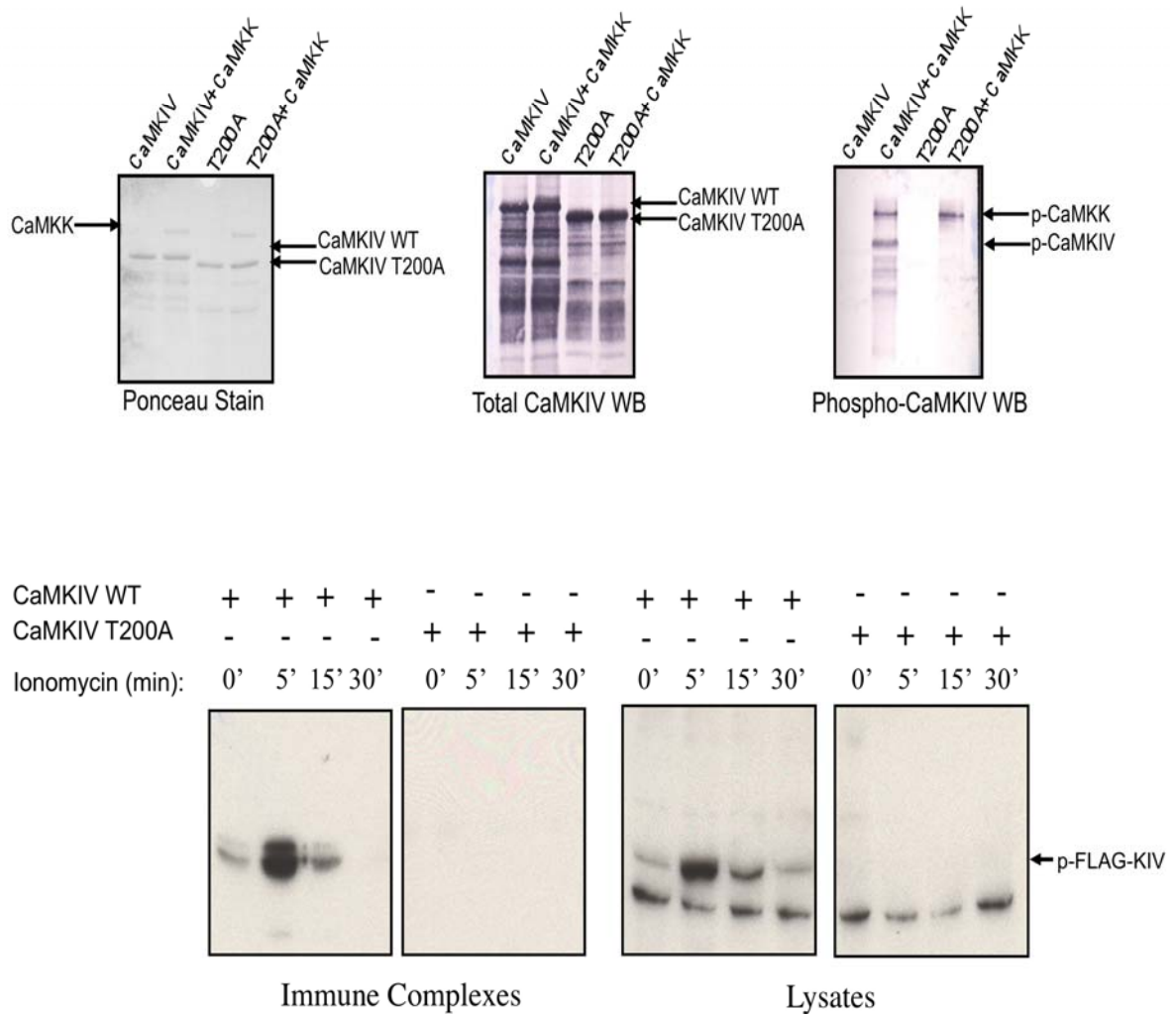
Figure 9: Characterization of the p-T200 (NEP) antibody. *Top panel,* Purified, recombinant wildtype CaMKIV (CaMKIV WT) or a T200A CaMKIV mutant (CaMKIV T200A) were utilized as substrates for recombinant CaMKK in an *in vitro* kinase reaction. Proteins were resolved by SDS-PAGE and subjected to Western blot (WB) analysis using the p-T200 (NEP) or rabbit anti-CaMKIV antibodies, and alkaline phosphatase secondaries. *Bottom panel,* HEK 293A cells transfected with FLAG-CaMKIV WT or FLAG-CaMKIV T200A were either untreated or stimulated for 5, 15, or 30 min with 2 μM ionomycin. Lysates and FLAG immune complexes were probed for CaMKIV T200 phosphorylation using the p-T200 (NEP) antibody and horseradish peroxidase-conjugated secondary antibodies (5).

(top left panels) and the corresponding western blots (top middle and right panels). The p-T200 (NEP) antibody detected both CaMKK and CaMKIV phosphorylation; the cross-reactivity of this antibody with CaMKK is likely due to the conservation of amino acid sequence between CaMKIV and CaMKK in the region to which the p-T200 was generated. In order to determine if this antibody could detect phosphorylated CaMKIV in cells, HEK293A cells were transfected with FLAG-tagged CaMKIV (CaMKIV WT or a FLAG-tagged CaMKIV mutant, CaMKIV T200A). HEK293A cells were used in most of our experiments because our analyses of several cell lines showed that HEK293A cells express a significant amount of endogenous CaMKIV (data not shown). Following transfection, cells were untreated or stimulated with the calcium ionophore, ionomycin, for 5, 15, or 30 min. Ionomycin increases intracellular Ca^{2+} levels and triggers activation of the upstream kinase, CaMKK, which in turn phosphorylates CaMKIV on Thr200 (23). Western analysis using the p-T200 (NEP) antibody revealed that CaMKIV WT was transiently phosphorylated on Thr200, with a peak at 5 min following the addition of ionomycin, and a return to near baseline levels by 15 min. No signal was observed for CaMKIV T200A, which served as a negative control and illustrated the specificity of the antibody (Figure 9; bottom panel). Although the p-T200 (NEP) antibody was able to recognize ectopic phosphorylated CaMKIV, it failed to detect endogenous phosphorylated CaMKIV in the HEK293A cells (data not shown). Therefore, we worked with a different company, Bethyl Laboratories, to develop a new CaMKIV phospho-specific antibody, p-T200 (BL). To characterize the affinity-purified antibody from Bethly Laboratories, HEK293FT cells expressing empty vector, FLAG-CaMKIV, or FLAG-T200A were either untreated or treated with ionomycin and then subjected to

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western analyses (23). As shown in Figure 10 (left panel), the p-T200 (BL) antibody detected CaMKIV phosphorylation in ionomycin-treated cells expressing FLAG-CaMKIV. However, no signal was observed in untreated cells expressing FLAG-CaMKIV or ionomycin-treated cells expressing the FLAG-T200A mutant, both of which served as negative controls. Furthermore, addition of the CaMKK inhibitor, STO609, remarkably reduced the levels of ionomycin-induced CaMKIV phosphorylation detected by the p-T200 (BL) antibody. Identical results were observed when the immuno-purified FLAG-tagged proteins were subjected to western analysis (Figure 10, right panels). These findings demonstrate that this p-T200 antibody specifically reacts with overexpressed CaMKIV phosphorylated at T200.

The next issue was to determine if the p-T200 (BL) antibody would recognize phosphorylated endogenous CaMKIV. HEK293A cells were incubated overnight in the absence or presence of the CaMKK inhibitor. The next day cells were pre-treated with okadaic acid, in order to prevent ongoing dephosphorylation by PP2A, followed by stimulation with ionomycin for 5 minutes. The cell lysates were then subjected to Western analysis. As shown in Figure 11, the p-T200 (BL) antibody recognized phosphorylated endogenous CaMKIV, and the addition of the CaMKK inhibitor significantly reduced the phosphorylation levels. Thus, the availability of this antibody allowed us to examine the phosphorylation state of both exogenous (Figure 10) and endogenous (Figure 11) CaMKIV in target cells.
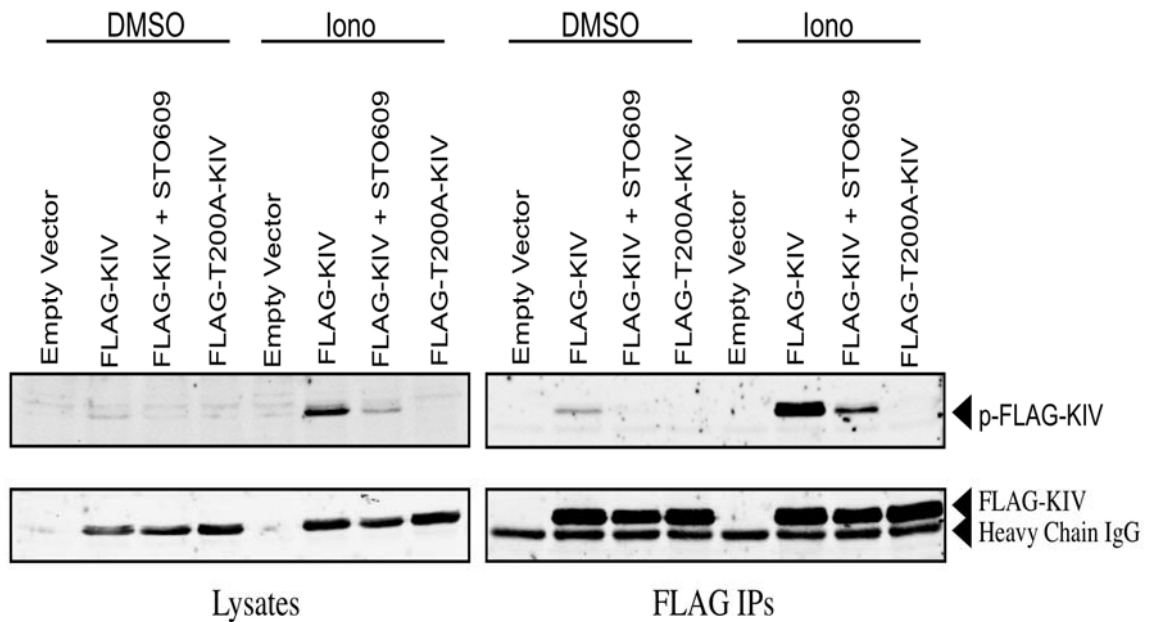
Figure 10: Characterization of the p-T200 (BL) antibody. HEK 293FT cells were transiently transfected with empty vector, FLAG-CaMKIV (FLAG-KIV), or a FLAG-CaMKIV T200A mutant. Twenty-four hours post-transfection, cells were treated overnight with vehicle (DMSO) or 5 μM STO-609 (CaMKK inhibitor). The next day, cells were treated for 5 min with vehicle (DMSO) or 2 μM ionomycin (Iono), followed by cell lysis and FLAG immunoprecipitations. Aliquots of the cell lysates and the corresponding FLAG immune complexes were subjected to Western analysis using the p-T200 (BL) and (top panels) and anti-FLAG antibodies (bottom panels).
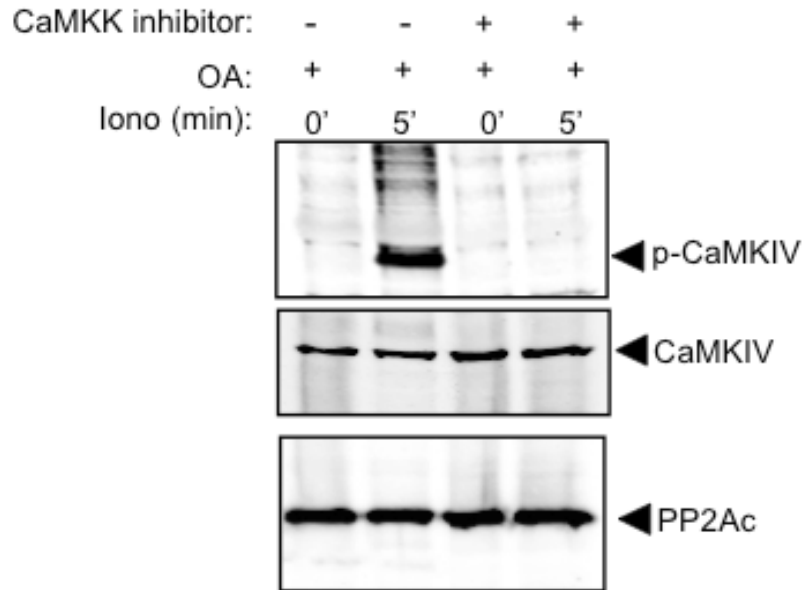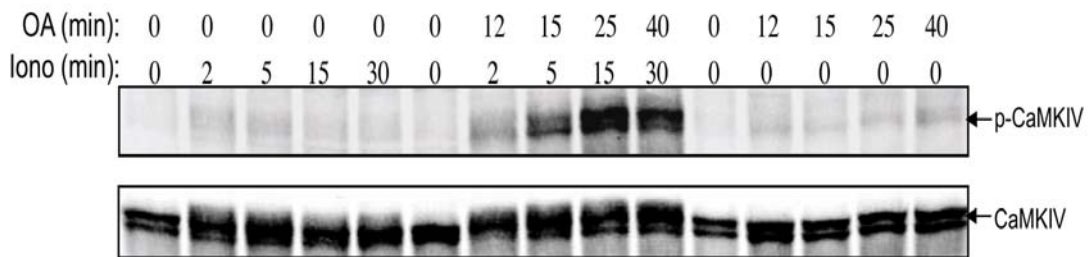
Figure 11: CaMKIV p-T200 antibody detects endogenous CaMKIV phosphorylation. HEK293A cells were treated overnight in the absence or presence of 5 uM STO-609 (CaMKK inhibitor). The next day, cells were pre-treated for 10 min with 1 μM okadaic acid (OA), followed by stimulation with 2μM ionomycin (Iono) for 5 min.
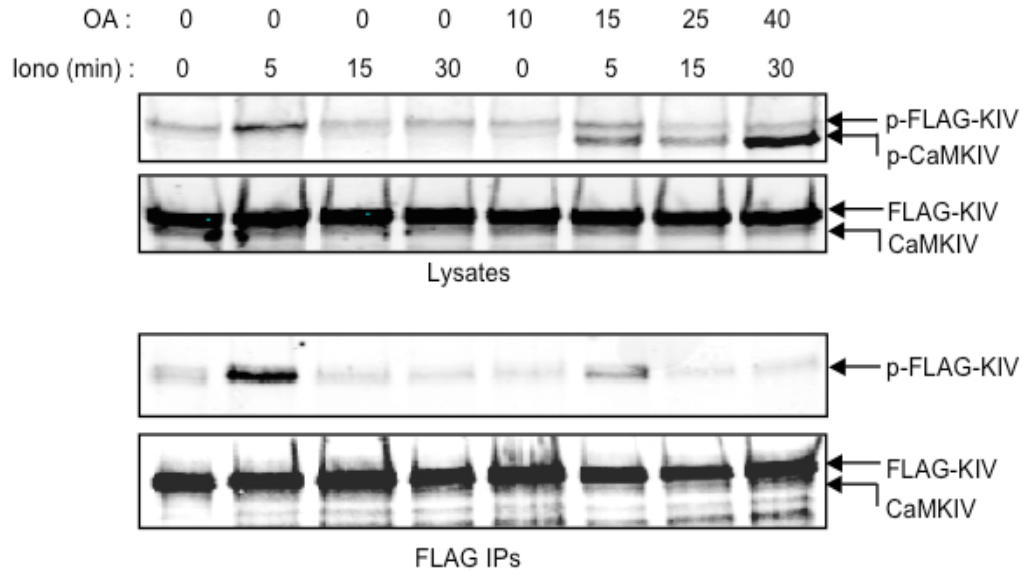
The Phosphorylation of Endogenous and Ectopic CaMKIV is Differentially Regulated by PP2A

