Heptad Repeats Regulate Protein Phosphatase 2A Recruitment to I-κB Kinase γ/NF-κB Essential Modulator and Are Targeted by Human T-lymphotropic Virus Type 1 Tax

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The switching on-and-off of I-κB kinase (IKK) and NF-κB occurs rapidly after signaling. How activated IKK becomes down-regulated is not well understood. Here we show that following tumor necrosis factor-α stimulation, protein phosphatase 2A (PP2A) association with IKK is increased. A heptad repeat in IKKγ, helix 2 (HLX2), mediates PP2A recruitment. Two other heptad repeats downstream of HLX2, termed coiled-coil region 2 (CCR2) and leucine zipper (LZ), bind HLX2 and negatively regulate HLX2 interaction with PP2A. HTLV-1 transactivator Tax also binds HLX2, and this interaction is enhanced by CCR2 but reduced by LZ. In the presence of Tax, PP2A-IKKγ binding is greatly strengthened. Interestingly, peptides spanning CCR2 and/or LZ disrupt IKKγ-Tax and IKKγ-PP2A interactions and potently inhibit NF-κB activation by Tax and tumor necrosis factor-α. We propose that when IKK is resting, HLX2, CCR2, and LZ form a helical bundle in which HLX2 is sequestered. The HLX2-CCR2-LZ bundle becomes unfolded by signal-induced modifications of IKKγ or after Tax binding. In this conformation, IKK becomes activated. IKKγ then recruits PP2A via the exposed HLX2 domain for rapid down-regulation of IKK. Tax-PP2A interaction, however, renders PP2A inactive, thus maintaining Tax-PP2A-IKK in an active state. Finally, CCR2 and LZ possibly inhibit IKK activation by stabilizing the HLX2-CCR2-LZ bundle.

The kinetics of the NF-κB regulatory pathway, from its activation to its return to the resting state, is rapid. Within minutes after treatment with inducers such as interleukin-1, tumor necrosis factor-α (TNF-α)3, or bacterial lipopolysaccharide, I-κB kinase (IKK) is activated. I-κBα and I-κBβ in turn become serine-phosphorylated by IKK and are targeted for polyubiquitination and degradation by proteasome (for a recent review, see Ref. 1). This leads to nuclear accumulation of NF-κB and activation of mRNA transcription of a large array of cellular genes, including that of I-κBα. The surge in mRNA and protein synthesis of I-κBα is then accompanied by its transport to the nucleus and the redistribution of NF-κB/I-κBα complex back to the cytoplasm (1, 2). Recent studies have indicated that IKK activity peaks at 10 min and declines to 25% of peak activity at 30 min post-TNF-α induction (3, 4). Coincident with the rapid rise and fall of IKK activity, I-κBα level becomes undetectable due to IKK phosphorylation and proteasome degradation at 20 min and reappears at 40 min post-induction as a consequence of de novo synthesis (3, 4).

The core IKK consists of two highly homologous catalytic subunits α and β of 85 and 87 kDa in sizes, respectively, and a 48-kDa regulatory subunit, IKKγ/NF-κB essential modulator (NEMO, referred to as IKKγ herein) (5, 6). Both IKKα and IKKβ contain NH2-terminal kinase domains followed by leucine zippers (LZ) and helix-loop-helix domains that mediate protein-protein interactions important for IKK oligomerization and kinase activity (6, 7). IKKγ also contains extensive helical regions and leucine zipper domains that are involved in protein-protein interaction (5, 6). In vivo, the IKK holoenzyme exists as a large protein complex of at least 700–900 kDa in size (6, 7). It is not clear what other protein components are present in the holo-IKK enzyme complex in addition to IKKα, IKKβ, and IKKγ (6). It has been proposed that the stoichiometry of IKK holoenzyme is α1β1γ2 or β2γ2, with each IKK holoenzyme containing two IKKγ subunits (8). Finally, tetramerization of IKKγ has also been reported to be important for IKK activation (9).

Activation of IKK requires serine phosphorylation of its activation loop by autophosphorylation and/or by upstream kinases such as MEKK and TAK1 (1, 6, 10, 11). Polyubiquitination of IKKγ/NF-κB essential modulator by the ubiquitin ligase, TRAF6, also plays a critical role in initiating IKK activation (12, 13). TRAF6 acts upstream of TAK1 and may be responsible for TAK1 recruitment to IKK (12, 13). Although the mechanisms for IKK activation have been extensively studied, the molecular events that control rapid IKK down-regulation are not well
understood. Many lines of evidence suggest that the major serine/threonine protein phosphatase, PP2A, is involved (14–16). HTLV-1 Tax activates IKK constitutively (17–22). This is due in part to a direct interaction between Tax and IKKγ (20, 22, 24). While IKKγ is essential for IKK activation, the mechanism through which IKKγ controls IKK activity remains unclear. We have shown that via a tripartite interaction, Tax, PP2A, and IKKγ form a stable ternary complex, wherein PP2A activity is inhibited or diminished due to its interaction with Tax (16). In essence, IKK is activated by serine phosphorylation upon extracellular stimulation. Phospho-IKK then becomes rapidly inactivated by IKKγ-associated PP2A, returning it to the resting state. These results suggest that in HTLV-I infected or transformed cells, PP2A inhibition by IKKγ-bound Tax maintains IKK in an active, phosphorylated state, causing constitutive phosphorylation and degradation of IκB (16).

