Regulation of IkB Kinase Complex by Phosphorylation of γ-Binding Domain of IkB Kinase β by Polo-like Kinase 1

Received for publication, August 13, 2008, and in revised form, October 24, 2008. Published, JBC Papers in Press, October 27, 2008, DOI 10.1074/jbc.M806258200

Tomoyasu Higashimoto1, Nymph Chan5, Yung-Kang Lee5, and Ebrahim Zandi†1
From the 4Department of Molecular Microbiology and Immunology and the 5Department of Pathology, University of Southern California/Norris Comprehensive Cancer Center, Keck School of Medicine and the 6Department of Molecular Pharmacology and Toxicology, School of Pharmacy, University of Southern California, Los Angeles, California 90089-9176

IkB kinase (IKK) complex is a key regulator of NF-κB pathways. Signal-induced interaction of the IKKγ (NEMO) subunit with the C-terminal IKKγ/NEMO-binding domain (γBD) of IKKβ is an essential interaction for IKK regulation. Underlying regulatory mechanism(s) of this interaction are not known. Phosphorylation of γBD has been suggested to play a regulatory role for IKK activation. However, a kinase that phosphorylates γBD has not been identified. In this study, we used a C-terminal fragment of IKKB as substrate and purified Polo-like kinase 1 (Plk1) from HeLa cell extracts by standard chromatography as a γBD kinase. Plk1 phosphorylates serines 733, 740, and 750 in the γBD of IKKβ in vitro. Phosphorylating γBD with Plk1 decreased its affinity for IKKγ in pulldown assay. We generated phosphoantibodies against serine 740 and showed that γBD is phosphorylated in vivo. Expressing a constitutively active Plk1 in mammalian cells reduced tumor necrosis factor (TNF)-induced IKK activation, resulting in decreased phosphorylation of endogenous IkBε and reduced NF-κB activation. To activate endogenous Plk1, cells were treated with nocodazole, which reduced TNF-induced IKK activation, and increased the phosphorylation of γBD. Knocking down Plk1 in mammalian cells restored TNF-induced IKK activation in nocodazole-treated cells. Activation of Plk1 inhibited TNF-induced expression of cyclin D1. In cells in which Plk1 was knocked down, TNFα increased expression of cyclin D1 and the proportion of cells in the S phase of the cell cycle. Taken together, this study shows that phosphorylation regulates the interaction of γBD of IKKβ with IKKγ and therefore plays a critical role for IKK activation. Moreover, we identify Plk1 as a γBD kinase, which negatively regulates TNF-induced IKK activation and cyclin D1 expression, thereby affecting cell cycle regulation. Untimely activation of cyclin D1 by TNFα can provide a potential mechanism for an involvement of TNFα in inflammation-induced cancer.

This work was supported, in whole or in part, by National Institutes of Health Grant R01 MG05325 (to E. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

1 2001 PEW Scholar in Biomedical Sciences. To whom correspondence should be addressed: USC/Norris Comprehensive Cancer Center, 1441 Eastlake Ave., Norris 6429, Los Angeles, CA 90033. Tel.: 323-865-0644; Fax: 323-865-0645; E-mail: Zandi@usc.edu.

2 The abbreviations used are: IKK, IkB kinase complex; γBD, IKKγ/NEMO-binding domain; TNF, tumor necrosis factor; HLH, helix-loop-helix; DTT, dithiothreitol; siRNA, short interfering RNA; EMSA, electromobility shift assay; GST, glutathione S-transferase; HA, hemagglutinin; IL, interleukin; MEF, mouse embryo fibroblast.
autophosphorylated within the complex, and their phosphorylation did not prolong activation kinetics of the IKK complex in a null background of IKKβ in MEF (20). Using the yeast system and MEFβ−/−, we also showed that mimicking phosphorylation of serine 740 within γBD and serine 750 in IKKβ significantly reduced IKKγ-induced activation of the IKK complex (20). Thus, the phosphorylation events at the C terminus of IKKβ can be divided into the autophosphorylation of the 10 serines adjacent to the HLH domain and phosphorylation of the serines within and adjacent to the γBD. This conclusion is consistent with the May et al. findings (19) and suggests that phosphorylation of the γBD may play a role in signal-induced IKK regulation.

A third region of IKKβ that plays an essential role in signal-induced IKK regulation is the γBD (also called NEMO binding domain) (8, 19–21). May et al. (19) showed that a small conserved region containing the hexapeptide, LDWSWL, in the C terminus of IKKα and IKKβ is a site of interaction for IKKγ (γBD) and is critical for the activation of IKK by TNFα (19, 21). It is important to note that γBD is the immediate C terminus of the 10 serines discussed above.

Mutating serines 740 (within the γBD) and 750 (C-terminal to γBD) to glutamic acid significantly reduced activation of the IKK complex in an IKKγ-dependent manner in mammalian cells and in reconstituted IKK in yeast (8, 19, 20). This indicates that phosphorylation of the γBD and the adjacent serine 750 may play a role in IKK regulation. Recently, the cryo-crystal structure of the C terminus of IKKβ with the N terminus of IKKγ (NEMO) was solved, and it shows that phosphorylation of serine 740 could play an important role in determining the affinity of the interaction (22).

In this study we identify Plk1 as a γBD kinase, which negatively regulates IKK and inhibits TNF-induced expression of cyclin D1. We show that serine 740 of IKKβ is indeed phosphorylated in vivo, and it regulates the interaction of γBD of IKKβ with IKKγ, and therefore plays a critical role for IKK activation. The mechanism of inhibition of IKK by Plk1 provides a paradigm for how phosphorylation of γBD of IKKβ is involved in regulation of the IKK complex.

**MATERIALS AND METHODS**

**Cell Culture, Cell Transfection, and siRNA—COS-7 and HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum and maintained at 37 °C, 5% CO2, 1 × 106 cells were seeded into each well of a 6-well plate for transfection using Lipofectamine Plus reagent (Invitrogen) as recommended by the manufacturer. Cell transfection for siRNAs was done using Oligofectamine reagent (Invitrogen) as described previously (23). The sequence of three sense and antisense oligonucleotides targeting three different regions of human Plk1 are as follows: Plk1 number 1, sense (5’-GAUACCCCUCCUAAAUATT) and antisense (5’-UUAAUUAAGGAGGUGAUCTT); Plk1 number 2, sense (5’-CACAACAAAGUGCAUUAGATT) and antisense (5’-UCUAUUCGACUUUGGUGT); and Plk1 number 3, sense (5’-AGACCUACCCCGGAUAAATT) and antisense (5’-UGUACCGGAGGUAGCUATT). Cells were stimulated 48 h after transfection with 20 ng/ml TNFα or 10 ng/ml IL-1β unless otherwise stated. After stimulation, media were removed, and cells were washed with phosphate-buffered saline. Cells were lysed in lysis buffer, pH 7.6, containing the following: 1× all purpose buffer, 1% Triton X-100, 300 mM NaCl, 40 mM Tris, 40 mM NaF, 40 mM β-glycerophosphate, 1 mM Na3VO4, 5 mM metabisulfite, 10 mM benzamide, 2 mM EDTA, 1 mM EGTA, 10% glycerol, 20 µg/ml aprotinin, 2.5 µg/ml leupeptin, 8.5 µg/ml bestatin, 2 µg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 13 mM p-nitrophenyl phosphate, and 2 mM DTT.