To monitor the kinetics of endogenous CaMKIV phosphorylation, we initially exploited Jurkat T cells as these cells express relatively high levels of CaMKIV (53, 137). Jurkat T cells were either untreated, or stimulated for varying time points with ionomycin alone, or with both ionomycin and okadaic acid. The treated cells were subjected to immunoblot analysis using the p-T200 (BL) antibody and an antibody recognizing total CaMKIV. Interestingly, very little CaMKIV phosphorylation could be detected unless okadaic acid was present; the presence of okadaic acid led to sustained detectable phosphorylation of the endogenous CaMKIV enzyme (Figure 12). The fact that phosphorylation of the endogenous enzyme could only be seen when PP2A was inhibited indicates that endogenous CaMKIV is tightly regulated by the phosphatase. In order to compare the phosphorylation profile of ectopic versus native CaMKIV, HEK293A cells overexpressing FLAG-CaMKIV were pre-treated with okadaic acid followed by ionomycin treatment for 5, 10, or 15 min. CaMKIV immune complexes and aliquots of the cell lysates were analyzed by immunoblot analysis. Similar to the phosphorylation profile observe in Jurkat T cells (Figure 12), there was a robust and sustained increase in endogenous CaMKIV phosphorylation that could only be seen in the presence of both ionomycin and okadaic acid (Figure 13). In contrast, we observed an ionomycin-induced peak in the phosphorylation of FLAG-CaMKIV at 5 min, with a subsequent decrease thereafter. Surprisingly, this dephosphorylation was not sensitive to okadaic acid treatment, as a similar FLAG-CaMKIV phosphorylation profile was observed in cells treated with ionomycin alone or with both ionomycin and okadaic acid. The phosphorylation profile of the ectopic enzyme was verified by Western blot analyses of

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OA (min): 0   0   0   0   0   0   12 15 25 40 0   12 15 25 40
Iono (min): 0   2   5   15 30 0   2   5   15 30 0   0   0   0   0

←p-CaMKIV

←CaMKIV

Figure 12: Endogenous CaMKIV phosphorylation is tightly regulated by PP2A. Jurkat T cells were treated with 2 µM ionomycin (Iono), 1 µM okadaic acid (OA), or a combination of both reagents for the indicated times. An aliquot of each cell lysate (15 µg of protein) was immunoblotted for phospho-T200-CaMKIV (p-CaMKIV) and total CaMKIV using the p-T200 (BL) and CaMKIV monoclonal antibody, respectively.
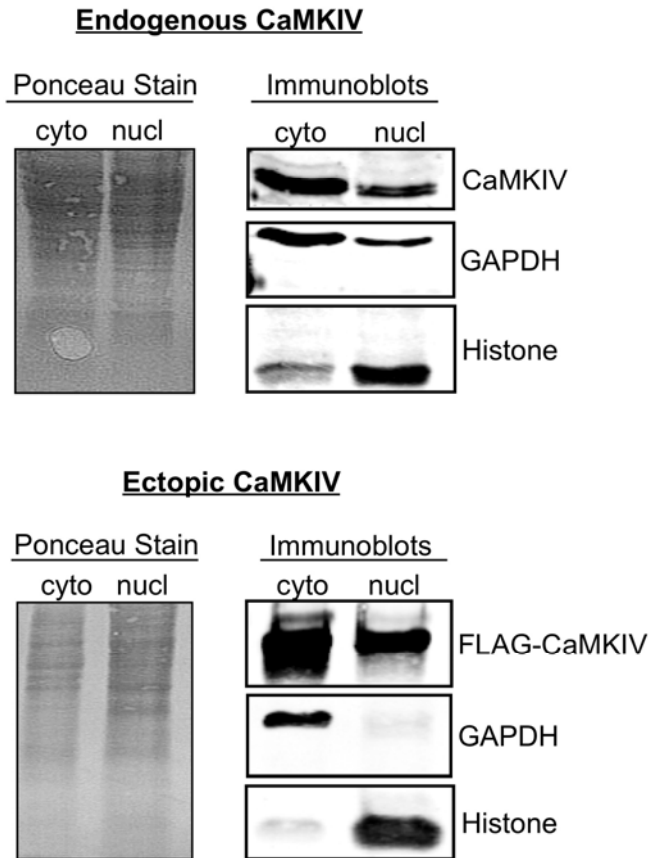
Figure 13: Inhibition of PP2A enhances ionomycin-induced phosphorylation of endogenous CaMKIV but has no effect on ectopic CaMKIV phosphorylation. HEK293A cells transfected with FLAG-CaMKIV were treated with 2 µM ionomycin or both ionomcyin and 1 µM okadaic acid (10 min pre-treatment). FLAG immune complexes were isolated from the resulting cell lysates, and bound proteins were eluted with FLAG peptide. Immunoblot analysis was carried out with the p-T200 (BL) antibody and a FLAG monoclonal antibody to visualize phospho-T200-CaMKIV (p-CaMKIV) and total CaMKIV, respectively. The migration of FLAG-CaMKIV and native CaMKIV are indicated with arrowheads.

FLAG-CaMKIV immune complexes (Figure 13, bottom panel). These data revealed that there is a profoundly different response of endogenous and ectopic CaMKIV phosphorylation to PP2A inhibition with okadaic acid. The ectopic kinase is insensitive to okadaic acid treatment, suggesting that exogenous CaMKIV is not tightly regulated by endogenous PP2A, whereas the dephosphorylation of endogenous CaMKIV is a tightly coupled event, which is readily reversed by treatment with okadaic acid.

Subcellular Localization of Ectopic and Endogenous CaMKIV

To test whether these differences in regulation by PP2A were due to differences in the subcellular localization of ectopic and endogenous CaMKIV, we examined their subcellular distribution in cells. HEK293A cells expressing endogenous CaMKIV alone or both FLAG-tagged and endogenous CaMKIV were fractionated into cytoplasmic or nuclear fractions. Equal amounts of the subcellular fractions were separated by SDS-PAGE, as can be seen in the Ponceau stain of the corresponding blot (Figure 14, left panel). Immunoblot analysis was carried out with antibodies recognizing CaMKIV, as well as GAPDH to show enrichment of the cytoplasmic fraction, and histone to show enrichment of the nuclear fraction. As shown in Figure 14, ectopic (bottom panels) and endogenous CaMKIV (top panels) exhibited similar distribution patterns and could be detected in both the cytosolic and the nuclear fractions. Analyses of the subcellular localization of FLAG-CaMKIV and endogenous CaMKIV by laser confocal immunofluorescence microscopy also revealed similar localization patterns, as both

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Endogenous CaMKIV

Ponceau Stain | Immunoblots

cyto nucl | cyto nucl

CaMKIV

GAPDH

Histone

Ectopic CaMKIV

Ponceau Stain | Immunoblots

cyto nucl | cyto nucl
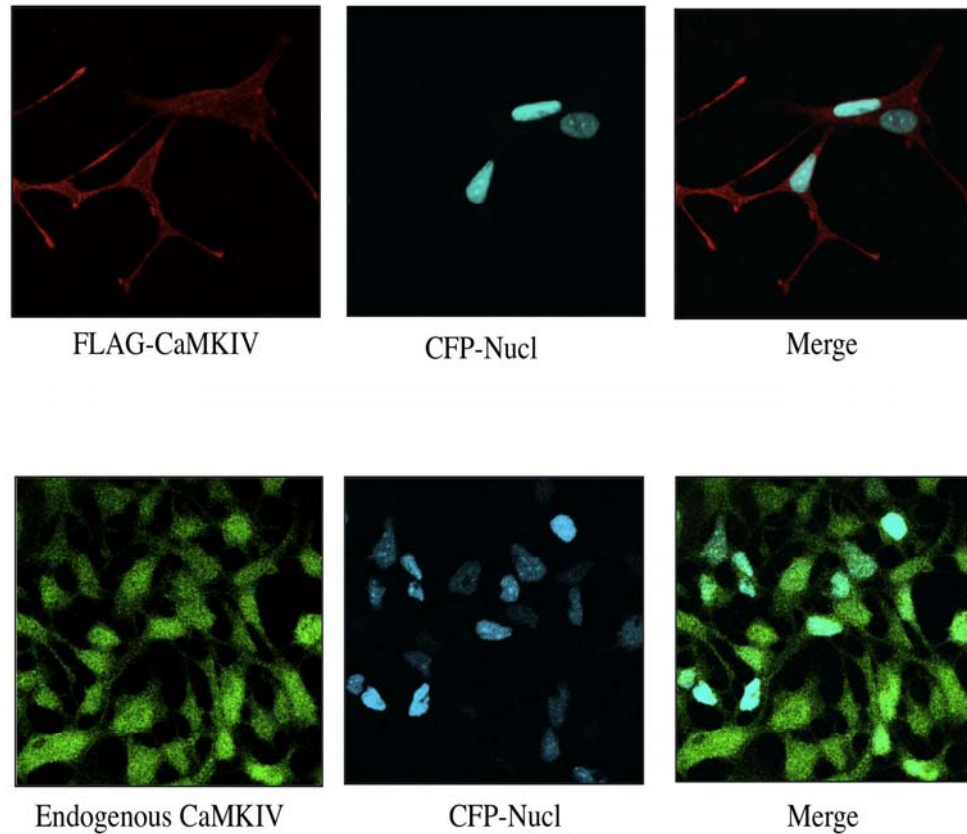
FLAG-CaMKIV

GAPDH

Histone

Figure 14: Analysis of endogenous and exogenous CaMKIV distribution by subcellular fractionation. HEK293A cells were either untransfected (top panel) or transfected with FLAG-CaMKIV (bottom panel). At 48 h post-transfection, cells were fractionated as described under *Materials and Methods*. The cysosolic (cyto) and nuclear (nucl) extracts were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The blot was stained with Ponceau S (left panels) and subsequently immunoblotted with antibodies recognizing CaMKIV, GAPDH, and histone (right panels).

ectopic and endogenous CaMKIV were detected in both the nucleus and cytoplasm (Figure 15). Little to no signal was detected in control experiments where either secondary antibody alone was used, or untransfected cells were probed with the FLAG antibody (data not shown).