IKKγ contains multiple α-helical heptad repeats that include HLX1 (helix-loop-helix 1), CCR1 (coiled-coil region 1), HLX2, CCR2, and LZ (leucine zipper). HLX1 and CCR1 regions are important for IKKγ interaction with IKKα and IKKβ (25, 26). In this study, the protein–protein interaction of IKKγ, PP2A, and Tax is investigated. We have localized HLX2 heptad repeat of IKKγ to be the primary binding domain of PP2A and Tax and have shown additionally that HLX2, CCR2, and LZ interact with one another dynamically to modulate PP2A recruitment and Tax interaction. Our results suggest that the structures of HLX2, CCR2, and LZ possibly alternate between a helical bundle (closed/inactive conformation) and an extended α-helix (open/active conformation) to regulate the accessibility of HLX2 to PP2A. Tax, through its interaction with HLX2 and CCR2, maintains IKKγ in an open/active conformation. Although this structure avidly recruits PP2A, the latter is inhibited by Tax, thereby keeping IKK constitutively active. CCR2 and LZ peptides possibly stabilize the HLX2-CCR2-LZ bundle, thereby keeping IKKγ in a closed conformation and preventing PP2A and Tax from binding IKKγ. These data indicate that the dynamic interaction of HLX2-CCR2-LZ heptad repeats modulates the recruitment of PP2A and allows IKKγ to function as a molecular switch that can turn IKK on or off rapidly. HTLV-1 Tax Interaction with IKKγ and PP2A keeps the switch at the “on” position and IKK constitutively active.

**EXPERIMENTAL PROCEDURES**

*Yeast Two- and Three-hybrid Assays and β-Galactosidase Assay*—Yeast two-hybrid assays were performed in strain L40 (MATa, his3A200, trpl–901, leu2–3112, ade2, lys2::lexA-HIS3, ura3::lexA-lacZ) where LacZ and HIS3 are driven by minimal GAL1 promoters fused to eight and four lexA boxes, respectively (27). A 2-μm plasmid, BTM116 (TRP), containing the LexA DNA-binding domain (27) was used to produce LexA-IKKγ fusions. The coding sequences for full-length IKKγ were similarly constructed. The coding sequences for CCR2 (IKKγ 250–307), LZ (IKKγ 300–360), and CCR2-LZ (IKKγ 250–360) were generated by PCR using appropriate oligonucleotides and fused to the VP16 activation domain via EcoRI and BamHI sites. Specific information concerning the oligonucleotide sequence is available upon request. For the yeast three-hybrid assays, tax cDNA was placed under the control of the Met-25 promoter on HO-pMET-poly-KanMX4-HO vector (28) and integrated at the mating type locus after transformation. The activities of β-galactosidase were measured using an “enhanced β-galactosidase assay kit (CPRG)” purchased from Gene Therapy Systems (San Diego, CA) as prescribed by the vendor and normalized against the absorbance unit of each culture. Relative β-galactosidase activities are plotted.

*Antibodies*—Mouse hybridoma antibody 4C5 reacts with amino acid residues 333–353 of Tax. Anti-PP2A C subunit monoclonal antibody reacts with the COOH-terminal amino acid residues 295–309 of the catalytic subunit of human PP2A and was from Santa Cruz Biotechnology, Inc., as well as antibodies against PP2A A subunit, actin, VP16, IKKβ, and IKKγ. Antibodies against HA, KT3, and EE epitopes were obtained from Covance Inc.

*Glutathione S-transferase Pulldown Assays*—For production of GST-IKKγ fusion proteins, the coding sequences for full-length IKKγ and truncated variants were generated using standard PCR methods as above. Constructs were ligated into the Ncol/EcoRI insertion site of pGEX-2T. Bacterial lysis, recombinant protein purification, and binding to glutathione beads was done as described previously (16).

For GST pulldown experiments, 500 ng each of purified GST, GST-IKKγ, or GST-IKKγ truncated variants were incubated for 30 min at 30 °C with 1.0 ml of cell lysate from 293T cells transfected with the EE-PP2A Aa expression plasmid. Cell lysate was prepared by incubating ∼7 × 10⁶ cells with 1 ml of a buffer solution containing 25 mM HEPES (pH 7.9), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM EDTA, 1 mg/ml bovine serum albumin, 10% glycerol, 0.15% Nonidet P-40, 0.25 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Incubation of the lysate with the beads, washing, and subsequent immunoblots were done as reported previously (16), except that 120 μl of a 50% slurry of prewashed glutathione-Sepharose 4B was added to each binding reaction.

*S-Agarose PullDown*—Human embryonic kidney (HEK) 293T cells were transfected with one or more of the following constructs: H6/S-PP2Aα, EE-PP2Aα, HA-IKKγ, and PP2Aαc. PCR was used to generate the hexa-histidine and RNase S-peptide-tagged (H6/S)-PP2Aα cDNA, which was ligated into the EcoRI/NotI insertion site of pTriEx-4Neo. Cell lysates from the transfected 293T cells were used for the S-agarose pulldown assay. Lysis buffer contained 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and complete protease inhibitor mixture (as prescribed by Roche Applied Science). Cell lysate, obtained by incubating 7 × 10⁶ cells with 1 ml of lysis buffer, was mixed with 0.2 ml of S-protein-agarose beads (Novagen) at 4 °C for 2 h. Beads from each sample were washed four times with a buffer solution contain-

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ing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, then boiled for 7 min with SDS-PAGE sample buffer and analyzed by immunoblotting as described previously (16).

Co-immunoprecipitation—For IKKγ-HA immunoprecipitation, transfected 293T cells were harvested and washed twice with cold PBS, lysed with 1 ml of lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and complete protease inhibitor mixture (Roche Applied Science), and incubated on ice for 15 min. Cell debris was removed by centrifugation at 12,000 rpm for 15 min at 4 °C. Lysates were precleared by incubation with 20 μl of 50% slurry of protein G-agrose (Sigma) for 1 h at 4 °C. Cell lysates were clarified by centrifugation at 12,000 rpm for 30 min at 4 °C. Anti-HA-agarose (Covance) was mixed with the precleared cell lysates and incubated at 4 °C for 2 h. The reaction mixtures were then washed three times with cold lysis buffer, boiled for 7 min with SDS-PAGE sample buffer, and analyzed by immunoblots as described (16). The IKKγ immunoprecipitation was done similarly, except that IKKγ antibody was used. Immunoprecipitation of whole cell lysates prepared from Jurkat and MT4 cells was similar as above except an anti-PP2A A subunit antibody and 107 cells each were used; and the lysis buffer contained only 0.1% Triton X-100.

