MOLECULAR AND CELLULAR BIOLOGY, May 1995, p. 2809–2818 Vol. 15, No. 5
0270-7306/95/$04.00+0

Coupling of a Signal Response Domain in IκBα to Multiple Pathways for NF-κB Activation

JEFFREY A. BROCKMAN, DAVID C. SCHERER, TIMOTHY A. MCKINSEY, SARAH M. HALL, XIAOXIA QI, WHA YOUNG LEE, AND DEAN W. BALLARD*

Howard Hughes Medical Institute and Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee

Received 7 November 1994/Returned for modification 25 January 1995/Accepted 13 February 1995

The eukaryotic transcription factor NF-κB plays a central role in the induced expression of human immunodeficiency virus type 1 and in many aspects of the genetic program mediating normal T-cell activation and growth. The nuclear activity of NF-κB is tightly regulated from the cytoplasmic compartment by an inhibitory subunit called IκBα. This cytoplasmic inhibitor is rapidly phosphorylated and degraded in response to a diverse set of NF-κB-inducing agents, including T-cell mitogens, proinflammatory cytokines, and viral transactivators such as the Tax protein of human T-cell leukemia virus type 1. To explore these IκBα-dependent mechanisms for NF-κB induction, we identified novel mutants of IκBα that uncouple its inhibitory and signal-transducing functions in human T lymphocytes. Specifically, removal of the N-terminal 36 amino acids of IκBα failed to disrupt its ability to form latent complexes with NF-κB in the cytoplasm. However, this deletion mutation prevented the induced phosphorylation, degradative loss, and functional release of IκBα from NF-κB in Tax-expressing cells. Alanine substitutions introduced at two serine residues positioned within this N-terminal regulatory region of IκBα also yielded constitutive repressors that escaped from Tax-induced turnover and that potently inhibited immune activation pathways for NF-κB induction, including those initiated from antigen and cytokine receptors. In contrast, introduction of a phosphoserine mimetic at these sites rectified this functional defect, a finding consistent with a causal linkage between the phosphorylation status and proteolytic stability of this cytoplasmic inhibitor. Together, these in vivo studies define a critical signal response domain in IκBα that coordinately controls the biologic activities of IκBα and NF-κB in response to viral and immune stimuli.

The eukaryotic transcription factor NF-κB participates in the activation and controlled proliferation of T lymphocytes during immune and inflammatory responses (for a review, see references 31, 32, and 46). The prototypical form of NF-κB is a heterodimeric complex containing two DNA binding subunits, termed p50 and RelA, both of which belong to the Rel family of transcription factors (5, 16, 28, 43, 54, 60, 67). When expressed in the nucleus, this inducible complex stimulates gene transcription via a potent transactivation domain in the C-terminal half of RelA (7, 68, 70). However, in resting T lymphocytes, the nuclear import of NF-κB is prevented because of high-affinity association of its RelA subunit with a labile cytoplasmic inhibitor called IκBα (3–5, 11, 27). This IκBα-dependent mechanism for the cytoplasmic retention of NF-κB is uncoupled by many extracellular signals, including T-cell receptor ligands, tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1) (for a review, see reference 32). During normal T-cell activation, nuclear translocation of NF-κB is preceded by the rapid phosphorylation and degradation of IκBα (9, 10, 19, 35, 80, 81). However, the role, if any, that phosphorylation plays in regulating the degradation of IκBα remains unclear.

In contrast to its transient nuclear expression in normal T lymphocytes, NF-κB is constitutively activated in cells expressing the Tax protein of human T-cell leukemia virus type 1 (HTLV-1) (6, 47, 69). This virus-host interplay confers Tax inducibility to a set of NF-κB-responsive cellular genes that are normally transcribed at high levels in response to T-cell activation signals (for reviews, see references 31 and 32). This set includes the transcription units encoding IL-2 and the alpha subunit of its high-affinity receptor (IL-2Rα), which together play a key role in normal growth signal transduction (21, 40, 44, 51, 76). The Tax protein has also been shown to activate transcription from the 5′ long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1), which contains two tandem NF-κB binding sites (15, 55). Recent studies have shown that Tax-induced activation of NF-κB is temporally associated with the phosphorylation and degradation of IκBα (42, 82). In addition, Schreck et al. have demonstrated that the activation of NF-κB by Tax is partially inhibited by select antioxidants that block physiologic NF-κB induction in human T cells (71, 72).