Endogenous, but not ectopic CaMKIV, binds to PP2Ac

The observed differences in regulation by PP2A wee not due to differences in the subcellular localization of ectopic and endogenous CaMKIV, as the two enzymes had similar distribution profiles (Figures 14 and 15). Therefore, we tested the hypothesis that differences in kinase regulation may be due to differential PP2Ac binding. To this end, we immunoprecipitated CaMKIV from lysates of parental HEK293A cells and HEK293A cells expressing FLAG-CaMKIV, and determined the ability of PP2Ac to bind to the endogenous or exogenous protein. Specifically, cell extracts were incubated with normal rabbit IgG (control), a CaMKIV rabbit polyclonal antibody, or anti-FLAG agarose beads, and the resulting control and immune complexes were subjected to Western analysis. As shown in Figure 16, no CaMKIV came down in the control immunoprecipitations. The CaMKIV antibody brought down both endogenous and FLAG-CaMKIV, whereas only FLAG-CaMKIV came down in the FLAG immunoprecipitations. Interestingly, Western analysis of the immune complexes showed that PP2Ac bound endogenous CaMKIV; however, we did not detect any increase in PP2Ac associated with FLAG-CaMKIV in either the CaMKIV or FLAG immune complexes from lysates of cells expressing FLAG-CaMKIV, as would have been expected if endogenous PP2Ac was binding to the exogenous enzyme (Figure 16). These

Figure 15: Analysis of endogenous and exogenous CaMKIV subcellular localization by confocal immunofluorescence microscopy. HEK293A cells were transfected with CFP-nucl (a marker for nuclei) alone (bottom panel) or FLAG-CaMKIV and CFP-nucl (top panel). At 40 h post-transfection, the subcellular localization of FLAG-CaMKIV or endogenous CaMKIV was analyzed by laser confocal immunofluorescence microscopy using the 60x objective. FLAG monoclonal and CaMKIV polyclonal (Bethyl Labs #440) antibodies were used to visualize ectopic an endogenous CaMKIV, respectively.
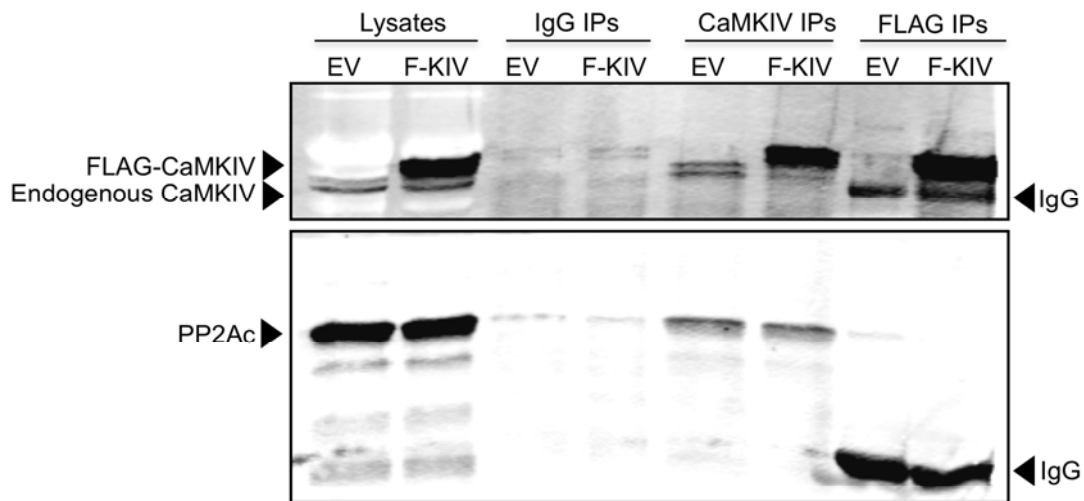
Figure 16: PP2Ac associates with endogenous, but not ectopic CaMKIV. HEK293A cells were transfected with empty vector (EV) or FLAG-CaMKIV (F-KIV), and lysed 48 h post-transfection. Immunoprecipitations were performed from the cell lysates using normal rabbit IgG and protein A-Sepharose (IgG IPs), a CaMKIV polyclonal rabbit antibody and protein A-Sepharose (CaMKIV IPs), or anti-FLAG agarose beads (FLAG IPs). Bound proteins were eluted with SDS buffer followed by immunoblot analysis with antibodies recognizing CaMKIV or PP2Ac. An aliquot of each cell lysate (30 μg of protein) was similarly analyzed.

data demonstrate that in contrast to endogenous CaMKIV, which is bound to PP2Ac and appears to be tightly and regulated by the associated phosphatase, the insensitivity of ectopic CaMKIV to regulation by PP2A is likely due to the fact that PP2Ac does not bind to the exogenous enzyme.

Discussion

Activation of CaMKIV requires Ca^{2+}/CaM binding and phosphorylation by CaMKK on T200, whereas the deactivation of CaMKIV is due to dephosphorylation by PP2A (5, 137). Studies presented in this chapter reveal that PP2A dephosphorylates the phospho-T200 residue within CaMKIV. In addition, we show that the regulation of endogenous CaMKIV by PP2A is remarkably different from that of ectopic CaMKIV, both temporally and in their differential sensitivity to okadaic acid. Specifically, we demonstrate that in the presence of the calcium ionophore, ionomycin, very little endogenous CaMKIV phosphorylation can be seen, apparently because PP2A associates with the endogenous kinase and tightly controls its phosphorylation at T200. Okadaic acid, however, inhibits the catalytic activity of PP2A, leading to sustained ionomycin-induced CaMKIV phosphorylation over time (Figures 12 and 13). Surprisingly, this pattern of phosphorylation is not reflected with the exogenous kinase. Treatment of cells expressing FLAG-CaMKIV with ionomycin alone, caused a peak in ectopic CaMKIV phosphorylation at 5 min with a subsequent decrease thereafter. This phosphorylation profile remained unchanged in the presence of okadaic acid (Figure 13), apparently due to the failure of ectopic CaMKIV to bind to PP2Ac (Figure 16). Thus, it appears that the differential regulation of endogenous versus exogenous CaMKIV is due to differences in

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their ability to associate with PP2A, as exogenous CaMKIV associates poorly with PP2A in comparison to the endogenous enzyme.

Our subcellular fractionation and immunofluorescence studies provide potentially interesting insights into the localization and regulation of CaMKIV in the cell. CaMKIV has long been regarded as a primarily nuclear protein, in part because it is responsible for Ca^{2+}-dependent gene transcription through the phosphorylation of several transcription factors, such as CREB (85, 137). Therefore, CaMKIV needs to be able to reach the nuclear compartment where the phosphorylation of CREB occurs. In support of this idea, immunostaining of brain slices and primary granule cells have revealed that endogenous CaMKIV is concentrated in the nucleus of neurons (35, 98, 133). Likewise, immunofluorescence studies from cultured cells expressing ectopic CaMKIV also showed nuclear staining (79). On the other hand, there are several reports demonstrating that endogenous and ectopic CaMKIV are present in the cytoplasm as well as the nucleus (65, 85, 102, 145). Our present studies show that endogenous and exogenous CaMKIV exist in both the nucleus and cytoplasm of HEK293A cells (Figures 14 and 15). The presence of CaMKIV in the cytoplasm is consistent with previous studies showing that inactive CaMKIV is retained in the cytoplasm, whereas the autonomously active form of CaMKIV translocates the nucleus where it participates in the regulation of transcription (27, 104). In addition, the existence of notable levels of CaMKIV in the cytoplasm indicates that this kinase may have physiological functions other than the phosphorylation of transcription factors. In support of this idea, one study showed that oncoprotein 18 is a major cytosolic target for activated CaMKIV (84), and another report

demonstrated that CaMKIV is involved in regulating cytoplasmic events associated with cell differentiation and survival (102).

Surprisingly, however, we found that in some experiments CaMKIV appeared to be primarily nuclear (Figure 17), consistent with the reports demonstrating that CaMKIV is a primarily nuclear enzyme (35, 98, 133). This variability in CaMKIV localization that we and others have observed, indicates that there is most likely a complex and dynamic regulation of CaMKIV localization that may be influenced by the levels of the kinase (such as in the case of transient transfections), the state of the cell, and/or even the cell cycle. In addition, our data show that the Bα subunit of PP2A is also localized in both the cytoplasm and the nucleus (Figure 17); therefore, it is plausible that PP2A could dephosphorylate CaMKIV in both compartments.

In summary, the studies presented in this chapter demonstrate that PP2A tightly regulates the phosphorylation state of endogenous CaMKIV via targeting the phospho-Thr200 residue. However, PP2A has little effect on the phosphorylation state of the ectopically-expressed kinase. This differential regulation of endogenous versus ectopic CaMKIV by PP2A does not appear to be due to differences in the subcellular localization of the kinases, as the distribution of both forms parallel one another in the cytoplasm and nucleus. Rather, we show that whereas endogenous CaMKIV associates with the PP2A catalytic subunit (PP2Ac), the ectopic kinase has very little PP2Ac associated with it (Figure 15). These data are consistent with earlier findings from our laboratory, which showed that 43% of native CaMKIV is complexed with PP2Ac (137). Together, our findings support a model in which PP2A associates with the endogenous kinase and tightly controls its phosphorylation at T200; however, this association and regulation by
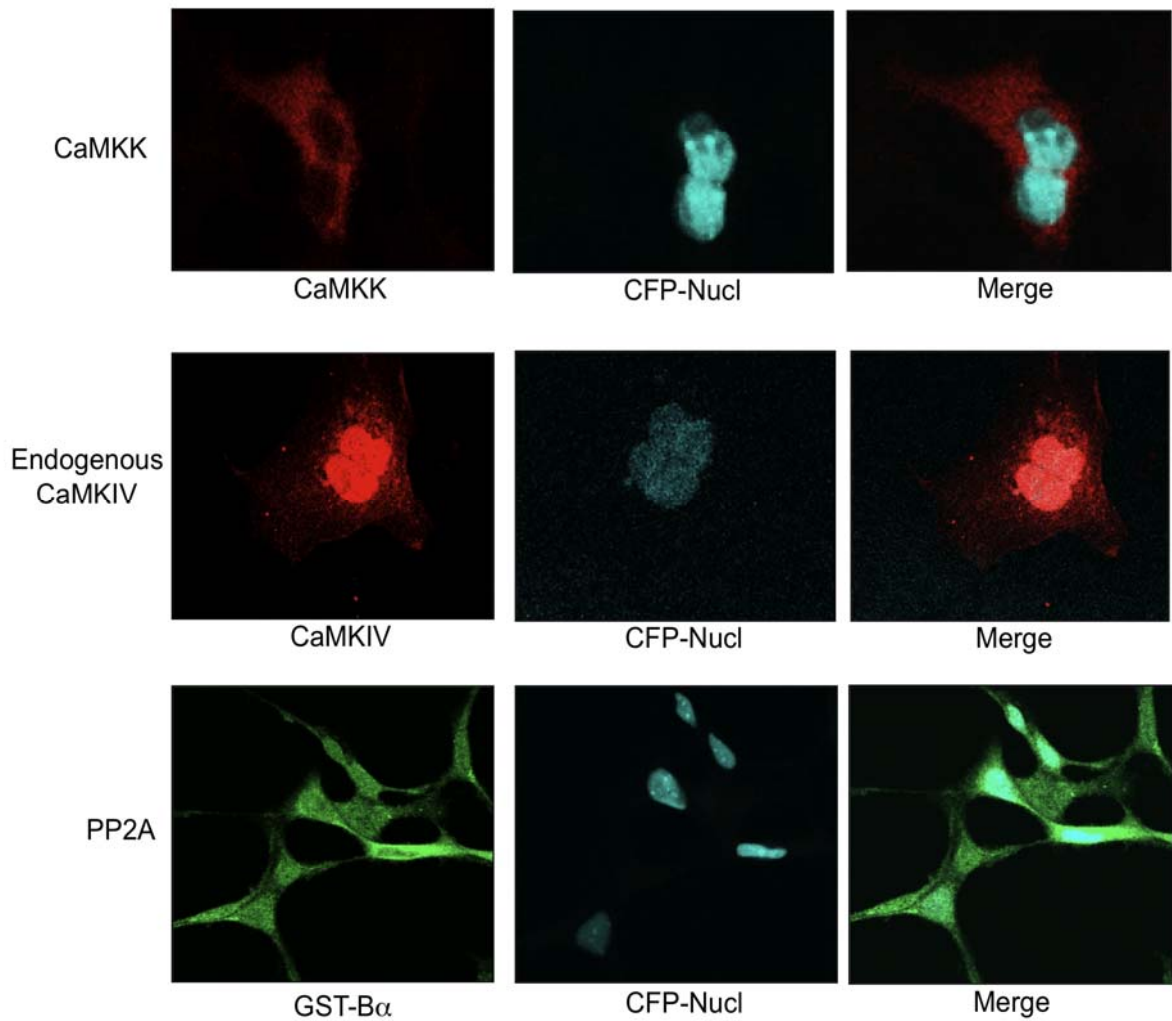
Figure 17: Analysis of the subcellular localization of the key components of the CaMKIV signaling pathway by confocal immunofluorescence microscopy. HEK293A cells were transfected with CFP-nucl alone (middle panel), or co-transfected with CaMKK and CFP-nucl (top panel), or GST-Bα and CFP-nucl (bottom panel). At 40 h post-transfection, the subcellular localization of CaMKIV, CaMKK, and GST-Bα was analyzed by laser confocal immunofluorescence microscopy using the 60x objective. CaMKK, CaMKIV, and GST antibodies were used in this experiment.

