Site-specific Tyrosine Phosphorylation of IκBα Negatively Regulates Its Inducible Phosphorylation and Degradation*

(Received for publication, August 9, 1996)

Sanjaya Singh‡§, Bryant G. Darnay‡, and Bharat B. Aggarwal¶

From the Cytokine Research Laboratory, Department of Molecular Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

The transcription factor NF-κB is retained in the cytoplasm by its interaction with the inhibitory subunit known as IκB. Signal-induced serine phosphorylation and subsequent ubiquitination of IκBα target it for degradation by the 26 S proteasome. Recently, pervanadate, a protein-tyrosine phosphatase inhibitor, was shown to block the degradation of IκBα, thus inhibiting NF-κB activation. We investigated the mechanism by which pervanadate inhibits the degradation of IκBα. Western blot analysis of IκBα from tumor necrosis factor-treated cells revealed a slower migrating IκBα species that was subsequently degraded. However, pervanadate-treated cells also revealed a slower migrating species of IκBα that appeared in a time- and dose-dependent manner and was not degraded by tumor necrosis factor. The slower migrating species of IκBα from pervanadate-treated cells was tyrosine-phosphorylated as revealed by cross-reactivity with anti-phosphotyrosine antibodies, by the ability of the specific tyrosine phosphatase PTP1B to dephosphorylate it, and by phosphoamino acid analysis of IκBα immunoprecipitated from 32P-labeled cells. By site-specific mutagenesis and deletion analysis, we identified Tyr-42 on IκBα as the phosphoacceptor site. Furthermore, in an in vitro reconstitution system, tyrosine-phosphorylated IκBα was protected from degradation. Our results demonstrate that inducible phosphorylation and degradation of IκBα are negatively regulated by phosphorylation at Tyr-42, thus preventing NF-κB activation.

The transcription factor NF-κB regulates the expression of many genes that play essential roles in immune and inflammatory responses including the type I human immunodeficiency virus (1–4). Like all members of the Rel/NF-κB transcription factor family, NF-κB has the unique property of being sequestered in its inactive state in the cytoplasm by a noncovalent association with inhibitory proteins called IκB (4). In mammalian species, at least seven structural homologs of IκB have been identified (4), but only the IκBα is needed but not sufficient for its degradation by the proteasome. Ubiquitination of lysines 21 and 22 (11, 19) follows inducible serine phosphorylation of IκBα, leading to the accumulation of a phosphorylated form of IκBα while still bound to NF-κB (12–18). These findings suggest that induced phosphorylation of IκBα is needed but not sufficient for its degradation by the proteasome. Recent reports (21, 22) demonstrated that TNF-mediated NF-κB activation is completely abolished by the protein-tyrosine phosphatase inhibitor pervanadate (PV), which blocks the degradation of IκBα. In this report, we examined the mechanism by which PV blocks the degradation of IκBα. We demonstrate that inhibition of protein-tyrosine phosphatase activity by PV results in site-specific tyrosine phosphorylation of IκBα, which prevents its inducible phosphorylation and degradation and hence inhibits the activation of NF-κB.

EXPERIMENTAL PROCEDURES

Materials—The cell lines employed in this study included ML-1a, a human myelomonoblastic leukemia cell line kindly provided by Dr. Ken Takeda (Showa University, Showa, Japan). U937, a human histiocytic lymphoma cell line, and HeLa, an epithelial carcinoma cell line, were obtained from the American Type Culture Collection (Rockville, MD). ML-1a and U937 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. HeLa cells were cultured in minimum Eagle's medium supplemented with 10% fetal calf serum.

1 The abbreviations used are: TNF, tumor necrosis factor; PV, pervanadate; ALLN, N-acetylleucylleucynorleucinal; PAGE, polyacrylamide gel electrophoresis.

* This work was supported by the Clayton Foundation for Research. 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.

‡ Contributed equally to this work.

§ Present address: Dept. of Biochemistry and Molecular Biology, P. O. Box 117, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

¶ To whom correspondence should be addressed: Cytokine Research Lab., Dept. of Molecular Oncology, P. O. Box 41, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-3503/6459; Fax: 713-794-1613.