DNA Transfection, TNF-α Induction, and Luciferase Reporter Assay—The coding sequences for influenza HA-VP16 activation-domain fusion (HAVP), HAVP-CCR2, HAVP-LZ, and HAVP-CCR2-LZ were generated by PCR and ligated into the EcoRI/BamHI insertion site of pcDNA 3.1. HEK293T cells were seeded in 24-well plates (2.5 × 104 cells/well) and transfected using the calcium phosphate (Invitrogen) method with 1 μg of E-selectin-luciferase reporter, 0.5 μg of pRL-TK (Renilla luciferase internal control), and the expression plasmid for the respective IKKγ peptides (0.25, 0.5, 1.0, and 2 μg). The total amount of DNA was normalized by the addition of pcDNA3.1. For TNF-α induction, at 40 h post-transfection, 2 ng/ml human TNF-α (Sigma) was added, and incubation was continued for 8 h. At the end of TNF-α treatment, the recipient cells were lysed with a reporter lysis buffer (luciferase reagent, Promega). Luciferase activity was detected with a luminometer after mixing 10 μl of extract with 25 μl of luciferase substrate (Promega). For NF-κB activation by Tax, 0.5 μg of Tax expression vector was transfected into 293T cells.

RESULTS

PP2A Associates with Activated IKK—We have shown previously that the catalytic C subunit of PP2A (PP2Ac), Tax, and IKK form a stable ternary complex (16). To investigate the physiological role of PP2A in regulating IKK, we used co-immunoprecipitation to measure their association in the presence or absence of extracellular stimulation. As shown in Fig. 1, only weak IKK-PP2A binding was detectable in 293T cells that were resting (Fig. 1, lane 3). Interestingly, when 293T cells were stimulated with TNF-α, concurrent with IKK activation and IκB degradation, PP2A binding to IKK was greatly increased (Fig. 1, lane 4). We interpret these data to suggest that the recruitment of PP2A to IKK is responsible for the rapid down-regulation of IKK following TNF-α signaling.

To determine whether Tax and IKKγ interact with the PP2A holoenzyme, 293T cells were transiently transfected with expression plasmids for HA-tagged IKKγ, EE-tagged PP2A Aα (A subunit), and His/S-peptide-tagged PP2A Ca (C

FIGURE 1. PP2A interacts with activated IKK. HEK293T cells were treated with TNF-α (10 ng/ml, 15 min) or not. Shown are immunoblots (IB) (lanes 1 and 2) or anti-IKKγ immunoprecipitates (IP) (lanes 3 and 4) probed with indicated antibodies.

FIGURE 2. IKKγ interacts with PP2A holoenzyme. A, immunoblots (IB) of anti-HA-IKKγ immunoprecipitates (IP: aHA) (top panel) or corresponding input whole cell lysates (bottom panel) from 293T cells transfected with various combinations of expression plasmids for hexahistidine-S-peptide-tagged PP2Ac subunit (H6/S-PP2ACa), EE-tagged PP2A Aα subunit (EE-PP2Aαa), and HA-tagged IKKγ (HA-IKKγ) (lanes 1–3). HA-tagged IKKγ was substituted with untagged IKKγ as a control (lane 4). B, immunoblots (IB) of RNase S-protein pulldown (top panel) or corresponding input whole cell lysates (bottom panel) from 293T cells transfected with various combinations of the same plasmids as A, except H6/S-PP2ACa was substituted with untagged PP2Acα in lane 4 as a negative control. C, immunoblots (IB) of anti-HA immunoprecipitates (IP) (top panel) or corresponding whole cell lysates (bottom panel) from 293T cells transfected with expression plasmids for KT3-tagged PP2A B subunits: KT3-PP2AB72, KT3-PP2AB72 γ, and KT3-PP2ABαα, respectively together with an expression plasmid for HA-IKKγ. In all panels the antibodies used in the immunoblots (IB) are indicated.
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subunit) in different combinations. Co-immunoprecipitation with HA antibody indicated that both PP2ACA and PP2Aα interact with IKKγ (Fig. 2A). Likewise, pull down of PP2A Cα using agarose beads conjugated with RNase-S-protein showed that PP2A Cα interacted with both IKKγ and PP2A αα (Fig. 2B). As might be expected, IKKβ was also detected in the pull-down of PP2A Cα.

We next examined interactions between PP2A B subunits and IKKγ. Here again, 293T cells were transiently transfected with expression plasmids for HA-IKKγ and each of three KT3-tagged PP2A B subunits: Bα, Bγ, and B72, respectively. Immunoprecipitation with HA antibody indicated that IKKγ associated with all three B subunits (Fig. 2C). This suggests that IKKγ probably interacts with multiple isoforms of PP2A holoenzyme containing different B subunits and is consistent with previous data showing the C subunit to be the direct mediator of the interaction.

