The NF-κB pathway is an important control point in the immune and inflammatory response. One of the critical events in the activation of this pathway is the stimulation of the IκB kinases (IKKs) by cytokines such as tumor necrosis factor-α and interleukin-1. Although the mechanisms that modulate IKK activation have been studied in detail, much less is known about the processes that down-regulate its activity following cytokine treatment. In this study, we utilized biochemical fractionation and mass spectrometry to demonstrate that protein phosphatase 2Cβ (PP2Cβ) can associate with the IKK complex. PP2Cβ association with the IKK complex led to the dephosphorylation of IKKβ and decreased its kinase activity. The binding of PP2Cβ to IKKβ was decreased at early times post-tumor necrosis factor-α treatment, and it was restored at later times following treatment with this cytokine. Experiments utilizing siRNA directed against PP2Cβ demonstrated an in vivo role for this phosphatase in decreasing IKK activity at late times following cytokine treatment. These studies are consistent with the ability of PP2Cβ to down-regulate cytokine-induced NF-κB activation by altering IKK activity.

The NF-κB pathway is a critical regulator of the cellular response to a variety of stimuli including cytokines such as TNFα and interleukin-1, bacterial and viral infection, and double-stranded RNA (1–7). Cytokines lead to a rapid increase in the activity of the IκB kinases, and this is followed by a subsequent decrease in the activity of these kinases, suggesting both positive and negative regulation of the NF-κB pathway. A better understanding of the NF-κB pathway will be important in defining how these factors modulate the host immune and inflammatory response and prevent apoptosis (1–7).

Although IKKα and IKKβ have a similar domain structure (7–12), IKKβ is at least 20-fold more active in the phosphorylation of the IκB proteins as compared with IKKα (9, 14, 23, 24). Studies using fibroblasts isolated from IKKα (25, 26), and IKKβ (27) knock-out mice confirm that IKKβ is the dominant kinase in regulating NF-κB activity. Activation of these kinases is associated with increased phosphorylation of serine residues in their activation loop at positions 176 and 180 in IKKα and 177 and 181 in IKKβ (9, 28). Mutation of these serine residues to alanine markedly decreases IKK activity, whereas replacement of these serine residues with glutamates results in the generation of constitutively active kinases (9). Both increased autoprophosphorylation and phosphorylation by upstream MAP3 kinases such as NF-κB-inducing kinase (NIK), TAK1, and MEKK1 are probably important in regulating IKK activity (1–7).

Although a number of studies have been reported on the mechanisms that lead to IKK activation, much less is known about the factors such as phosphatases that may down-regulate its activity. Previous studies suggest that the phosphatases PP2A and PP2B can negatively regulate the NF-κB pathway (29–32). However, the identity of phosphatases that control IKK activity remains to be determined. Four classes of serine/threonine phosphatases have been categorized according to their substrate specificity, divergent cation requirement, and sensitivity to inhibitors. PP1, PP2A, and PP2B (calcineurin) have ~40% amino acid identity in their catalytic domains, whereas PP2C does not share significant sequence homology (33). PP1, PP2A, and PP2B (calcineurin) are present in oligomeric complexes associated with their regulatory subunits and are sensitive to the phosphatase inhibitor okadaic acid. In contrast, PP2C is active as a monomer and is insensitive to okadaic acid.

In this study, we present evidence that PP2Cβ can associate with the IKK complex to result in IKKβ dephosphorylation and
reductions in its kinase activity. PP2Cβ-mediated reductions in IKKβ activity were also associated with decreases in NF-κB activity. These results suggest that PP2Cβ may down-regulate the NF-κB pathway at late times following cytokine stimulation.

EXPERIMENTAL PROCEDURES

Analysis of IKK-associated Proteins—CMV expression vectors encoding FLAG-tagged IKKα/NEMO and Myc-tagged IKKβ were transfected into 293 cells. At 48 h post-transfection, the cells were harvested and homogenized in Tris-buffered saline (50 mM Tris·HCl, pH 7.4, and 250 mM NaCl). After centrifugation at 12,000 × g for 10 min, the supernatant was applied to the M2 FLAG affinity gel column (Sigma), and the bound FLAG-tagged proteins were washed extensively with Tris-buffered saline and eluted with FLAG peptide (Sigma). The eluted proteins were then dialyzed against buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol).