This paper is available on line at http://www-jbc.stanford.edu/jbc/
bovine serum and antibiotics. Recombinant bacterium-derived purified human IFN-β (1000 units/ml) was a gift of Genentech, Inc. (South San Francisco, CA). Pervanadate was prepared fresh each time as described previously (21). Affinity-purified rabbit anti-ixBa anti-serum directed against the N and C termini and full-length IxBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio-Rad. Calpain inhibitor I (N-acetyl-L-leucyl-L-leucyl-L-leucinal (ALLN)), biotinylated horseradish peroxidase antibodies, and horseradish peroxidase-conjugated anti-biotin were obtained from Sigma. Anti-FLAG antibody (monoclonal antibody M2) and anti-FLAG antibody-conjugated agarose were obtained from Eastman Kodak Co. Goat anti-mouse IgG conjugated to horseradish peroxidase was obtained from Transduction Laboratories (Lexington, KY). Okadaic acid was obtained from LC Laboratory. Half-milliliter alkaline phosphatase was obtained from Life Technologies, Inc.

Plasmids—The plasmid pBS(SK)-IxBα was a generous gift of Dr. P. Chiao (University of Texas M. D. Anderson Cancer Center, Houston, TX), and the plasmid pGEX-PTP1B-His of Dr. J. Weiner (University of Texas M. D. Anderson Cancer Center). The plasmids pCMV4-FI-agarose, pCMV4-FI-agarose-Y42F, and pCMV4-FI-agarose-ΔC (lacking residues 243–317) (23) were kindly provided by Dr. B. Ballard (Vanderbilt University School of Medicine, Nashville, TN). HintIII and SmalI fragments generated from pCMV4-FI-agarose (wild type), pCMV4-FI-agarose-Y42F, and pCMV4-FI-agarose-ΔC were cloned into the HintIII and SmalI sites of pBS(SK)− (Stratagene, La Jolla, CA) for preparing 35S-labeled, epitope-tagged IxBα protein.

Transfections—Confluent HeLa cells (106) were transiently transfected into 50% confluent HeLa cells (1 µg of plasmid/100-mm dish) by Lipofectamine (Life Technologies, Inc.) as described by the manufacturer. After 6 h, the medium was replaced by complete medium and left overnight. The transfected cells were pooled from plates and plated in 60-mm dishes overnight at 37 °C. Approximately 40 h after transfection, the cells were treated as indicated, and cytoplasmic extracts were prepared by the addition of lysis buffer containing 20 mM HEPES, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After 30 min on ice, the samples were cleared by centrifugation for 10 min. Protein was estimated by the method of Bradford (24). Approximately 40 µg of lysate was subjected to 10% SDS-PAGE. Western blot analysis was performed using a monoclonal anti-FLAG antibody at 1 µg/ml and horseradish peroxidase-conjugated goat anti-mouse IgG; the blots were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

Western Blotting of IxBα—ML-1a or U937 cells (2 × 106) were treated as indicated, collected by centrifugation, and washed two times with PBS and then resuspended in balanced salt solution. Unless otherwise stated, experiments with perevanadate were for 30 min at 37 °C using a concentration of 100 µM. Lysates were prepared as described previously (21). Protein was estimated by the method of Bradford (24). Approximately 40 µg of lysate was subjected to 10% SDS-PAGE. Western blot analysis was performed using the indicated antibodies, and blots were visualized by ECL.

Immunoprecipitation of IxBα from Orthophosphate-labeled Cells—U937 cells were labeled with 32P-orthophosphate (DuPont NEN) and immunoprecipitated as described earlier (22) except that a mixture of antibodies generated against the N terminus, C terminus, and full-length IxBα was used. In vitro transcribed and translated 35S-labeled IxBα (see below) was used as a control for the migration of IxBα on SDS-PAGE. For phosphoamino acid analysis, immunoprecipitates were resolved by 10% SDS-PAGE (a large gel apparatus, 18 × 20 cm) and electrobotted onto polyvinylidine difluoride membranes. The membrane containing phosphorylated IxBα was identified by autoradiography, excised, and subjected to acid hydrolysis followed by two-dimensional electrophoretic separation on thin-layer chromatography plates as described (25).