Although these findings suggest a convergent mechanism for NF-κB induction involving the inactivation of IκBα, an explanation for how this cytoplasmic inhibitor integrates seemingly disparate viral and immune activation signals has remained unknown. To address this question, we devised an expression system that permits functional incorporation of ectopic forms of IκBα into the endogenous NF-κB signaling pathway of human T lymphocytes. Using this in vivo approach, we have identified a signal response (SR) domain in the N-terminal region of IκBα that is required not only for constitutive activation of NF-κB mediated by HTLV-1 Tax but also for transient activation of NF-κB via signal transduction pathways linked to T-cell antigen and cytokine receptors. Mutations introduced at two serine residues within this regulatory domain produced constitutive repressors of NF-κB-directed transcription, despite the presence of agonists that normally induce the

* Corresponding author. Mailing address: Howard Hughes Medical Institute, Vanderbilt University School of Medicine, 802 Rudolph Light Hall, Nashville, TN 37232-0295. Phone: (615) 343-1548. Fax: (615) 343-4726. Electronic mail address: ballardw@ctrvax.vanderbilt.edu.

degradation of IκBα and the nuclear translocation of NF-κB. This dominant-negative phenotype was associated with the escape of IκBα from proteolytic breakdown, suggesting that the phosphorylation status of the SR domain may affect the induced entry of IκB into its degradative pathway.

MATERIALS AND METHODS

Plasmids. Complementary DNAs encoding full-length wild-type and mutant forms of HTLV-1 Tax (77), human RelA (67), and human IκBα (33) were cloned into the polylinker of the eukaryotic expression vector pCMV4 (2) immediately downstream of the cytomegalovirus immediate-early promoter. Chloramphenicol acetyltransferase (CAT) reporter plasmids contained either the full-length HTLV-1 LTR (78), HIV-1 LTR (78), c-*fos* promoter (29), IL-2Rα promoter (nucleotides −317 to +109) (49), or HIV κB enhancer cassette linked to a heterologous TATA box (79). Deletion mutants of IκBα encoding amino acids 37 to 317 (IκBαΔN) and 1 to 242 (IκBαΔC) were constructed by PCR using specific oligonucleotide primers (5′-GGGAAGCTTCTCGTCCGCGCCATGAAAGACGAGGAGTACGAG-3′ and 5′-GGTCTAGATCATAACGTCAGACGCTGGCCT-3′ for IκBαΔN; 5′-CCCCAAGCTTCTCGTCCGCGCCATGTTCCAG-3′ and 5′-GGTCTAGATCAATCAGCCCCACACTTCAACAG-3′ for IκBαΔC) and the wild-type IκBα (IκBαWT) cDNA (33) as a template. Amplified products were digested with *Hin*dIII and *Xba*I, purified on 1% agarose gels, and ligated into the pCMV4 polylinker. Site-directed mutations were introduced into the full-length IκBα cDNA by using the phosphorothioate method and oligonucleotide primers that introduced diagnostic restriction sites (56). Epitope-tagged derivatives of select IκBα mutants were constructed by PCR-assisted amplification with 5′ primers that fused sequences encoding the FLAG epitope (13, 64) in frame with N-terminal coding sequences of IκBα (5′-CCCAAGCTTCCACCATGGACTACAAAGACGATGACGATAAAATGTTCCAGGCGGCCGAGCGC-3′ for IκBαWT, IκBαΔC, IκBαS32A, and IκBαS32E; 5′-CCCAAGCTTCCACCATGGACTACAAAGACGATGACGATAAAATGAAAGACGAGGAGTACGAGC-3′ for IκBαΔN). Amplified products were cloned into the *Hin*dIII and *Xba*I polylinker sites of pCMV4.