**Purification and Identification of Polo-like Kinase 1—**

HeLa S3 cells were grown in suspension at the Norris Cancer Center Tissue Culture and Reagent Core. Untreated or TNFα (20 ng/ml for 10 min)-treated cells (for each case about 1010 cells) were harvested, and lysates in buffer A (20 mM Tris–HCl, 20 mM NaF, 20 mM β-glycerophosphate, 19 mM p-nitrophenyl phosphate, 500 µM Na3VO4, 2.5 mM metabisulfite, 5 mM benzamide, 1 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, pH 7.6) were supplemented with 2 µl DTT, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml bestatin, and 150 mM NaCl as described previously (5). The S-100 fractions were purified over a 70-ml Q-Sepharose FF column equilibrated with buffer A. After washing the column with buffer A, the column was eluted with a linear 0.15–1 M NaCl gradient using buffer B (buffer A plus 1 M NaCl). Flow-through fractions were pooled and applied to a HiPrep 16/10 heparin FF column (Amersham Biosciences). After washing with buffer A, the column was eluted with a linear 0.15–1 M NaCl gradient. Fractions with peak kinase activity were pooled and diluted 1:4 in buffer A and applied to 25 ml of CM-Sepharose FF (Amersham Biosciences) column. After the column was washed with buffer A, the column was eluted with a linear 0.1–1 M NaCl gradient. Peak fractions containing kinase activity were diluted 1:4 in phosphate buffer with 50 mM NaCl and 10% glycerol and loaded onto a 5-ml poly(U)-Sepharose 4B (Amersham Biosciences) column and washed with phosphate buffer with 50 mM NaCl and 10% glycerol. The column was eluted isocratically with phosphate buffer containing 570 mM NaCl and 10% glycerol. Fractions with kinase activity were pooled, diluted 1:4 in 50 mM phosphate buffer, and applied to a 1-ml substrate affinity column consisting of His-Myc-35C-WT cross-linked to CNBr-activated Sepharose 4B (GE Healthcare). The column was washed with 50 mM phosphate buffer containing 10% glycerol, and proteins were eluted with a linear 0–1 M NaCl gradient.

**Mass Spectrometry Identification of Plk1 and PAK4—**

Because the fraction containing the peak Plk activity from the substrate affinity column contained many polypeptides (see Fig. 1E), proteins were digested in solution by trypsin, and the peptides were identified by an on-line reverse phase chromatography with mass spectrometry at the Stanford Mass Spectrometry Core. Two protein kinases Plk1 and PAK4 were among the top 50 identified proteins.

**Plasmid Construction and Expression of IKKβ C-terminal Fragments—**

The C-terminal fragments, with or without the HLH domain, of IKKβ were amplified by PCR and cloned into the bacterial expression vector pET-Hel-N1 using Ncol and Notl cloning sites (24). In this vector, all constructs were fused in-frame at their C termi-
Regulation of IKK by Plk1

nus to six histidines and a Myc epitope. The fragments with the HLH domain contain IKKβ amino acid 588 to its C terminus. The 3SC fragments (lacking the HLH region) contain IKKβ amino acid 660 to its C-terminal end. IKKβ wild type and IKKβM10 (18) were used as PCR templates. The following PCR primers were used for PCR amplification of the HLH wild type and M10 mutant: forward primer 5'-AAACCATGGCTATCCAGAGCTTGCCGCAGC-3' and reverse primer 5'-AAGCGGCCGCTAGGCGGCTTCTCAGGCGCACTG-3'. To generate HLH-cS14A, cS12A, cS10L2A, and M10 was used as template and amplified with forward primer p1 in combination with reverse primers containing the corresponding serine to alanine mutations. The reverse primers are as follows: HLH-cS14A, 5'-AAGCGGCCGCTAGGCGGCTTCTCAGGCGCACTG-3'; HLH-cS12A, AAGCGGCCGCTAGGCGGCTTCTCAGGCGCACTG-3'; HLH-cS10L2A, AAGCGGCCGCTAGGCGGCTTCTCAGGCGCACTG-3'; HLH-cS10L2A, AAGCGGCCGCTAGGCGGCTTCTCAGGCGCACTG-3'. The underlined bases show the serine to alanine mutations. For amplification of the IKKδ C-terminal fragments lacking the HLH domain (3SC constructs), the forward primer 5'-AAAACCATGGAGATTGCTTGTAGCAAGGTCCGT-3' was combined with the same reverse primers used for HLH containing constructs. These fragments were expressed in Escherichia coli BL21(DE3)pLysS and purified by nickel-nitrilotriacetic acid-agarose chromatography (Qiagen).

Cloning of plk1 and PAK4 and in Vitro Transcription/Translation—After identification of Plk1 (GenBank™ accession number U01038) and PAK4 (GenBank™ accession number NM_005884), their corresponding cDNAs were amplified with forward primer p1 in combination with reverse primers used as PCR templates. The following PCR primers were used for HLH wild type and M10 restriction sites. The pBS-HA-WT-Plk1 and HA-PAK4 was used as template for site-directed mutagenesis using a similar method as described in the QuickChange site-directed mutagenesis kit (Stratagene).

Mammalian expression vectors were constructed for the Plk1 wild type and mutants and PAK4 by subcloning the corresponding cDNAs into the pRC-β-actin (5) and pRC-CMV (Invitrogen) vectors using HindIII and NotI restriction sites. The pBS-HA-WT-Plk1 and HA-PAK4 was used as template for in vitro transcription/translation using TnT (T3)-coupled reticulocyte lysate system (Promega).

Construction, Bacterial Expression, and Affinity Purification of Glutathione S-Transferase (GST) Fusion IKKβ—GST-IKKβ were constructed by digesting pRC-β-actin IKKβ wild type and S70A/S750A mutant by BstXI (New England Biolabs) and blunt ending the 5' end. The 3' end was digested with NotI (New England Biolabs). HA IKKβ insert was then cloned into pGEX-KG (Amersham Biosciences) plasmid and was digested with SmaI and XbaI. The GST fusion proteins were expressed in E. coli BL21(DE3) pLys using 1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Cells were spun down and resuspended in phosphate-buffered saline containing 1% (v/v) Triton X-100. Cells were sonicated and centrifuged for 20 min at 20,000 relative centrifugal force. Supernatant was incubated with GST-Sepharose (Amersham Biosciences) and eluted according to manufacturer's protocol.

In Vitro Kinase Assay—Cell lysates (50–100 μg) were immunoprecipitated with rabbit polyclonal IKKγ (IKK kinase activity), goat polyclonal Plk1(for Plk1 activity) (Santa Cruz Biotechnology), JNK1 (for JNK activity) (BD Biosciences), or monoclonal HA antibody (Tissue Culture and Reagent Core, University of Southern California) and protein G-Sepharose (Amersham Biosciences) in lysis buffer. The precipitates were washed once with lysis buffer and once with 1× kinase buffer containing the following: 20 mM Tris and 20 mM MgCl₂, pH 7.6. The precipitates were then incubated with kinase mixture containing 2 mM DTT, 20 mM ATP, 0.1 μCi of [³²P]ATP for 15 min at 30 °C, and 4 μg of IKKβ 3SCWT (for Plk1 activity, unless otherwise noted), 2 μg of GST-IkBa-(1–54) (for IKK activity), or 2 μg of GST-c-JUN-(1–79) (for JNK activity) were used as substrates in the kinase reaction. The kinase reaction was quenched with 6× SDS containing loading dye, resolved by SDS-PAGE, and detected and analyzed by PhosphorImager (GE Healthcare).

Generation of Phosphoantibody against γ-Binding Domain—Phosphoantibodies were generated in rabbit against phosphorylated EQDQSFTADWP5LQTEE peptide using an outside vendor (Antagene Inc.). The nonphosphoantibodies were removed by passing the sera through the nonphosphorylated peptide affinity column. Phosphoantibodies were then purified and enriched on phosphopeptide affinity column.

Western Blot—In general for most Western blots, 20 μg of cell lysates were loaded and resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and probed with the indicated antibodies. IKKβ (1:500 dilution) was pur-
chased from Abgent (San Diego, CA). IKKα antibody (1:1000) was purchased from Pharmingen. HA and Myc antibodies (1:1000 dilution) were purchased from Tissue Culture and Reagent Core (University of Southern California, CA). Plk1 antibody (1:1000 dilution) was purchased from Zymed Laboratories Inc. β-Actin (1:10,000 dilution) and FLAG antibody (1:1000 dilution) were purchased from Sigma. IKKγ antibody was purchased from Santa Cruz Biotechnology. IκBα and phospho-IκBα antibodies (1:1000 dilution) were purchased from Cell Signaling Technology (Beverly, MA). All Western blots were imaged and quantified using the Fluor-S max quantification system (Bio-Rad).

**Electrophoretic Mobility Shift Assay (EMSA)**—Whole cell lysates (4 μg) were incubated with labeled NF-κB or Oct-1 (octamer factor 1) oligonucleotides (25, 28) in gel shift mixture containing the following: 4% Ficoll-400, 1 mM EDTA, 1 mM DTT, 50 μg/ml poly(dl-dC), and 25 μg/ml single-stranded DNA. The complexes were resolved on a 6% native polyacrylamide gel and analyzed by a PhosphorImager (GE Healthcare).