PP2A does not occur in the context of the exogenous kinase (Figures 12 and 15). The data presented here are also pertinent to the interpretation of future studies exploring CaMKIV•PP2A complexes, and indicate that novel tools may be needed for studying the regulation and function of this complex in heterologous systems. Such tools will be further elaborated upon in the 'Future Directions' section of Chapter IV.

CHAPTER III

**THE REGULATORY B SUBUNITS OF PP2A ARE CRITICAL MODULATORS
OF THE CAMKIV•PP2A SIGNALING COMPLEX**

Introduction

Protein kinases recognize many of their substrates via primary sequence motifs that surround phosphorylatable residues; however, consensus sequences appear to contribute little to substrate recognition by protein phosphatases (70). Thus, the mechanisms by which these enzymes are targeted to specific cellular substrates and various subcellular compartments have been an important subject of recent explorations. In the case of PP2A, studies have focused on the role that the regulatory B subunits play in targeting the phosphatase to different effectors and/or and cellular compartments. As described in Chapter I under "PP2A structure" and "PP2A regulation", there are four families of B subunits; each family is comprised of homologous gene products, but there is little sequence similarity between distinct families, even though they recognize similar repeat segments of the A subunits. The B subunits exhibit tissue-specific expression patterns, distinct subcellular distributions, and developmentally regulated patterns of expression (reviewed in 63). Furthermore, several studies have shown that the B subunits dictate substrate specificity of PP2A holoenzymes *in vitro*. For instance, PR55/Bα is required for PP2A to efficiently dephosphorylate vimentin, an intermediate filament protein (135). Bα-containing PP2A heterotrimers also dephosphorylate substrates phosphorylated by the p34cdc2 cyclin-dependent kinase (86). The diversity of the B subunit expression, together with the substrate selectivity of different PP2A holoenzymes

observed in some *in vitro* assays, indicates that the B subunits likely modulate the substrate specificity of PP2A towards its target proteins *in vivo* as well.

The idea that the B subunits dictate the substrate specificity of PP2A *in vivo* has indeed been supported by several studies. For example, one report showed that PP2A holoenzymes containing the PR61/B′ subunit mediate PP2A functions in the Wnt/β-catenin signaling cascade, a signal transduction pathway necessary for vertebrate axis formation in early embryogenesis (114). It has also been reported that the PR55/Bα regulatory subunit mediates dephosphoryation of the cytoskeletal proteins tau and vimentin by PP2A (121, 122, 135), and Bβ-containing PP2A holoenzymes target PP2A to mitochondria to modulate apoptosis (32). Moreover, several reports have revealed that specific B subunits are important in controlling the function of protein kinase•PP2A signaling complexes. For instance, a study from our laboratory showed that Bα- and Bδ-containing PP2A holoenzymes positively regulate the ERK/MAPK signaling pathway by targeting the phosphatase to Raf1 where it dephosphorylates inhibitory phospho-sites (2). In addition, it has been reported that multiple B subunit-containing holoenzymes target Akt and appear to control the phosphorylation of different sites within the kinase (78).

Given the data demonstrating the importance of the B subunits in the control of PP2A function, we hypothesized that one or more B subunits play a critical role in facilitating the assembly of the CaMKIV•PP2A complex and modulation of CaMKIV activity. Lending credence to this idea was the fact that the initial purification of the CaMKIV•PP2A complex revealed the presence of a Bα regulatory subunit (137). These studies also revealed an enhancement of CaMKIV-mediated transcription when CaMKIV was coexpressed with SV40 small t antigen (137), which inhibits PP2A by displacing the

B subunit from specific ABC holoenzymes (143). In a subsequent study, it was demonstrated that the coexpression of small t with CaMKIV led to the partial disruption of the CaMKIV•PP2A complex, as well as an increase in CaMKIV phosphorylation on Thr200 (5). Although both of these studies implicated a B subunit as an important component of the CaMKIV•PP2A complex, neither study examined the B subunits directly or confirmed their potential importance in the assembly and function of this complex. Herein, we show that Bα- and Bδ-containing holoenzymes can form complexes with CaMKIV. We also provide evidence demonstrating that Bα and Bδ are capable of facilitating the interaction between CaMKIV and PP2A. Finally, our *in vitro* data demonstrate that ABαC and ABδC holoenzymes directly dephosphorylate phospho-T200 within CaMKIV.

Materials and Methods

Antibodies and Reagents

Anti-FLAG M2-agarose, FLAG peptide (DYKDDDDK), and rabbit and mouse anti-FLAG antibodies were obtained from Sigma (St. Louis, MO). Mouse monoclonal antibodies recognizing CaMKIV and PP2A catalytic subunit were from BD Biosciences Pharmingen (San Diego, CA). The affinity-purified CaMKIV phospho-T200 antibody (BL 1839) was from Bethyl Laboratories, Inc. (Montgomery, TX). The generation and characterization of affinity-purified Bα̃δ antibodies were as reported previously (126). Secondary antibodies for fluorescence detection were obtained from Rockland (Gilbertsville, PA) or Molecular Probes (Eugene, OR). The Block IT oligonucleotide was also obtained from Invitrogen. Plasmid purification kits were obtained from Qiagen

(Valencia, CA). Okadaic acid and ionomycin were purchased from Alexis Biochemicals (San Diego, CA) and Sigma (St. Louis, MO), respectively. Lipofectamine 2000 and Dharmafect was purchased from Invitrogen (Carlsbad, CA) and Dharmacon (Lafayette, CO), respectively. The Odyssey blocking buffer was from LI-COR (Lincoln, NE).

Plasmid Constructs

Generation of FLAG-tagged Bα and Bδ mammalian expression plasmids were described previously (2). Mammalian expression plasmids for FLAG-Bβ, FLAG-Bγ, FLAG-B′β, FLAG-B′γ, and FLAG-B′ε were generous gifts from Dr. Stefan Strack (University of Iowa). Mammalian expression plasmids for FLAG-CaMKIV and FLAG-CaMKIV T200A were kindly provided by Dr. Tony Means (Duke University). The HA-CaMKIV expression plasmid was generated by subcloning human CaMKIV cDNA into the pCMV-HA vector (Clontech, Palo Alto, CA). Specifically, PCR cloning was used to amplify the CaMKIV gene from the FLAG-CaMKIV construct with a BglII and NotI site at the 5' and 3' ends of the coding sequence. The following primers were used in the PCR reaction: sense, 5'-ACCGAGATCTCTATGCTCAAAGTCACG-3'; antisense, 5' TGCGGCCGCTTAGTACTCTGGCAG-3'. The PCR-amplified product was then ligated in-frame into the BglII and NotI sites located within the multiple cloning region of the pCMV-HA vector. Proper construction of the plasmids were verified by automated DNA Sequencing (Vanderbilt University DNA Core Facility). Three different Bα duplex siRNA oligonucleotides were used in our experiments; all were manufactured by Invitrogen (Carlsbad, CA). Bα siRNA #579 was composed of the sense oligonucleotide 5'-GCCTATGGATCTAATGGTTGA-3' and the antisense oligonucleotide 5'-

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TCAACCATTAGATCCATAGGC-3'; Bα siRNA #688 was composed of the sense oligonucleotide 5'-GCAGATGATTTGCGGATTAAT-3' and the antisense oligonucleotide 5'-ATTAATCCGCAAATCATCTGC-3'; Bα siRNA #839 was composed of the sense oligonucleotide 5'-GCAGCAGTAAAGGAACTATTC-3' and the antisense oligonucleotide 5'-GAATAGTTCCTTTACTGCTGC-3'.

Cell Culture and Transfection

The human embryonic kidney cell line QBI-293A (HEK293A) was a gift from Dr. Tony Means (Duke University). Cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 110 mg/L sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK293A cells grown to ~90-95% confluency in 100 mm or 60 mm dishes were transfected with the appropriate expression vectors using Lipofectamine 2000 according to the manufacturer's directions.. Transfections with siRNA were performed in HEK293A cells using the Dharmafect reagent or Lipofectamine 2000 according to the manufacturer's directions.

RNAi-Mediated Knockdown of Bα

For analysis of Bα knockdown on CaMKIV phosphorylation, HEK293A cells were transfected with 100 nM Bα siRNA #839 using Dharmafect. Following a 72 hour incubation, siRNA-transfected cells were pre-treated with 1 μM okadaic acid for 10 min, followed by treatment with 2 μM ionomycin for the indicated time points. Cells were then washed once with PBS and harvested from 60 mm dishes in 200 μl of ice-cold lysis

buffer (20 mM Tris, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 0.5% Igepal, 20 mM $Na_4P_2O_7$, 40 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1 mM PMSF, 3 mM benzamidine, 5 mM pepstatin, and 5 μg/ml leupeptin). The resulting lysates were subjected to immunoblot analysis.

Coimmunoprecipitations

For immunoprecipitions using antibody-conjugated agarose beads, cells were washed once with phosphate-buffered saline (PBS), and then harvested in ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.6, 0.1% Igepal CA-630, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenlymethlysulfonyl fluoride, 17 μg/ml aprotinin, and 5 μg/ml leupeptin. Cell lysates were transferred to a 1.5-ml Eppendorf tube, incubated on ice for 20 min, and centrifuged at 14,000 x g for 10 min at 4°C. The clarified lysates were then incubated with 20 μl of a 50% slurry of anti-FLAG agarose beads for 4-16 h at 4˚C. After two washes in 1 ml of lysis buffer, and one wash in 1 ml of Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 3 mM KCl), bound proteins were eluted from the anti-FLAG agarose beads by incubation in 40 μl of TBS containing 300 ng/μl FLAG peptide at 4˚C for either 1 h or overnight. The eluted protein was recovered by centrifugation at 14,000 x g for 1 min, and the supernatant was removed and filtered through a disk (Kontes/Kimble #420162-0000) to remove the FLAG resin. The filtrate was then subjected to SDS-PAGE and immunoblot analysis. In some experiments, bound proteins were directly eluted from the anti-FLAG agarose beads in SDS sample buffer.

Purification of FLAG-Tagged Proteins

To purify the PP2A holoenzymes, HEK293A cells grown to about 90-95% confluency in 10 cm plates were transfected with the indicated FLAG-tagged B subunit expression plasmid using Lipofectamine 2000. Forty-eight hours post-transfection, the cells were washed once with PBS and harvested from the dishes in 500 μl of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.6, 0.1% Igepal CA-630, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenlymethlysulfonyl fluoride, 17 μg/ml aprotinin, and 5 μg/ml leupeptin. Following centrifugation for 10 min at 14,000 x g, the clarified lysates were incubated with 20 μl of a 50% slurry of anti-FLAG agarose for 4 hours. Bound proteins were washed twice with PAN buffer (10 mM PIPES, pH 7.0, 100 mM NaCl, and 17 μg/ml aprotinin) containing 0.5% Igepal, once with PAN buffer, and once in phosphatase assay buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1mM dithiothreitol, and 0.25 mg/ml bovine serum albumin). Bound proteins were eluted by incubation for 30 min at 4°C in phosphatase assay buffer (100 μl) containing 300 μg/ml FLAG peptide. Glycerol was added to the eluates to a final concentration of 50%. Aliquots of the purified holoenzymes were either assayed for phosphatase activity or subjected to SDS-PAGE followed by immunoblot analysis.

To purify phosphorylated FLAG-CaMKIV, HEK293FT cells were transfected with FLAG-CaMKIV. Forty-eight hours post-transfection, cells were stimulated for 5 min by replacing the media with serum-free DMEM containing 2 μM ionomycin. The cells were then washed once with PBS and harvested from the dish in 200 μl of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.0, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 0.5% Igepal, 20 mM $Na_4P_2O_7$, 40 mM β-glycerol-phosphate, 1 mM Na_3VO_4, 1 mM

PMSF, 3 mM benzamidine, 5 mM pepstatin, and 5 μg/ml leupeptin. Following centrifugation at 14,000 x g for 10 min at 4°C, clarified cell lysates were incubated overnight at 4°C on a rotator with 20 μl of a 50% slurry of anti-FLAG agarose beads. After three washes with lysis buffer, and one wash with phosphatase assay buffer, bound proteins were eluted by incubation for 1 hour at 4°C in 50 μl phosphatase assay buffer containing 100 μg/ml FLAG peptide. Glycerol was added to the eluates to a final concentration of 30%.

Preparation of Phosphorylated Substrates and Phosphatase Assays

Radiolabeled substrate (i.e. cAMP-dependent protein kinase-phosphorylated histone H1) was prepared as described previously (126). Purified FLAG-tagged PP2A holoenzymes (10 ng) were assayed for phosphatase activity in a 50-μl reaction containing phosphatase assay buffer and ^{32}P-histone H1. Following a 15 min incubation at 30°C, the reactions were terminated by the addition of trichloroacetic acid (20% final concentration). The samples were incubated on ice for 10 min, and proteins were pelleted by centrifugation at 13,000 x g for 10 min. Supernatants were collected and quantified for ^{32}P$_i$ release by liquid scintillation counting.

