The 90-kDa Ribosomal S6 Kinase (pp90rsk) Phosphorylates the N-terminal Regulatory Domain of IκBα and Stimulates Its Degradation in Vitro*

(Received for publication, January 3, 1997, and in revised form, May 20, 1997)

Lucy Ghodasă§§, Xin Lin§§, and Warner C. Greene¶¶

From the §University of Colorado Health Sciences Center, Department of Pharmacology, School of Medicine, Denver, Colorado 80262 and the ¶¶Gladstone Institute of Virology and Immunology and Departments of Medicine and Microbiology and Immunology, University of California, San Francisco, San Francisco General Hospital, San Francisco, California 94141-9100

The 90-kDa Ribosomal S6 Kinase (NF-κB) is a eukaryotic member of the Rel family of transcription factors whose biological activity is post-translationally regulated by its assembly with various ankyrin-rich cytoplasmic inhibitors, including IκBα. Expression of NF-κB in the nucleus occurs after signal-induced phosphorylation, ubiquitination, and proteasome-mediated degradation of IκBα. The induced proteolysis of IκBα unmasks the nuclear localization signal within NF-κB, allowing its rapid migration into the nucleus, where it activates the transcription of many target genes. At present, the identity of the IκB kinase(s) that triggers the first step in IκBα degradation remains unknown. We have investigated the potential function of the 90-kDa ribosomal S6 kinase, or pp90rsk, as a signal-inducible IκBα kinase. pp90rsk lies downstream of mitogen-activated protein (MAP) kinase in the well characterized Ras-Raf-MEK-MAP kinase pathway that is induced by various growth factors and phorbol ester. We now show that pp90rsk, but not pp70S6K or MAP kinase, phosphorylates the regulatory N terminus of IκBα principally on serine 32 and triggers effective IκBα degradation in vitro. When co-expressed in vivo in COS cells, IκBα and pp90rsk readily assemble into a complex that is immunoprecipitated with antibodies specific for either partner. While phorbol 12-myristate 13-acetate produced rapid activation of pp90rsk, in vivo, other potent NF-κB inducers, including tumor necrosis factor α and the Tax transactivator of human T-cell lymphotropic virus, type I, failed to activate pp90rsk. These data suggest that more than a single IκBα kinase exists within the cell and that these IκBα kinases are differentially activated by different NF-κB inducers.

Nuclear factor κB (NF-κB) is a transcription factor whose function is regulated by a family of cytoplasmic inhibitors termed the IκBs (reviewed in Refs. 1 and 2). At present, nine IκB family members have been identified (IκBα, IκBβ, IκBγ, IκBδ, IκBε, p105, p100, Bcl-3, and Cactus), each distinguished by the presence of multiple ankyrin repeats. The prototypic and best studied of the IκBs is IκBα (3), which binds to the heterodimeric NF-κB complex (p50/Rel A) (4), masks the nuclear localization signal present in Rel A (5, 6), and sequesters NF-κB in the cytoplasm (4–6). When appropriate inductive signals are delivered to the cell, phosphorylation of IκBα ensues (7–10), followed by the conjugation of multiple ubiquitin molecules and the degradation of the ubiquitinated IκBα phosphoprotein by the 26 S proteasome complex (11–13). Of note, IκBα degradation proceeds while the inhibitor is still physically associated with the NF-κB phosphoprotein by the 26 S proteasome complex (11–13). Note, the NF-κB complex is ultimately liberated, allowing its rapid translocation into the nucleus, where it engages cognate enhancer elements and alters the transcriptional activity of various target genes.

Although phosphorylation of IκBα is required for its proteinolysis and the subsequent activation of NF-κB, the nature of the cellular protein kinase(s) mediating this reaction remains unknown. Signal-induced phosphorylation involves two serine residues located at positions 32 and 36 near the N terminus of IκBα. Substitution of these serines with alanine residues generates a constitutively acting IκBα repressor that readily binds to NF-κB but fails to undergo signal-induced phosphorylation and degradation (7, 8, 10, 18).

Studies in Drosophila have also yielded valuable insights into the biology of the Rel proteins and their control by the IκBs. In the dorsal-ventral signal transduction pathway of Drosophila, dorsalizing signals mediated through the receptor Toll (an IL-1 receptor homologue) target Cactus (a member of the IκB family) for degradation and result in activation of Dorsal (a Rel family member) (19). In this pathway, Pelle, a serine/threonine protein kinase, regulates the degradation of Dorsal (a Rel family member) (20). Recently, a human IL-1 receptor-associated kinase has been cloned that is homologous to Pelle (21). This kinase appears to participate in the IL-1-induced signaling pathway leading to NF-κB induction, but no evidence yet exists for its direct phosphotransfer to IκBα.

Casein kinase II (CKII) also phosphorylates IκBα in vivo (22–24). However, phosphopeptide mapping of phosphorylated virus; TNF-α, tumor necrosis factor α; PAGE, polyacrylamide gel electrophoresis; CaMKII, Ca2+-calmodulin-dependent protein kinase II; CREB, cyclic AMP response element-binding protein; PP2A, protein phosphatase 2A.

* This work was supported by National Institutes of Health Grants P30 AI27763-08 Supplement (to W. C. G.) and 5RO1 GM49055, American Cancer Society Research Award VM-91, a Junior Faculty Research Award of the American Cancer Society (to L. G.), and Grant P30 AI27763 from the National Institutes of Health/University of California, San Francisco, Center for AIDS 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.

§ These authors contributed equally to this work.

