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The B α and B δ Regulatory Subunits of PP2A are Necessary for Assembly of the CaMKIV•PP2A Signaling Complex

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Abstract

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) is a serine/threonine kinase that is important in synaptic plasticity and T cell maturation. Activation of CaMKIV requires calcium/calmodulin binding and phosphorylation at T200 by CaMK kinase. Our previous work has shown that protein serine/threonine phosphatase 2A (PP2A) forms a complex with CaMKIV and negatively regulates its activity. Here we demonstrate that PP2A tightly regulates the T200 phosphorylation of endogenous CaMKIV, but has little effect on the phosphorylation of the ectopically-expressed kinase. This differential regulation of endogenous versus exogenous CaMKIV is due to differences in their ability to associate with PP2A, as exogenous CaMKIV associates poorly with PP2A in comparison to endogenous CaMKIV. However, overexpression of the B α or B δ regulatory subunits of PP2A causes the recruitment of PP2Ac to ectopic CaMKIV, leading to formation of a CaMKIV•PP2A complex. Together, these data indicate that the B subunits are essential for the interaction of PP2A with CaMKIV.

Keywords

CaMKIV; PP2A; phosphorylation; regulatory subunit

Introduction

CaMKIV is a serine-threonine protein kinase that functions as a potent mediator of calcium-induced gene expression, primarily through its ability to phosphorylate and activate a variety of transcription factors [1]. This kinase is enriched in the brain and thymus where it plays an important role in long-term potentiation and T cell activation, respectively [2,3]. Activation of CaMKIV occurs in response to increased intracellular calcium levels, which induces calcium/calmodulin binding to both CaMKK and CaMKIV. The binding of calcium/calmodulin to CaMKIV leads to removal of the autoinhibitory segment of CaMKIV from the catalytic core, thereby exposing its active site. CaMKK then phosphorylates CaMKIV on T200 within the activation loop [4,5]. The activation of CaMKIV is very transient because an associated protein serine/threonine phosphatase 2A (PP2A) dephosphorylates phospho-T200, thereby extinguishing CaMKIV activity and abrogating its ability to drive transcription [6,7].

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PP2A is a major serine/threonine phosphatase that has been implicated in the control of numerous biological processes including development, cell growth, differentiation, and apoptosis [8]. It predominantly exists in cells as a heterotrimeric holoenzyme consisting of a catalytic C subunit (PP2A_C), a structural A subunit, and a variable B subunit. Four B subunit families have been identified (B or PR55, B' or PR61, B'' or PR72, and B''' or PR93/PR110) and each family encodes multiple genes, with multiple splice variants. These B subunits exhibit differential subcellular localization as well as tissue-specific and developmentally-regulated patterns of gene expression [9]. The variability in their expression pattern and cellular localization allows the B subunits to confer substrate specificity to PP2A by directing the enzyme to different intracellular locations, thereby facilitating the dephosphorylation of specific substrates in distinct cellular compartments [9–11].

In this study, we further examined the CaMKIV•PP2A complex with special emphasis on the role of the PP2A regulatory B subunits. We initially 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 found that this differential regulation was due to the fact that endogenous CaMKIV associated with PP2A_C, whereas ectopic CaMKIV had very little associated PP2A_C. However, overexpression of the B α or B δ regulatory subunits facilitated formation of a CaMKIV•PP2A complex. Together, our results suggest that the B α and B δ regulatory subunits of PP2A provide the molecular basis for assembly of the CaMKIV•PP2A signaling complex. Our data also raise concerns of whether heterologous systems are reliable for the study of CaMKIV regulation by PP2A, as ectopic CaMKIV regulatory mechanisms do not mimic those of the endogenous kinase in the absence of a co-expressed PP2A regulatory B subunit.