The affinity-purified FLAG-IKKα/NEMO and associated proteins were precipitated with trichloroacetic acid and resuspended in 100 mM ammonium bicarbonate containing 5% acetonitrile. The purified proteins were reduced, alkylated with dithiothreitol and iodoacetamide, and digested with trypsin. The peptide mixture was loaded onto an on-line capillary HPLC system (Waters, Milford, MA), equilibrated in 0.5% acetic acid, and the peptides were eluted using a linear gradient of 0–40% acetonitrile over 60 min followed by 40–60% over 10 min at a flow rate of 0.3 ml/min. The eluted peptides were analyzed using an LCQ-DECA ion trap mass spectrometer (Finnigan, San Jose, CA) (34). All tandem spectra were searched against the University of Washington human data base using the SEQUEST algorithm (35). Data processing of the SEQUEST files to identify proteins associated with IKKα/NEMO was then performed (36).

DNA Constructs—The human PP2Cβ cDNA was isolated from total HeLa RNA followed by reverse transcriptase-PCR using a SuperScript kit (Invitrogen) as suggested by the manufacturer’s protocol. The primers used for the cloning of the PP2Cβ cDNA were 5′-GTCGGCAAGCT- TACTAGTTGGAATACCC-3′ and 5′-GGCTTAGTCTGATTGATGAC- CATATTCTTACCACATCCT-3′. The resulting PCR product was cloned into the HindIII and XbaI cloning sites of the pCMV-FLAG and pCMV-Myc expression vectors and confirmed by DNA sequencing. The resulting constructs were used for the cloning of the PP2Cβ protein, coding amino acids 381–480 of PP2Cβ was cloned into the HindIII and XbaI cloning sites of the pCMV-FLAG and pCMV-Myc expression vectors and confirmed by DNA sequencing. FLAG-tagged wild type IKKα, IKKβ, IKKβ/NEMO, and the constitutively active IKKβ Ser-Ser → Glu-Glu construct (0.5 μg) were transfected either alone or in the presence of Myc-tagged wild type or Arg → Gly mutant of PP2Cβ (3.0 μg) into 293T cells and harvested 30 h post-transfection. Extracts (300 μg) in PD buffer were immunoprecipitated overnight at 4°C with 1–2 μg of FLAG antibody, followed by the addition of protein A-agarose for 1–3 h at 4°C, and extensively washed with PD buffer. In vitro kinase assays were performed for 20 min at 30°C in kinase buffer containing 1.0 mM dithiothreitol, 10 μM ATP with 10 μCi of [γ-32P]ATP and the GST-IκBα substrate (5.0 μg) followed by analysis by SDS-PAGE and autoradiography.

To assay the ability of PP2Cβ to dephosphorylate IKKβ, FLAG-IKKβ protein was immunoprecipitated from extracts with the FLAG antibody, and autophosphorylation assays were carried out in kinase buffer containing [γ-32P]ATP. The radiolabeled IKKβ protein was washed extensively and incubated with or without FLAG antibody column-purified wild type or Arg → Gly mutant of PP2Cβ at 30°C for 30 min followed by SDS-PAGE and autoradiography.

In Vivo Phosphorylation Assay—HeLa cells at 60% confluence were grown in Dulbecco’s modified Eagle’s medium lacking phosphate (Invitrogen) in the presence of 50 μM of [32P]orthophosphate (5.0 μCi/ml) (PerkinElmer Life Sciences) for 4 h. The cells were then treated with TNFα (10 ng/ml) (Roche Applied Science) for the indicated times and harvested in PD buffer. The extracts (200 μl) were immunoprecipitated with antibody directed against IKKα/β, and the radiolabeled IKKβ proteins were resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

Quantitative Real Time PCR—Quantitative PCR was utilized to evaluate the efficiency of siRNA-mediated knock-down of PP2Cβ (40). cDNAs were prepared from the control and RNAi-transfected HeLa cells using primers for PP2Cβ forward (5′-CTACCGCACAATCTTCTG- GAGGAG-3′) and reverse (5′-TCGAAGAAGTAGCTGTGGCAG-3′) and 18 S RNA forward (5′-AGGATTGACGGAGGGCAG-3′) and reverse (5′-GAGCTTGAAAGGGCATCA-3′). Each PCR was carried out in triplicate in a 20-μl volume using Syber Green Mastermix (Applied Biosystems) in the ABI Prism 7700 Sequence Detection System (40). Quantitation of PP2Cβ mRNA levels was determined using the ABI dissociation curve and normalized to the amount of 18 S RNA present in each sample.