In Vitro **CaMKIV Dephosphorylation Assays**

Purified phosphorylated FLAG-CaMKIV was incubated with purified FLAG-tagged ABαC or ABδC holoenzymes (60 ng) in the absence or presence of 500 nM OA for 30 min at 37°C. Dephosphorylation reactions were terminated by the addition of SDS sample buffer and subjected to immunoblot analysis using antibodies recognizing

phospho-CaMKIV (p-T200-BL) and total CaMKIV (anti-FLAG). In some assays, the PP2A holoenzymes were normalized for equivalent activity. In other assays, equivalent amounts of PP2A catalytic subunit (PP2Ac) were used; in these cases, the blots were also probed with a PP2Ac antibody to ensure that equivalent amounts of the phosphatase catalytic subunit were added to the dephosphorylation reactions.

Western Analysis

Protein samples were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred to 0.45 μm nylon-supported nitrocellulose membranes in 10 mM CAPS, pH 11, containing 10% methanol (1 h at 1 A). Proteins on the membrane were visualized with Ponceau S, followed by washing in TTBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, and 0.2% Tween 20). Membranes were blocked in Odyssey buffer (LI-COR, Lincoln, Nebraska), incubated for 1 h with the appropriate primary antibody (1/1000 dilution), washed 4 times with TTBS (5 min each), followed by incubation for 1 h with the corresponding fluorophore-conjugated secondary antibody (1:20,000 dilution). All incubations and washes were done at room temperature, and all antibodies were diluted in Odyssey blocking buffer. After washing 3 times (10 min each), visualization and quantification of the immunolabeled proteins was accomplished using the Odyssey^{TM} infrared imaging system and Odyssey software, which measures integrated pixel intensity.

Bα- and Bδ-Containing PP2A Holoenzymes Associate with CaMKIV

The tissue-specific expression pattern and differential subcellular localization of the PP2A regulatory B subunits have led to the postulate that these subunits direct PP2A to its cell-specific substrates; this substrate-specifying role of the B subunit has been shown in the case of several different PP2A holoenzymes (121, 122, 135). Thus, we sought to determine if the B subunits are responsible for the interaction of CaMKIV with PP2A. Since previous studies revealed the presence of Bα in the purified endogenous CaMKIV•PP2A complex (137), we began our studies by testing the ability of ectopic Bα-containing CaMKIV•PP2A complexes to form. To this end, HA-tagged CaMKIV and FLAG-tagged Bα were co-expressed in HEK293A cells, followed by FLAG immunoprecipitations from the cell lysates. Western analyses of the FLAG immune complexes revealed that HA-CaMKIV and endogenous PP2Ac associated with FLAG-Bα (Figure 18).

We then looked at the ability of another isoform in the B family, namely Bδ, to bind to CaMKIV. Our rationale for doing this was based on the finding from our laboratory that the Bδ isoform shares a high degree of sequence homology with Bα, including the epitope used to generate what was previously thought to be a "Bα-specific" antibody (125). The amino acid sequence of these N-termini of Bα and Bδ are depicted in Figure 19 (top panel), and the boxed amino acids indicate the polypeptide that was used to generate what was thought at the time to be Bα-specific antibodies. The specificity of this antibody for Bα and Bδ was examined by performing Western analysis
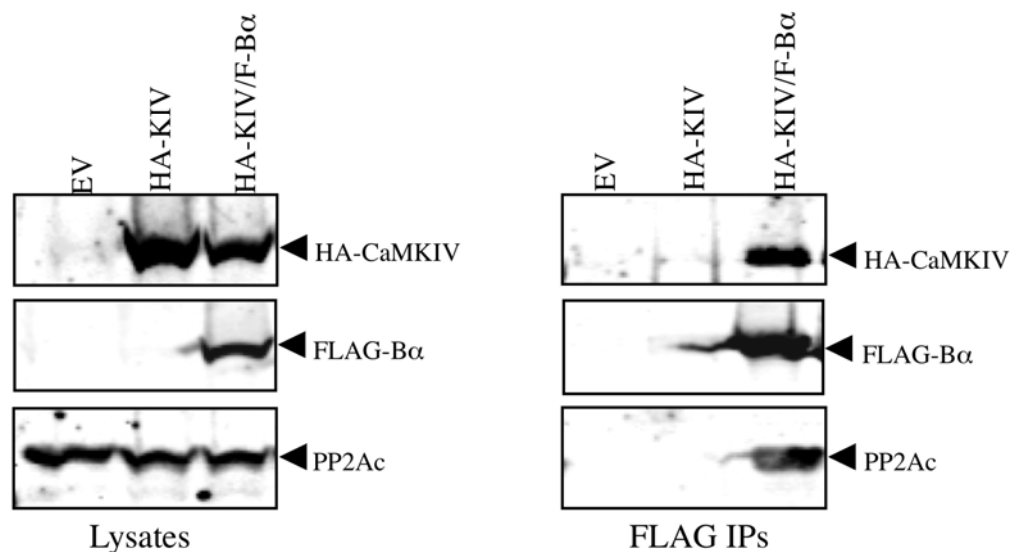
Figure 18: Formation of an ectopic Bα-containing CaMKIV•PP2A complex. HEK293FT cells were transfected with empty vector (EV), HA-CaMKIV, or FLAG-Bα. Cells were lysed 48 h post-transfection, followed by isolation of protein complexes with anti-FLAG agarose beads (FLAG IPs). Bound proteins were eluted with sample buffer and resolved by SDS-PAGE. Immunoblot analysis of the cell lysates (15 μg of protein) and FLAG IPs was carried out with antibodies recognizing HA, FLAG, and PP2Ac.

Bα and Bδ subunit alignment

```
Bδ  MAGAGGGGCPTGGNDFQWCF SQVKGAVDEDVAE //  [ 100% ]
Bα  MAGAGGG......NDIQWCF SQVKGAVDDDVAE //  [  89% ]
```
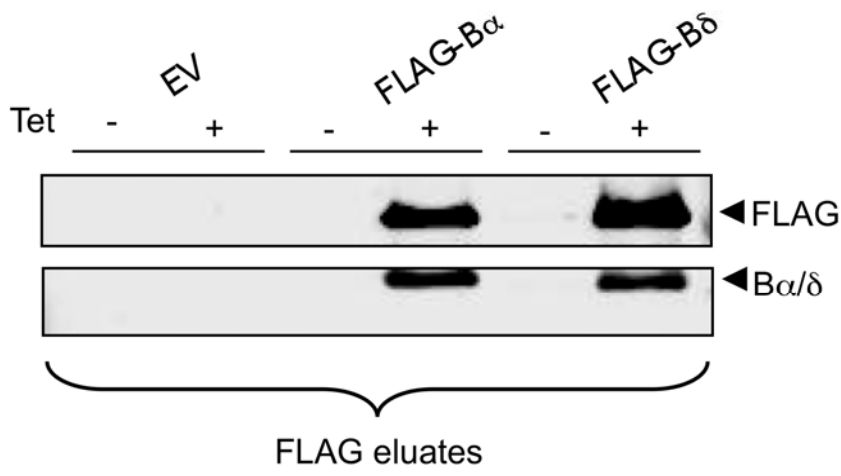

Figure 19: Characterization of the Bα and Bδ antibodies. *Top panel;* Amino acid sequence alignment of the N-termini of the Bα and Bδ subunits is depicted. The boxed area represents the epitope to which the "Bα-specific" antibody was directed (see text for details). The underlined sequence is the region to which the Bδ antibody was directed. The shaded boxed area indicates the percent amino acid sequence identity relative to Bδ. *Bottom panel;* FLAG-Bα, FLAG-Bδ, or empty vector (EV) were overexpressed in HEK T-rex cells under the control of a tetracycline-inducible promoter. FLAG immune complexes were subjected to Western analyses using a FLAG monoclonal antibody, or the "Bα-specific" antibody which recognizes both Bα and Bδ (2).

of FLAG immune complexes from cells expressing empty vector, FLAG-Bα or FLAG-Bδ. As shown in Figure 19 (bottom panel), the antibody recognized both Bα and Bδ. An antibody recognizing a unique sequence in Bδ was also tested and shown to specifically recognize the Bδ subunit. These results demonstrated that the "Bα" antibody used in our early analyses of the purified CaMKIV•PP2A complex actually recognizes both Bα and Bδ, thus raising the possibility that Bδ-containing PP2A holoenzymes may also associate with CaMKIV. To determine whether ABαC and/or ABδC interact with CaMKIV, HEK293A cells were co-transfected with untagged CaMKIV and FLAG-tagged Bα or Bδ regulatory subunits. Western analysis of the FLAG immune complexes isolated from these cells revealed both Bα- and Bδ-containing holoenzymes bound to CaMKIV; no PP2A subunits were detected in the control immunoprecipitations (Figure 20). These findings indicate that two different heterotrimeric forms of PP2A, ABαC and ABδC, can associate with CaMKIV.

The B subunits recruit PP2Ac to CaMKIV

In order to determine if the Bα and Bδ subunits are important in the assembly of the CaMKIV•PP2A complex, we co-transfected HEK293A cells with FLAG-CaMKIV and untagged Bα or Bδ regulatory subunits. Proteins bound to FLAG-CaMKIV were isolated from the cellular extracts using anti-FLAG-agarose beads, and eluted from the beads with a FLAG peptide. Western analyses of the FLAG peptide eluates revealed that FLAG-CaMKIV brought down very little PP2Ac from lysates of cells only transfected with FLAG-CaMKIV (Figure 21). However, there was a significant increase in the amount of PP2Ac bound to FLAG-CaMKIV when immunoprecipitations were performed

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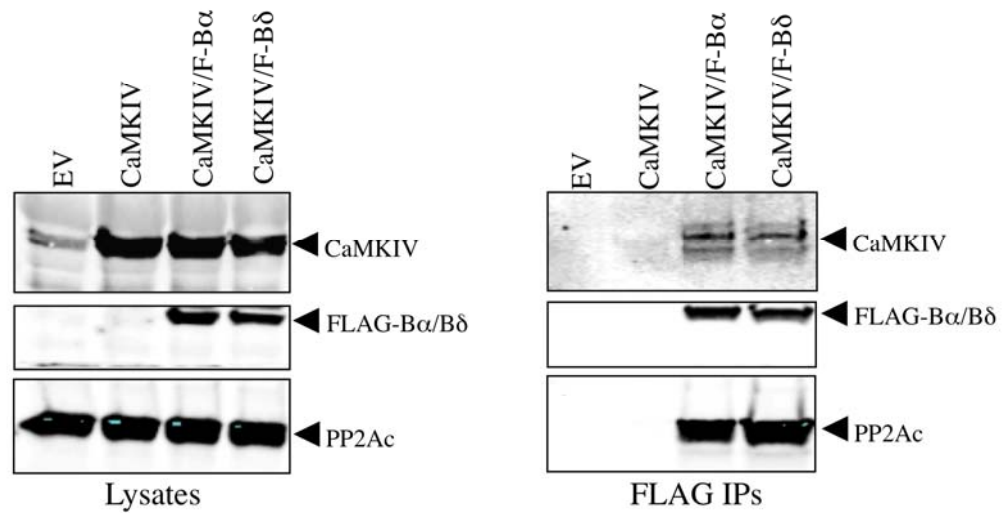
Figure 20: CaMKIV binds to both the Bα-and Bδ-containing PP2A holoenzymes. HEK293A cells were transfected with empty vector (EV), untagged CaMKIV or FLAG-tagged B subunit and CaMKIV. At 48 h post-transfections, cells were lysed an FLAG immune complexes were isolated from the cell lysates. Bound proteins were eluted with SDS sample buffer and subjected to Western analysis using antibodies recognizing CaMKIV, the FLAG epitope, and PP2Ac.
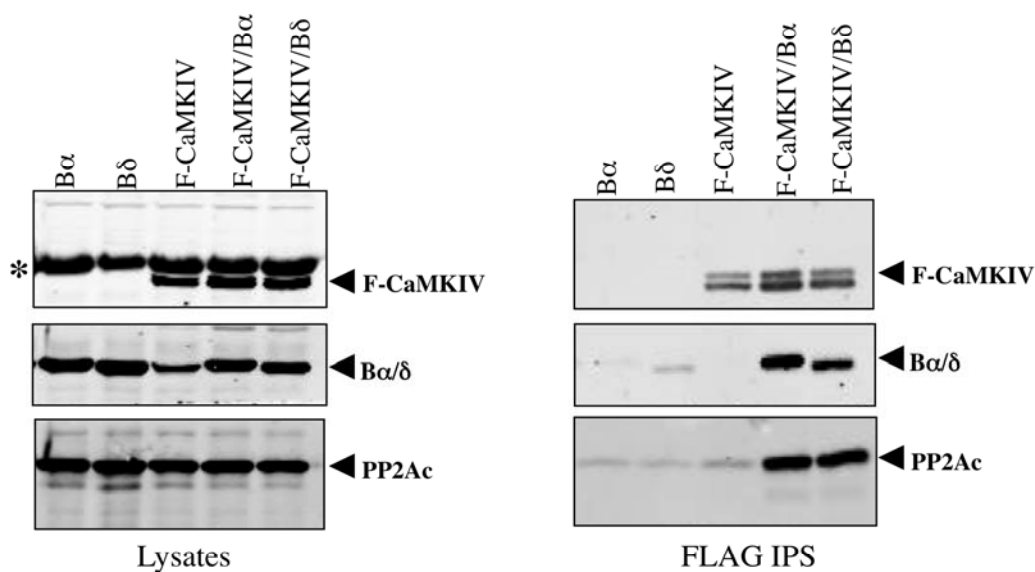
Figure 21: Bα and Bδ recruit PP2Ac to CaMKIV. HEK 293A cells were transfected with untagged Bα or Bδ alone, FLAG-CaMKIV alone, or a combination of FLAG-CaMKIV and untagged Bα or Bδ. Cells were lysed, followed by immunoprecipitation with anti-FLAG agarose beads, and protein elution from the beads with the FLAG peptide. The cell lysates (45 μg) and FLAG peptide eluates were resolved by SDS-PAGE, and immunoblot analysis was carried out with antibodies recognizing CaMKIV, Bα/Bδ, and PP2Ac. *, denotes a non-specific band recognized by the secondary antibody used in these experiments.

from cells co-expressing Bα or Bδ. These results indicate that the B subunits are essential in recruiting PP2Ac to CaMKIV. Moreover, these data show that the B subunits are necessary for the interaction of CaMKIV with PP2A.