To confirm a direct interaction between PP2A holoenzyme with IKKγ in vivo, immunoprecipitation was carried out for cell lysates derived from an HTLV-1-transformed T cell line, MT4, and an HTLV-1-unrelated T cell line, Jurkat, using the PP2A A subunit antibody. As expected, PP2A A antibody co-precipitated both the PP2A A and C subunits (Fig. 3A, lanes 3 and 4). Importantly, IKKγ also co-precipitated with the PP2A A subunit (Fig. 3A, lanes 3 and 4). Consistent with the notion that PP2A binding to IKK becomes increased in the presence of Tax, a higher level of IKKγ was found to associate with PP2A in the MT4 cell lysate (compare Fig. 3A, lanes 3 and 4). Finally, purified PP2A enzyme comprising largely of A and C subunits (from Upstate Biotechnology Inc.; Fig. 3B, lane 5) was mixed with GST-IKKγ or GST in the presence or absence of purified, bacterially derived Tax protein (16) and incubated with glutathione-agarose beads to pull down GST-IKKγ or GST and the associated proteins. As shown in Fig. 3B, there was no interaction between PP2A with GST was detectable irrespective of the addition of Tax (lanes 1 and 3). While a weak association between PP2A and GST-IKKγ could be seen (lane 2), a much stronger binding of both PP2A A and C subunits to GST-IKKγ was observed in the presence of Tax. Together, these results support the idea that PP2A holoenzyme becomes recruited to TNF-α-activated or Tax-activated IKK.

The HLX2 Domain of IKKγ Interacts with PP2A—To localize the domain(s) of IKKγ that interacts with PP2A, a series of IKKγ COOH-terminal truncations were made wherein the various helical domains of IKKγ were successively deleted. These truncations were designed based on the pairwise and multicolour programs of Bonnie Berger (29). The deletions were fused with GST to facilitate their purification and analysis. Extracts from 293T cells transfected transiently expressing EE-PP2A Aα were then incubated with glutathione-Sepharose beads charged with purified GST-tagged IKKγ-1–86, IKKγ-1–188, IKKγ-1–253, IKKγ-1–307, or full-length IKKγ (IKKγ-1–419). Purified GST and GST fusions were incubated with cell lysate from 293T cells transfected with the EE-PP2A Aα expression plasmid. Shown is the pull down immunoblotted with an anti-EE antibody (bottom panel).

In a complementary approach, the coding sequences of IKKγ deletions and PP2A Cα were fused to the LexA DNA-binding domain (BD) and the VP16 activation domain (AD), respectively, and introduced into a Saccharomyces cerevisiae reporter strain, L40, for two-hybrid analysis (27, 30). In agreement with the results above that point to the importance of IKKγ-1–253, the two-hybrid system also revealed the affinity of interaction between IKKγ derivatives and PP2A Cα in an order similar to the pulldown results, i.e., BDIIKKγ-1–253 > BDIIKKγ-1–307 > BDIIKKγ-1–419 > BDIIKKγ-1–188 and BDIIKKγ-1–188 > BDIIKKγ-1–419 (Fig. 5A, solid bars). Neither BDIIKKγ-1–86 nor BDIIKKγ-1–151–307 interacted with PP2A Cα (Fig. 5A, solid bars). Because the protein sequence and overall structure of S. cerevisiae and

FIGURE 3. Tax increases association of PP2A holoenzyme with IKKγ. A, immunoblot (IB) of PP2A A and C subunits and IKKγ. Lanes 1 and 2 represent input lysates of Jurkat (HTLV-1-unrelated) and MT4 (HTLV-1-transformed, Tax-expressing) T cell lines, respectively. Lanes 3 and 4 represent anti-PP2A A immunoprecipitates of Jurkat and MT4, respectively. B, GST pull down was carried out as described previously (16). Purified GST and GST-IKKγ (were incubated with purified PP2A (comprising largely of A and C subunits, lane 5) in the presence or absence of bacterially derived Tax purified on nickel-nitrilotriacetic acid-Sepharose via a COOH-terminal hexahistidine tag.

FIGURE 4. PP2A binds the HLX2 heptad repeat of IKKγ. Anti-GST immunoblot (IB) of purified GST and GST fusions containing various IKKγ COOH-terminal truncations (top panel (left to right)) IKKγ-1–86, IKKγ-1–188, IKKγ-1–253, and IKKγ-1–307, and full-length IKKγ (IKKγ-1–419). Purified GST and GST fusions were incubated with cell lysate from 293T cells transfected with the EE-PP2A Aα expression plasmid. Shown is the pull down immunoblotted with an anti-EE antibody (bottom panel).
heptad repeats negatively modulate the accessibility of HLX2 to PP2A.

Tax Enhances PP2A-IKKγ Binding by Interacting with HLX2 and CCR2 Domains—A similar yeast two-hybrid analysis was performed to map the Tax-binding domain in IKKγ. In contrast to the results described above, BD-IKKγ-1–307, which contained both the HLX2 and CCR2 region, was found to bind Tax the strongest, followed by BD-IKKγ-1–253 and BD-IKKγ-1–419 (Fig. 5A, open bars). Weak interaction was detected for BD-IKKγ-1–188, -151–419, and -151–307 (Fig. 5A, open bars). Little or no interaction between Tax and IKKγ-1–86 was found. These results suggest that Tax and PP2A bind to distinct, but overlapping, regions of IKKγ. Here again, the LZ domain exerted a negative influence on Tax-IKKγ binding also, but unlike PP2A-IKKγ interaction, CCR2 increased Tax-IKKγ binding (compare Fig. 5A, BD-IKKγ-1–253 and BD-IKKγ-1–307, open bars).