RESULTS

PP2Cβ Is Associated with the IKK Complex—First, we investigated whether proteins in addition to IKKα, IKKβ, IKKγ/NEMO, and Hsp90/Cdc37 were associated with the IKK complex (8–13, 15–17). For these studies, 293 cells were cotransfected with CMV expression vectors encoding FLAG-tagged IKKγ/NEMO and Myc-tagged IKKβ, and extracts were prepared. The FLAG-tagged IKKγ/NEMO was then isolated...
using FLAG affinity chromatography. After extensive washing of the column to remove nonspecific associated proteins, the remaining proteins were eluted with FLAG peptide and subjected to trypsin digestion followed by HPLC and LCQ-DECA ion trap mass spectrometry. In addition to IKKa and IKKβ, three peptides corresponding to the serine/threonine protein phosphatase 2Cβ (PP2Cβ) were identified. These results suggested that PP2Cβ could potentially associate with IKKγ/NEMO and IKKβ (Table I).

Characterization of IKK Interactions with PP2Cβ—To confirm the association of PP2Cβ with the IKK complex, communoprecipitation experiments were performed utilizing G418-resistant 293 cell lines that either stably expressed FLAG-tagged PP2Cβ or did not express this epitope-tagged protein. Western blot analysis with the FLAG antibody confirmed the presence of FLAG-tagged PP2Cβ in the stably transfected cell line and its absence in the control cell line (Fig. 1A, lanes 1 and 2).

To characterize the association of PP2Cβ with IKKα, IKKβ, and IKKγ/NEMO (Fig. 1B), extracts prepared from the 293 cells stably expressing FLAG-tagged PP2Cβ were immunoprecipitated with either normal mouse IgG (Fig. 1B, lane 1) or the FLAG antibody (Fig. 1B, lane 2) followed by Western blot analysis with the IKK antibodies. PP2Cβ was associated with all three subunits of the IKK complex including IKKα (Fig. 1B, top), IKKβ (Fig. 1B, middle), and IKKγ/NEMO (Fig. 1B, bottom). There was no association of PP2Cβ with these proteins when the extracts were immunoprecipitated with normal mouse IgG (Fig. 1B, lane 1). To further define the association of PP2Cβ with components of the IKK complex, 293T cells were transfected with a CMV expression vector alone (Fig. 1C, lane 1) or CMV vectors encoding Myc-tagged PP2Cβ with either FLAG-tagged IKKα (Fig. 1C, lane 2), IKKβ (Fig. 1C, lane 3), or IKKγ/NEMO (Fig. 1C, lane 4). Following immunoprecipitation of the FLAG-tagged IKKαs with either the FLAG antibody (Fig. 1C, top) or normal mouse IgG (Fig. 1C, middle), Western blot analysis was performed with the Myc antibody to detect Myc-PP2Cβ. These results demonstrated that there were similar interactions of PP2Cβ with IKKα, IKKβ, and IKKγ/NEMO. Western blot analysis demonstrated similar levels of expression of IKKα, IKKβ, IKKγ/NEMO, and PP2Cβ (Fig. 1C, bottom).

To analyze the association of the endogenous PP2Cβ with components of the IKK complex (Fig. 1D), extracts prepared from the HeLa cells were immunoprecipitated with either normal mouse IgG (Fig. 1D, lane 1) or a murine polyclonal antibody directed against human PP2Cβ (Fig. 1D, lane 2) followed by Western blot analysis with the antibodies directed against components of the IKK complex. PP2Cβ was associated with components of the IKK complex including IKKα (Fig. 1D, top), IKKβ (Fig. 1D, middle), and IKKγ/NEMO (Fig. 1D, bottom). There was no association of PP2Cβ with these proteins when the extracts were immunoprecipitated with normal mouse IgG (Fig. 1D, lane 1, top). The murine antibody directed against PP2Cβ reacted with both endogenous and transiently overexpressed FLAG-PP2Cβ (Fig. 1D, bottom). These results demonstrate that both endogenous and overexpressed PP2Cβ interacts with one or more components of the IKK complex.