¶¶ To whom correspondence should be addressed: Gladstone Institute of Virology and Immunology, P. O. Box 41900, San Francisco, CA 94141-9100. Tel.: 415-695-3800, Fax: 415-826-1817; E-mail: Warner_Greene@quicmail.ucsf.edu.

The abbreviations used are: NF-κB, nuclear factor κB; PMMA, phorbol 12-myristate 13-acetate; IL, interleukin; CKII, casein kinase II; MEK, MAP/ERK kinase kinase; MAP, mitogen-activated protein; HA, hemagglutinin; DTT, dithiothreitol; HTLV, human T-cell lymphotropic virus; TNF-α, tumor necrosis factor α; PAGE, polyacrylamide gel electrophoresis; CaMKII, Ca2+-calmodulin-dependent protein kinase II; CREB, cyclic AMP response element-binding protein; PP2A, protein phosphatase 2A.

This paper is available on line at http://www.jbc.org

Vol. 272, No. 34, Issue of August 22, pp. 21281–21288, 1997

Printed in U.S.A.
pp90^rsk Triggers Degradation of IkBa in Vitro

IkBa has shown that residues within the C-terminal PEST region, rather than the N-terminal serines, are targeted by CKII. Phosphorylation of the C terminus of IkBa by CKII or other kinases may play a role in the constitutive degradation of uncomplexed IkBa. Additionally, CKII-mediated phosphorylation appears important for the accelerated turnover of IkBa and the persistent induction of NF-κB observed following HIV infection of macrophages (24). Immunodepletion of CKII from these cell extracts results in an inhibition of IkBa degradation in vitro (24).

Recently, a novel ubiquitination-stimulated protein kinase has been identified that phosphorylates IkBa in a serine 32/36-dependent manner (25). This kinase resides in a large 700-kDa multiprotein complex, and ubiquitination of some component of the complex results in increased IkBa phosphorylating activity. Whether ubiquitination directly activates the kinase or, alternatively, acts indirectly to alter another component of the complex remains unresolved.

We have investigated the potential role of a known intracellular protein kinase in the Ras-Raf-MEK-MAP kinase signaling pathway as an intracellular protein kinase in the Ras-Raf-MEK-MAP kinase signaling pathway (26, 27). We now show that pp90^rsk phosphorylates the N terminus of IkBa principally on serine 32 and functionally induces IkBa degradation in vitro. We further show that pp90^rsk and IkBa can physically associate in vivo. Finally, we show that only a subset of the known NF-κB-inducing signals leads to the activation of pp90^rsk. These findings suggest that rather than a single IkBa kinase, a family of IkBa kinases may exist within the cell that are differentially activated by different inducers of NF-κB.

MATERIALS AND METHODS

cDNA Vectors and Expression of Proteins—The expression vector containing a hemagglutinin-epitope-tagged pp90^rsk cDNA (pMT2-HA-RSK1) was provided by Dr. Joseph Avruch (Harvard University and Massachusetts General Hospital, Boston, MA). Wild type IkBa cDNA provided by Dr. A. Baldwin (University of Colorado, Chapel Hill, NC) was cloned into the HindIII and XbaI sites of the pcMV4 eukaryotic expression vector provided by Dr. Mark Stinski (University of Iowa, Iowa City, IA). For in vitro translation, wild type IkBa or mutant IkBa containing alanine for serine substitutions at position 32 and/or 36 (4) were cloned into the HindIII and XbaI or Xmal sites of the bScript SK(+)-vector using上下游 tagged, biologically radiolabeled IkBa or its mutant analogues were synthesized by transcription-coupled in vitro translation in wheat germ extracts (Promega).

A bacterial expression plasmid encoding hexahistidine (His)-tagged IkBa was constructed by cloning the IkBa cDNA into the pTrcHisC vector (Invitrogen). The N-terminal Δ1–36 IkBa deletion mutant was cloned into the pSET2C vector (identical to the pTrcHisC vector except in the promoter region). Wild type IkBa and the S32/36A IkBa containing alanine for serine substitutions at both positions 32 and 36 were isolated from Escherichia coli lysates by purification on a nickel chelate column (Ni-NTA, Qiagen). Following an initial wash in high salt buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl), successive washes were performed with elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing increasing concentrations of imidazole (0, 10, 50, 100 mM). The His-tagged proteins were eluted in buffer containing 200 mM imidazole. The fractions containing the desired protein were dialyzed overnight in 50 mM Tris-HCl, pH 7.5, and 2 mM DTT.

Cell Lines and Tissue Culture Conditions—Jurkat cells and Jurkat cells stably expressing either HTLV-I Tax or Tax antisense cDNA constructs and a neomycin resistance gene were maintained in RPMI 1640 supplemented with 10% fetal calf serum and penicillin/streptomycin at 37 °C in 5% CO2. 800 μg/ml G418 was added to the Jurkat-Tax and anti-Tax cell culture media. Cells were treated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) or TNF-α (50 ng/ml) for various periods of time. Vehicle controls corresponding to the amounts of added MeSO for PMA and water for TNF-α were performed in parallel. The cells were washed once with ice-cold phosphate-buffered saline and lysed in ELB buffer (50 mM HEPEs, pH 7.4, 250 mM NaCl, 0.2% Nonidet P-40, 5 mM EDTA, 0.5 mM DTT, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture containing 0.75 μg/ml bestatin; 0.5 μg/ml each of aprotinin, antipain, leupeptin, and trypsin inhibitor; 0.4 μg/ml phosphorylase; and 0.05 μg/ml pepstatin). The cell lysates were clarified by centrifugation at 4 °C for 15 min at 15,000 × g, and the supernatant was used for immunoprecipitation as described below.