In Vitro Reconstitution Assay for IxBα Degradation—We used an in vitro reconstitution system similar to that described by Chen et al. (8). U937 cells were treated as described in the figure legends, and cytoplasmic extracts were prepared as described for Western blotting of IxBα except that phosphoric acid was added to a final concentration of 0.4 M and the supernatant was stored at −80 °C. The typical protein concentration of these extracts was 10−20 µg/ml. In vitro transcribed and translated 35S-labeled, FLAG-tagged IxBα and mutants were generated using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) using pBS-IxBα or pBS-FI-agarose plasmids as the template in a 50-µl reaction mixture containing [35S]methionine (Amersham Corp). The reconstitution system contained 1−5 µl of 35S-labeled, FLAG-tagged IxBα that was incubated with cell extracts (200−400 µg) in the presence of an ATP-regenerating system (50 mM Tris, pH 7.6, 5 mM MgCl2, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, and 30 mM okadaic acid). In one experiment, we omitted okadaic acid from the lysates and the reaction mixture, but included the kinase inhibitor isopentyladenine (26) at a concentration of 500 µM to inhibit the serine phosphorylation of IxBα. The reaction mixtures were incubated at 37 °C for the indicated times, and the reactions were stopped by the addition of lysis buffer (20 mM HEPES, pH 7.4, 250 mM NaCl, 0.1% Nonidet P-40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and immunoprecipitated with anti-FLAG antibody-conjugated agarose. The immunoprecipitates were washed twice with lysis buffer, and the protein was eluted by the addition of SDS sample buffer and boiling for 5 min. The samples were subjected to 10% SDS-PAGE, and the dried gels were visualized by a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) or by autoradiography and quantitated by ImageQuant software (Molecular Dynamics, Inc.).

Purification of PTP1B—The plasmid pGEX-PTP1B-His was used to transform Escherichia coli strain BL21. An overnight culture containing the transformed cells was used to inoculate 250 ml of Luria broth, grown to an A600 of 0.8, and induced with 0.5 mM isopropylthiogalactoside for 3 h at 37 °C. The cells were collected and ruptured by lysis buffer (20 mM Tris, pH 8.0, 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) containing 500 µg of lysozyme for 30 min on ice. The lysate was sonicated and clarified by centrifugation at 100,000 × g for 30 min, and the supernatant was loaded into 0.5 ml of glutathione-agarose batch chromatography in the presence of 25 mM imidazole for 1 h at 4 °C. The Ni2+-agarose was washed with 12 ml of lysis buffer containing 1 mM NaCl and 25 mM imidazole and then with 2 ml of buffer containing 20 mM HEPES, pH 7.4, and 50 mM NaCl. The protein was eluted with 2 ml of elution buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 10% glycerol, and 1 mM imidazole). The eluted protein (GST-PTP1B-His) was dialyzed overnight at 4 °C against storage buffer (50 mM HEPES, pH 7.9, 0.1% 2-mercaptoethanol, and 50% glycerol) and stored at −20 °C.

Protein-tyrosine Phosphatase Assay—U937 cells were treated and lysates were prepared as described (21). Lysates (20 µg) were mixed with 1 µg of purified GST-PTP1B-His or storage buffer and assayed in a 30-µl volume of protein-tyrosine phosphatase buffer containing 50 mM Tris, pH 7.4, 50 mM NaCl, and 1 mM dithiothreitol in the presence or absence of 2 mM orthovanadate for 30 min at 37 °C. The reactions were stopped with SDS sample buffer, and the samples were boiled for 5 min and subjected to 10% SDS-PAGE and immunoblotted with anti-IxBα antibodies as described above.

RESULTS AND DISCUSSION

Previously, we (21) and others (22) reported that inhibitors of protein-tyrosine phosphatases block the activation of NF-κB by a variety of inducers. In this report, we investigated the mechanism by which PV blocks this activation. We used human ML-1a, U937, and HELa cells in this study since their response to various inducers of NF-κB is well characterized (8, 21, 22, 27). We did not find any differences among these cell lines with regard to the inducible phosphorylation and degradation of IxBα.