PP2Cβ Dephosphorylates IKKβ in Vivo and in Vitro—Cyto kinase treatment increases the activity of the MAP3 kinase TAK1, which has been demonstrated to function as an upstream kinase that stimulates IKKβ and activates the NF-κB pathway (40, 41). PP2Cβ has previously been demonstrated to associate with TAK1 and dephosphorylate this kinase to result in reduced stress-activated protein kinase activity (41). Since IKKβ is the critical kinase involved in cytokine-induced NF-κB activation and can associate either directly or indirectly with PP2Cβ, we addressed whether PP2Cβ might dephosphorylate IKKβ and thus reduce NF-κB activity.

HeLa cells were transfected with either wild type or the Arg→Gly mutant of PP2Cβ and labeled in vivo with [32P]orthophosphate either in the presence or absence of TNFα (Fig. 2A). Following immunoprecipitation of endogenous [32P]-labeled IKKβ, SDS-PAGE and autoradiography were performed. There was no detectable IKKβ phosphorylation in untreated cells (Fig. 2A, lanes 1–3, top), but a marked increase in IKKβ and likely IKKα phosphorylation...
Western blot analysis was performed with these unlabeled extracts using HA and Myc antibodies (bottom unlabeled cellular extracts demonstrated similar levels of endogenous FLAG-IKK or treated with TNFα). 293T cells were transfected with a CMV expression vector alone (lanes 1 and 4) or with vectors encoding Myc-tagged wild type (2.0 μg), (lanes 2 and 5) or Arg→Gly (R/G) mutant of PP2Cβ (lanes 3 and 6). Cells were incubated with serum-free Dulbecco’s modified Eagle’s medium lacking phosphate followed by the addition of [32P]orthophosphate for 4 h and then either untreated (lanes 1–3) or treated with TNFα (10 ng/ml) (lanes 4–6) for 15 min. Following immunoprecipitation (IP) of the extracts with an IKKα/β antibody, SDS-PAGE and autoradiography were then performed (top). Western blot analysis with the IKKα/β antibody (middle) or a Myc antibody (bottom) was also performed with a portion of these unlabeled samples. B, 293T cells were transfected with a CMV expression vector alone (2.5 μg) (lane 1) or CMV expression vectors encoding HA-IKKβ (0.5 μg) or HA-MEKK1 (0.5 μg) (lanes 2 and 5) in the absence and presence of wild type or Arg→Gly mutant of PP2Cβ (2.0 μg) (lanes 3, 4, 6, and 7). Samples were 32P-labeled in vivo, immunoprecipitated with the HA antibody, and processed as in A (top). Western blot analysis was performed with these unlabeled extracts using HA and Myc antibodies (bottom). C, 293T cells were transfected with a FLAG-IKKβ expression vector, and this protein was immunoprecipitated with a FLAG antibody and incubated in the presence of [γ-32P]ATP for 20 min at 30 °C. The phosphorylated IKKβ protein was incubated with either phosphatase buffer alone (lane 1) or phosphatase buffer containing the FLAG affinity column-purified wild type (lane 2) or Arg→Gly mutant of PP2Cβ (lane 3). The samples were separated by SDS-PAGE and visualized by autoradiography (top). Extracts containing IKKβ and the purified FLAG-tagged wild type or Arg→Gly mutant of PP2Cβ were also analyzed by Western blot analysis with the FLAG antibody (bottom).

was noted following TNFα treatment (Fig. 2A, lane 4, top). The phosphorylation of IKKβ and probably IKKα was significantly reduced in TNFα-treated cells transfected with wild type PP2Cβ (Fig. 2A, lane 5, top) but not with the PP2Cβ Arg→Gly (R/G) mutant (Fig. 2A, lane 6, top). Western blot analysis of a portion of the unlabeled cellular extracts demonstrated similar levels of endogenous IKKα/β and the transfected Myc-tagged PP2Cβ proteins (Fig. 2A, bottom).