**Luciferase Assay**—The transcriptional activity of NF-κB was measured using the IL-8 luciferase reporter gene (5). HEK 293 cells were co-transfected with 25 ng of IL-8 luciferase, 25 ng of pRL-CMV (Promega), and 50 ng of plasmid constructs. COS-7 cells were transfected with 50 ng of IL-8 luciferase, 50 ng of pRL-CMV (Promega), and 50 ng of plasmid constructs. 24 h after transfection, the cells were treated with 20 ng/ml of TNFα for 12 h and harvested. Effect of Plk1 expression on NF-κB transcriptional activity was determined by luciferase assay normalized by the expression of Renilla luciferase.

**RESULTS**

**Identification of Polo-like Kinase 1 as an IKKβ C-terminal Kinase**—Studies from our laboratory and others (19, 20) suggest that phosphorylation of the yBD and the adjacent serines on IKKβ may play a role in the regulation of the IKK complex. To test this hypothesis, we biochemically searched for a kinase activity in HeLa cell extracts that could phosphorylate the C terminus of IKKβ. We also asked whether such kinase activity is modulated by TNFα.

We constructed bacterial expression vectors for the C terminus of IKKβ fused in-frame to six histidines and an Myc epitope (Fig. 1A). The fragments with the HLH include amino acid 652–756, and fragments lacking the HLH include amino acids 660–756 of the human IKKβ. Wild type and various serine-to-alanine mutants of each fragment were constructed (Fig. 1A). These fragments were expressed in BL21 (DE3) *E. coli* and purified by nickel affinity pulldown. To specifically identify a kinase or kinases that phosphorylate the yBD (serine 740) and the adjacent serines 733 and 750 in the C terminus of IKKβ, the 3SCS10A fragment, in which all serines from 633 to 704 are changed to alanine, was used as the substrate. Because fractionating cell extracts proved to be a useful strategy in the identification and isolation of IKK (5), we first fractionated resting or TNFα-stimulated (15 min) HeLa cell extracts on a Q-Sepharose anion exchange column. We then examined the fractions for kinase activity toward the 3SCS10A fragment. The 3SCS10A substrate was phosphorylated in fractions that were eluted by 150–250 mM NaCl in extracts of resting HeLa cells (Fig. 1B).

In fractions from TNF-stimulated cells, the level of this phosphorylation decreased significantly only in corresponding fractions that were eluted by 200–250 mM NaCl (designated TR for TNF-responsive fractions) but not in fractions eluting at 150–200 mM NaCl (designated CA for constitutively active fractions, Fig. 1B). We first focused on the TNF-responsive kinase activity (TR fractions) and made several attempts to further purify and identify the kinase. Thus far, we have not been able to identify this TNF-responsive kinase. However, we were able to further purify and identify the constitutively active (CA) kinase, which was also present in much larger quantities in the flow-through of the Q-Sepharose column (Fig. 1, C and D).

Both the activity bound to Q-Sepharose (CA fractions) and the flow-through activity were tested to determine their specificity for serines in the C terminus of IKKβ. Wild type and serine to alanine mutations of C-terminal fragments of IKKβ (Fig. 1A) were used as substrates. As shown in Fig. 1, C and D, both activities phosphorylated wild type, S10A, L35A, and S10L2A but not S12A and S14A mutants. The data indicate that the kinase strongly phosphorylates serines 733 and 740 and to some degree serine 750. Fig. 1, C and D, do not quantitatively compare the kinase activities of the bound fractions to the flow-through; an ~5-fold shorter exposure time is shown for the Fig. 1C.

The kinase was partially purified by several chromatography steps (Fig. 1E). Even after these chromatography steps and a final substrate affinity step, several proteins co-purified with the kinase activity (Fig. 1E). To identify the kinase, the fraction with the peak kinase activity from the substrate affinity column was digested with trypsin, and the peptides were identified by mass spectrometry. Two serine/threonine protein kinases, the Polo-like kinase 1 (Plk1) and p21-activated kinase 4 (PAK4) (30), were identified among the top 50 proteins that were sequenced (see under “Materials and Methods”).

To confirm that Plk1 is the kinase responsible for phosphorylation of IKKβ C-terminal fragments, we depleted Plk1 from the peak fraction of the substrate affinity column by an anti-Plk1 antibody (Fig. 1F). Depleting Plk1 from the fraction significantly reduced the kinase activity toward 3SCS10A (Fig. 1F). The majority of the activity was retained by the anti-Plk1 in the immunocomplex (Fig. 1F). The Plk1 is a cell cycle-regulated kinase, whose protein levels and activity are very low at G0/G1 phases of cell cycle. The protein levels and the activity are increased during S phase and peak at G2/M phases. Isolating active Plk1 from exponentially growing HeLa cells is not surprising as statistically one-third of exponentially growing cells in culture are in G2/M phase.

Plk1 and PAK4 were then cloned by PCR and tagged to the HA epitope. Both kinases were expressed *in vitro* using rabbit reticulocyte lysates and immunoprecipitated by HA antibodies for immune kinase complex assay (Fig. 2A). The HA-Plk1 phosphorylated SCS10A but not SCS12A mutant, indicating a similar specificity to the partially purified Plk1 (Fig. 2A). The HA-PAK4 did not phosphorylate these substrates (Fig. 2A). We further examined phosphorylation by Plk1 of bacterially expressed full-length GST-tagged IKKβ-KA and GST-IKKβ-KA-CTAA, in which serines 740 and 750 are mutated to alanines (Fig. 2B). GST-IKKβ-KA is a kinase-defective mutant of...
IKKβ (7). The Plk1 phosphorylated GST-IKKβ-KA but to a lesser extent (2-fold) GST-IKKβ-KA-CTAA (Fig. 2B). The phosphorylation of GST-IKKβ-KA-CTAA by Plk1 is most likely due to the phosphorylation of the serine 733. Together, the data indicate that Plk1 phosphorylates C-terminal serines 733, 740, and 750 in IKKβ.

Expression of a Constitutively Active Form of Plk1 Down-regulates TNFα-induced IKK Activation—To examine the regulation of IKK by Plk1 in mammalian cells, we constructed a FLAG-tagged mammalian expression vector for Plk1 (see under “Materials and Methods”). We expressed the FLAG-tagged wild type Plk1 in HEK 293 cells and measured the basal

FIGURE 1. Identification of Polo-like kinase 1 as an IKKβ C-terminal kinase. A, schematic diagram of wild type and serine to alanine mutations of the IKKβ C-terminal fragments with or without the HLH domain (not drawn to scale). B, identification of a kinase activity that phosphorylates the C terminus of IKKβ in fractionated HeLa cell extracts. HeLa S3 cells treated with or without TNFα were lysed, and S-100 fractions were fractionated over a Q-Sepharose FF as described under “Materials and Methods.” Fractions were tested for kinase activity toward the 3SCS10A fragment of the C terminus of IKKβ. INP, input; CA, constitutive kinase activity; TR, TNF-responsive kinase activity. C and D, comparison of the kinase activities toward different serine to alanine mutants of the C terminus of IKKβ in the Q-Sepharose FF-bound CA fractions (C) and Q-Sepharose FF flow-through (D). Wild type and mutant forms of HLH containing C-terminal fragments (see A) were used as substrates for kinase assay (KA). The protein levels of substrates were monitored by immunoblot (IB) using a Myc-antibody.

E, chromatography steps for the identification of the kinase activity. HeLa S3 S100 lysate was fractionated as described under “Materials and Methods.” From each fractionation step as indicated at the bottom of the silver stain gel, 2 µg of total proteins was loaded on a SDS-PAGE, and then silver-stained. The same fractions were tested for kinase activity toward 3SCwt substrate. The position of the Plk1 protein that was identified by mass spectrometry is indicated at the left. F, immunoaffinity depletion of Plk1 from the peak fraction of the substrate affinity column confirms that Plk1 is the IKKβ C-terminal kinase. Fraction (50 µl) with peak kinase activity was immunoprecipitated using either anti-HA or anti-Plk1 as described under “Materials and Methods.” After extensive washing, kinase activities in the immunocomplexes and supernatants were determined toward the SCS10A fragment of IKKβ. Because of the larger volume of supernatant, only 15% of the input, supernatant, and immunocomplexes were used for kinase assay (KA). The degree of Plk1 depletion was determined by immunoblotting (IB) using anti-Plk1 antibody.
expected, as we anticipated that at least one-third of cells
Thus, overexpression of wild type Plk1 without any activating
substrates were determined by immunoblot (IB) using anti-HA and anti-Myc,
respectively. B, Plk1 phosphorylates full-length IKKβ. GST-IKKβ (kinase-defective)
and GST-IKKβCTAA were expressed in E. coli and purified as described
under "Materials and Methods," and the kinase activity (KA) of partially purified
Plk1 toward GST-IKKβ (1 μg) proteins was determined. The GST-IKKβ protein
levels were determined by immunoblot (IB).

levels and TNFα-induced activities of the endogenous IKK complex. In parallel transfections with GFP, we estimated the transfection efficiency to be more than 60% (data not shown). Expression of FLAG-Plk1 changed neither the basal levels nor the TNFα-induced activation of endogenous IKK (Fig. 3A).