In Vitro Assay of Protein Kinase Activity—Phosphorylation of bacterially expressed IkBa (0.5 μg) was performed in 50-μl reaction mixtures incubated for 15 min at room temperature. For Xenopus laevis egg-derived MAP kinase, X. laevis egg-derived pp90^rsk (4), and mouse pp70^s6k, the reaction buffer contained 20 mM HEPEs, pH 7.0, 10 mM MgCl2, 2 mM ATP, 0.05 mM EGTA, 0.5 mM bovine serum albumin, 40 μCi of [γ-32P]ATP (specific activity 3000 Ci/μmol). Myelin basic protein (0.1 mg/ml, Sigma) or 40 S ribosomal subunits (0.05 mg/ml) generously provided by Dr. James Maller (Howard Hughes Medical Institute and University of Colorado Health Sciences Center, Denver, CO) were used as positive controls for these kinase reactions. Aliquots of the reactions were mixed in SDS-PAGE sample buffer, heated to 98 °C, and microcentrifuged at 15,000 × g for 2 min, followed by analysis of the supernatants by SDS-PAGE. Alternatively, IkBa was immunoprecipitated from the reactions with peptide-specific polyclonal rabbit antisera (2.5 μg) recognizing the C-terminus of IkBa. Immune complexes were reacted with formalin-fixed Staphylococcus aureus cells (Pansorbin A, 50 μl, Calbiochem) and collected by centrifugation. The Protein A- bound immune complexes were washed three times until similarly analyzed by SDS-PAGE and autoradiography. In the case of pp90^rsk, three independently purified preparations were tested. The specific activities of these pp90^rsk preparations were 4.6, 8.9, and 11.2 nmol of ATP incorporated/min/mg of protein assayed using Kemptide as substrate.

V8 Digestion of in Vitro Phosphorylated IkBa—Bacterially produced His-tagged IkBa was phosphorylated with purified pp90^rsk as described above and subjected to mild V8 proteolysis at room temperature for 10 min. The protease inhibitor N-α-tosyl-l-lysine chloromethyl ketone was added to the reaction mixture together with 100 μg/ml (final concentration) of bovine serum albumin to terminate proteolytic cleavage. IkBa peptides containing the N-terminal His-epitope were immuno- precipitated from the digest using his-tag-specific antibodies. The immunoprecipitates were analyzed with Tris-Tricine gels by the method of Schagger and von Jagow (28).

In Vitro Degradation of IkBa—In vitro synthesized [35S]-radiolabeled IkBa was phosphorylated with pp90^rsk in the presence of unlabeled ATP as described. The phosphorylated or control IkBa proteins (25 μl) were incubated in 100 μl of reticulocyte lysates containing 5 mM DTT, 2.5 mM ATP, 1 mM creatine phosphate, and 200 μg/ml creatine phosphokinase. Samples were removed at various times and quickly frozen in liquid nitrogen. The samples were then immunoprecipitated with C-terminal-specific anti-IkBa antibodies and analyzed by SDS-PAGE followed by autoradiography.

Coimmunoprecipitation Assays—Monkey kidney COS57 cells, maintained in complete Dulbecco’s modified Eagle’s medium, were transfected with pMT2-RA-HSK1 (encoding pp90^rsk) or pCMV4-HA-PP2A (encoding a regulatory subunit of protein phosphatase 2A) and CMV4-IkBa using LipofectAMINE (Life Technologies, Inc.). After 48 h, the cells were starved for 1 h in methionine/cysteine-free Dulbecco’s modified Eagle’s medium and then metabolically radiolabeled with [35S]methionine and [35S]cysteine for 2 h. Whole-cell extracts were prepared by lysis in ELB buffer, followed by immunoprecipitation analyses as described above using either anti-HA-epitope antibody (BabCo, Berkeley, CA) or anti-IkBa antisera specific for the C terminus of this inhibitor. Nonradioactive COS cell lysates were also prepared and immunoprecipitated with the anti-HA or IkBa antibodies followed by immunoblotting with anti-pp90^rsk-specific antibodies. These immunoblots were developed with a horseradish peroxidase-conjugated secondary antibody and enhanced chemoluminescence (ECL) as described by the manufacturer (Amersham).

Immunocomplex-associated Protein Kinase Assay—Cell lysates derived from about 5 × 10⁸ Jurkat cells were precleared for 1 h and incubated with 10 μl of anti-pp90^rsk antibody (Santa Cruz Biotechnol- ogy) at 4 °C for 1 h. 30 μl of Protein A-agarose (Boehringer Mannheim) was then added to the mixture, and the incubation was continued for an additional 1 h. The mixture was centrifuged at 5000 rpm for 3 min in ELB buffer. Immunoprecipitated complexes were washed three times with kinase buffer (25 mM glycerol-2-phosphate, 20 mM HEPEs, pH 7.4, 10 mM MgCl2, 4 mM NaF, 2 mM MnCl2, 1 mM dithiothreitol, 0.1 mM Na3VO4, and either 20 μM ATP (specific activity 3000 Ci/μmol for [32P]labeling of recombinant IkBa) or 100 μM ATP (for phosphorylation of [35S]-labeled IkBa). 30 μl of the kinase buffer containing 0.3 μg of bacterially expressed IkBa or 2 μl of...
wheat germ-translated reactions of wild type or mutant IkBα was added to the immune complexes, and the mixtures were incubated at 30 °C for 30 min for [35S]P radiolabeling of IkBα or 2 h for phosphorylation of [35S]labeled IkBα. The reaction products were then analyzed by SDS-PAGE and autoradiography.