The ability of PP2Cβ to dephosphorylate another kinase such as MEK1, which has been implicated in activating the NF-κB pathway, was next addressed. Expression vectors encoding HA-tagged IKKβ or MEK1 alone or together with either Myc-tagged wild type or the Arg→Gly mutant of PP2Cβ were transfected into 293T cells followed by in vivo labeling with [32P]orthophosphate. Immunoprecipitation of HA-tagged IKKβ demonstrated that its phosphorylation was markedly reduced in the presence of wild type PP2Cβ (Fig. 2B, lanes 2 and 3, top) but not in the presence of PP2Cβ Arg→Gly mutant (Fig. 2B, lane 4, top). There were no changes in the phosphorylation of HA-tagged MEK1 in either the presence of wild type or the Arg→Gly mutant of PP2Cβ (Fig. 2B, lanes 6 and 7, top). Western blot analysis demonstrated similar expression levels of epitope-tagged IKKβ, MEK1, and PP2Cβ (Fig. 2B, bottom). Similar studies indicated that there was no effect of PP2Cβ on the in vivo phosphorylation of NIK, another kinase implicated in activating the NF-κB pathway (data not shown).

Finally, we addressed whether PP2Cβ could dephosphorylate IKKβ in in vitro assays. FLAG-tagged IKKβ expressed in 293T cells was immunoprecipitated and autophosphorylated in vitro by incubation with [γ-32P]ATP. The 32P-labeled IKKβ protein was then incubated with either FLAG affinity-purified wild type or the Arg→Gly mutant of PP2Cβ and analyzed following SDS-PAGE and autoradiography (Fig. 2C). There was markedly reduced phosphorylation of IKKβ in the presence of wild type PP2Cβ (Fig. 2C, lane 2, top) but not in the presence of the PP2Cβ Arg→Gly mutant (Fig. 2C, lane 3, top). Western blot analysis demonstrated similar expression of IKKβ and PP2Cβ (Fig. 2C, bottom). Taken together, both in vivo and in vitro assays demonstrated that PP2Cβ could dephosphorylate IKKβ.

**PP2Cβ Inhibits IKKβ Kinase Activity**—Treatment with cytokines such as TNFα and interleukin-1 leads to increases in the phosphorylation of serine residues 177 and 181 in the IKKβ activation loop to stimulate its kinase activity (28, 38). Mutation of serine residues 177 and 181 to alanine reduces IKKβ kinase activity, whereas substitution of these residues with glutamates, which mimics phosphorylation, results in a constitutively active kinase (42). Next we addressed whether PP2Cβ could dephosphorylate serine residues 177 and 181 in IKKβ to decrease its kinase activity. For these studies, 293T cells were transfected with FLAG-tagged wild type IKKβ (Fig. 3A) or the constitutively active IKKβ Ser-Ser→Glu-Glu (SS/EE) mutant (Fig. 3B) either alone or together with the Myc-tagged wild type or Arg→Gly mutant of PP2Cβ. FLAG-tagged IKKβ was immunoprecipitated from these extracts and assayed in in vitro kinase assays with a GST-IκBα substrate. The kinase activity of wild type IKKβ was markedly reduced in the presence of wild type PP2Cβ (Fig. 3A, lane 3, top) but not by the PP2Cβ Arg→Gly mutant (Fig. 3A, lane 4, top). In contrast, the kinase activity of IKKβ Ser-Ser→Glu-Glu mutant was not significantly altered in the presence of either wild type or the Arg→Gly mutant of PP2Cβ (Fig. 3B, lanes 2–4, top). Western blot analysis demonstrated similar levels of expression of IKKβ and the IKKβ Ser-Ser→Glu-Glu mutant in addition to the wild type and Arg→Gly mutant of PP2Cβ (Fig. 3, A and B, lower panels). These results suggested that the PP2Cβ-mediated reductions in IKKβ kinase activity could potentially be explained by its ability to dephosphorylate serine residues in the IKKβ activation loop.
Fig. 3. PP2Cβ inhibits IKKβ activity. A and B, 293 cells were transfected with a CMV expression vector alone (3.0 μg) (lane 1) or CMV expression vectors encoding either FLAG-tagged wild type IKKβ (A) or a constitutively active IKKβ Ser-Ser → Glu-Glu (SS/EE) mutant (B) (0.3 μg) either alone (lane 2) or together with Myc-tagged wild type PP2Cβ (3.0 μg) (lane 3) or the PP2Cβ Arg → Gly (R/G) mutant (3.0 μg) (lane 4). Extracts (200 μg) were immunoprecipitated (IP) with FLAG antibody, followed by in vitro kinase assays with a GST-IκBα substrate and SDS-PAGE and autoradiography (top). Extracts were also analyzed by Western blot analysis for IKKβ and PP2Cβ expression (bottom).