FIGURE 2. Comparison of the kinase activities of partially purified Plk1 and
cloned Plk1. A, HA-IKKα, HA-PAK4, and HA-Plk1 were transcribed and
translated in vitro as described under "Materials and Methods." Kinases were
immunoprecipitated using HA antibody, and kinase activity (KA) was determined
using [γ32P]ATP and [γ32P]cS12A. Protein levels of HA-tagged kinases and
substrates were determined by immunoblot (IB) using anti-HA and anti-Myc,
respectively. B, Plk1 phosphorylates full-length IKKβ. GST-IKKβ (kinase-defective)
does not change the basal levels of JNK activity. Thus, overexpression of wild type Plk1 without any activating signal did not have an effect on IKK activity. This result was unexpected, as we anticipated that at least one-third of cells expressing Plk1 to be in G2/M phase and have active Plk1. However, this was not the case, and to examine whether constitutively active mutants of Plk1 would have an effect on IKK, we mutated serine 137 and threonine 210 individually to glutamic acid. Phosphorylation of these serines was reported to activate constitutively active mutants of Plk1 would have an effect on IKK, we expressed these Plk1 constructs in HEK 293 cells and tested their activities by immune complex kinase assay using the IKKβ C-terminal fragment 3SCwt (Fig. 3, B and C). Protein expression of these constructs was determined by Western blot (Fig. 3B). The wild type Plk1 has some level of activity, which is not seen in K82M mutant. The Plk1-S137D has the highest activity toward 3SCwt. The Plk1-T210D phosphorylated 3SCwt to a similar degree as the wild type. The expression levels of Plk1-S137D and Plk1-T210D were lower than the wild type and the Plk1-K82M mutant (Fig. 3B). This experiment shows that constitutively active Plk1-S137D has increased activity toward the C terminus of IKKβ.

We then examined the effect of expression of K82M, S137D, and T210D Plk1 mutants on the TNFα-induced IKK activity (Fig. 3C). Cells were transfected with expression vectors for Plk1-K82M, Plk1-S137D, or Plk1-T210D with an efficiency of more than 60%. Cells were left untreated or treated with TNFα for 15 min. The activity of endogenous IKK was measured by immune complex kinase assay using IKKγ antibodies. The K82M and T210D Plk1 mutants did not have a noticeable effect on either basal levels or TNFα-induced IKK activities (Fig. 3C).

Regulation of IKK by Plk1

FIGURE 2. Comparison of the kinase activities of partially purified Plk1 and
cloned Plk1. A, HA-IKKα, HA-PAK4, and HA-Plk1 were transcribed and
translated in vitro as described under "Materials and Methods." Kinases were
immunoprecipitated using HA antibody, and kinase activity (KA) was determined
using [γ32P]ATP and [γ32P]cS12A. Protein levels of HA-tagged kinases and
substrates were determined by immunoblot (IB) using anti-HA and anti-Myc,
respectively. B, Plk1 phosphorylates full-length IKKβ. GST-IKKβ (kinase-defective)
does not change the basal levels of JNK activity. Thus, overexpression of wild type Plk1 without any activating signal did not have an effect on IKK activity. This result was unexpected, as we anticipated that at least one-third of cells expressing Plk1 to be in G2/M phase and have active Plk1. However, this was not the case, and to examine whether constitutively active mutants of Plk1 would have an effect on IKK, we mutated serine 137 and threonine 210 individually to glutamic acid. Phosphorylation of these serines was reported to activate constitutively active mutants of Plk1 would have an effect on IKK, we expressed these Plk1 constructs in HEK 293 cells and tested their activities by immune complex kinase assay using the IKKβ C-terminal fragment 3SCwt (Fig. 3, B and C). Protein expression of these constructs was determined by Western blot (Fig. 3B). The wild type Plk1 has some level of activity, which is not seen in K82M mutant. The Plk1-S137D has the highest activity toward 3SCwt. The Plk1-T210D phosphorylated 3SCwt to a similar degree as the wild type. The expression levels of Plk1-S137D and Plk1-T210D were lower than the wild type and the Plk1-K82M mutant (Fig. 3B). This experiment shows that constitutively active Plk1-S137D has increased activity toward the C terminus of IKKβ.

We then examined the effect of expression of K82M, S137D, and T210D Plk1 mutants on the TNFα-induced IKK activity (Fig. 3C). Cells were transfected with expression vectors for Plk1-K82M, Plk1-S137D, or Plk1-T210D with an efficiency of more than 60%. Cells were left untreated or treated with TNFα for 15 min. The activity of endogenous IKK was measured by immune complex kinase assay using IKKγ antibodies. The K82M and T210D Plk1 mutants did not have a noticeable effect on either basal levels or TNFα-induced IKK activities (Fig. 3C).

On the other hand, expression of Plk1-S137D reduced TNFα-induced IKK activity. All three mutants of Plk1 were expressed at similar levels (Fig. 3C, F-Plk1, IB). Immunoblot analysis showed that expression of Plk1-S137D resulted in a slight reduction of IKKβ and IKKγ proteins, which may account for some of the inhibitory effect of this Plk1 mutant on IKK activity. Normalizing the IKK kinase activity based on the immunoblot signal intensity of IKKβ showed that indeed Plk1-S137D decreased the activation of IKK by TNFα by about 45% (Fig. 3D). Thus, the activated form of Plk1, Plk1-S137D, reduces IKK activation by TNFα. Under similar experimental conditions, expression of Plk1-S137D also reduced IKK activation by IL-1β (data not shown).

Treatment of cells with TNFα activates not only the IKK/ NF-κB pathway but also the JNK pathway (33). To examine the specificity of IKK inhibition by Plk1-S137D, we measured its effect on JNK activation by TNFα (Fig. 3E). HEK 293 cells were transfected with vector, Plk1-K82M, or Plk1-S137D. After 48 h, cells were left untreated or treated with TNFα for 10 and 30 min. Cells were harvested, and JNK activity was measured by immune complex kinase assay using GST-c-Jun as a substrate (5). Expressing Plk1-K82M or Plk1-S137D did not change the basal levels of JNK activity (Fig. 3E). The Plk1-S137D prolonged TNF-induced activation of JNK. After 30 min of TNF treatment JNK activity is higher in Plk1-S137D expressing cells versus in vector or Plk1-K82M expressing cells (Fig. 3E). Thus Plk1-S137D inhibits IKK but not JNK downstream of TNF signaling.

Expression of Plk1-S137D Reduces TNF-induced Phosphorylation and Degradation of Endogenous IκBα—To assess the effect of the Plk1-S137D expression on the cellular activity of IKK, we determined the TNF-induced phosphorylation and protein levels of IκBα in HEK 293 cells. As phosphorylated IκBα at serines 32 and 36 (IκB target sites) is rapidly ubiquitinated and degraded, we blocked proteasome activity by MG132 (33, 34). HEK 293 cells were transfected with vector only, Plk1-K82M, or Plk1-S137D expression vectors for 24 h. Cells were then incubated with MG132 for 2 h and treated with TNFα for 5, 7, and 15 min. Immunoblot analysis of cell extracts using phospho-specific anti-IκBα showed that in vector and Plk1-K82M-transfected cells, 5 min of TNFα treatment increased the level of phosphorylation of IκBα to a similar degree. At 15 min, large molecular weight bands were detected, which is an indication of poly-ubiquitinated IκBα (Fig. 4A, p-IκBα blot). Expression of Plk1-S137D, on the other hand, reduced the phosphorylation of IκBα (Fig. 4A). Large molecular weight bands of IκBα were not observed in Plk1-S137D-expressing cells. The slightly lower levels of IκBα proteins in Plk1-S137D-transfected cells are most likely because of a chronic down-regulation of NF-κB caused by expression of Plk1-S137D; NF-κB activation is required for transcriptional induction of IκBα (3).
In the same extracts, we also examined IKK activity by immune complex kinase assay (Fig. 4B). Similar to data in Fig. 3C, although Plk1-K82M did not inhibit TNFα-induced IKK activity, Plk1-S137D expression reduced IKK activation by TNFα (Fig. 4B). Consistently, expression of Plk1-S137D reduced IKKβ protein levels to some degree, but normalization of IKK activity based on the IKKβ immunoblot signal intensity showed that it also inhibited IKK activation by TNFα (Fig. 4C). Taken together, the data indicate that expression of Plk1-S137D reduces IKK activity in cells.