Materials and Methods

Antibodies and Reagents

Anti-FLAG M2-agarose, FLAG peptide, and rabbit and mouse anti-FLAG antibodies were from Sigma (St. Louis, MO). Monoclonal CaMKIV and PP2A_C antibodies were from BD Biosciences Pharmingen (San Diego, CA). CaMKIV phospho-T200 and CaMKIV polyclonal antibodies were from Bethyl Laboratories, Inc. (Montgomery, TX). Generation and characterization of affinity-purified B α /B δ antibodies were reported previously [12,16]. Secondary antibodies for fluorescence detection were from Rockland (Gilbertsville, PA) or Molecular Probes (Eugene, OR). Normal rabbit IgG was from The Jackson Laboratory (Bar Harbor, ME), and protein A-Sepharose was from Zymed Laboratories Inc. (San Francisco, CA). Lipofectamine 2000 and *TransIT*-Express transfection reagents were from Invitrogen (Carlsbad, CA) and Mirus (Madison, WI), respectively. Okadaic acid and ionomycin were 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).

Plasmid Constructs

Generation of FLAG-tagged B α and B δ mammalian expression plasmids were described previously [13]. Mammalian expression plasmids for FLAG-CaMKIV and FLAG-CaMKIV T200A were kindly provided by Dr. Tony Means (Duke University). The HA-CaMKIV construct was generated by subcloning human CaMKIV cDNA into the BglII and NotI sites of the pCMV-HA vector (Clontech, Palo Alto, CA). Proper construction of the plasmid was verified by automated DNA Sequencing (Vanderbilt DNA Core Facility).

Cell culture and transfection

The human embryonic kidney cell line QBI-293A (HEK293A) was a gift from Dr. Tony Means (Duke University), and QBI-293FT (HEK293FT) was from Quantum Biotechnologies (Montreal, Canada). Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics; 110 mg/L sodium pyruvate was also added to the HEK293A media. Jurkat T cells were maintained in RPMI media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. HEK293A cells grown to ~90% confluency in a 100 mm dish were transfected with the appropriate expression vectors using Lipofectamine 2000. HEK293FT cells grown to 45% confluency in 60 mm dishes were transfected with the appropriate expression vectors using *TransIT-Express*.

Coimmunoprecipitations

For immunoprecipitations using antibody-conjugated agarose beads, cells were harvested in 500 µl lysis buffer (25 mM NaH₂PO₄, 2 mM EDTA, 2 mM EGTA, 10 µg/ml leupeptin, 100 µg/ml Pefabloc, and 100 nM okadaic acid). Lysates were incubated on ice for 30 min and centrifuged at 13,000 × g for 10 min at 4°C. Clarified lysates were then incubated with 20 µl of a 50% slurry of anti-FLAG agarose beads overnight at 4°C, and bound proteins were eluted with 300 ng/µl FLAG peptide, followed by 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 shown in Fig. 2, 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. Clarified lysates were incubated overnight at 4°C with 2 µg of rabbit polyclonal CaMKIV or 2 µg of rabbit IgG, and then incubated for 1 h with 20 µl of a 50% slurry of Protein A-Sepharose beads. Bound proteins were eluted in SDS sample buffer and subjected to Western analysis.

Analysis of CaMKIV phosphorylation

For characterization of the CaMKIV phospho-T200 antibody, HEK293FT cells were transfected with pcDNA3 (empty vector), FLAG-CaMKIV (FLAG-KIV), or a FLAG-CaMKIV construct containing a single threonine to alanine mutation (FLAG-KIV-T200A). Twenty-four h post-transfection, the media was replaced with serum-free DMEM containing 5 µM STO-609 or an equivalent volume of vehicle (DMSO). Following overnight incubation, cells were treated for 5 min with serum-free DMEM containing 2 µM ionomycin or an equivalent volume of DMSO. Cells were washed once with PBS and harvested from the dish in 200 µl lysis buffer (20 mM Tris, pH 7.0, 0.5% Igepal, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 20 mM Na₄P₂O₇, 40 mM β-glycerol-phosphate, 1 mM Na₃VO₄, 1 mM PMSF, 3 mM benzamidine, 5 mM pepstatin, and 5 µg/ml leupeptin). Clarified cell lysates were incubated with anti-FLAG agarose beads and bound proteins were eluted with SDS sample buffer. To analyze endogenous CaMKIV phosphorylation, Jurkat T cells were serum-starved 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 ionomycin stimulation. To compare endogenous versus ectopic CaMKIV phosphorylation in response to stimuli, HEK293A cells were transfected with FLAG-CaMKIV. Forty-eight h post-transfection, cells were stimulated for the indicated time points with ionomycin alone or a combination of okadaic acid and ionomycin as described above. Clarified cell lysates were incubated with anti-FLAG agarose beads, and bound proteins were eluted from the resin with 300 ng/µl FLAG peptide. Aliquots of the cell lysates and FLAG immune complexes were subjected to Western analysis.