Phosphatase Treatment of Phosphorylated Proteins—Following the phosphorylation reactions, IkBα was immunoprecipitated with anti-IkBα antiserum specific for the C-terminus and Protein A-agarose. The bound immune complexes were washed three times in dephosphorylation buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA) followed by the addition of 10 units of calf intestinal alkaline phosphatase (Boehringer Mannheim). The samples were then incubated at 37 °C for 30 min and analyzed by SDS-PAGE and autoradiography.

RESULTS

In Vitro Phosphorylation of IkBα by pp90rsk—Phorbol ester is both a potent inducer of NF-κB and a known activator of the MAP kinase pathway. To determine whether kinases positioned along this or related pathways are capable of phosphorylating IkBα, the ability of MAP kinase, pp70S6K, and pp90rsk to phosphorylate IkBα in vitro was examined. These kinases phosphorylate IkBα residues that lie within specific consensus sequences. For example, MAP kinase phosphorylates serines and threonines that precede proline residues (proline-directed kinase consensus sequence), while both pp90rsk and pp70S6K phosphorylate serines or threonines within the consensus sequence Arg-X-Ser/Thr (29).

Recombinant His-tagged IkBα was used as the substrate in these in vitro kinase assays. Following the kinase reaction, IkBα was immunoprecipitated from the reaction using polyclonal anti-IkBα antibodies (30). Immunoprecipitation was performed to ensure that the phosphorylated protein was indeed recombinant IkBα. As shown in Fig. 1, pp90rsk (lanes 7 and 8), but not pp70S6K (lanes 3 and 4) or p42 MAPK (lanes 11 and 12), phosphorylated recombinant His-tagged IkBα in vitro. The failure of the pp70S6K and p42 MAPK preparations to phosphorylate IkBα was not due to inactivation of these enzymes during their purification, as each capably phosphorylated known protein substrates, including the 40S ribosomal subunit for pp70S6K (lanes 1 and 2) and myelin basic protein for p42 MAPK (lanes 9 and 10). Of note, while both pp90rsk and pp70S6K recognize the same consensus phosphoacceptor site and phosphorylate the S6 protein of the 40S ribosome, only pp90rsk phosphorylated IkBα. Another kinase, Ca2+-calmodulin-dependent protein kinase II (CamKII) also shares the same consensus phosphoacceptor site as pp90rsk phosphorylated IkBα. However, like pp70S6K CamKII failed to phosphorylate IkBα in vitro, although it did phosphorylate one of its physiological substrates, synapsin II (data not shown). These data highlight the ability of pp90rsk to utilize IkBα as a substrate for phosphorylation in vitro.

Immunoprecipitation of a Phosphorylated N-terminal Fragment of IkBα—To ascertain whether pp90rsk phosphorylates IkBα at either of the two critical N-terminal serine residues located in positions 32 and 36, a 6xHis-IkBα and a similarly epitope-tagged S32/36A IkBα mutant in which both of these serines were substituted with alanine, was subjected to in vitro phosphorylation as described above. The protein was then subjected to mild V8 (endoprotease Glu C) proteolysis with a sequencing-grade protease. The cleaved proteins were immunoprecipitated with antisera specific for the N-terminal histidine epitope. This antisera recognizes both wild type IkBα and the S32/36A His-tagged mutants. Phosphorylation of IkBα at either serine 32 or 36 should result in a fragment of 84/87 amino acids (including the epitope tag) when exposed to V8 protease, which cleaves at residue 48 or 51. Fig. 2A depicts the recombinant IkBα protein showing the signal-dependent N-terminal phosphorylation sites in relation to the V8 cleavage sites. The smallest band in the immunoprecipitate shown in the leftmost two lanes of Fig. 2B, indicated by the arrow, migrates between the 8.16- and 10.6-kDa myoglobin fragment, consistent with the molecular size of the 84/87 N-terminal fragment of IkBα. In contrast, the S32/36A IkBα protein failed to yield a similarly sized band (Fig. 2, fourth and fifth lanes). The even smaller band detected with these samples was also detected in the absence of added V8 protease. These results thus demonstrate that pp90rsk is capable of phosphorylating the regulatory N terminus of IkBα involving serine 32 and/or 36.

pp90rsk-mediated Phosphorylation Promotes IkBα Degradation in Vitro—Although a variety of kinases can phosphorylate IkBα in vitro, the critical functional issue is whether these kinases promote IkBα degradation. Using an in vitro degradation system, we studied whether pp90rsk-mediated phosphorylation of IkBα triggers its destruction. The reticulocyte lysate degradation system employed in these experiments contains all of the component proteins and macromolecules necessary for ubiquitin-dependent and -independent 26S proteasome-mediated degradation (31). A variety of proteins have been shown to be degraded in this reticulocyte lysate system, including ornithine decarboxylase, antizyme, and the transcription factors Fox, Jun, and Myc (32–38).