siRNA Directed against PP2Cβ Increases TNFα-induced IKK Activity—In order to address whether endogenous PP2Cβ is involved in regulating TNFα-induced IKK activity, siRNA directed against PP2Cβ was utilized to determine whether it altered IKK activity following TNFα treatment. HeLa cells were transfected with Oligofectamine alone or Oligofectamine containing siRNAs directed against either PP2Cβ or the HTLV-1 tax gene as a control. At 48 h post-transfection, cells were treated with TNFα and harvested at 0, 5, 15, 30, 60, or 120 min. To determine the ability of siRNA to reduce PP2Cβ mRNA, RNA prepared from these cells was analyzed by quantitative real time PCR (Fig. 4A). Real time PCR analysis demonstrated an ~70% inhibition of PP2Cβ mRNA levels in the presence of PP2Cβ siRNA (Fig. 4A, lanes 7–12) as compared with cells transfected with Oligofectamine alone or Tax siRNA (Fig. 4A, lanes 1–6 and 13–18). These results suggested that siRNA transfection could efficiently decrease the amount of PP2Cβ mRNA in HeLa cells.

Next we determined whether PP2Cβ siRNA altered the kinetics of TNFα-mediated increases in IKK activity (Fig. 4B). Endogenous IKK proteins were immunoprecipitated and assayed using in vitro kinase assays with a GST-IκBα substrate. TNFα treatment for 5 and 15 min markedly increased IKKβ activity in control and PP2Cβ- and Tax siRNA-transfected cells (Fig. 4B, lanes 2, 3, 8, 9, 14, and 15, top). However, at later times post-TNFα treatment (30–120 min), there was increased IKKβ activity in the PP2Cβ siRNA-treated cells (Fig. 4B, lanes 10–12, top) as compared with that seen in the control and Tax siRNA-transfected cells (Fig. 4B, lanes 4–6 and 16–18, top). Western blot analysis demonstrated similar levels of IKKα and IKKβ expression (Fig. 4B, bottom).

The extracts from this experiment were also analyzed for the levels of phospho-IκBα, IκBα, endogenous PP2Cβ, IKKβ, and actin (Fig. 4C). There was enhanced phosphorylation of IκBα at 5 min post-TNFα treatment in control and PP2Cβ and Tax siRNA-treated cells (Fig. 4C, lanes 2, 8, and 14, top). At 15 min post-TNFα treatment, phospho-IκBα levels were decreased in the control as well as in the siRNA-treated cells (Fig. 4C, lanes 3, 9, and 15, top). However, there was no detectable phosphorylation of IκBα in the extracts prepared from the control and Tax siRNA-transfected cells between 30 and 120 min post-TNFα treatment (Fig. 4C, lanes 4–6 and 16–18, top). In contrast, there were significant levels of phospho-IκBα in the extracts prepared from the PP2Cβ siRNA-transfected cells at these times (Fig. 4C, lanes 10–12, top). Total IκBα levels were decreased at 15 min post-TNFα treatment in extracts prepared from both the control and the siRNA-treated cells and increased by 60 min post-TNFα treatment (Fig. 4C, middle). A slight increase in IκBα levels at 60 min post-TNFα treatment was consistently seen in extracts prepared from the PP2Cβ siRNA-treated cells as compared with that seen in the control and Tax siRNA-treated cells. The PP2Cβ siRNA resulted in a 70% reduction in endogenous PP2Cβ levels (Fig. 4C, lanes 7–12, middle) as compared with control and Tax siRNA-transfected cells (Fig. 4C, lanes 1–6 and 13–18, middle). Similar levels of IKKβ and actin were noted (Fig. 4C, bottom). These results, which were seen in three independent experiments, suggested that siRNA directed against PP2Cβ could result in a prolonged increase in TNFα-mediated IKKβ activity.