Western Analysis

Western analysis was carried out as previously described [14]. All membranes were blocked in Odyssey buffer for detection with the Odyssey Infrared Imaging System (LI-COR, Lincoln, Nebraska).

Results

Characterization of a new CaMKIV phospho-T200-specific antibody

Our interest in exploring the PP2A-CaMKIV interaction has been directed at understanding the molecular mechanism by which CaMKIV is negatively regulated by the associated PP2A, with special emphasis on the role of the PP2A regulatory B subunits. In order to assess the impact of PP2A on CaMKIV, we needed a tool to monitor T200 phosphorylation in CaMKIV, as phosphorylation of this residue is required for kinase activation [5]. In a previous study, we utilized a phospho-T200 antibody to monitor the phosphorylation of FLAG-CaMKIV in HEK293A cells [6]; however, this antibody failed to detect phosphorylated endogenous CaMKIV (Reece & Wadzinski, unpublished observation). In the current study, we exploited a new CaMKIV phospho-T200 (p-T200) antibody, which allowed us to examine the phosphorylation state of both endogenous and ectopic CaMKIV. To test the specificity of the p-T200 antibody, HEK293FT cells expressing empty vector, FLAG-CaMKIV, or FLAG-T200A were either untreated or treated with ionomycin to increase intracellular calcium levels. As shown in Fig. 1A, the p-T200 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 antibody. Identical results were observed when the immuno-purified FLAG-tagged proteins were subjected to Western analysis (Fig. 1A). These findings demonstrate that the p-T200 antibody specifically recognizes CaMKIV phosphorylated at T200.

Differential regulation of endogenous and exogenous CaMKIV phosphorylation by PP2A

The p-T200 antibody was then used to monitor the phosphorylation state of endogenous CaMKIV. We initially exploited Jurkat T cells as these cells express relatively high levels of CaMKIV [15]. Jurkat T cells were treated for the indicated time points with ionomycin alone, okadaic acid alone (a potent PP2A inhibitor), or both ionomycin and okadaic acid. Interestingly, no endogenous CaMKIV phosphorylation could be detected unless the PP2A inhibitor was present; the presence of okadaic acid led to sustained phosphorylation of the endogenous CaMKIV enzyme (Fig. 1B). Similar results were obtained using primary rat neuronal cultures (data not shown). This profile of endogenous CaMKIV phosphorylation could also be seen in HEK293A cells. As shown in Fig. 1C (left panel), there was a robust and sustained increase in endogenous HEK293A CaMKIV phosphorylation that could only be observed in the presence of ionomycin and okadaic acid. In contrast, in transfected HEK293A cells, we observed an ionomycin-induced peak in the phosphorylation of FLAG-CaMKIV at 5 min, with a subsequent decrease thereafter. Surprisingly, the dephosphorylation of FLAG-CaMKIV was not sensitive to okadaic acid treatment, as a similar FLAG-CaMKIV profile could be seen in cells treated with ionomycin alone, or treated with both ionomycin and okadaic acid (Fig. 1C, left panel). The phosphorylation profile of the ectopic enzyme was verified by Western analyses of FLAG-CaMKIV immune complexes (Fig 1C, right panel). These data reveal a profoundly different response of ionomycin-induced endogenous and exogenous phosphorylation following PP2A inhibition with okadaic acid. The FLAG-tagged kinase is insensitive to okadaic acid treatment, suggesting that exogenous CaMKIV is not tightly regulated by endogenous PP2A. In contrast, the endogenous kinase exhibits exquisite sensitivity to okadaic

acid, indicating that its dephosphorylation is a tightly coupled event that can be readily blocked by PP2A inhibition.