We next investigated how Tax affects IKKγ interaction with PP2A. To this end, a yeast three-hybrid system was employed wherein tax gene was placed under the control of the Met25 promoter (kindly provided by J. M. Egly; Ref. 28). The Met25-tax expression cassette was then integrated into the mating type locus of the respective L40 reporter strains that already harbored AD-PP2A Cα and each of the BD-IKKγ constructs. As indicated in Fig. 5B, Tax greatly promoted IKKγ interaction with PP2A Cα. Interestingly, in the presence of Tax (methionine starvation), the profile of the affinities of PP2A Cα for various IKKγ deletions was skewed toward that of Tax-IKKγ interaction, with IKKγ-1–307 binding PP2A Cα the best (compare Figs. 5A and 4B). This skewing of the interaction profile was observed even when methionine was supplemented in the growth media to suppress tax expression. The repression of the Met-25 promoter was, however, incomplete, as a low but detectable level of Tax remained (data not shown). The leaky expression of Tax is most likely responsible for the deviation of the Tax-IKKγ-PP2A interaction profile in Fig. 5B (open bars) from those in Figs. 4 and 5A. These results suggest that IKKγ-1–307 contains the domains required for strong Tax-IKKγ-PP2A ternary complex formation. Furthermore, Tax facilitates PP2A binding to IKKγ by a direct interaction with

human PP2A Cα subunits are highly homologous, the human PP2A Cα expressed here most likely exists as a holoenzyme with the S. cerevisiae A and B subunits. The NH2-terminal helical domains of IKKγ have been shown to be critical for binding IKKα and IKKβ (25, 26). This domain may also be important for the integrity of IKKγ structure such that a deletion of IKKγ amino acid residues 1–150 drastically reduced binding to PP2A Cα (Fig. 5, A and B). In aggregate, these data suggest that the region spanning the HLX2 heptad repeat (amino acids 188–253) is critical for PP2A interaction. Importantly, inclusion of the COOH-terminal CCR2 (amino acids 254–303) and LZ (amino acids 304–365) heptad repeats significantly attenuated HLX2 interaction with PP2A (Fig. 5A, BD-IKKγ-1–307 and BD-IKKγ-1–419, respectively) suggesting that CCR2 and LZ domains such that a deletion of IKKγ amino acid residues 1–150 drastically reduced binding to PP2A Cα subunits. The NH2-terminal helical domains of IKKγ have been shown to be critical for binding IKKα and IKKβ (25, 26). This domain may also be important for the integrity of IKKγ structure such that a deletion of IKKγ amino acid residues 1–150 drastically reduced binding to PP2A Cα (Fig. 5, A and B). In aggregate, these data suggest that the region spanning the HLX2 heptad repeat (amino acids 188–253) is critical for PP2A interaction. Importantly, inclusion of the COOH-terminal CCR2 (amino acids 254–303) and LZ (amino acids 304–365) heptad repeats significantly attenuated HLX2 interaction with PP2A (Fig. 5A, BD-IKKγ-1–307 and BD-IKKγ-1–419, respectively) suggesting that CCR2 and LZ domains
both IKKγ and PP2A and possibly by converting IKKγ into a conforma tion favorable for PP2A binding. The protein interaction results are summarized in Fig. 5C.

The CCR2 and LZ Domains of IKKγ Interact with HLX2 and Modulate Its Interaction with PP2A and Tax—Because IKKγ truncations lacking the CCR2 and LZ heptad repeats showed greater affinity for PP2A and Tax than the full-length IKKγ, we surmised that CCR2 or LZ might negatively modulate the accessibility of HLX2 to PP2A. Because HLX2, CCR2, and LZ domains are extensively helical, it follows that CCR2 and/or LZ may directly bind HLX2 through intra- or intermolecular coiled-coil interaction, thereby restricting HLX access by PP2A.

To assess the interaction between CCR2 and LZ with HLX2, yeast two-hybrid analysis was again performed. As shown in Fig. 6, both CCR2 and LZ bound IKKγ-1–253 strongly but did not bind the empty vector control or LexA-IKKγ-1–188 at all (Fig. 6A). CCR2-LZ dual heptad repeats also interacted with HLX2 with significant affinity (Fig. 6A, ADIKKCCCR2-LZ). Because CCR2 and LZ have been shown to bind each other through coiled-coil interaction (31), and in light of the present results, we think it likely that HLX2, CCR2, and LZ may form a helical bundle in which HLX2 is sequestered from binding PP2A. To extend this line of reasoning further, we hypothesize that when the HLX2-CCR2-LZ bundle forms, IKKγ is in a closed and inactive conformation (Fig. 6B1). In this resting state, it is not necessary for IKK to recruit PP2A and HLX is blocked from PP2A access. The HLX2-CCR2-LZ interaction, however, can become disrupted after Tax binding to HLX2 and CCR2 or by signal (e.g. TNF-α)-induced post-translational modifications. When HLX2-CCR2-LZ bundle is unfolded, IKKγ and IKK assume an open and active conformation, which then recruits PP2A via the exposed HLX2 region for rapid IKK inactivation (Fig. 6B2). Tax binds IKKγ and converts HLX2-CCR2-LZ bundle into an active conformation, which recruits PP2A, but Tax-PP2A interaction renders PP2A inactive or less active, thereby maintaining IKKγ and IKK in a constitutively active state (Fig. 6B3).

CCR2, LZ, and CCR2-LZ Fusion Potently Inhibit NF-κB Activation by Tax and TNF-α—To test the hypothesis outlined above, we derived expression vectors for dual HA and VP16 activation domain (HA-VP16)-tagged CCR2, LZ, and CCR2-LZ fusions and co-transfected them individually with Tax, and E-sel-Luc, a luciferase reporter driven by the NF-κB-responsive E-selectin promoter. Indeed, in the forms of HA-VP16 fusions, CCR2 in particular, followed by LZ and CCR2-LZ, all drastically inhibited Tax-mediated (Fig. 7A) and TNF-α-induced NF-κB activation (Fig. 7B) in a dose-dependent manner. After HA-VP16-CCR2 expression plasmid was transfected into 293T cells, a direct binding of CCR2 to IKKγ could be readily detected (Fig. 7C). Finally, CCR2 added in trans by transfection reduced IKKγ-Tax and IKKγ-PP2A association in a dose-dependent manner (Fig. 7D, IP: HA panels, IB: Tax and PP2Ac, respectively) but did not affect IKKγ-IKKβ interaction (Fig. 7D IP: HA panels, IB: IKKβ). These results support the notion that CCR2, and possibly LZ as well, interacts with HLX2 to form a helical bundle and negatively regulates IKKγ access by PP2A (Fig. 6B4).