PP2Cβ Decreases NF-κB-directed Gene Expression—Next we addressed whether overexpression of PP2Cβ altered the kinetics of TNFα-mediated IκBα degradation and NF-κB-regulated gene expression. Parental cells and 293 cells stably expressing FLAG-tagged PP2Cβ were treated with TNFα for various times, and extracts were analyzed by Western blot for changes in the levels of phospho-IκBα and total IκBα levels. The cells stably expressing the FLAG-tagged PP2Cβ demonstrated slightly reduced levels of phospho-IκBα at both early (Fig. 5A, lanes 2 and 9, top) and later times (Fig. 5A, lanes 5–7 and 12–14, top) post-TNFα treatment as compared with parental cells. The cells stably expressing FLAG-tagged PP2Cβ also exhibited reduced degradation of IκBα at both early and late times post-TNFα treatment (Fig. 5A, lanes 3–7 and 10–14, middle). Western blot analysis demonstrated similar expression of IKKα, IKKβ, FLAG-PP2Cβ, and actin. These results indicated that overexpression of PP2Cβ reduced phospho-IκBα levels and IκBα degradation and was also associated with decreased resynthesis of IκBα.

To determine whether PP2Cβ overexpression altered NF-κB-mediated gene expression, the parental cells and cells stably expressing FLAG-tagged PP2Cβ were cotransfected with an NF-κB luciferase reporter and a Rous sarcoma virus-β-galactosidase expression vector. At 18 h post-transfection, the cells were either untreated or treated with TNFα for 6 h prior to assaying luciferase activity (Fig. 5B). NF-κB reporter activity was increased in both TNFα-treated parental cells and cells stably expressing FLAG-tagged PP2Cβ (Fig. 5B). However, TNFα-mediated increases in NF-κB activity were reduced by 60% in cells stably expressing FLAG-tagged PP2Cβ as compared with parental cells. These experiments were repeated three times, and the average of triplicate samples is shown. These studies indicated that PP2Cβ decreases TNFα-mediated NF-κB activation.

Finally, we addressed whether the interactions of FLAG-PP2Cβ and IKKβ were altered following TNFα stimulation. Parental cells and cells stably expressing FLAG-tagged PP2Cβ were treated with TNFα for various times, and extracts were prepared and immunoprecipitated with IKKα/β antibody or normal rabbit IgG followed by Western blot analysis with FLAG antibody (Fig. 5C). Significant interactions between FLAG-PP2Cβ and IKKα/β were demonstrated in the absence of TNFα (Fig. 5C, lane 6, top). However, these interactions were
and the PP2C was utilized to analyze the expression of min. Total RNA was prepared from each extracts from H9251 also analyzed by Western blot for IKK shown, with the average of triplicate samples is demonstrated similar expression of FLAG-PP2C (Fig. 5c, top and middle). Western blot analysis demonstrated similar expression of FLAG-PP2C, IKKα, and IKKβ (Fig. 5c, bottom) and TNFα-mediated IkBo degradation (Fig. 5c, bottom). These results indicated that the association of PP2Cβ and IKKαβ was regulated by TNFα treatment and that the kinetics of this association appeared to correlate with changes in IKK activity.

**DISCUSSION**

In this study, we present evidence suggesting that PP2Cβ negatively regulates the NF-κB pathway post-TNFα treatment by dephosphorylating IKKβ and thus reducing its kinase activity. Several lines of evidence substantiate these conclusions. First, we found that both endogenous and overexpressed PP2Cβ interacted with the IKK complex. Second, we observed that PP2Cβ dephosphorylated both endogenous and transiently expressed IKKβ but not other kinases including MEKK1 and NIK. Third, PP2Cβ reduced TNFα-mediated increases in wild type IKKβ activity while not changing the activity of the constitutively active IKKβ Ser-Ser → Glu-Glu mutant. Fourth, siRNA directed against PP2Cβ, but not the control tax gene, prolonged cytokine-induced IKKβ activity to result in increased phospho-IκBα levels. Finally, overexpression of PP2Cβ reduced TNFα-mediated IkBo degradation and resynthesis as well as the levels of phospho-IκBα, leading to decreases in NF-κB reporter activity. Collectively, these data are consistent with a role for PP2Cβ in down-regulating NF-κB activity at late times post-TNFα treatment by associating with the IKKαβ complex and dephosphorylating IKKβ.