[35S]-Radiolabeled wild type IkBα (Fig. 3, upper panel) and the S32/36A IkBα mutant (lower panel) were synthesized in vitro and preincubated with Xenopus egg-derived pp90rsk (lanes 1–4), mammalian pp70S6K (lanes 5–8), or control kinase buffer lacking an added kinase (lanes 9 and 10). The IkBα proteins were then incubated in a degradation-competent reticulocyte lysate that had specifically been pretreated with RNase or heparin. Hemin is known to inhibit the 26S proteasome but is often added to reticulocyte lysates to improve translation since it prevents premature peptide chain termination (39). Degradation of [35S]-IkBα was monitored by immunoprecipitation with specific anti-IkBα antisera over the course of a 120-min incubation conducted in the presence of an ATP-regenerating system. As shown in lanes 1–4 of the upper panel, pp90rsk-treated wild type IkBα was significantly degraded during the 2-h incubation. However, IkBα treated with pp70S6K (lanes 5–8) or control buffer (lanes 9 and 10) was not degraded. In contrast to wild type IkBα, the S32/36A IkBα mutant was not degraded when incubated with pp90rsk (lower panel, lanes 1–4), indicating that IkBα degradation in this in vitro system is dependent on the presence of these N-terminal regulatory serines, as it is in vivo. Together, these results suggest that pp90rsk-mediated phosphorylation of IkBα is biologically relevant since it leads to serine 32- and/or serine 36-dependent degradation of wild type IkBα protein in vitro.
**pp90^sk** Triggers Degradation of IxBα in Vitro

**pp90^sk** Phosphorylates IxBα by pp90^sk Principally on Serine 32—Previous in vivo studies have shown that both S32 and S36 at the N terminus of IxBα are required for signal-induced phosphorylation and degradation of this inhibitor (7–10, 18). Additionally, both of these serine residues are directly phosphorylated in vivo by an unknown kinase(s) following cellular stimulation with PMA or TNF-α (45). Phosphorylation at these sites in vivo results in a slower electrophoretic mobility for the IxBα protein that is readily detectable on SDS-PAGE gels. In contrast, no mobility shift is observed when cells containing the S32/36A IxBα mutant is similarly induced. To assess whether pp90^sk mediates phosphorylation on S32, S36, or both sites, pp90^sk-generated fragments were examined by trypsin digestion of IxBα lysates containing [35S]methionine/cysteine, followed by SDS-PAGE and autoradiography, and the resulting autoradiogram is shown in Fig. 2A, schematic of IxBα protein including N-terminal serines 32 and 36, ankyrin repeats, and C-terminal PEST sequences. Endoprotease Glu C (V8) digestion of this His-tagged IxBα protein substrate is predicted to yield N-terminal IxBα fragments that are 84 and 87 amino acids long and span serines 32 and 36 and the N-terminal His-epitope tag. B, autoradiogram of a Tris-Tricine SDS-PAGE gel (10–20% gradient) containing anti-N-terminal IxBα immunoprecipitations (first, second, fifth, and sixth lanes) performed on pp90^sk-phosphorylated and V8-proteolized wild type His-tagged IxBα (duplicate samples in first and second lanes) or the IxBα S32/36A mutant (fourth and fifth lanes). Proteolytic digestions were performed as described under “Materials and Methods.” Undigested phosphorylated wild type and S32/36A IxBα proteins are shown in the third and sixth lanes, respectively. The arrow indicates an appropriate-sized fragment for the N-terminal peptide uniquely generated with wild type IxBα but not with the S32/36A mutant of IxBα. The even smaller band obtained with IxBα S32/36A likely corresponds to a spontaneous degradation product, as it is also present in the untreated control sample.

**Phosphatase Treatment of Phosphorylated IxBα Reverses the Shift in Mobility on SDS-PAGE**—To confirm that the observed mobility shifts reflect phosphorylation of IxBα, pp90^sk-treated wild type and mutant S32/36A IxBα proteins were incubated with calf intestinal alkaline phosphatase (Fig. 4B). The regained mobility of pp90^sk-treated wild type IxBα (lane 3) was lost following treatment with phosphatase (lane 4). In contrast, phosphatase treatment of the S32/36A IxBα mutant, which did not display a mobility shift in the presence of pp90^sk, did not alter its electrophoretic mobility (compare lanes 7 and 8). These results confirm that pp90^sk-mediated phosphorylation of IxBα is responsible for the altered migration of the wild type IxBα.
pp90<sup>rk</sup> Triggers Degradation of I<sub>Ba</sub> in Vitro

Times physically associate with each other. Some protein kinases associate with their substrates under circumstances where the kinase is inactive or where ATP is limiting. If the association between a protein kinase and its substrate is sufficiently avid, their interaction may be detected by coimmunoprecipitation of the two proteins. To assess whether I<sub>Ba</sub> can physically associate with pp90<sup>rk</sup> in vivo, COS cells were cotransfected with expression vectors encoding HA-tagged pp90<sup>rk</sup> or control HA-tagged protein phosphatase-2A regulatory subunit (HA-PP2A) and I<sub>Ba</sub>. Following transfection, proteins were metabolically radiolabeled with <sup>35</sup>S-methionine and cysteine, and the resultant cell lysates were immunoprecipitated with nonspecific preimmune, anti-HA, or anti-I<sub>Ba</sub> antibodies. Immunoprecipitation of HA-PP2A and I<sub>Ba</sub> transfected cells with anti-HA antibodies revealed a major band corresponding to HA-PP2A but no coimmunoprecipitation of I<sub>Ba</sub> (Fig. 5A, compare lanes 1 and 2). Similarly, addition of anti-I<sub>Ba</sub> antibody immunoprecipitated I<sub>Ba</sub> but not PP2A (compare lane 3 to lanes 1 and 2). In contrast, cotransfection of cells with HA-pp90<sup>rk</sup> and I<sub>Ba</sub> led to coimmunoprecipitation of both of these molecules using either the HA- or I<sub>Ba</sub>-specific antibodies (lanes 5 and 6). The in vivo association of pp90<sup>rk</sup> and I<sub>Ba</sub> was confirmed in experiments involving initial immunoprecipitation with anti-HA or anti-I<sub>Ba</sub> followed by immunoblotting with an anti-pp90<sup>rk</sup> antibody (Fig. 5B). As shown in lanes 2 and 3, anti-I<sub>Ba</sub>-immunoprecipitated significant amounts of pp90<sup>rk</sup> in these cotransfected cells, while nonspecific preimmune sera yielded no detectable pp90<sup>rk</sup> (lane 1). Together, these results indicate that I<sub>Ba</sub> can physically associate with pp90<sup>rk</sup> in vivo and thus provide further support for the possibility that pp90<sup>rk</sup> functions as a physiologically relevant I<sub>Ba</sub> kinase.