DISCUSSION

In this study, we have shown that PP2A holoenzyme interacts with IKKγ. We have further demonstrated that the interaction between PP2A and IKK is tightly controlled. In resting cells, IKK binds PP2A weakly, but upon TNF-α stimulation, IKK undergoes a structural change, possibly as a result of post-translational modifications and/or interaction with other regulatory factors, that facilitates recruitment of PP2A. We have mapped the domains in IKKγ that interact with PP2A and Tax, respectively. As might be expected, the PP2A- and Tax-binding domains in IKKγ overlap and are localized to amino acid residues 188–253 (HLX2) and 188–307 (HLX2-CCR2), respectively. The latter observation agrees in part with a previous report, which also showed the HLX2 region to be critical for Tax binding (32). The present study has further revealed the importance of HLX2 in binding PP2A and how its interaction with PP2A and Tax is regulated by downstream CCR2 and LZ heptad repeats. Although both CCR2 and LZ negatively control HLX2-PP2A binding, CCR2 is needed for strong HLX2-Tax interaction. In the presence of Tax, the binding between IKKγ and PP2A via the exposed HLX2 region for rapid IKK inactivation (Fig. 6B2). Tax binds IKKγ and converts HLX2-CCR2-LZ bundle into an active conformation, which recruits PP2A, but Tax-PP2A interaction renders PP2A inactive or less active, thereby maintaining IKKγ and IKK in a constitutively active state (Fig. 6B3).
activation by Tax and TNF-α interaction with Tax and PP2A. A, CCR2 and LZ heptad repeats block NF-κB activation by Tax. HEK293T cells were transfected with 1 μg of E-selectin-Luc, 0.5 μg of pRL-TK, with or without 0.5 μg of Tax expression vector and, in increasing concentrations, expression plasmids for HA-VP16 (HAVP) alone or HA-VP16 fusions containing CCR2, LZ, and CCR2-LZ repeats (labeled as CCR2, LZ, and CCR2-LZ, respectively). The open, dotted, densely dotted, hatched, and solid bars represent without Tax, and Tax plus 0.25, 0.5, 1.0, and 2.0 μg of HVP, CCR2, and CCR2-LZ expression plasmids, respectively. The total DNA amount was held constant by the addition of pcDNA3.1. Cells were harvested for luciferase assays 48 h after transfection. B, CCR2 and LZ heptad repeats block NF-κB activation by Tax. HEK293T cells were transfected as described for A except that the Tax expression construct was not included. Forty hours post-transfection, human TNF-α (2 ng/ml) was added and incubated for 8 h. Cells were harvested at the end of the TNF-α treatment for luciferase assays. Results in A and B are means ± S.E. from triplicate determinations. C, CCR2 binds IKKγ in vivo. HEK293T cells were transiently transfected with 2 μg of pcDNA or pcDNA-HAVP-CCR2, respectively. Cells were harvested 48 h post-transfection and immunoprecipitated with HA antibody-conjugated agarose or an anti-IKKγ antibody followed by protein G pulldown. IKKγ and CCR2 were detected by anti-IKKγ (IB: IKKγ) and anti-VP16 (IB: VP16) antibodies, respectively. D, CCR2 disrupts PP2A and Tax binding to IKKγ. HEK293T cells were transiently transfected with 0.5 μg of pcDNA-HA-IKKγ, 1.5 μg of CMV-Tax, and increasing amounts (0.25, 0.5, and 1 μg) of pcDNA-HAVP-CCR2. Cells were harvested 48 h post-transfection and immunoprecipitated with HA antibody-conjugated agarose. IKKγ, IKKγ-Tax, PP2Ac, and CCR2 were detected by immunoblots (IB) with appropriate antibodies. The left and right panels represent immunoblots for the whole cell lysates (lysate) and those of HA-IKKγ immunoprecipitates (IP: HA), respectively.

FIGURE 7. CCR2, LZ and CCR2-LZ heptad repeats expressed in trans potently inhibit NF-κB activation by TNF-α or Tax and disrupt IKKγ interaction with Tax and PP2A. A, CCR2 and LZ heptad repeats block NF-κB activation by Tax. HEK293T cells were transfected with 1 μg of E-selectin-Luc, 0.5 μg of pRL-TK, with or without 0.5 μg of Tax expression vector and, in increasing concentrations, expression plasmids for HA-VP16 (HAVP) alone or HA-VP16 fusions containing CCR2, LZ, and CCR2-LZ repeats (labeled as CCR2, LZ, and CCR2-LZ, respectively). The open, dotted, densely dotted, hatched, and solid bars represent without Tax, and Tax plus 0.25, 0.5, 1.0, and 2.0 μg of HVP, CCR2, and CCR2-LZ expression plasmids, respectively. The total DNA amount was held constant by the addition of pcDNA3.1. Cells were harvested for luciferase assays 48 h after transfection. B, CCR2 and LZ heptad repeats block NF-κB activation by Tax. HEK293T cells were transfected as described for A except that the Tax expression construct was not included. Forty hours post-transfection, human TNF-α (2 ng/ml) was added and incubated for 8 h. Cells were harvested at the end of the TNF-α treatment for luciferase assays. Results in A and B are means ± S.E. from triplicate determinations. C, CCR2 binds IKKγ in vivo. HEK293T cells were transiently transfected with 2 μg of pcDNA or pcDNA-HAVP-CCR2, respectively. Cells were harvested 48 h post-transfection and immunoprecipitated with HA antibody-conjugated agarose or an anti-IKKγ antibody followed by protein G pulldown. IKKγ and CCR2 were detected by anti-IKKγ (IB: IKKγ) and anti-VP16 (IB: VP16) antibodies, respectively. D, CCR2 disrupts PP2A and Tax binding to IKKγ. HEK293T cells were transiently transfected with 0.5 μg of pcDNA-HA-IKKγ, 1.5 μg of CMV-Tax, and increasing amounts (0.25, 0.5, and 1 μg) of pcDNA-HAVP-CCR2. Cells were harvested 48 h post-transfection and immunoprecipitated with HA antibody-conjugated agarose. IKKγ, IKKγ-Tax, PP2Ac, and CCR2 were detected by immunoblots (IB) with appropriate antibodies. The left and right panels represent immunoblots for the whole cell lysates (lysate) and those of HA-IKKγ immunoprecipitates (IP: HA), respectively.