Cell culture, transfections, and CAT assays. Jurkat T lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Approximately 10^7 cells suspended in RPMI (300 μl) were transfected by electroporation (250 V, 960 μF) (25) with the indicated effector and reporter plasmids, using a Bio-Rad Gene Pulser equipped with a capacitance extender. Electroporated cells were placed on ice for 10 min, transferred to 5 ml of complete medium, and then expanded for 48 h. Where indicated, cultures were treated after 24 h of growth with either TNF-α (300 U/ml; Genzyme) or combinations of phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μM; Calbiochem) for 20 h prior to harvest. In some experiments, cell cultures were pretreated with the proteasome inhibitor MG132 (62) (kindly supplied by MyoGenics, Inc.) before addition of the NF-κB agonist. Whole-cell extracts were prepared from transfectants, normalized for protein concentration (18), and assayed for CAT activity, using the diffusion-based liquid scintillation counting method described by Neumann et al. (59).

Immunoprecipitation and immunoblotting. Cytosolic extracts were prepared from transfected cells as described previously (73) except that the detergent lysis buffer was supplemented with an extensive cocktail of protease inhibitors (8). Lysates were clarified by centrifugation and equilibrated in ELB buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet-P40). Epitope-tagged IκBα was isolated from these cytosolic extracts by incubation with 20 μl of agarose beads conjugated to monoclonal anti-FLAG M2 antibody (IBI-Kodak). Immunoprecipitates were washed three times with ELB buffer, heat denatured in 4% sodium dodecyl sulfate (SDS)–10% β-mercaptoethanol, fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), and electrophoretically transferred to a polyvinylidene difluoride membrane (Dupont). Membranes were blocked (1 h at room temperature) with Tris-buffered saline containing 0.1% Tween 20 and 5% powdered milk (BLOTTO) and then incubated with a rabbit antipeptide antiserum specific for either IκBα (amino acids 1 to 28 and 229 to 317) or RelA (amino acids 529 to 551). Immunoreactive polypeptides were detected by using donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase in an enhanced chemiluminescence system as specified by the manufacturer (Amersham).

Gel retardation assays. Nuclear fractions were prepared from Jurkat T-cell transfectants by high-salt extraction (73) in the presence of protease inhibitors (8). Gel mobility shift assays were performed by using a ^{32}P-radiolabeled oligonucleotide duplex derived from κB enhancer sequences in the IL-2Rα promoter (5′-CAACGGCAGGGGAATTCCCCTCTCCTT-3′) (8). DNA binding reaction mixtures (20 μl) contained 5 μg of Jurkat nuclear extract, 2 μg of double-stranded poly(dI-dC), and 10 μg of bovine serum albumin buffered in 20 mM HEPES (pH 7.9)–5% glycerol–1 mM EDTA–1% Nonidet P-40–5 mM dithiothreitol. Approximately 10 fmol (200,000 cpm) of radiolabeled probe was used per reaction. Resultant nucleoprotein complexes were resolved on native 5% polyacrylamide gels and detected by autoradiography as previously described (14).

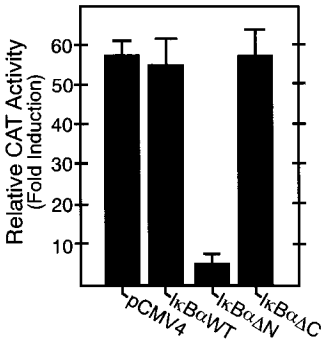

FIG. 1. Identification of a Tax-responsive domain in IκBα. Jurkat T cells were cotransfected with HIV-κB-CAT (5 μg) and equivalent doses (2.5 μg) of the indicated pCMV4-based IκBα expression vectors, in the presence or absence of a Tax expression vector (5 μg). After 48 h of culture, whole-cell extracts were prepared, normalized for total protein, and assayed for CAT activity. Results from five independent transfections are reported as the mean fold induction (± standard error of the mean) of CAT activity in Tax-expressing versus Tax-deficient cells.