Expression of Plk1-S137D Reduces DNA Binding and Transcriptional Activity of NF-κB—We next examined the effect of expression of the constitutively active Plk1-S137D on TNFα-induced DNA binding and transcriptional activity of NF-κB. For DNA binding measurements, HEK 293 cells were transfected with vector, Plk1-K82M, or Plk1-S137D for 24 h. Cells were left untreated or treated with TNFα for 30 min. DNA binding activity of NF-κB was measured by EMSA in nuclear extracts of cells. For control, the DNA binding of Oct-1 was measured in the same extracts. In vector-transfected cells, the DNA binding of

In the same extracts, we also examined IKK activity by immune complex kinase assay (Fig. 4B). Similar to data in Fig. 3C, although Plk1-K82M did not inhibit TNFα-induced IKK activity, Plk1-S137D expression reduced IKK activation by TNFα (Fig. 4B). Consistently, expression of Plk1-S137D reduced IKKβ protein levels to some degree, but normalization of IKK activity based on the IKKβ immunoblot signal intensity showed that it also inhibited IKK activation by TNFα (Fig. 4C). Taken together, the data indicate that expression of Plk1-S137D reduces IKK activity in cells.

Expression of Plk1-S137D Reduces DNA Binding and Transcriptional Activity of NF-κB—We next examined the effect of expression of the constitutively active Plk1-S137D on TNFα-induced DNA binding and transcriptional activity of NF-κB. For DNA binding measurements, HEK 293 cells were transfected with vector, Plk1-K82M, or Plk1-S137D for 24 h. Cells were left untreated or treated with TNFα for 30 min. DNA binding activity of NF-κB was measured by EMSA in nuclear extracts of cells. For control, the DNA binding of Oct-1 was measured in the same extracts. In vector-transfected cells, the DNA binding of

Cell lysates were immunoprecipitated using anti-IKK antibody for in vitro kinase assay (KA, top) using GST-(1–54)-IκBα as a substrate. FLAG-Plk1, IKKβ, and IKKγ levels were assessed by immunoblotting (IB). B, for comparison of activities of wild type and mutant plk1 in HEK 293 cells, cells were transfected with expression vectors for empty vector or wild type, K82M, S137D, and T210D Plk1. Cells were harvested and immunoprecipitated using anti-FLAG antibody for in vitro kinase assay (KA, top) using 3SCwt as a substrate. FLAG-Plk1 expression levels and substrate levels were assessed by immunoblotting (IB). C, effect of expression of constitutively active Plk1 on IKK activity. Cells were transfected with expression vectors for indicated Plk1 mutants as described above. After 48 h, cells were stimulated or not with 20 ng/ml TNFα for 10 min. Cell lysates were immunoprecipitated using anti-IKK antibody for in vitro kinase assay (KA, top) using GST-IκBα-(1–54) as a substrate. FLAG-Plk1, IKKα, IKKβ, and IKKγ levels were assessed by immunoblotting (IB). D, specific kinase activity of IKK was calculated for the experiment in C. Kinase activity was quantitated by using ImageQuant software. IKKβ levels on the immunoblot were quantitated by using Quantity One software. Ratio of signal intensities of kinase activity divided by the signal intensity of immunoblot of IKKβ protein levels are presented as specific kinase activities. E, expression of constitutively active Plk1 does not reduce TNFα-induced JNK activation. Cells were transfected with expression vectors for K82M or S137D Plk1 as described above and stimulated with TNFα for indicated times. Lysates were immunoprecipitated using anti-JNK1 antibody and GST-c-Jun-(1–79) was used as substrate (KA, top). Protein levels of FLAG-Plk1 and JNK1 was determined by immunoblotting (IB).
NF-κB was increased by TNFα treatment (Fig. 5A). Transfection of Plk1-K82M did not reduce the TNF-induced NF-κB DNA binding. Expression of Plk1-S137D, on the other hand, reduced TNF-induced NF-κB binding to DNA (Fig. 5A). DNA binding of Oct-1 did not change as a result of TNF treatment or expression of Plk1 constructs in cells (Fig. 5A). Thus, expressing Plk1-S137D, which resulted in inhibition of IKK and stabilization of IκBα (Fig. 4), ultimately reduced NF-κB DNA binding.

We further examined the effect of the expression of Plk1-S137D on the transcriptional activation of NF-κB using a luciferase reporter gene under the control of the human IL-8 promoter (5). As an internal control for transfection efficiency and luciferase assay in all transfections, the pRL-Renilla construct was co-transfected. The IL-8-luciferase construct and pRL-Renilla were co-expressed along with vector, Plk1-wt, Plk1-K82M, or Plk1-S137D into HEK 293 or COS-7 cells in triplicate. After 24 h of transfection, cells were left untreated or treated with 20 ng/ml TNFα for 12 h. The activities of luciferase and Renilla were measured in cell extracts, and the ratios of luciferase/Renilla were determined for each sample. In vector-transfected HEK 293 cells, IL-8 promoter was activated by TNFα by 3-fold (Fig. 5B). Expressing Plk1-wt or Plk1-K82M in HEK 293 cells reduced both basal levels and TNF-induced activation of IL-8 promoter. However, the fold induction by TNFα in both cases remained 3-fold (Fig. 5B). On the other hand, expressing Plk1-S137D not only reduced the basal level but also the TNF-induced activation of IL-8 promoter (Fig. 5B). Considering that expression of Plk1-S137D was much lower than Plk1-wt and Plk1-K82M (Fig. 5C), proportionally, the inhibitory effect of Plk1-S137D on IL-8 promoter should be much higher than measured in Fig. 5B. The same reporter gene experiment was carried out in COS-7 cells to further confirm the effect of Plk1-S137D expression on NF-κB activity (Fig. 5B, COS cells panel). In vector-transfected COS-7 cells, TNFα treatment resulted in a 3.5-fold increase in IL-8 promoter activation. Expressing Plk1-wt reduced basal level activity of the IL-8 promoter to some degree, but TNFα induced the promoter activity to a similar level as the vector-transfected cells (Fig. 5B, COS cells panel). Interestingly in COS-7 cells, expressing Plk1-K82M increased TNF-induced activity of the IL-8 promoter by about
50% above the vector and Plk1-wt-transfected cells. The Plk1-S137D expression, however, reduced both basal levels and TNF-induced activity of the IL-8 promoter to a significant degree (Fig. 5B, COS panel). The TNF-induced activation of IL-8 promoter in Plk1-S137D expressing COS-7 cells was reduced to a level similar to the basal level activity of the promoter in vector-transfected cells. Western blot analysis showed that Plk1-wt and Plk1-K82M are expressed at higher levels.
compared with Plk1-S137D (Fig. 5C, right panel). Thus, in two independent cell lines, Plk1-S137D reduced significantly both basal and TNF-induced transcriptional activity of NF-κB.

Phosphorylation of the C-terminal Fragment of IKKβ (β3SC) by Plk1 Reduces Its in Vitro Interaction with IKKγ—We have shown that mimicking the phosphorylation state of the γ-binding region in IKKβ maintains IKK in a low state of activity (20). Here we examined the effect of phosphorylation of serine 740 and 733 in the C terminus of IKKβ on its in vitro interaction with IKKγ by co-immunoprecipitation. Constitutively active (Plk1-DD) and kinase-defective (K82M) Plk1 were transfected and immunoprecipitated from COS-7 cells and used to phosphorylate the 3SCWT (see Fig. 6A) prior to interaction study with two fragments of IKKγ (Fig. 6A). The IKKγ fragment, ΔC-204, which contains the N-terminal IKKβ-interaction domain, co-immunoprecipitated with 3SCWT when Plk1-K82M was used as the kinase (Fig. 6B). The association was diminished when 3SCWT was phosphorylated with Plk1-DD prior to co-immunoprecipitation (Fig. 6B). As a control, ΔN-257, which lacks the N-terminal interaction domain with IKKβ, did not associate with 3SCWT under either condition (Fig. 6B). In summary, the data in Fig. 6 indicate that phosphorylated C-terminal γ-binding domain of IKKβ has reduced affinity for IKKγ.