PV Treatment Induces Phosphorylated IxBα That Is Not Degraded—To investigate the mechanism by which PV blocks the degradation of IxBα, we analyzed IxBα from ML-1a cells by Western blotting with IxBα-specific antibodies. Inducible phosphorylation of serines 32 and 36 of IxBα appears as a slow migrating band as analyzed by SDS-PAGE and immunoblotting of cytoplasmic extracts (8, 12, 23). As expected, Western blot analysis showed that TNF caused a slower migrating species of IxBα to appear within 2 min, to be degraded in 5 min, and to reappear after 30 min (Fig. 1A, upper panel). However, when cells were pretreated with PV, the slower migrating species of IxBα appeared (Fig. 1A, lower panel), and this form of IxBα was not degraded upon further stimulation with TNF (lower panel). A dose-response analysis indicated that a PV

1B. G. Darnay, S. Singh, and B. B. Aggarwal, unpublished observations.
Tyrosine Phosphorylation of IκBα

PV treatment causes the tyrosine phosphorylation of IκBα. A, anti-phosphotyrosine Western blot analysis of IκBα immunoprecipitated from PV-treated cells. IκBα was immunoprecipitated from untreated (Control) or PV-treated U937 cells, resolved by 10% SDS-PAGE, and immunoblotted with biotinylated anti-phosphotyrosine antibodies as described under "Experimental Procedures." B, the slower migrating species of IκBα from PV-treated cells is phosphorylated in vivo. Untreated (Control) or PV-treated U937 cells were labeled with [32P]orthophosphate, and lysates were prepared and immunoprecipitated with either IκBα antibodies or preimmune serum (PIS), resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and autoradiographed. In vitro transcribed and translated [35S]-labeled IκBα (Inst.) is shown as a control for the migration of IκBα. C, phosphoamino acid analysis reveals that IκBα from PV-treated cells is tyrosine-phosphorylated in vivo. After autoradiography of the membrane shown in B, the IκBα band from the control (n) and the slower migrating band from PV-treated cells (s) were excised from the membrane and subjected to phosphoamino acid analysis and two-dimensional thin-layer electrophoresis as described under "Experimental Procedures." The TLC plate was analyzed by a PhosphorImager, and the migration of phosphoamino acid standards was visualized by ninhydrin as indicated.

Fig. 2. PV treatment causes the tyrosine phosphorylation of IκBα. A, anti-phosphotyrosine Western blot analysis of IκBα immunoprecipitated from PV-treated cells. IκBα was immunoprecipitated from untreated (Control) or PV-treated U937 cells, resolved by 10% SDS-PAGE, and immunoblotted with biotinylated anti-phosphotyrosine antibodies as described under "Experimental Procedures." B, the slower migrating species of IκBα from PV-treated cells is phosphorylated in vivo. Untreated (Control) or PV-treated U937 cells were labeled with [32P]orthophosphate, and lysates were prepared and immunoprecipitated with either IκBα antibodies or preimmune serum (PIS), resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and autoradiographed. In vitro transcribed and translated [35S]-labeled IκBα (Inst.) is shown as a control for the migration of IκBα. C, phosphoamino acid analysis reveals that IκBα from PV-treated cells is tyrosine-phosphorylated in vivo. After autoradiography of the membrane shown in B, the IκBα band from the control (n) and the slower migrating band from PV-treated cells (s) were excised from the membrane and subjected to phosphoamino acid analysis and two-dimensional thin-layer electrophoresis as described under "Experimental Procedures." The TLC plate was analyzed by a PhosphorImager, and the migration of phosphoamino acid standards was visualized by ninhydrin as indicated.