Induction of pp90<sup>rk</sup> Kinase Activity by Phorbol Ester but Not by TNF-<alpha> or HTLV-I Tax—Studies were next performed to assess whether pp90<sup>rk</sup> is activated by various well known inducers of NF-<kappa>B. When I<sub>Ba</sub> was monitored either by a very small but perceptible change in its electrophoretic mobility, reflecting autophosphorylation (Fig. 6A), or by its ability to phosphorylate recombinant I<sub>Ba</sub> when the latter was added as an exogenous substrate to an in vitro kinase assay performed with anti-pp90<sup>rk</sup> immunoprecipitates (Fig. 6B). PMA stimulation of Jurkat cells for 5 or 15 min resulted in the rapid activation of pp90<sup>rk</sup> (panel A, lanes 3 and 4) and phosphorylation of I<sub>Ba</sub> (panel B, lanes 3 and 4). In contrast, two other recognized inducers of NF-kB, TNF-<alpha> and HTLV-I Tax, did not significantly activate pp90<sup>rk</sup> autophosphorylation (panel A, lanes 5–8) or induce phosphorylation of I<sub>Ba</sub> in the in vitro kinase assay (panel B, lanes 5–8). However, this particular preparation of TNF-<alpha> (see panel C, lanes 5–7) and HTLV-I Tax (data not shown) stimulated phosphorylation and degradation of I<sub>Ba</sub>. These findings indicate that only a subset of the known inducers NF-kB leads to the activation of pp90<sup>rk</sup>, suggesting that other kinases are likely activated by different NF-kB inducers, such as TNF-<alpha> and HTLV-I Tax. This result argues against the notion of a single cellular I<sub>Ba</sub> kinase.

DISCUSSION

Phosphorylation and dephosphorylation represents a general strategy frequently employed for the dynamic regulation of eukaryotic transcription factor function. The enhancer-binding protein is often the specific target of such post-translational modifications that lead to an alteration in its DNA binding or functional activity. However, in the NF-kB system, primary regulation is exerted through phosphorylation of I<sub>Ba</sub>, an ankyrin-rich inhibitor that sequesters the NF-kB complex in the cytoplasm. Phosphorylation targets I<sub>Ba</sub> for ubiquitination.
and subsequent degradation by the 26 S proteasome. Although a necessary step in the activation of transcription, phosphorylation alone does not result in the release of IkBα from the NF-κB complex and thus is insufficient for activation of NF-κB-mediated transcription. Thus far, the identity of the kinase(s) responsible for coupling cellular activation to phosphorylation of IkBα or other members of the IkB family has remained elusive.

In the current paper, we have explored the possible function of pp90Rsk as a stimulus-coupled IkBα kinase. In quiescent cells, inactive pp90Rsk resides in the cytoplasm and is partially complexed with its upstream regulator, p42/44MAPK (41). Cellular activation mediated by various growth factors operating through the Ras-Raf-MEK-MAPK pathway or phorbol ester leads to the activation of MAP kinase, the phosphorylation and activation of pp90Rsk, and the import of these kinases into the nucleus. Activated forms of pp90Rsk have already been implicated in the regulation of various nuclear transcription factors, including c-Fos (40) and Nur77 (42, 43). Recently, pp90Rsk has been reported to produce both positive and negative effects on another family of inducible transcription factors, the cyclic AMP response element-binding proteins (CREB). Specifically,
pp90\textsuperscript{ak}, one of three closely related \textit{rsk} genes (\textit{rsk1}, \textit{rsk2}, \textit{rsk3}), has been shown to function as a stimulus-coupled \textit{CREB} kinase modulating \textit{CREB} activity by phosphorylating this factor on a key regulatory serine located at position 133 (44). Conversely, pp90\textsuperscript{ak} appears to oppose \textit{CREB} action by inducibly but stably associating with the \textit{CREB}-binding protein and blocking the interaction of this co-activator with \textit{CREB} (45). Of note, the enzymatic function of pp90\textsuperscript{ak} is not required for these latter inhibitory effects. Together, these various results provide an experimental precedent for the participation of pp90\textsuperscript{ak} as a regulatory interface between the signals induced by the ligation of various growth factor receptors on the membrane and specific transcription factors that modulate the activity of target genes within the nucleus.

Using a purified activated enzyme preparation in initial \textit{in vitro} kinase assays, we found that pp90\textsuperscript{ak} mediates phosphorylation of I\textit{xB}a. Furthermore, based on V8 protease analysis, this phosphorylation involves the N-terminus of I\textit{xB}a, where two critical residues for signal-induced phosphorylation, Ser-32 and Ser-36, reside. pp90\textsuperscript{ak}-mediated phosphorylation of I\textit{xB}a proved biologically relevant since this post-translational modification stimulated proteasome-dependent degradation of I\textit{xB}a. In contrast, the S32/36A double-substitution mutant of I\textit{xB}a was not degraded in the presence of activated pp90\textsuperscript{ak}. The stoichiometry of I\textit{xB}a phosphorylation by pp90\textsuperscript{ak} appeared quite high, as assessed by the ability of pp90\textsuperscript{ak} to elicit a gel mobility shift for I\textit{xB}a. By this criterion, one-third to one-half of the I\textit{xB}a molecules displayed an altered mobility in the presence of pp90\textsuperscript{ak}. Interestingly, the closely related S6 kinase, pp70\textsuperscript{G6K}, which recognizes the same consensus phosphoacceptor site, Arg-X-X-Ser/Thr, as pp90\textsuperscript{ak} is incapable of phosphorylating I\textit{xB}a. Likewise, CaMKII, a calmodulin-dependent kinase that also phosphorylates within the same consensus phosphoacceptor site as pp90\textsuperscript{ak} and pp70\textsuperscript{G6K}, fails to phosphorylate I\textit{xB}a. These findings indicate that the recognition of I\textit{xB}a by these protein kinases involves determinants beyond the consensus phosphoacceptor site. It is likely that the overall three-dimensional structures of I\textit{xB}a and the kinase play a pivotal role in the effectiveness of this protein-protein interaction.