Serine 740 in the γ-Binding Domain of IKKβ Is Phosphorylated in Vivo—To examine the state of phosphorylation of the γ-binding domain in vivo, we generated phosphoantibodies against a γ-binding domain phosphorylated peptide (see under “Materials and Methods”). The anti-phosphoantibody (hereafter named p740) recognized wild type IKKβ but has much diminished affinity for the IKKβCTAA mutant, in which serines 740 and 733 are changed to alanine (Fig. 7A) (20). Furthermore, the immunoblot detection of endogenous IKKβ by p740 antibody was significantly reduced when IKKβ was treated with calf intestinal phosphatase (Fig. 7B). At high levels of IKKβ protein, the p740 antibody can also detect nonphosphorylated IKKβ (data not shown). It needs to be noted that because of sensitivity issues, phosphorylation of endogenous IKKβ was determined by p740 antibody after immunoprecipitation of the IKK complex. Therefore, for all the following experiments to detect phosphorylated Ser-740 by the p740 antibody, the IKK complex was first immunoprecipitated by the anti-IKKγ antibody.

Activation of Plk1 by Nocodazole Sustains Phosphorylation of Ser-740 in TNF-treated Cells—In experiments designed to investigate cytokine-induced IKK regulation at different stages of the cell cycle, we observed that treatment of cells with nocodazole, an inducer of Plk1 (27), significantly reduced TNF-

FIGURE 6. Phosphorylation of the C-terminal fragment of IKKβ (β3SC) by Plk1 reduces its in vitro interaction with IKKγ. A, schematics of IKKβ and IKKγ fragments used for co-immunoprecipitation in B. B, constitutively active (Plk1-DD) and kinase-defective (K82M) Plk1 were transfected and immunoprecipitated from COS-7 cells and used to phosphorylate the β3SCWT prior to the interaction study with two fragments of IKKγ. The IKKγ fragment, ΔC-204, which contains the N-terminal IKKβ-interaction domain, co-immunoprecipitated with β3SCWT when Plk1-K82M was used as the kinase. The association was diminished when β3SCWT was phosphorylated with Plk1-DD prior to co-immunoprecipitation. As a control, ΔN-257, which lacks N-terminal interaction domain with IKKβ, did not associate with β3SCWT under either condition. IB, immunoblot.
Regulation of IKK by Plk1

![Figure 7: Nocodazole inhibits dephosphorylation of serine 740 in the γ-binding domain of IKKβ. A and B, validation of phosphoantibody against serine 740. Polyclonal phosphoantibody against serine 740 was generated as described under "Material and Methods." A, anti-serine 740 phosphoantibody (p740) recognized wild type (WT) IKKβ but has much diminished affinity for the IKKβCTAA mutant (AA), in which serines 740 and 733 are changed to alanine. Equal total wild type and IKKβCTAA protein levels were determined by HA Western blot. B, immunoblot detection of endogenous IKKβ by p740 antibody was significantly reduced when IKKβ was treated with calf intestinal phosphatase (CIP) prior to Western blot analysis. C, nocodazole inhibits in vivo phosphorylation of serine 740. HeLa cells were treated with DMSO (vehicle) or nocodazole (200 ng/ml) for 16 h. Then cells were treated with TNFα for different times as indicated. Phosphorylation of serine 740 was examined by p740 antibody after the IKK complex was immunoprecipitated by anti-IKKγ antibody. Increased expression of Plk1 after nocodazole treatment was determined by Western blot using Plk1 antibody. Activation of IKK by TNFα was examined by determining phosphorylation state of endogenous IκBα (pIκBα).]

induced IKK activation. Here we used the p740 antibody to first examine the phosphorylation pattern of serine 740 in IKKβ during TNF induction and second to examine the effect of nocodazole on this phosphorylation. First, HeLa cells were treated with DMSO (vehicle) or nocodazole (200 ng/ml) for 16 h. Then cells were treated with TNFα for different times as indicated in Fig. 7C. Phosphorylation of serine 740 was examined by p740 antibody after the IKK complex was immunoprecipitated by anti-IKKγ antibody. Strong phosphorylation of serine 740 was observed in cells that were not treated with TNFα under both DMSO or nocodazole treatment (Fig. 7C). In DMSO-treated cells, TNFα treatment within the first 5 min reduced the phosphorylation of serine 740. However, the phosphorylation increased within 10–15 min of TNFα treatment. There was a decrease of phosphorylation at 30 min and another increase at the 60-min time point. In nocodazole-treated cells, TNF reduced serine 740 phosphorylation within 5 min. This reduction was significantly lower when compared with DMSO-treated cells (Fig. 7C). More importantly, nocodazole treatment sustained the phosphorylation of serine 740 during TNFα treatment and prevented TNF-induced dynamic dephosphorylation of serine 740 seen in DMSO-treated cells. As expected, Plk1 was induced by nocodazole (Fig. 7C, PLK panel). IκBα phosphorylation of these samples was determined as a measure for IKK activation. IκBα phosphorylation peaked at 5–10 min of TNF induction in DMSO-treated cells and was significantly reduced in nocodazole-treated cells (Fig. 7C, pIκBα panel). Together, the data indicate that serine 740 undergoes dynamic dephosphorylation and phosphorylation during TNF induction; nocodazole sustains the phosphorylation state.

Knocking Down Plk1 Restores TNF-induced IKK Activation and Reduces Phosphorylation of Serine 740 in Nocodazole-treated Cells—Activated Plk1 down-regulates IKK. Plk1 can be activated by nocodazole. Therefore, we examined whether activation of Plk1 by nocodazole results in down-regulation of IKK and increased phosphorylation of serine 740. We knocked down Plk1 in COS-7 cells using three specific siRNAs, each targeting different sequences (see under "Materials and Methods"), and we determined TNF-induced IKK activation in the presence and absence of nocodazole. The data using Plk1 siRNA1 are shown in Fig. 8A, and the data using siRNA2 and -3 are shown supplemental Fig. 1. For each experiment, COS-7 cells were transfected with a control siRNA or Plk1-specific siRNAs (see under "Materials and Methods" for the sequences of the siRNAs). Four hours post-transfection, cells were treated either with DMSO (the solvent for nocodazole) or with 200 ng/ml nocodazole for 18 h. Before harvest, cells were left untreated or treated with 20 ng/ml TNFα, and the activity of endogenous Plk1 and TNF responsiveness of endogenous IKK were measured by immune kinase complex assays (Fig. 8A). In cells transfected with control siRNA, treatment with DMSO did not prevent TNFα-induced IKK activation, and the basal level activity of Plk1 remained unchanged (Fig. 8A). In cells transfected with control siRNA, nocodazole treatment reduced TNF-induced IKK activation (Fig. 8A). As shown previously, nocodazole activated Plk1 in these cells both at the protein level and activity (27). Each of the three Plk1-siRNAs reduced Plk1 expression and nocodazole-induced activity to comparable degrees (Fig. 8A and supplemental Fig. 1). In cells transfected with Plk1-siRNAs and treated with DMSO, basal levels or TNF-induced activation of IKK were reduced by 10% (siRNA1) and 50% (siRNA2 and -3). On the other hand, knocking down Plk1 restored TNF-induced IKK activation in the presence of nocodazole (Fig. 8A). In the cells transfected with Plk1-siRNA1 and treated with DMSO, IKK was activated by TNFα to 6.9-fold, and nocodazole treatment reduced it to 4.2-fold (Fig. 8A). In cells transfected with Plk1-siRNA2 or -3, which were more effective in knocking down Plk1, nocodazole did not reduce TNF-induced IKK activation at all, when compared with the DMSO-treated cells (supplemental Fig. 1). In all cases, protein levels of IKKβ, IKKγ, and β-actin remained unchanged. It is interesting to note that transfection of cells with Plk1-siRNAs by itself reduced TNF-induced IKK activation by about 2-fold.