ABαC- and ABδC-Mediated Dephosphorylation of CaMKIV

Once we had established a role for the Bα and Bδ subunits in the assembly of the CaMKIV•PP2A complex, we next tested whether the respective B subunit-containing holoenzymes directly desphosphorylate phospho-CaMKIV. To address this question, we utilized FLAG-tagged Bα- or Bδ-containing holoenzymes and phosphorylated FLAG-CaMKIV (see Materials and Methods). Western and silver stain analyses of the purified holoenzymes revealed that the FLAG-tagged B subunits bound to the endogenous PP2A catalytic and A subunits in a near stoichiometric manner (2). To test whether the isolated PP2A holoenzymes exhibited phosphatase activity towards a generic phosphatase substate, they were assayed for catalytic activity towards [^{32}P]histone H1. Both holoenzymes exhibited significant phosphatase activity (Figure 22, top panel). These data, together with our biochemical analyses of the purified PP2A holoenzymes (2), provided convincing evidence that we had successfully isolated functional PP2A holoenzymes from cells expressing a FLAG-tagged PP2A regulatory subunit. The purified holoenzymes were normalized for equal concentrations of PP2A catalytic subunit and subsequently incubated with purified phosphorylated FLAG-CaMKIV in the absence or presence of the PP2A inhibitor okadaic acid. Immunoblot analysis of the reaction mixtures revealed that both holoenzymes dephosphorylated phospho-Thr200 in an okadaic-acid sensitive fashion (Figure 22, bottom panel). These results indicate that
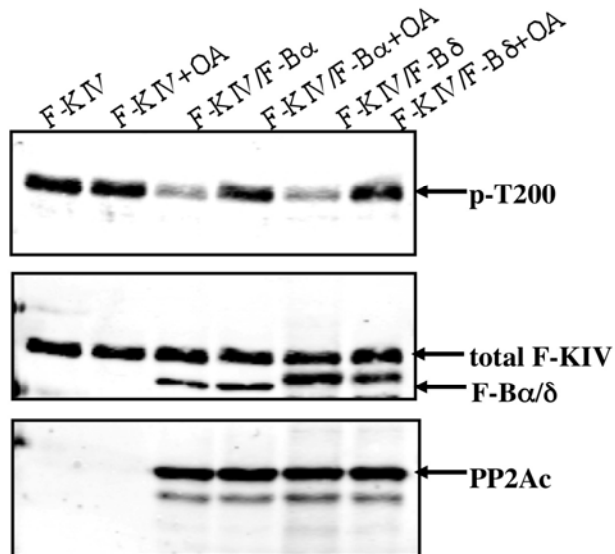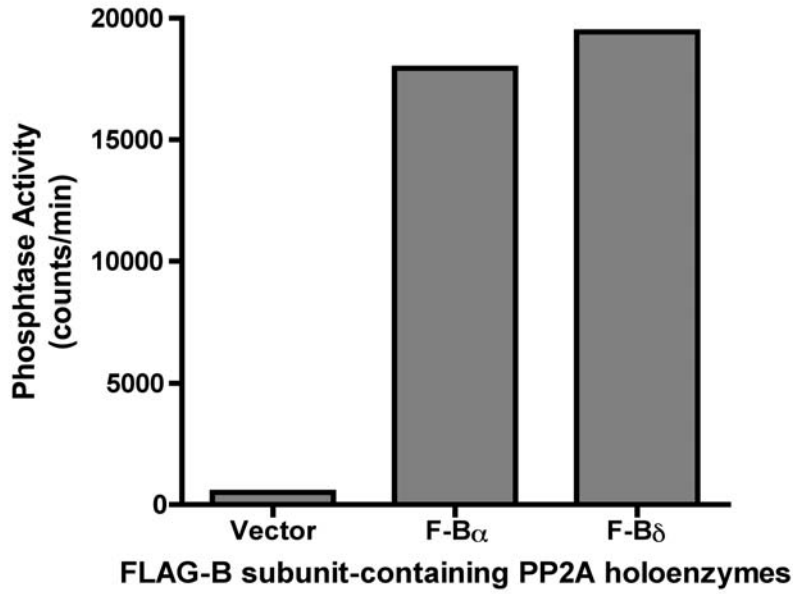
Figure 22: ABαC and ABδC-mediated dephosphorylation of CaMKIV. *Top panel;* FLAG-tagged Bα- and Bδ-containing holoenzymes were assayed for phosphatase activities using [^{32}P]histone H1 as a substrate. FLAG immune complexes from cells transfected with empty vector served as a control. The assays were done in duplicate and the activity is represented as the average counts per min. *Bottom panel;* Phosphorylated FLAG-CaMKIV was incubated with purified FLAG-tagged Bα or Bδ-containing holoenzymes in the absence or presence of okadaic acid (OA) for 1 h. The reactions were terminated by the addition of SDS sample buffer and subjected to Western analysis using antibodies recognizing phospho-CaMKIV (p-T200 BL), PP2Ac, and the FLAG epitope (to detect FLAG-CaMKIV and FLAG-Bα/Bδ).

both Bα- and Bδ-containing holoenzymes can directly dephosphorylate phospho-Thr200 in CaMKIV.

Multiple B subunit-containing holoenzymes dephosphoryate CaMKIV

To test the possibility that other B subunit-containing holoenzymes. in addition to ABαC and ABδC, are capable of mediating CaMKIV dephosphorylation, we performed *in vitro* dephosphorylation assays using several different purified PP2A holoenzymes. In these experiments, the purified holoenzymes were normalized for roughly equivalent catalytic activity towards phospho-histone (Figure 23, bottom panel), as our initial assays revealed significant differences in the activities of these holoenzymes when they were assayed under conditions of approximately equal catalytic subunit (Figure 23, top panel). Immunoblot analysis showed that although each of the holoenzymes tested was able to dephosphorylate CaMKIV, the greatest degree of dephosphorylation occurred with the ABαC and ABδC heterotrimers (Figure 24), indicating that these holoenzymes could be the primary regulators of CaMKIV activity in a cellular system.

To see if we could confirm our *in vitro* data, which indicated that the Bα̃ and Bδ-containing holoeznymes are the primary modulators of CaMKIV activity, we exploited an RNAi strategy to knockdown Bα in HEK293 cells. We focused on the Bα subunit because our studies demonstrated that Bα-containing holoenzymes can associate with CaMKIV and, more importantly, Bα appears to be a major PP2A regulatory subunit in these HEK293A cells. We predicted that if ABαC holoenzymes are the primary form of PP2A catalyzing the dephosphorylation of CaMKIV, then the absence of Bα would lead to endogenous CaMKIV phosphorylation after cellular stimulation with ionomcyin alone,
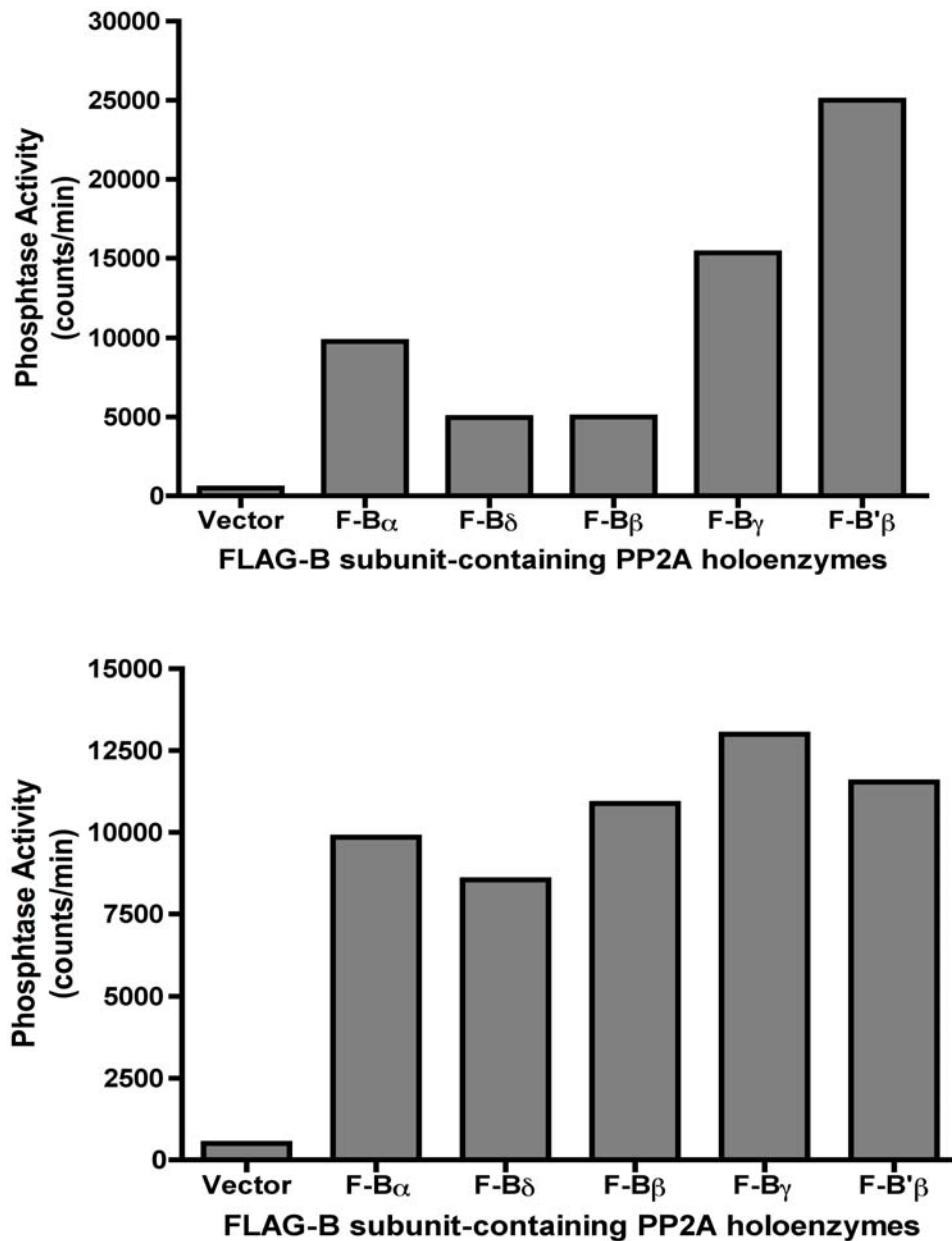
Figure 23: Phosphatase activity of several FLAG-tagged B subunit containing holoenzymes. *Top panel;* The indicated FLAG-tagged B subunit-containing holoenzymes were normalized for equivalent levels of PP2Ac and assayed for phosphatase activities using [^{32}P]histone H1 as a substrate. FLAG immune complexes from cells transfectted with empty vector served as a control. The assays were done in duplicate and the activity is represented as the average counts per min. *Bottom panel;* The purified holoenzymes were normalized for equivalent catalytic activity towards phospho-histone.
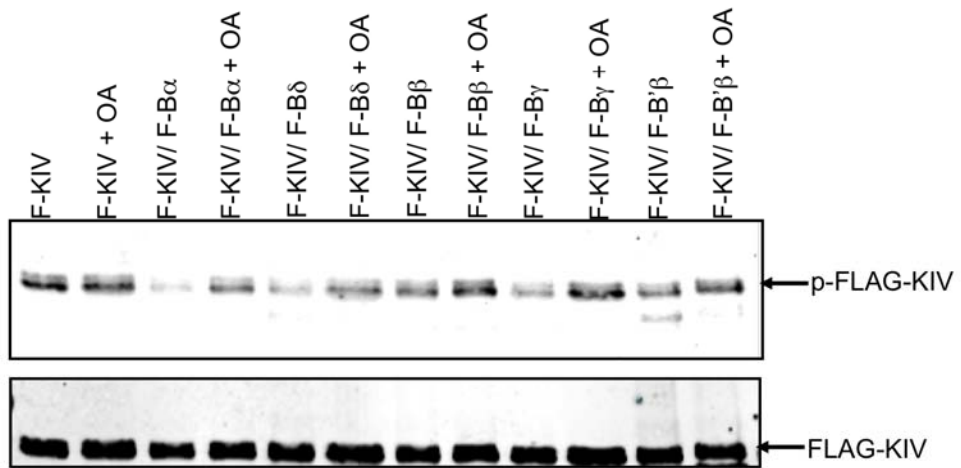
Figure 24: Multiple B subunit-containing holoenzymes can dephosphorylate CaMKIV *in vitro*. The purified holoenzymes (normalized for equivalent [^{32}P] histone H1 phosphatase activity) were incubated with phosphorylated FLAG-CaMKIV in the absence or presence of okadaic acid (OA) for 1 h. The reactions were terminated by the addition of SDS sample buffer and subjected to Western analysis using antibodies recognizing phospho-CaMKIV (p-T200-BL) and total CaMKIV (anti-FLAG).

similar to what occurs following cellular incubation with ionomcycin and okadaic acid. (Figures 12 and 13). We began these studies by testing several different Bα-targeted siRNA oligonucleotides for their ability to knock down endogenous Bα in HEK293A cells. Bα siRNA oligonucleotide #839 resulted in the most pronounced decrease in total signal detected with the antibody that recognizes both Bα and Bδ (Figure 25, top panel). The weak signal remaining after Bα knockdown represents either residual Bα (due to incomplete knockdown) or the levels of Bδ in these cells. However, given that that the Bα/δ antibody recognizes both B subunit isoforms equally well, these results indicate that Bδ is likely expressed at very low levels in HEK293A cells.