Since a "purified" pp90\textsuperscript{ak} preparation was used in these \textit{in vitro} studies, we considered the possibility that the I\textit{xB}a phosphorylation might be due to a contaminating kinase. However this possibility is unlikely because: 1) each of three independently purified pp90\textsuperscript{ak} preparations displayed the same I\textit{xB}a kinase activity in at least three assays; 2) overloading of an SDS-PAGE gel with the pp90\textsuperscript{ak} preparation followed by silver staining revealed only pp90\textsuperscript{ak} and no other bands; and 3) autophosphorylation reactions with the pp90\textsuperscript{ak} preparation revealed no other bands. The observed I\textit{xB}a kinase activity in the pp90\textsuperscript{ak} preparations thus appears to reflect the activity of pp90\textsuperscript{ak} rather than a contaminant.

Since serines 32 and 36 located near the N terminus of I\textit{xB}a are key regulatory sites that must be directly phosphorylated to trigger subsequent ubiquitination and degradation of this inhibitor (7–10, 18, 46), potential phosphorylation of these sites by pp90\textsuperscript{ak} was studied. Serine 32 is embedded within a sequence that conforms to the consensus phosphoacceptor site for phosphorylation by pp90\textsuperscript{ak}; however, serine 36 is not. With wild type I\textit{xB}a or the S32A and S36A single-substitution mutants of this inhibitor as substrates, pp90\textsuperscript{ak} was shown to readily phosphorylate I\textit{xB}a proteins containing serine 32. In contrast, as judged by mobility shift, serine 36 functioned as a very poor substrate for pp90\textsuperscript{ak}. Since both serine 32 and serine 36 must be directly phosphorylated for subsequent degradation (46), these findings suggest that the action of pp90\textsuperscript{ak} alone may not be sufficient to trigger the subsequent ubiquitination and degradation of I\textit{xB}a. It is possible that a second, yet unidentified, kinase present in the reticulocyte lysate mediates phosphorylation at serine 36, thus promoting I\textit{xB}a degradation. Although unproven, it is intriguing to consider the possibility that phosphorylation \textit{in vivo} at the first serine site by one kinase may facilitate sequential phosphorylation at the second serine site by a different kinase. Alternatively, pp90\textsuperscript{ak} phosphorylation at serine 32 may enhance its activity at serine 36, producing a polarity to the sequence of modifications. Precedence for regulation by such sequential phosphorylation is found in the case of both pp90\textsuperscript{ak} and pp70\textsuperscript{G6K} phosphorylation of S6 peptide (47). Finally, degradation of I\textit{xB}a \textit{in vitro} may proceed with phosphorylation at serine 32 only.

To test the potential \textit{in vivo} relevance of pp90\textsuperscript{ak} as an I\textit{xB}a kinase, we investigated whether these proteins can physically associate within a cell. Using COS cells for cotransfection with HA epitope-tagged pp90\textsuperscript{ak} and I\textit{xB}a expression vectors, we demonstrated that these two proteins, but not similarly epitope-tagged control proteins, are communoprecipitated using either anti-HA or anti-I\textit{xB}a-specific antibodies. Using mutants of I\textit{xB}a to study this interaction further, our preliminary results indicate that the N terminus of I\textit{xB}a spanning the regulatory serines at positions 32 and 36 is not required for I\textit{xB}a binding to pp90\textsuperscript{ak}. In contrast, deletion of ankyrin repeats 1–5 of I\textit{xB}a severely impairs the interaction of I\textit{xB}a with pp90\textsuperscript{ak}.

Our final series of studies explored what NF-\textit{xB}-inducing signals operate through pp90\textsuperscript{ak}-mediated phosphorylation of I\textit{xB}a. These studies revealed that PMA induced activation of pp90\textsuperscript{ak}, phosphorylation of I\textit{xB}a, and induction of NF-\textit{xB}. In sharp contrast, neither TNF-\textalpha nor the Tax trans-activator protein of HTLV-I activated pp90\textsuperscript{ak}. However, both of the laters inducers potently stimulated phosphorylation and degradation of I\textit{xB}a and activated nuclear NF-\textit{xB} expression. This result clearly indicates that not all NF-\textit{xB} inducers operate through pp90\textsuperscript{ak} activation. These results predict the likely existence of multiple I\textit{xB}a kinases that are differentially coupled to various signaling pathways. Thus, the critical kinase(s) ultimately responsible for phosphorylating serines 32 and 36 may vary, depending on the particular NF-\textit{xB}-inducing signal. We propose that pp90\textsuperscript{ak} represents one such kinase in a larger set of enzymes that regulate I\textit{xB}a phosphorylation.