and PP2A became greatly increased, consistent with our published results that Tax, PP2A, and IKK form a stable ternary complex (16).

What is particularly interesting is that the CCR2, LZ, and CCR2-LZ fusion bind HLX2 directly and negatively affect HLX2 access by PP2A and Tax. The propensity for HLX2 to form coiled coil is predicted to be lower than that for CCR2 and LZ (20% versus 90–100% based on the multicoil program; Ref. 29). This may be due in part to its critical role in dynamic interactions with multiple protein partners, wherein a flexible and less structured motif is more desirable. Remarkably, CCR2, LZ, and CCR2-LZ fusion potently inhibited NF-κB activation by Tax and TNF-α, and CCR2 disrupted PP2A and Tax binding with IKKγ. Based on these results, we hypothesize that, in its resting/inactive state, CCR2 and LZ heptad repeats may interact with HLX2 in a helical bundle and sequester HLX2 from access by PP2A. When IKK becomes activated, HLX2 assumes an open conformation, which avidly and rapidly recruits PP2A to turn off IKK activity. This open form of IKKγ is stabilized or induced by Tax. The HLX2 region of the Tax-associated IKKγ is exposed and avidly recruits PP2A. However, the interaction between Tax and PP2A inhibits PP2A activity and prevents PP2A from inactivating IKK. The current data do not rule out the possibility that CCR2, LZ and CCR2-LZ fusion peptides may also affect intermolecular IKKγ interaction (oligomerization), thereby affecting Tax-PP2A binding. Detailed biochemical analyses using purified components are currently in progress to address this issue. Finally, a recent study has indicated that the CCR2-LZ region of IKKγ contains a K63-polyubiquitin-binding domain and its interaction with K63-polyubiquitin is required for IKK activation (33). It is possible that binding of K63-polyubiquitin to CCR2-LZ also alters IKKγ and IKK structures in a manner similar to Tax-CCR2-HLX2 interaction and thereby induces IKK to assume an open conformation.

It has been reported recently that a deletion of amino residues 121–179 of IKKγ prevented PP2A recruitment by IKKγ and attenuated IKK activity (34). In the same study, okadaic acid, a PP2A inhibitor, was found to attenuate TNF-α-induced degradation of IκB (34). These data were taken to suggest that IKKγ-121–179 constitutes the PP2A-binding domain, and PP2A plays a positive role in IKK activation (34). The major PP2A-binding domain of IKKγ that we have mapped is localized to the HLX2 region in residues 189–253 and differs from the previous report. In our analyses, even though the COOH-truncation mutant IKKγ-1–188 did bind PP2A somewhat, its extent of binding is negligible compared with that of the HLX2-containing IKKγ-1–253. The fact that Tax-enhanced PP2A binding was observed for IKKγ-1–253 but not for IKKγ-1–188, again, supports the notion that HLX2 is the bona fide PP2A-binding site. It is possible that the loss of PP2A binding from the IKKγ-121–179 deletion may have resulted in a structural disruption to the coiled-coil region 1 of IKKγ, which secondarily affected the conformation of HLX2. Whether PP2A plays a negative, positive, or dual role in regulating IKK activity also remains to be fully resolved. Okadaic acid, a PP2A inhibitor, has been shown by multiple investigators to potently activate IKK and IκB degradation (14, 15). Published literature suggests that...
once IKK is activated by T-loop phosphorylation, it undergoes further hyperphosphorylation and becomes attenuated in its activity. PP2A may play a role in removing all phosphate moieties from IKK, thereby returning IKK to its fully resting state, or perhaps under some circumstances, removing only the inhibitory phosphates while leaving the activating phosphate intact. Although the IKK activity of Tax-IKK-PP2A complex appears constitutively active as judged by the constant phosphorylation and total destruction of IκB in Tax-expressing cells, the status of IKK phosphorylation in the complex is unclear. It is possible that under the influence of Tax, a spectrum of T-loop-phosphorylated and hyperphosphorylated forms of IKK are represented. The data reported here do not rule out a positive role of PP2A in IKK activation.

The exact nature of the putative helical bundle formed by HLX2-CCR2-LZ is not clear at present. Several reports have already suggested a similarity between CCR2-LZ of IKKγ to the helical domains in HIV gp41, influenza virus HA2, and other viral fusion proteins (31, 35). The heptad repeats of gp41 and HA2 alternate between a six-helix bundle formed by trimeric α-helical hairpins and three fully extended α-helices to facilitate fusion peptide insertion into target cell membrane and subsequent viral and host cell membrane fusion. It has been proposed recently that the stoichiometry of IKK holoenzyme is $\alpha_1\beta_1\gamma_2$ or $\beta_2\gamma_2$, with each IKK holoenzyme containing two IKKγ subunits (8). Based on the results above, we speculate that the HLX2, CCR2, and LZ heptad repeats of the two IKKγ subunits may also alternate between a six-helix bundle and two fully extended helices as a function of post-translational modifications or interaction with K63-polyubiquitin or with HTLV-I Tax (Fig. 6B). Additional helical interactions between the fully extended helices may maintain IKK in a helical structure and allow IKK catalytic subunits to undergo auto-phosphorylation and activation. Finally, in light of the recent success of HIV fusion inhibitors, the activity of IKK and NF-κB may also be modulated using peptides with sequences spanning HLX2, CCR2, and LZ heptad repeats as reported here and elsewhere (23).