PV Causes Tyrosine Phosphorylation of IκBα—Since PV inhibits protein-tyrosine phosphatases and increases total protein tyrosine phosphorylation in cells (Ref. 22 and data not shown), it may directly induce the tyrosine phosphorylation of IκBα. To explore this possibility, immunoprecipitated IκBα from control and PV-treated cells was analyzed by Western blotting using anti-phosphotyrosine antibodies. IκBα immunoprecipitated from PV-treated cells, but not from control cells, was indeed tyrosine-phosphorylated (Fig. 2A). The tyrosine-phosphorylated band was confirmed to be the slower migrating species of IκBα by Western blot analysis using anti-IκBα antibodies (data not shown). Additionally, immunoprecipitation of IκBα from [32P]-labeled cells revealed a slower migrating species of IκBα only from cells treated with PV (Fig. 2B). Furthermore, phosphoamino acid analysis revealed that IκBα was constitutively phosphorylated at serine residues (Fig. 2C, left panel), concentration of 25 μM caused the slower migrating band to appear, and 100 μM PV converted all IκBα to the slower migrating species (Fig. 1B, upper panel). A time course analysis with 100 μM PV indicated that a 5-min treatment was sufficient to convert all IκBα to the slower migrating species (Fig. 1B, lower panel). The retardation of the migration of IκBα from PV-treated cells was attributed to phosphorylation since calf intestine alkaline phosphatase increased its mobility on SDS-PAGE (Fig. 1A). These observations indicated that PV treatment caused the phosphorylation of IκBα, which thus protected it from TNF-induced degradation.

FIG. 1. PV prevents TNF-induced degradation of IκBα. A, PV decreases the mobility of IκBα and inhibits its degradation by TNF. A, PV decreases the mobility of IκBα and inhibits its degradation by TNF. ML-1a cells pretreated without (upper panel) or with (lower panel) PV (100 μM, 30 min, 37°C) were incubated in the presence of various concentrations of calf intestine alkaline phosphatase (CIP) for 10 min at 37°C and then assayed for IκBα as described for A, s, n, and f represent the slower, normal, and faster migrating species of IκBα, respectively.
most likely by casein kinase II (28–30); but in PV-treated cells, the slower migrating species was additionally phosphorylated at a tyrosine residue (Fig. 2C, right panel). Thus, the pervanadate-induced decrease in the mobility of IκBα was due to tyrosine (not serine) phosphorylation.

Tyrosine Phosphorylation of IκBα Prevents TNF-induced Serine Phosphorylation—To further examine the effects of tyrosine phosphorylation on TNF-induced serine phosphorylation, we performed a Western blot analysis. However, like TNF-induced phosphorylation, PV-induced phosphorylation reduces the mobility of IκBα, thus making it difficult to distinguish the serine- and tyrosine-phosphorylated forms by Western blotting. To differentiate the phosphorylation of serine from that of tyrosine, we used a specific tyrosine phosphatase, PTP1B (32), which should reverse only the PV-induced dephosphorylation. Where or mutants (Y42F and ΔC) were incubated with cell lysates prepared from PV-treated U937 cells in the presence of an ATP-regenerating system at 37°C for 45 min and immunoprecipitated with anti-FLAG antibody-conjugated agarose as described under “Experimental Procedures.” Where indicated, the immunoprecipitates were mixed with PTP1B, and a phosphatase assay was performed as described under “Experimental Procedures.” Proteins were eluted by the addition of SDS sample buffer, boiled, resolved by 10% SDS-PAGE, and analyzed by a PhosphorImager. C, cell transfection of FLAG-tagged IκBα implicates Tyr-42 as the phosphorylation site induced by PV in vivo. HeLa cells were transfected with epitope-tagged wild-type IκBα (WT) or the Y42F mutant as described under “Experimental Procedures.” Cells were left untreated or were treated with PV, and cytoplasmic lysates were prepared. Fifty micrograms of lysates was subjected to 8.5% SDS-PAGE (large gel apparatus), immuno-blotted onto polyvinylidene difluoride membranes, and probed with anti-FLAG antibodies as described under “Experimental Procedures.” s and n represent the slower and normal migrating species of IκBα, respectively.