E. Zandi, unpublished data.
This could be the result of the toxicity of the Plk1-siRNAs or, alternatively, because down-regulation of Plk1 can affect cell cycle progression, which may affect IKK regulation.

Next, we examined phosphorylation of serine 740 in control siRNA and in cells transfected with Plk1 siRNA. In cells transfected with control siRNA and treated with DMSO, phosphorylation of serine 740 was reduced after TNF treatment (Fig. 8B). Nocodazole treatment of these cells resulted in sustained phosphorylation of serine 740 after TNF treatment (Fig. 8B). In cells transfected with Plk siRNA, there was a reduction of phosphorylation of serine 740 regardless of DMSO or nocodazole treatment (Fig. 8B), indicating that Plk1 phosphorylates serine 740 of IKKβ in cells. Taken together, the data in Fig. 8 indicate that activation of endogenous Plk1 results in phosphorylation of serine 740 in IKKβ. This in turn reduces TNF-induced IKK activation.

**Plk1 Inhibits TNF-induced Expression of Cyclin D1**—TNF stimulates cell proliferation in regenerating liver, for example (35). A key mechanism of TNF-induced cell proliferation is the expression of cyclin D1 through IKK/NF-κB (36, 37). Cyclin D1 expression and nuclear translocation are increased in the G₁ phase of the cell cycle, where it associates with CDK4 and CDK6 to induce the S phase entry of cells. Cyclin D1 is degraded in S phase, and its protein levels are kept low until cells enter the G₂ phase (38). Cyclin D1 is overexpressed in a number of tumors (38). It is reported that TNF accelerates S phase in tumor cells (39). TNF is also implicated in inflammation-induced cancer (40). However, as TNF is produced in response to infection and injury, and many cells at different stages of cell cycle are exposed to it, it is reasonable to assume that mechanism(s) must exist to prevent TNF-induced cyclin D1 expression in the S and possibly G₂/M phases of the cell cycle. Otherwise, TNF would cause untimely expression of cyclin D1 and an irregular cell cycle. We have shown that TNF-induced activation of IKK/NF-κB is suppressed during the S phase (Higashimoto et al., 50). Here we examined whether Plk1 regulates TNF-induced cyclin D1 expression. First, treating HeLa cells with TNFα resulted in an increase of cyclin D1 (Fig. 9A). Treating cells with nocodazole reduced both basal and TNF-induced cyclin D1 levels (Fig. 9A). Activation of Plk1 by nocodazole correlated well with the reduction of cyclin D1 in HeLa cells (Fig. 9A). We then examined whether reducing Plk1 would affect TNF-induced expression of cyclin D1. HeLa cells transfected with control siRNA or Plk1 siRNA and treated with or without TNF as described under "Materials and Methods." The levels of cyclin D1 (Fig. 9A). The levels of cyclin D1 (Fig. 9A). We then examined whether Plk1 regulates TNF-induced cyclin D1 expression. First, treating HeLa cells with TNFα resulted in an increase of cyclin D1 (Fig. 9A). Treating cells with nocodazole reduced both basal and TNF-induced cyclin D1 levels (Fig. 9A). Activation of Plk1 by nocodazole correlated well with the reduction of cyclin D1 in HeLa cells (Fig. 9A). We then examined whether reducing Plk1 would affect TNF-induced expression of cyclin D1.
Regulation of IKK by Plk1

cyclin D1 expression. As shown in Fig. 9B, HeLa cells transfected with a control siRNA, TNFα, increased the level of cyclin D1. In HeLa cells transfected with Plk1 siRNA, there is a reduction in cyclin D1, which is most likely because of the cell cycle arrest at S/G2/M (see Fig. 9C). Cyclin D1 expression is also reduced when cells are treated with nocodazole (see Fig. 9A). On the other hand, TNFα treatment increased cyclin D1 significantly in cells transfected with Plk1 siRNA (Fig. 9B). This increased level of cyclin D1 is higher than the corresponding levels in control siRNA-transfected cells. To investigate whether the TNF-and Plk1-dependent cyclin D1 regulation has an effect on the cell cycle, we transfected HeLa cells either with control or Plk1 siRNA, and we examined the cell cycle in the presence or absence of TNFα. As shown in Fig. 9C, TNFα did not have any discernible effect on the cell cycle of cells transfected with control siRNA. Knockdown of Plk1, however, increased the cell population at S and G2/M phases (Fig. 9C). This was expected because Plk1 is required for S/G2 progression and mitosis. Interestingly, treatment of these cells with TNFα decreased the cell population in G1 phase while increasing the cell population in S and G2/M phases. These data are explained by the fact that cyclin D1 is a key factor for G to S phase transition, and TNFα increases the cyclin D1 levels in cells when Plk1 levels are reduced by siRNA (Fig. 9B). This is an interesting observation as it establishes a new pathway for down-regulation of cyclin D1 expression by Plk1. This pathway may be a mechanism to prevent inappropriate expression of cyclin D1 when cells are exposed to TNFα. Chronic exposure of cells to TNFα during chronic inflammation coupled with low activity of Plk1 could be a mechanism for accelerated and uncontrolled cell proliferation.

DISCUSSION

Phosphorylation of the C-terminal region of IKKβ plays a key role in signal-induced regulation of IKK. It has been shown that serines spanning between HLH and γBD are autophosphorylation sites (18). However, whether serine 740 within the γBD is phosphorylated in vivo and whether this phosphorylation would play a role in regulation of IKK and the corresponding kinase(s) were not known. Here we show that Plk1 is a γBD kinase, which phosphorylates serines 733 and 740 in vitro. By using specific phosphoantibodies we show that serine 740 in IKKβ is phosphorylated in vivo. Serine 740 is phosphorylated in resting cells and undergoes dynamic de-phosphorylation and re-phosphorylation in response to TNFα. Nocodazole, which activates Plk1, prevents TNF-induced de-phosphorylation of serine 740 in IKKβ. Knocking down Plk1 restores TNF-induced de-phosphorylation of serine 740 in nocodazole-treated cells.

IKKγ is an essential component of the IKK complex, and it is required for signal-induced activation of IKKβ. In different experimental systems, IKKγ expression has been shown to induce or prevent IKK complex activity (8, 41–44). As proposed by May et al. (19), IKKγ could have a dual function, and phosphorylation of the γBD may play a role in IKK regulation. Using our yeast reconstitution system of the IKK complex, we have shown that mimicking phosphorylation of the γBD in IKKβ reduces significantly the activity of IKK (20). We hypothesized that in resting cells IKKγ-induced activation of the IKK complex may be prevented by phosphorylation of the γBD of IKKβ. Data presented here provide experimental evidence to support our hypothesis. This mode of regulation for IKK is also supported by the reports that phosphatase inhibitors activate NF-κB through IKK complex (5, 45–47). Furthermore, the recent crystal structure of the γBD of IKKβ with IKKγ reveals a critical intramolecular hydrogen bond within γBD between serine 740 and aspartate 738 (22). Based on the crystal structure, this hydrogen bond is necessary for correct interaction of tryptophans 739 and 741 of γBD with IKKγ (22). Rushe et al. (22) discussed that breaking this hydrogen bond by phosphorylation of serine 740, for example, would reduce the affinity of γBD for IKKγ. In Fig. 6 it is shown that phosphorylated γBD has significantly reduced affinity for IKKγ. This model suggests that, within the IKK complex, IKKγ interacts with the γBD of IKKβ in a dynamic manner, and phosphorylation of serine 740 and perhaps 733 regulates this dynamic interaction. It is important to note that because IKKγ is associated with the catalytic subunits, IKKβ and/or IKKα, in resting cells, and cell stimulation does not seem to change this association in ways that would alter the composition of the complex (25, 26, 44), IKKγ must therefore be tethered to the catalytic subunits by additional nonregulatory interactions other than γBD. In support of this, we have shown that deletion of the γBD of IKKβ, although it inhibits IKK activation, does not prevent complex formation between IKKγ and IKKβ in a yeast reconstitution system (8). This model is further supported by the data that a peptide encompassing the γDB can break up the interaction of IKKγ with the catalytic subunits in vitro and prevent stimulus-dependent IKK activation in vivo (19, 21).

Data in Fig. 7C show that a rapid de-phosphorylation of IKKβ is associated with its activation. This highlights the role of phosphatases in activation of the IKK complex. The serine/threonine phosphatase PP2A has been reported to both activate and inactivate the IKK complex (48). One study indicates that PP2A associates with the IKK complex via the N terminus of IKKγ and is involved in TNF-induced activation of IKK (48). Whether PP2A is the phosphatase that de-phosphorylates the C terminus of IKKβ during TNF induction remains to be determined.