Acknowledgments—We thank Dr. Joseph Avruch, Dr. Al Baldwin, Dr. Dean Ballard (Vanderbilt University, Nashville, TN), Dr. James Maller and Dr. Michael Browning (University of Colorado Health Sciences Center, Denver, CO), and Dr. Mark Stinski for reagents, Dr. Dean Ballard and Dr. James Maller for helpful scientific discussions, and Mark Dettle for preparation of the manuscript. We also thank Richard Ruben and David Brand for technical assistance.

REFERENCES
1. Beg, A. A., and Baldwin, A. J. (1993) \textit{Genes Dev.} 7, 2064–2070
2. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) \textit{Genes Dev.} 9, 2723–2725
3. Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. (1991) \textit{Cell} 65, 1281–1289
4. Baeuerle, P. A., and Baltimore, D. (1988) \textit{Cell} 53, 211–217
5. Ganchi, P., Sun, S. C., Greene, W. C., and Ballard, D. W. (1992) \textit{Mol. Biol. Cell} 3, 1339–1352
6. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskill, S., Rosen, C. A., and Baldwin, A. S., Jr. (1992) \textit{Genes Dev.} 6, 1899–1913
7. Brown, K., Gersberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995) \textit{Science} 267, 1488–1489
8. Bruckman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., and Ballard, D. W. (1995) \textit{Mol. Cell. Biol.} 15, 2899–2918
9. Traenckner, E. B., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) \textit{EMBO J.} 14, 2876–2883
10. Sun, S. C., Elwood, J., Beraud, C., and Greene, W. C. (1994) \textit{Mol. Cell. Biol.} 14, 7377–7384
11. Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) \textit{Genes Dev.} 9, 1586–1597
12. Baldi, L., Brown, K., Franzoso, G., and Siebenlist, U. (1996) \textit{J. Biol. Chem.} 271, 21287

pp90\textsuperscript{ak} Triggers Degradation of I\textit{xB}a \textit{in Vitro}
pp90^rk Triggers Degradation of IxBa in Vitro

376–379
13. Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11259–11263
14. DiDonato, J. A., Mercurio, F., and Karin, M. (1995) Mol. Cell. Biol. 15, 1302–1311
15. Finco, T. S., Beg, A. A., and Baldwin, A. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11884–11888
16. Lin, Y. C., Brown, K., and Siebenlist, U. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 552–556
17. Traenckner, E. B., Wilk, S., and Baeuerle, P. A. (1994) EMBO J. 13, 5433–5441
18. Belvin, M. P., Jin, Y., and Anderson, K. V. (1995) Genes Dev. 9, 783–793
19. Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996) Mol. Cell. Biol. 16, 1401–1409
21. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Cell 84, 853–862
22. Blenis, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5869–5892
23. Chen, R. H., Tung, R., Abate, C., and Blenis, J. (1993) Biochem. Soc. Trans. 21, 895–900
24. Davis, J. J., Hazel, T. G., Chen, R. H., Blenis, J., and Lau, L. F. (1993) Mol. Endocrinol. 7, 953–964
25. Chen, Z. J., Parent, L., and Maniatis, T. (1996) Cell 84, 853–862
26. Blenis, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5869–5892
27. Sturgill, T. W., Ray, L. B., Erikson, K., and Maller, J. L. (1998) Nature 334, 715–718
28. Schagger, H., and von Jagow, J. G. (1987) Anal. Biochem. 166, 368–379
29. Pearson, R. B., and Kemp, B. E. (1991) Methods Enzymol. 200, 62–81
30. Sun, S. C., Ganchi, P., Ballard, D. W., and Greene, W. C. (1993) Science 259, 1912–1915
31. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–848
32. Hermida-Matsumoto, M.-L., Chock, P. B., Curran, T., and Yang, D. C. H. (1996) J. Biol. Chem. 271, 4930–4936
33. Li, X., and Coffino, P. (1994) Mol. Cell. Biol. 14, 87–92
34. Lottspeicher, P., Pratt, G., and Reichsteiner, M. (1991) J. Biol. Chem. 266, 11213–11220
35. Lu, L., Stanley, B. A., and Pegg, A. E. (1991) Biochem. J. 277, 671–675
36. Papavassiliou, A. G., Treier, M., Chavrier, C., and Bohmann, D. (1992) Science 258, 1941–1944
37. Rosenberg, H. Y., Bercovich, Z., and Kahana, C. (1991) Biochem. J. 277, 683–685
38. Rosenberg, H. Y., Strumpf, D., and Kahana, C. (1991) Eur. J. Biochem. 197, 419–424
39. Gross, M. (1980) Mol. Cell. Biol. 31, 25–36
40. Chen, R. H., Tung, R., Abate, C., and Blenis, J. (1993) Biochem. Soc. Trans. 21, 895–900
41. Hsiao, K. M., Chou, S. Y., Shih, S. J., and Ferrell, J. E., Jr. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5480–5484
42. Davis, J. J., Hazel, T. G., Chen, R. H., Blenis, J., and Lau, L. F. (1993) Mol. Endocrinol. 7, 953–964
43. Fisher, T. L., and Blenis, J. (1996) Mol. Cell. Biol. 16, 1212–1219
44. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959–963
45. Nakajima, T., Fukamizu, A., Takahashi, J., Gage, P. H., Fisher, T., Blenis, J., and Montminy, M. R. (1996) Cell 86, 465–474
46. DiDonato, J., Mercurio, F., Rosette, C., Wu, L. J., Suyang, H., Ghosh, S., and Karin, M. (1996) Mol. Cell. Biol. 16, 1295–1304
47. Wettenhall, R. E. H., Rosette, C., and Maller, J. L. (1992) J. Biol. Chem. 267, 9021–9027