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REFERENCES

1. Hayden, M. S., and Ghosh, S. (2004) Genes Dev. 18, 2195–2224
2. Ghosh, S., and Karin, M. (2002) Cell 109, (suppl.) S81–S96
3. Cheong, R., Bergmann, A., Werner, S. L., Regal, J., Hoffmann, A., and Levchenko, A. (2006) J. Biol. Chem. 281, 2945–2950
4. Werner, S. L., Barken, D., and Hoffmann, A. (2005) Science 309, 1857–1861
5. Yamazaki, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) Cell 93, 1231–1240
6. Karin, M., and Ben Neriah, Y. L. (2000) Annu. Rev. Immunol. 18, 621–663
7. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
8. Miller, B. S., and Zandi, E. (2001) J. Biol. Chem. 276, 36320–36326
9. Tegzethoff, S., Behlke, I., and Scheideireit, C. (2003) Mol. Cell. Biol. 23, 2029–2041
10. Yin, M. J., Christerson, L. B., Yamamoto, Y., Kwak, Y. T., Xu, S., Mercurio, F., Barbosa, M., Cobb, M. H., and Gaynor, R. B. (1998) Cell 93, 875–884
11. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
12. Sun, L., Deng, L., Ka, C. K., Xia, Z. P., and Chen, Z. J. (2004) Mol. Cell 14, 289–301
13. Ea, C. K., Sun, L., Inoue, J., and Chen, Z. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 15318–15323
14. Sung, S. J., Walters, J. A., and Fu, S. M. (1992) J. Exp. Med. 176, 897–901
15. Schmidt, K. N., Traenckner, E. B., Meier, B., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 27136–27142
16. Fu, D. X., Kuo, Y. L., Liu, B. Y., Jeang, K. T., and Giam, C. Z. (2003) J. Biol. Chem. 278, 1487–1493
17. Yamaoka, S., Elwood, J., Beraud, C., and Greene, W. C. (1994) Mol. Cell. Biol. 14, 7377–7384
18. Good, L., and Sun, S. C. (1996) J. Virol. 70, 2730–2735
19. Sun, S. C., and Ballard, D. W. (1999) Oncogene 18, 6948–6958
20. Xiao, G., and Sun, S. C. (2000) Oncogene 19, 5198–5203
21. Chu, Z. L., Di Donato, J. A.,HAViger, J., and Ballard, D. W. (1998) J. Biol. Chem. 273, 15891–15894
22. Chu, Z. L., Shin, Y. A., Yang, J. M., Di Donato, J. A., and Ballard DW, L. H. (1999) J. Biol. Chem. 274, 15297–15300
23. Agou, F., Courtois, G., Chiaravalli, J., Baleux, F., Coic, Y. M., Traincard, F., Israel, A., and Veron, M. (2004) J. Biol. Chem. 279, 54248–54257
24. Jin, D. Y., Giordano, V., Kibler, K. V., Nakano, H., and Jeang, K. T. L. H. (1999) J. Biol. Chem. 274, 17402–17405
25. May, M. I., Marienfeld, R. B., and Ghosh, S. (2002) J. Biol. Chem. 277, 4992–46000
26. May, M. I., D’Acquisto, F., Madge, L. A., Glockner, J., Popper, J. S., and Ghosh, S. (2000) Science 289, 1550–1554
27. Hollegen, S. M., Sternglans, R., Cheng, P. F., and Weintraub, H. (1995) Mol. Cell. Biol. 15, 3813–3822
28. Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Egly, J. M. (1998) Mol. Cell 2, 22995–22999
29. McDonnell, A. V., Jiang, T., Keating, A. E., and Berger, B. (2006) Bioinformatics 22, 356–358
30. Shih, H. M., Goldmann, P. S., De Maggio, A. I., Hollegen, S. M., Goodman, R. H., and Hoekstra, M. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13896–13901
31. Agou, F., Traincard, F., Vinolo, E., Courtois, G., Yamaoka, S., Israel, A., and Veron, M. (2004) J. Biol. Chem. 279, 27861–27869
32. Iha, H., Kibler, K. V., Yedavalli, V. R., Peloponese, J. M., Haller, K., Miyazato, A., Kasai, T., and Jeang, K. T. (2003) Oncogene 22, 8912–8923
33. Ea, C. K., Deng, L., Xia, Z. P., Pineda, G., and Chen, Z. J. (2006) Mol. Cell 22, 245–257
34. Kray, A. E., Carter, R. S., Pennington, K. N., Gomez, R. J., Sanders, I. E., Llanes, J. M., Khan, W. N., Ballard, D. W., and Wadzinski, B. E. (2005) J. Biol. Chem. 280, 35974–35982
35. Filipio-Santos, O., Bustamante, J., Havermamp, K. M., Vinolo, E., Ku, C. L., Puel, A., Frucht, D. M., Christel, K., von Bernuth, H., Jouanguy, E., Feinberg, J., Durandy, A., Seunach, B., Chappier, A., Vogt, G., de Beauchef, L., Fieschi, C., Picard, C., Garfa, M., Chemlili, J., Bejaoui, M., Tsolia, M. N., Kutukculer, N., Pellen, A., Notarangelo, L., Bodemer, C., Geissmann, F., Israel, A., Veron, M., Knackstedt, M., Barbouche, R., Abel, L., Magdork, K., Gendre, D., Agou, F., Holland, S. M., and Casanova, J. L. (2006) J. Exp. Med. 203, 1745–1759