Identification of Plk1 as a γBD kinase was a surprise. The fact that protein levels and activity of Plk1 are cell cycle-regulated raises the question of whether Plk1 is a general negative regulator of IKK, or alternatively it only down-regulates IKK under certain conditions in cells, for example cells at a specific stage of cell cycle. For the first time we show (Fig. 8A) that nocodazole, which arrests cells at G2/M phase of cell cycle, reduced TNF-induced IKK activation. Knocking down Plk1 restored TNF-induced activation in nocodazole-treated cells (Fig. 8A). Significance of regulation of TNF/IKK/NF-κB pathway by Plk1 is shown in Fig. 9, where Plk1 inhibits TNF-induced expression of cyclin D1. Overexpression of cyclin D1 is associated with cancer in several tissues (49). Expression of cyclin D1 is increased in the G1 phase, which activates CDK4 and CKD6 to phosphorylate retinoblastoma protein and activate transcription of S phase genes for entry into the S phase of the cell cycle. At the end of the G1 phase, cyclin D1 is degraded, and
its expression remains low during S, G<sub>2</sub>, and M phases. We have shown that TNF-induced activation of IKK is down-regulated during the S phase of the cell cycle (50). Down-regulation of the TNF/IKK/NF-κB pathway during S phase may prevent untimely expression of cyclin D1. The precise mechanism of how overexpression of cyclin D1 plays a role in cancer is not known. Given the strong association of overexpression of cyclin D1 in many cancers, it is conceivable to assume that constitutive and untimely activation of cyclin D1 by TNF/IKK/NF-κB/IKK pathway could provide a mechanism for TNFα to induce uncontrolled cell growth and initiate cell transformation. We have shown that TNFα can cause DNA damage and chromosomal abnormalities that are blocked by an IKK inhibitor (50). In a broader context, these observations provide a mechanism for the involvement of TNF/IKK pathway in inflammation-induced cancer.

Taken together, the data presented in this work show that serine 740 of IKKβ is indeed phosphorylated in vivo, and it regulates the interaction of γBD of IKKβ with IKKγ and therefore plays a critical role for IKK activation. Moreover, we identify Plk1 as a γBD kinase, which negatively regulates IKK and inhibits TNF-induced expression of cyclin D1. The mechanism of inhibition of IKK by Plk1 provides a paradigm for how phosphorylation of γBD of IKKβ is involved in regulation of the IKK complex. There may be other kinases that phosphorylate the γBD of IKKβ and down-regulate the IKK complex through its C-terminal γBD.

Acknowledgment—We thank Dr. Michael Lieber for reading and commenting on the manuscript.

REFERENCES

1. Ghosh, S., and Karin, M. (2002) Cell 109, 581–596
2. Yaron, A., Hatzubai, A., Davis, M., Lavon, I., Amit, S., Manning, A. M., Andersen, J. S., Mann, M., Mercurio, F., and Ben-Neriah, Y. (1998) Nature 396, 590–594
3. Siebenlist, U., Franzoso, G., and Brown, K. (1994) Annu. Rev. Cell Biol. 10, 405–455
4. Strack, P., Caligiuri, M., Pelletier, M., Boisclair, M., Theodoras, A., Beer-Parker, P., Brizuela, L., and Rolfe, M. (2000) Nature Cell Biol. 2, 441–450
5. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554
6. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
7. Zandi, E., Chen, Y., and Karin, M. (1998) Science 281, 1360–1363
8. Miller, B. S., and Zandi, E. (2001) J. Biol. Chem. 276, 36320–36326
9. Delhase, M., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (2003) Oncogene 22, 2217–2227
10. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2004) Mol. Cell 14, 289–301
11. Krapfmann, D., and Scheidereit, C. (2005) EMBO Rep. 6, 321–326
12. Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) Science 284, 309–313
13. May, M. J., Marienfeld, R. B., and Ghosh, S. (2002) J. Biol. Chem. 277, 45992–46000
14. Schomer-Miller, B., Higashimoto, T., Lee, Y. K., and Zandi, E. (2006) J. Biol. Chem. 281, 15268–15276
15. May, M. J., D’Acquisto, F., Madge, L. A., Glockner, J., Poher, J. S., and Ghosh, S. (2000) Science 289, 1550–1554
16. Rushe, M., Silvian, L., Bixler, S., Chen, L. L., Cheung, A., Bowes, S., Cuervo, H., Berkowitz, S., Zheng, T., Guckian, K., Pellegrini, M., and Lugovskoy, A. (2008) Structure (Lond.) 16, 798–808
17. Wang, L. C., Okitsu, C. Y., and Zandi, E. (2005) J. Biol. Chem. 280, 7634–7644
18. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60–89
19. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 395, 297–300
20. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
21. Ying, Y. J., Ma, S., Terada, Y., and Erikson, R. L. (2002) J. Biol. Chem. 277, 44115–44120
22. Zandi, E., Tran, T. N., Chamberlain, W., and Parker, C. S. (1997) Genes Dev. 11, 1299–1314
23. Clay, F. J., McEwen, S. J., Bertoncello, L., Wilks, A. F., and Dunn, A. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4882–4886
24. Abo, A., Qu, I., Cammarano, M. S., Dan, C., Fritsch, A., Baud, V., Belisle, B., and Minden, A. (1998) EMBO J. 17, 6527–6540
25. Tsvetkov, L., and Stern, D. F. (2005) Cell Cycle 4, 166–171
26. Jackman, M., Lindon, C., Nigg, E. A., and Pines, J. (2003) Nat. Cell Biol. 5, 143–148
27. Westwick, J. K., Weitzel, C., Minden, A., Karin, M., and Brenner, D. A. (1994) J. Biol. Chem. 269, 26396–26401
28. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773–785
29. Fausto, N. (2000) J. Hepatol. 32, 19–31
30. Widera, D., Milkenberg, I., Elvers, M., Kalt schmidt, C., and Kalt schmidt, B. (2006) BMC Neurosci. 7, 64
31. Gutfri tt, D. C., Alban ese, C., Reuth er, J. Y., Pestell, R. G., and Baldwin, A. S., Jr. (1999) Mol. Cell. Biol. 19, 5785–5799
32. Gauth eschi, O., Ratschiller, D., Gugger, M., B ettic her, D. C., and Heyw arth, I. (2007) Lung Cancer 55, 1–14
33. Volland, S., Amtmann, E., and Sauer, G. (1994) Int. J. Cancer 56, 698–705
34. Fujiki, H., Suganuma, M., Okabe, S., Sueoka, E., Saga, K., Imai, K., and Nakachi, K. (2000) Cancer Detect. Prev. 24, 91–99
35. Yamamoto, Y., Kim, D. W., Kwak, Y. T., Prajapati, S., Verma, U., and Gaynor, B. R. (2001) J. Biol. Chem. 276, 36327–36336
36. Ye, J., Xie, X., Tarassishin, L., and Horwitz, M. S. (2000) J. Biol. Chem. 275, 9882–9889
37. Li, Y., Kang, J., Friedman, J., Tarassishin, L., Ye, J., Kovalenko, A., Wallach, D., and Horwitz, M. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1042–1047
38. Li, X. H., Fang, X., and Gaynor, R. B. (2001) J. Biol. Chem. 276, 4494–4500
39. Harhaj, E. W., and Sun, S. C. (1997) J. Biol. Chem. 272, 5409–5412
40. Sun, S. C., Maggirwar, S. B., and Harhaj, E. (1995) J. Biol. Chem. 270, 18347–18351
41. Traenckner, E. B., Pahl, H. L., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) EMBO J. 14, 2876–2883
42. Kray, A. E., Carter, R. S., Pennington, K. N., Gomez, R. J., Sanders, L. E., Llanes, J. M., Khan, W. N., Ballard, D. W., and Wadzinski, B. E. (2005) J. Biol. Chem. 280, 35974–35982
43. Liao, D. J., Thakur, A., Wu, J., Biliran, H., and Sarkar, F. H. (2007) Crit. Rev. Oncog. 13, 93–158
44. Higashimoto, T., Panopoulos, A., Hsieh, C. L., and Zandi, E. (2006) Cytokine 34, 39–50