Next, we monitored ionomycin- and ionomycin/okadaic acid-induced phosphorylation of endogenous CaMKIV in cells transfected with empty vector or the Bα siRNA oligonucleotide. When the control cells were treated with ionomcyin alone, very little CaMKIV phosphorylation could be seen, which is likely due to the tight regulation that occurs at T200 by PP2A. Only when these cells were treated with both ionomycin and okadaic acid could phosphorylation of the endogenous kinase be detected (Figure 25, bottom panel), akin to what we see in both Jurkat T cells (Figure 12) and HEK293A cells (Figure 13). Interestingly, the CaMKIV phosphorylation profile in the Bα-knockdown cells mirrored that of the empty vector-transfected cells. When the Bα-knockdown cells were treated with ionomycin alone, little CaMKIV phosphorylation could be seen be seen. However, in the presence of both ionomycin and okadaic acid, there was profound CaMKIV phosphorylation that was sustained over time (Figure 25,
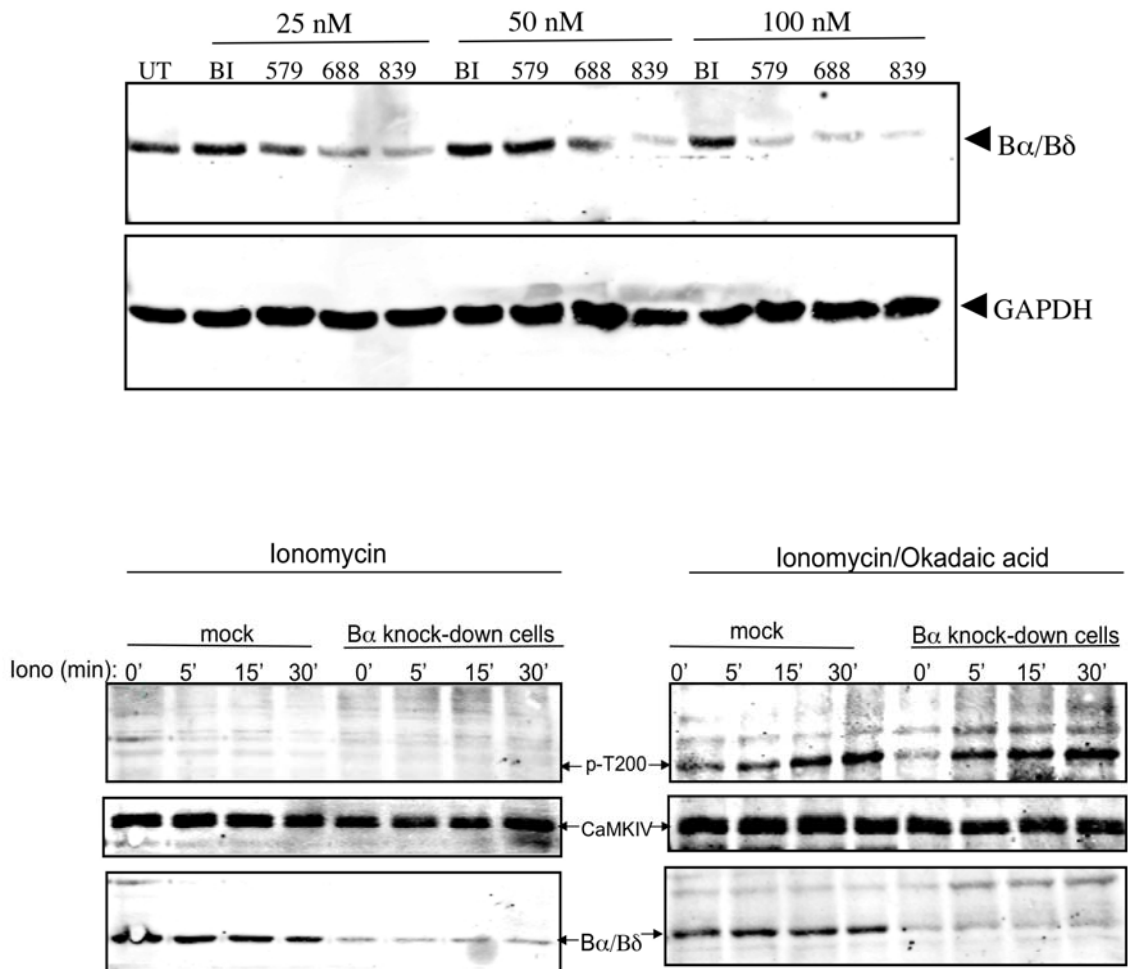
Figure 25: Knockdown of Bα does not effect CaMKIV phosphorylation. *Top panel;* HEK293A cells were transfected with 25 nM, 50 nM, or 100 nM of the indicated Bα-targeted siRNA oligonucleotides using Dharmafect. Fifty-six hours post-transfection, cells were lysed and subjected to immunoblot analysis with our Bα/Bδ and GAPDH antibodies. *Bottom panel;* HEK293A cells were transfected with empty vector (mock transfections) or with 100 nM of the #839 Bα siRNA oligonucleotide. Ninety-six h post-transfection, both sets of cells were either treated with 2 μM ionomycin alone, or pre-treated with 1 uM okadaic acid (OA), followed by treatment with ionomcyin for the indicated time points. Immunoblot analysis of the aliquots of each cell lysate (15 μg of protein) was carried out with the p-T200 (BL), a CaMKIV monoclonal antibody, and our Bα/Bδ antibodies.

bottom panel). The fact that ionomcyin alone failed to induce CaMKIV phosphorylation in the Bα-knockdown cells, indicates that PP2A was still present and tightly regulating CaMKIV phosphorylation at T200. This explains why the addition of a PP2A inhibitor (okadaic acid) was necessary in order to visualize phosphorylation. In conclusion, it does appear that the ABαC and ABδC heterotrimers are the primary modulators of CaMKIV signaling based on our *in vitro* and cellular data. However, further studies will be needed to verify our tentative conclusions and such studies are outlined in Chapter IV

Discussion

The regulatory B subunits of PP2A dictate the subcellular localization and substrate specificity of the phosphatase, thereby controlling PP2A activity towards numerous target proteins in diverse cellular signaling pathways. In recent years, a number of signaling modules composed of PP2A and a protein kinase have been identified (Figure 5). Thus, it has become of particular interest to determine whether the regulatory B subunit is involved in the formation of these multimeric complexes and in the regulation of kinase activity. As we and others have successfully used overexpression and RNAi strategies to demonstrate the role of individual regulatory subunits in various signaling cascades, we exploited these strategies to address the role of two B subunits, Bα an Bδ, in the control of CaMKIV phosphorylation. Although previous studies had only identified the Bα subunit in the CaMKIV•PP2A complex (137), we present evidence here that the highly homologous B subunit, Bδ, is capable of interacting with

CaMKIV as well. Furthermore, we show that both of these regulatory B subunits are essential for the interaction of PP2A with CaMKIV and are capable of dephosphorylating CaMKIV *in vitro*. Based on these findings, we hypothesized that Bα- and Bδ-containing holoenzymes may be the primary forms of PP2A that regulates CaMKIV activity in cells. To determine whether other PP2A holoenzymes could also dephosphorylate CaMKIV, we expanded our *in vitro* dephosphorylation assays to test other B subunit-holoenzymes. We found that all of the holoenzymes tested were capable of dephosphorylating CaMKIV; however, the greatest degree of dephosphorylation occurred with the ABαC and ABδC heterotrimers. We then exploited a cellular approach in an attempt to confirm our *in vitro* data. We employed an RNAi strategy to knockdown Bα and then examined the effect of this genetic manipulation on CaMKIV phosphorylation. We found that in the absence of the pharmacological inhibitor of PP2A (okadaic acid), CaMKIV phosphorylation could not be seen in the Bα-knockdown cells.

These findings do not rule out the possibility that Bα- and/or Bδ-containing holoenzymes are key regulators of the CaMKIV signaling pathway, as it is possible that the residual Bα-containing holoenzymes that remain (due to incomplete Bα knockdown), may be sufficient to facilitate the dephosphorylation of CaMKIV. Another possibility to explain our observations is that Bδ-containing holoenzymes are the primary regulators of CaMKIV signaling. In addition, it is plausible that Bδ-containing holoenzymes may be able to substitute for Bα-containing holoenzymes in a compensatory fashion when ABαC heterotrimers are not present, as in the case of Bα-knockdown cells. Alternatively, both Bα- and Bδ-containing holoenzymes may dephosphorylate CaMKIV so both B subunits would need to be absent in order to see a

corresponding effect on CaMKIV phosphorylation. Finally, one cannot rule out the possibility that other B subunits are capable of dephosphorylating CaMKIV *in vivo*. There is no sequence similarity between B and B′ family members (64) and they are even structurally distinct; the B family of subunits fold into a β-propeller (32) whereas the B' regulatory subunits feature an α-helical repeat architecture that is similar to the PP2A/A subunit (26, 141). However, one feature that is conspicuously conserved between the ABαC and AB'γC heterotrimers is an extended regulatory subunit loop that touches the catalytic site in the PP2A/C subunit (110). This conserved feature may explain how B subunits from different families could confer similar substrate recognition to PP2A heterotrimers. Additional studies will be needed to conclusively determine which B subunits regulate CaMKIV signaling in cells; such studies are outlined in Chapter IV.

In summary, we demonstrate that Bα and Bδ recruit PP2Ac to CaMKIV to catalyze the dephosphorylation of CaMKIV *in vitro*. Although the knockdown of Bα knockdown did not lead to CaMKIV phosphorylation upon ionomycin treatment, it is still conceivable that Bα and/or Bδ are the major regulators of this signaling pathway. There have been other reports in the literature demonstrating the importance that the highly homologous Bα-and Bδ-containing PP2A holoenzymes play in the regulation of signaling. For example, one study showed that both ABαC and ABδC heterotrimers are important modulators of TGF-beta/Activin/Nodal signaling (12). In that report, the two regulatory subunits exerted opposite effects on this signaling pathway as the knockdown of Bα suppressed TGF-beta/Activin/Nodal-dependent responses, whereas the knockown of Bδ enhanced these responses (12). In contrast, our present studies indicate that the Bα and Bδ regulatory subunits likely exhibit redundant functions in the control of CaMKIV

dephosphorylation, at least *in vitro*, as we observed no major differences in their ability to dephosphorylate CaMKIV. If this is indeed the case *in vivo*, then the distinction between the two subunits may lie in their ability to differentially target PP2A to CaMKIV in distinct subcellular compartments. Consistent with that idea, Bα and Bδ exhibit some differences from one another in their subcellular localization (125).

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CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Summary

The studies outlined in this thesis began with the finding from this laboratory more than a decade ago, which showed that PP2A forms a complex with CaMKIV and functions as a negative regulator of CaMKIV signaling. That discovery provided a framework with which we worked within to fine-tune our understanding of the CaMKIV•PP2A signaling module. When the complex was first identified, both *in vitro* experiments and cellular studies using the SV40 small t antigen (outlined in Chapter I; "The Role of PP2A in CaMKIV signaling") indicated that PP2A causes the dephosphorylation and inactivation of the associated CaMKIV. What remained unknown, however, was the identity of the residue(s) in CaMKIV targeted by PP2A. CaMKIV requires phosphorylation at multiple sites, including Thr 200, Ser 11 and Ser 12, to become fully activated (reviewed in 3). Thus, we began our current studies by extensively characterizing a novel CaMKIV phospho-specific antibody that allowed us to investigate, for the first time, the kinetics of CaMKIV phosphorylation and dephosphorylation. Our cellular and *in vitro* data generated with this antibody demonstrated that PP2A inactivates CaMKIV by directly dephosphorylating its phospho-Thr200 residue (Figures 12, 13, 22, 24). Moreover, our studies revealed that the phosphorylation of endogenous CaMKIV is tightly regulated by the associated PP2A (Figures 12 and 13).