IKKβ exhibits maximum kinase activity within 5 min following cytokine stimulation, and its activity is decreased by 30–60 min post-TNFα stimulation. The mechanism by which IKKβ activity is decreased following cytokine stimulation has not been totally elucidated. In the current study, mass spectrometry and protein interaction studies demonstrated that IKK subunits can associate with the serine/threonine phosphatase PP2Cβ. The fact that PP2Cβ alters the activity of wild type IKKβ but not the constitutive IKKβ Ser-Ser → Glu-Glu mutant suggests that PP2Cβ probably acts on IKK itself or an upstream kinase to down-regulate the NF-κB pathway. PP2Cβ has been reported to dephosphorylate the upstream kinase TAK1, which stimulates IKK activity (40, 41). However, the effects of PP2Cβ were relatively specific in that this phospha-
that have been reported to activate the NF-κB pathway (4), and 240 (lanes 7 and 14) min. Western blot analysis was performed on extracts prepared from these cells using antibodies directed against phospho-IκBα (top) or IκBα, IKKα, IKKβ, the FLAG epitope, and actin (bottom). B, parental cells or cells stably expressing FLAG-tagged PP2Cβ were cotransfected with an NF-κB luciferase reporter vector (0.2 μg) and a Rous sarcoma virus-β-galactosidase expression vector (0.2 μg). At 18 h post-transfection, the cells were either left untreated or treated with TNFα (5 ng/ml) and harvested 6 h later. Luciferase and β-galactosidase activity were then determined. These experiments were repeated three times, and the average of triplicate samples is shown with error bars denoting the S.E.

Other serine/threonine protein phosphatases including PP2A and PP2C have been implicated in the negative regulation of signaling pathways including stress-activated protein kinase (41, 47, 50, 51). For example, the ectopic expression of mouse PP2Cα and PP2Cβ-1 can inhibit the stress-activated MKK3/6-p38 and MKK4/7-c-Jun N-terminal kinase pathways probably via dephosphorylation of TAK1, but they do not alter the mitogen-activated MKK1-ERK1 pathway (41, 47, 50, 51). For example, the ectopic expression of mouse PP2Cα and PP2Cβ-1 can inhibit the stress-activated MKK3/6-p38 and MKK4/7-c-Jun N-terminal kinase pathways probably via dephosphorylation of TAK1, but they do not alter the mitogen-activated MKK1-ERK1 pathway (41, 47, 50, 51). PP2Cα and PP2Cβ have also been demonstrated to dephosphorylate the cyclin-dependent kinases Cdk2 and Cdk6 and inhibit their activity (52). However, PP2C family members do not always function as negative regulators of signaling pathways. For example, PP2Cα can function as a positive regulator of Wnt signaling by dephosphorylating Axin (53). Thus, the PP2C family members can play both positive and negative roles in regulating signal transduction pathways.

There are likely multiple mechanisms that can down-regulate IKK activity following cytokine treatment. First, phosphatases such as PP2Cβ and PP2Cε may function to inhibit the activity of upstream kinases such as TAK1 that stimulate IKK.
Second, phosphatases such as PP2A can interact with IKK\(\gamma\)/NEMO to inhibit IKK activity (8, 30). Third, IKK can undergo autophosphorylation at multiple sites in its carboxyl terminus to down-regulate its activity (28). Finally, we demonstrate in this study that PP2C\(\beta\) associates with the IKK complex and leads to reduced IKK activity at late times following TNF\(\alpha\) treatment. These results suggest that multiple mechanisms, including phosphatases such as PP2C\(\beta\), are probably involved in both maintaining basal IKK activity and down-regulating IKK activity following cytokine treatment.

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