Fig. 4. PV causes the phosphorylation of Tyr-42 on IκBα. A, schematic diagram of human IκBα and the positions of all eight tyrosine residues. The amino-terminal region (residues 1–72) and ankyrin repeats (residues 73–242, shaded) are indicated. The C-terminal region contains an acidic region, PEST region, and phosphorylation sites for casein kinase II (CK II). All eight tyrosine residues are numbered. The two inducible phosphorylation (serines 32 and 36) and ubiquitination (lysines 21 and 22) sites are shown in an expanded region containing residues 18–47. A mutant of IκBα lacking residues 243–317 (ΔC) and a mutant in which Tyr-42 was changed to Phe (Y42F) were used in this study as described under “Experimental Procedures.” B, in vitro phosphorylation of Tyr-42 on IκBα from PV-treated cells. 32S-Labeled, FLAG-tagged wild-type IκBα (WT) or mutants (Y42F and ΔC) were incubated with cell lysates prepared from PV-treated U937 cells in the presence of an ATP-regenerating system at 37°C for 45 min and immunoprecipitated with anti-FLAG antibody-conjugated agarose as described under “Experimental Procedures.” Where indicated, the immunoprecipitates were mixed with PTP1B, and a phosphatase assay was performed as described under “Experimental Procedures.” Proteins were eluted by the addition of SDS sample buffer, boiled, resolved by 10% SDS-PAGE, and analyzed by a PhosphorImager. C, cell transfection of FLAG-tagged IκBα implicates Tyr-42 as the phosphorylation site induced by PV in vivo. HeLa cells were transfected with epitope-tagged wild-type IκBα (WT) or the Y42F mutant as described under “Experimental Procedures.” Cells were left untreated or were treated with PV, and cytoplasmic lysates were prepared. Fifty micrograms of lysates was subjected to 8.5% SDS-PAGE (large gel apparatus), immuno-blotted onto polyvinylidene difluoride membranes, and probed with anti-FLAG antibodies as described under “Experimental Procedures.” s and n represent the slower and normal migrating species of IκBα, respectively.

Besides inducing the tyrosine phosphorylation of IκBα, pervanadate could also either inactivate the IκBα serine kinase or block an upstream activator of the IκBα serine kinase. This would require the localization of the tyrosine residue on IκBα that undergoes phosphorylation in response to PV. The mutation of this tyrosine residue, then, should overcome the inhibition of PV treatment without interfering with the serine kinase.
epitope-tagged wild-type and Y42F IκBα genes into HeLa cells and analyzed the expression of the IκBα proteins by Western blotting using anti-FLAG antibodies: the FLAG-tagged Y42F IκBα mutant migrated faster than the wild type on SDS-polyacrylamide gels. Upon PV treatment, FLAG-tagged wild-type IκBα, but not the Y42F mutant, decreased in mobility (Fig. 4C), providing evidence that this site was tyrosine-phosphorylated in vivo. Because of overexpression, however, only a small portion of epitope-tagged IκBα was shifted in PV-treated cells (Fig. 4C). Interestingly, Tyr-42 is close to the inducible serine phosphorylation sites (serines 32 and 36) and ubiquitination sites (lysines 21 and 22) needed for the degradation of IκBα. Thus, it is possible that the phosphorylation of Tyr-42 stereocemically hindered subsequent phosphorylation by the inducible serine kinase.

Tyrosine-phosphorylated IκBα Is Protected from Inducible Degradation—Because the IκBα-overexpressing HeLa cells lacked TNF responsiveness, our attempts to utilize these cells expressing epitope-tagged wild-type IκBα and the Y42F mutant were unsuccessful. Therefore, we used an in vitro reconstitution assay that contained cellular extracts, an ATP-regenerating system, and okadaic acid with wild-type IκBα or the Y42F IκBα mutant. [35S]-Labeled, FLAG-tagged wild-type IκBα was phosphorylated and degraded in a time-dependent manner using this assay (Fig. 5A). In vitro translated [35S]-labeled Y42F IκBα was mixed with either untreated or PV-treated cell lysates for 60 min as described for Fig. 5A, subjected to SDS-PAGE, and analyzed by a PhosphorImager. The Y42F mutant was degraded similarly in extracts prepared from either untreated or PV-treated cells (Fig. 5B), suggesting a lack of effect of PV on the serine kinase.