Differential binding of PP2Ac to endogenous and exogenous CaMKIV

The observed differences in regulation by PP2A were not due to differences in the subcellular localization of ectopic and endogenous CaMKIV as the two enzymes had similar subcellular distribution profiles (Supplemental Figs. 1 and 2). Therefore, we tested the hypothesis that the 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 Fig. 2, no CaMKIV came down in the control immunoprecipitation. The CaMKIV antibody brought down both endogenous CaMKIV and FLAG-CaMKIV, whereas only FLAG-CaMKIV came down in the FLAG immunoprecipitations. Interestingly, Western analysis of the immune complexes showed that PP2Ac bound to 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 kinase (Fig. 2). These data demonstrate that the observed differences of ectopic and endogenous CaMKIV regulation by PP2A (Fig. 1C) are likely due to the fact that PP2Ac binds more efficiently to the endogenous enzyme.

B α and B δ -containing PP2A holoenzymes bind to exogenous CaMKIV

Since the regulatory B subunits of PP2A are thought to direct this enzyme to its cell-specific substrates [9–11], we sought to determine if the B subunits are responsible for the interaction of CaMKIV with PP2A. Our previous studies revealed the presence of a B α -containing PP2A holoenzyme in the partially purified CaMKIV•PP2A complex; however, given the high sequence homology between B α and B δ (Fig. 3A, top panel), we tested whether PP2A holoenzymes containing B δ could also interact with CaMKIV. HEK293A cells were cotransfected with untagged CaMKIV and FLAG-tagged B α or B δ regulatory subunits, followed by Western analyses of the resulting FLAG immune complexes. As shown in Fig. 3A (bottom panel), both B α - and B δ -containing holoenzymes bound CaMKIV; no PP2A subunits were detected in the control immunoprecipitations. Results from immunoprecipitation experiments using differentially tagged forms of CaMKIV and the B subunits confirmed these findings (data not shown).

The B subunits recruit PP2Ac to CaMKIV

In order to determine if these B subunits are important for the assembly of the CaMKIV•PP2A complex, we co-transfected HEK293A cells with FLAG-tagged CaMKIV and untagged B α or B δ regulatory subunits. Proteins bound to FLAG-CaMKIV were isolated from the cell extracts using anti-FLAG-agarose beads. 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 (Fig. 3B). However, there was a significant increase in the amount of PP2Ac bound to FLAG-CaMKIV when immunoprecipitations were performed from lysates of cells co-expressing B α or B δ (Fig. 3B). These results indicate that the B α and B δ regulatory subunits of PP2A are necessary for recruiting PP2Ac to CaMKIV.

Discussion

Activation of CaMKIV requires calcium/calmodulin binding and phosphorylation by CaMKK on T200; its inactivation is due to dephosphorylation of p-T200 by PP2A [6,7]. Our study reveals the interesting finding that the regulation of endogenous CaMKIV phosphorylation by PP2A is remarkably different from that of ectopic CaMKIV, both temporally and in their sensitivity to okadaic acid. Specifically, we found that in contrast to ectopic CaMKIV, PP2A associates with the endogenous kinase and tightly controls its phosphorylation at T200 (Fig. 1C & Fig. 2). However, when the ectopic kinase was co-expressed with B α or B δ , endogenous PP2Ac was recruited to the kinase, leading to the formation of a CaMKIV•PP2A complex (Fig. 3B).

The association of B α -containing PP2A holoenzymes with CaMKIV was not surprising to us, as our earlier analysis of partially purified CaMKIV•PP2A complexes from rat brain extracts revealed the presence of a B α subunit [7]. However, since that time, a novel B subunit isoform, B δ , was identified and shown to share a high degree of sequence homology with B α (Fig. 3A), including the epitope used to generate what was previously thought to be a “B α -specific” antibody [16]. Our subsequent report revealed that this antibody does in fact recognize both B α and B δ [13]. These observations raised the possibility that B δ -containing CaMKIV•PP2A complexes may also exist in cells. Indeed, our present data indicate that both B α - and B δ -containing PP2A holoenzymes can associate with CaMKIV (Fig. 3). These findings are interesting not only because they indicate that different heterotrimeric forms of PP2A may play a role in regulating CaMKIV activity, but also because they reveal a targeting role for these B subunits in recruiting PP2A to CaMKIV. Consistent with our observations, previous reports have shown that the regulatory B subunits target PP2A holoenzymes to a number of other protein kinases [13,17]. There is also precedence for the observation that different heterotrimeric forms of PP2A can bind to the same substrate. For example, both B α - and B δ -containing PP2A holoenzymes associate with Raf to positively regulate the MAPK signaling pathway [13], and both B' β - and B' δ -containing holoenzymes associate with the TrkA neurotrophin receptor to attenuate receptor desensitization and sustain the activity of neurotrophin effector kinases [18]. As in the case for the Raf•PP2A and TrkA neurotrophin receptor•PP2A complexes, further studies will be necessary to determine if the two different CaMKIV-associated PP2A holoenzymes (AB α C and AB δ C) regulate CaMKIV activity in a similar manner.