When we examined the kinetics of endogenous versus ectopic CaMKIV phosphorylation in cells, we made the surprising finding that their regulation by PP2A differed markedly - both temporally and in their sensitivity to okadaic acid (Figure 13). The differential regulation of an endogenous and ectopic is not without precendence. A 2008 report demonstrated that the endogenous and exogenous forms of Chfr, a checkpoint protein that plays an important function in cell cycle progression and tumor suppression, are localized and regulated differently in cells (19). The authors of this study found that the Chfr protein needed to be expressed at low levels in order for its localization and regulation to mimic that of the endogenous protein.

In our studies, we found that the differential regulation of ectopic and endogenous CaMKIV was due to the fact that, unlike endogenous CaMKIV, ectopic CaMKIV had very little PP2Ac associated with it (Figure 16). However, when CaMKIV was co-expressed with a PP2A regulatory B subunit, endogenous PP2Ac was recruited to the kinase, leading to the formation of a CaMKIV•PP2A complex. Future studies will be needed to determine if this reconstituted complex is regulated by PP2A in a similar manner to the endogenous complex. In addition, our data showed that both Bα and Bδ were capable of recruiting the PP2A core dimer to CaMKIV, thus demonstrating that these two regulatory subunits facilitate the assembly of this complex (Figure 21).

When we tested the ability of Bα- and Bδ-containing PP2A holoenzymes to dephosphorylate CaMKIV *in vitro*, we found that both forms of PP2A were able to carry out this function (Figure 22). In addition, although we found that other PP2A holoenzymes were able to dephosphorylate CaMKIV to some degree, the greatest dephosphorylation occurred with ABαC and ABδC holoenzymes (Figure 23). We tried

confirming our *in vitro* experiments in a cellular system by examining the effect of RNAi-mediated knockdown of the Bα subunit on CaMKIV phosphorylation. However, little ionomycin-induced CaMKIV phosphorylation was evident in the absence of the Bα subunit, indicating that the low levels of Bα that remained after knockdown were still capable of dephosphorylating the kinase, or possibly that Bδ is the primary regulator of CaMKIV dephosphorylation. Other interpretations of these data, as well as further experimentation to address this issue, are outlined below in 'Future Studies' under 'What is the oligomeric composition of the CaMKIV•PP2A complex'.

In summary, our studies make several significant contributions to our understanding of the regulation of the CaMKIV•PP2A signaling complex. We show that PP2A negatively regulates the associated CaMKIV by directly dephosphorylating the kinase on its Thr200 residue. In addition, we show that this regulation is tight and acute in the context of the endogenous kinase. Our studies also demonstrate that both Bα- and Bδ-containing PP2A holoenzymes bind to CaMKIV and facilitate the assembly of the CaMKIV•PP2A complex. Finally, we show that ABαC and ABδC can modulate CaMKIV dephosphorylation *in vitro*, although it is clear that future studies will be needed to determine if these PP2A holoenzymes are the primary regulators of CaMKIV signaling in cells. Given the fact that CaMKIV signaling plays a key role in many physiological processes, such as neuronal growth and function, memory formation and maintenance, as well as the proper functioning of the immune system, understanding the mechanistic details of how this kinase is regulated is important. Our studies provide novel insights into the regulation of CaMKIV by PP2A and lays the foundation for future

studies directed towards understanding how the manipulation or intervention of this pathway would impact key biological processes.

Future Studies

The work described in this thesis adds to our current knowledge of the molecular mechanisms governing the inactivation of CaMKIV by PP2A. However, several questions remain, which need to be addressed in order to fully understand the biology of the CaMKIV•PP2A signaling module, and its role in the maintenance of cellular functions that are under the control of Ca^{2+}-dependent signaling pathways.

Does the reconstituted CaMKIV•PP2A complex mimic the endogenous complex?

Our studies demonstrate that in contrast to endogenous CaMKIV, which is tightly regulated by the associated PP2A (Figures 12 and 13), ectopic CaMKIV does not bind to PP2A (Figure 16) and is therefore insensitive to regulation by the PP2A enzyme (Figure 13). However, the coexpression of CaMKIV with the Bα or Bδ regulatory subunits recruits PP2A to the kinase, leading to the formation of a CaMKIV•PP2A complex (Figure 21). An important question that these data raise is whether or not the reconstituted CaMKIV•PP2A complex functions like the endogenous complex. This is an important question to address because this complex cannot be properly studied in a heterologous system until one can determine which tools are needed to achieve similar regulation of the ectopic and endogenous kinase. For example, in the case of the Chfr protein, the authors were able to determine that just adjusting the expression levels of the ectopic protein caused its regulation and localization to mimic that of the endogenous

protein. In our studies, overexpressing the Bα or Bδ subunits allows the ectopic CaMKIV•PP2A to be reconstituted (Figure 21), so these overexpressed B subunits may also be necessary in achieving similar regulation of the ectopic and endogenous complex.

One way to address this issue would be to examine stimulus-induced T200 phosphorylation of ectopic CaMKIV phosphorylation in cells that have been co-transfected with epitope-tagged forms of CaMKIV and Bα or Bδ. If the regulation of the ectopic CaMKIV•PP2A complex mimics that of the endogenous complex, one would expect the CaMKIV phosphorylation profiles to mirror one another in response to stimulus. The phosphorylation of ectopic CaMKIV should be weak or absent following cell treatment with ionomcyin alone, since it is being rapidly dephosphorylated by the associated phosphatase. However, as is the case for the endogenous kinase (Figure 12), phosphorylation of CaMKIV within the reconstituted complex should increase over time in the presence of both ionomycin and okadaic acid. An experiment that would address the functionality of the phosphatase-associated CaMKIV would be to isolate the complex from the appropriate cell lysate using an antibody that targets the epitope-tagged phosphatase subunit. These immune complexes could then be assayed for kinase activity using CREB as a substrate. In both approaches, achieving the proper stoichiometric ratio of the kinase and phosphatase would be necessary before any comparisons could be made to the endogenous complex. As previously mentioned, these studies will be important in guiding future experiments directed towards studying the CaMKIV•PP2A complex in heterologous systems.

Does the regulatory B subunit remain stably associated with CaMKIV?

In order to fully understand the CaMKIV•PP2A signaling module, it is key to comprehend the nature and regulation of the CaMKIV-PP2A interaction, and in fact, several studies have attempted to address this issue. For example, when the complex was first identified, it was thought that the interaction between the two proteins was stable, and not altered with activation (137). Thus it was postulated that in order for the phosphorylation of CaMKIV and CREB to occur, CaMKK might transiently outpace PP2A to ensure that CaMKIV remains active long enough to phosphorylate CREB. Alternatively, it was also hypothesized that PP2A could be inhibited by a Ca^{2+}-independent post-translational modifications such as phosphorylation. However, more recent studies have suggested that the interaction of CaMKIV with PP2A is dynamic. In one report, the authors observed that the increased intracellular Ca^{2+} concentration that accompanies mitochondrial dysfunction resulted in the disruption of the interaction between CaMKIV and PP2A (9). A subsequent study demonstrated that Ca^{2+}/CaM disrupted the CaMKIV•PP2A complex in cellular extracts by competing with PP2A for the same binding site within the autoregulatory domain of CaMKIV (5). Together, these reports support a model in which the PP2A holoenzyme is able to dissociate from CaMKIV in the presence of increased Ca^{2+}, leading to activation of the enzyme by CaMKK, which generates autonomous CaMKIV activity. Subsequently, Ca^{2+} levels decrease, enabling dissociation of Ca^{2+}/CaM and rebinding of PP2A, which dephosphorylates CaMKIV and attenuates its autonomous activity (104).

Our studies raise another interesting possibility regarding the nature of the CaMKIV-PP2A interaction. Namely, the possibility that the B subunit remains stably

associated with CaMKIV and only the A/C dimer dissociates from the kinase in the presence of Ca^{2+}/CaM. In our studies, we found that endogenous, but not ectopic CaMKIV, associates with PP2Ac in the absence of a coexpressed B subunit (Figures 16 and 21). However, one would expect that if CaMKIV and all three subunits of PP2A (ABC) are actively associating and dissociating from one another, then endogenous PP2Ac would be equally likely to bind to the ectopic or endogenous CaMKIV. Thus, it is possible that the B subunit remains stably associated with endogenous CaMKIV, even in the presence of activating stimuli, and functions to recruit the A/C dimer to the kinase to facilitate CaMKIV deactivation. In the cell lines used in our studies, there might be insufficient levels of B subunit in the cell to recruit A/C to the exogenous kinase, which would explain the necessity for coexpressing Bα or Bδ with CaMKIV in order to see binding of ectopic CaMKIV to PP2Ac (Figure 21). One way to address this hypothesis would be to immunopurify CaMKIV•PP2A complexes from cells treated with or without various stimuli (e.g. ionomycin) with an antibody that targets the kinase. This would be followed by immunoblot analysis with antibodies recognizing each of the PP2A subunits. If the A/C dimer dissociates from CaMKIV following cellular stimulation, then Western analysis of the immune complexes would show binding of the A and C subunits to CaMKIV only under resting conditions, whereas B subunit binding to CaMKIV would be detected in both unstimulated and stimulated cells. These studies could potentially provide novel insights into the regulation of the CaMKIV-PP2A interaction.

What is the oligomeric composition of the CaMKIV•PP2A signaling complex?

The existence of multiple families and isoforms of PP2A regulatory B subunits provides many possibilities for phosphatase regulation. Therefore, understanding the

precise oligomeric composition of the CaMKIV•PP2A complex is key in understanding its function and regulation. Furthermore, identifying the regulatory B subunits that are present in this signaling module will be important for future studies directed towards modulating PP2A activity both *in vitro* and *in vivo*.

In our studies, we were able to determine the partial oligomeric composition of the CaMKIV•PP2A complex, as our data demonstrates that both Bα- and Bδ-containing PP2A holoenzymes can bind to CaMKIV (Figure 20). Our *in vitro* data suggest that these are the primary regulators of CaMKIV signaling; however, this conclusion needs to be confirmed in cells, as our RNAi experiments did not show an effect on CaMKIV phosphorylation when Bα was knocked down (Figure 24). Thus, we are left with several possibilities regarding the B subunit composition of the CaMKIV•PP2A complex. One possibility is that Bα-containing holoenzymes may indeed be the primary regulators of CaMKIV signaling, and residual Bα-containing holoenzymes that remain after Bα knockdown are able to facilitate the dephosphorylation of CaMKIV, To address this, CaMKIV phosphorylation would need to be examined in a Bα-null cell line. If ABαC holoenzymes are the primary modulators of CaMKIV phosphorylation, then CaMKIV phosphorylation would be evident upon cellular stimulation. If this is not the case, then the results in this Bα-null cell line would be similar to what was seen in our studies - no CaMKIV phosphorylation would be evident in the absence of a PP2A inhibitor. Another possibility is that Bδ-containing holoenzymes could be the key modulator of CaMKIV signaling. The signal that can be detected by immunoblot analysis after Bα knockdown may represent the levels of Bδ in the cell, and those holoenzymes (ABδC) may be the primary form of PP2A catalyzing the dephosphorylation of CaMKIV. This possibility

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could be tested by knocking down Bδ in cells, followed by confirmation of knockdown by RT-PCR or immunoblot analysis using our Bδ-specific antibody. In addition, it is plausible that Bδ-containing holoenzymes may be able to substitute for Bα-containing holoenzymes in a compensatory fashion when ABαC heterotrimers are not present, as in the case of Bα-knockdown cells. Alternatively, both Bα- and Bδ-containing holoenzymes may dephosphorylate CaMKIV so both B subunits would need to be absent in order to see a corresponding effect on CaMKIV phosphorylation. A double knockdown of both Bα and Bδ would be informative in determining whether both B subunit-containing holoenzymes negatively regulate CaMKIV activity. Finally, one cannot rule out the possibility that the additional heterotrimeric forms of PP2A containing other B subunits or B′ subunits, bind CaMKIV and dephosphorylate Thr200. One way to address this possibility would be to systematically knock down individual and multiple B subunits and then monitor CaMKIV phosphorylation. However, it would be important to determine beforehand which B subunits are present in targeted cell lines. To confirm the importance of multiple B subunits in CaMKIV regulation, if that is in fact the case, the B subunits could be coexpressed with CaMKIV followed by analysis of ectopic CaMKIV phosphorylation to determine if its phosphorylation state parallels that of the endogenous kinase.

Which region of the B subunit(s) contains the binding domain for CaMKIV?

Several studies have focused on identifying the PP2A-interaction domain within CaMKIV (5, 27, 137) (depicted in Figure 6); however, the corresponding domain within PP2A that is important for binding to CaMKIV has not been identified. Identifying the

CaMKIV binding domain within PP2A will be very useful for future studies aimed towards defining the role that CaMKIV•PP2A complexes play in the control of physiological processes such as cell growth, differentiation, and survival. Since we have shown that Bα and Bδ recruit PP2A to CaMKIV (Figure 21), it is likely that the interaction domain resides within the B subunits. To map the binding domain, a panel of deletion/point mutants could be made within Bα, for example, and then tested for their ability to bind CaMKIV. Once the binding domain has been delineated, peptides that encompass the binding domain could be used to disrupt CaMKIV•PP2A complexes in the cell. Examining the phenotypic consequences of such manipulations would provide further insights into the role of this complex in physiological processes under the control of CaMKIV including long-term potentiation and T-cell activation.

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