To further confirm that tyrosine-phosphorylated IκBα is protected from degradation, we prepared a tyrosine-phosphorylated form of IκBα by first incubating [35S]-labeled, epitope-tagged IκBα in extracts from PV-treated cells (similar to Fig. 4B) and then immunoprecipitating it with anti-FLAG antibody-conjugated agarose; we used the immunoprecipitate as the substrate in the in vitro reconstitution system with lysates prepared from untreated (control) or PV-treated cells. Tyrosine-phosphorylated, [35S]-labeled IκBα was protected from degradation in lysates prepared from PV-treated cells (Fig. 5C). However, >50% of the [35S]-labeled IκBα was degraded in control lysates (Fig. 5C), the latter perhaps because tyrosine-phosphorylated IκBα first underwent dephosphorylation at tyrosine, leading to subsequent serine phosphorylation and degradation. These results are consistent with the thesis that site-specific tyrosine phosphorylation of residue 42 on IκBα protects it from inducible serine phosphorylation and thus inhibits its subsequent degradation. Since phosphorylation of serine residue 32 or 36 is a prerequisite for the subsequent ubiquitination of lysines 21 and 22 (8, 11, 19), which targets IκBα for degradation, the mechanism of inhibition by pervanadate appears to be upstream of the ubiquitin machinery. This may explain why ubiquitin-conjugated forms of IκBα were not observed in cell lysates prepared from (PV + TNF)-treated U937 cells (data not shown).

We have shown here that treatment of cells with PV results in site-specific tyrosine phosphorylation of IκBα and that this phosphorylation protects it from degradation and hence inhibits the activation of NF-κB. While PV blocks the degradation of IκBα induced by a wide variety of inducers (21, 22), the data suggest a common inhibitory role for this tyrosine phosphorylation with all these inducers. It is plausible that a futile cycle occurs in which a protein-tyrosine kinase and phosphatase catalyze the phosphorylation and dephosphorylation of IκBα in vivo. That would explain why we and others (22) were not able to detect tyrosine-phosphorylated IκBα in unstimulated cells. It is possible that the amount of tyrosine-phosphorylated IκBα in unstimulated cells is below the limits of detection due to an active protein-tyrosine phosphatase, but we were able to detect tyrosine-phosphorylated IκBα in vivo when protein-tyrosine phosphatase activity was abolished.

Although Tyr-42 is conserved among all IκBα gene products known thus far, IκBβ does not contain an analogous tyrosine positioned near its inducible phosphorylation sites (6). Additionally, IκBβ is not tyrosine-phosphorylated upon treatment with pervanadate. Like IκBα, however, IκBβ also appears to be regulated by phosphorylation and degradation (5, 6, 11, 35). While TNF is able to induce the degradation of IκBα and IκBβ in HeLa cells (11), it is not able to cause the degradation of IκBβ in U937 cells. Thus, it appears that the ability of inducers to cause the degradation of IκBβ could be cell type-specific (5, 6). The tyrosine phosphorylation of IκBα may suggest an additional mechanism of regulation, one that affects IκBα, but...
not IxBβ. The block of IxBα and IxBβ degradation by specific proteasome inhibitors is a viable strategy for targeting the unwanted activation of NF-kB in inflammatory and immune responses. The molecular identity of the putative protein-tyrosine kinase and phosphatase suggested here may allow for the design of pharmacological inhibitors of NF-kB activation.

Note Added in Proof—While our paper was in press, a report appeared by Imbert et al. (36) confirming our results that treatment of Jurkat cells with pervanadate causes tyrosine 42 phosphorylation of IxBk. They did not examine the effect of this phosphorylation on TNF-dependent NF-kB activation. Unlike our results, however, Imbert et al. (36) showed that tyrosine 42 phosphorylation of IxBk accompanies activation of NF-kB by pervanadate.

Acknowledgments—We thank Dr. P. J. Chiao for providing the IxBk cDNA, Dr. D. W. Ballard for providing pCMV4 FLAG-tagged IxBk and its mutants, Dr. J. Weiner for providing pGEX-PTP1B-His, Dr. D. Choube for providing anti-phosphotyrosine antibodies, Dr. S. A. G. Reddy for many delightful discussions, and W. Pagel for helpful suggestions in editing the manuscript.

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