In conclusion, our studies reveal that the differential regulation of endogenous and exogenous CaMKIV phosphorylation is due to the fact that endogenous CaMKIV binds PP2A much better than the exogenous enzyme. However, coexpression of the B α or B δ regulatory subunit facilitates formation of an ectopic CaMKIV•PP2A complex. Additional studies will be needed to determine if the regulation and function of the reconstituted CaMKIV•PP2A complex (i.e. coexpressed CaMKIV and B α /B δ) mimic that of the endogenous complex. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Inhibition of PP2A enhances ionomycin-induced phosphorylation of endogenous but not ectopic CaMKIV phosphorylation

A, HEK293FT cells were transfected with empty vector, FLAG-CaMKIV (FLAG-KIV), or a FLAG-CaMKIV mutant (FLAG-KIV-T200A). At 24 h post-transfection, cells were pre-treated overnight with STO-609 or vehicle (DMSO), and then treated with Ionomycin (Iono) or DMSO for 5 min. Lysates (15 μ g of protein) and FLAG immune complexes were subjected to Western analysis using antibodies recognizing phospho-T200-CaMKIV (p-FLAG-KIV) and the FLAG epitope (FLAG-KIV) (n=4). B, Jurkat T cells were treated with ionomycin, okadaic acid (OA), or a combination of both reagents for the indicated times as described in *Materials and Methods*. Cell lysates (15 μ g of protein) were immunoblotted for phospho-T200-CaMKIV (p-CaMKIV) and total CaMKIV (n=3). C, HEK293A cells transfected with FLAG-CaMKIV were treated with ionomycin or both ionomycin and okadaic acid as described in *Materials and Methods*. Lysates (30 μ g of protein) and FLAG immune complexes were subjected to Western analysis using antibodies recognizing phospho-T200-CaMKIV and total CaMKIV (n=3).

Fig. 2. PP2Ac associates with endogenous, but not ectopic CaMKIV

HEK293A cells were either untransfected (UT) or transfected with FLAG-CaMKIV (F-KIV). After 48 h, immunoprecipitations were performed from the cell lysates using rabbit IgG and protein A-Sepharose (IgG IPs), a CaMKIV 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 was similarly analyzed (n=3).

A

B α and B δ subunit alignment

B δ MAGAGGGGCPTGGNDFQWCFSSQVKGAVDEDVAE³⁴ 453
 B α MAGAGGGNDIQWCFSSQVKGAVDDDVAE²⁸ 447

B

Fig. 3. The B α and B δ subunits recruit PP2Ac to CaMKIV

A (top panel), amino acid sequence alignment of the N-termini of the B α and B δ subunits is depicted. The boxed area represents the epitope to which the “B α -specific” antibody was directed (see text for details). The shaded boxed area indicates the percent amino acid sequence identity relative to B δ . *A (bottom panels)*, HEK293A cells were transfected with empty vector (EV), untagged CaMKIV (KIV), or FLAG-tagged B subunit and KIV. At 48 h post-transfection, cells were lysed and FLAG immune complexes were isolated. Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and immunoblotting with antibodies recognizing CaMKIV, the FLAG epitope, and PP2Ac (n=9). *B*, HEK293A cells were transfected with untagged B α or B δ alone, FLAG-CaMKIV (F-KIV) alone, or a combination of F-KIV and untagged B α or B δ . Cells were lysed, followed by immunoprecipitation with anti-FLAG agarose beads, and protein elution from the beads with FLAG peptide. Cell lysates (45 μ g of protein) and FLAG peptide eluates were resolved by SDS-PAGE. Immunoblot analysis was carried out with antibodies recognizing CaMKIV, B α /B δ , and PP2Ac (n=4). *, denotes a non-specific band recognized by the secondary antibody.