RESULTS

IκBα is a cellular target of HTLV-1 Tax. Recent primary structural analyses of human IκBα have indicated that this cytoplasmic inhibitor contains a central domain (amino acids 73 to 242) composed of five ankyrin repeat motifs, a C-terminal PEST domain (amino acids 243 to 317) characteristic of many short-lived proteins, and an N-terminal domain of unknown biological function (33). To determine whether IκBα is required for Tax-induced activation of NF-κB, we initially examined the ability of Tax to mediate κB-specific transcription in cells reconstituted with deletion mutants of IκBα that contained a fully intact ankyrin repeat domain. For these studies, human Jurkat T lymphocytes were transfected with expression vectors encoding these truncated forms of IκBα, HTLV-1 Tax, and a CAT reporter plasmid under the transcriptional control of two NF-κB binding sites from the HIV enhancer (HIV-κB-CAT) (55, 79).

As shown in Fig. 1, Tax induced the activation of the HIV-κB reporter gene at least 50-fold over basal levels in the absence of ectopic IκBα. This activity persisted in the presence of either IκBαWT or a mutant of IκBα lacking the C-terminal PEST domain (amino acids 243 to 317; IκBαΔC). In sharp contrast to IκBαWT, identical doses (2.5 μg) of a deletion mutant lacking the N-terminal 36 amino acids (IκBαΔN) almost completely abolished the Tax response. Results of immunoblotting studies indicated that the IκBαWT and IκBαΔN proteins were expressed at comparable steady-state levels (see Fig. 3A). Furthermore, titration experiments indicated that 10- to 20-fold higher concentrations of IκBαWT than of IκBαΔN were required to achieve half-maximal inhibition of this response, thus precluding the possibility that these divergent functional effects were due to slight variations in effector plasmid input.

Conditional repression of Tax-dependent transcription. In addition to NF-κB, Tax can also interface with distinct transcription factors, including serum-responsive factor (26) and members of the cyclic AMP-responsive element-binding protein/activating transcription factor (CREB/ATF) family (83, 89), thus expanding the range of viral and cellular genes under Tax control. In light of these findings, the altered in vivo function of IκBα produced by this N-terminal deletion (Fig. 1) could reflect the inhibition of auxiliary components of the cellular transcriptional machinery other than NF-κB.

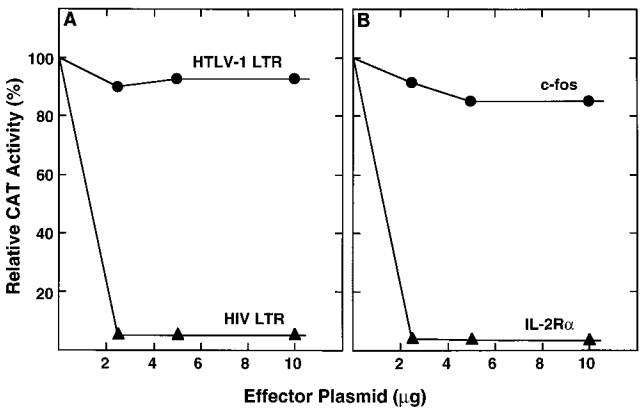

FIG. 2. Conditional repression of Tax-dependent transcription. Jurkat T cells were transfected with a Tax expression vector (5 μg), graded amounts of IκBαΔN effector plasmid, and a CAT reporter construct under transcriptional control of either the HIV LTR (1 μg), HTLV-1 LTR (0.5 μg), c-*fos* promoter (1 μg), or IL-2Rα promoter (5 μg). Input DNA for all transfections (20 μg) was normalized by addition of blank pCMV4 expression vector. For each titration point ($n \geq 3$), the increase in reporter gene activity over basal levels is expressed as the mean percentage of CAT activity measured in control cells lacking the IκBαΔN expression vector. In IκBαΔN-deficient cells, the Tax-dependent increases in CAT activity over basal levels (fold induction) were 21.6 ± 3.3 (HTLV-1 LTR), 8.0 ± 2.8 (HIV LTR), 10.6 ± 2.7 (c-*fos* promoter), and 10.7 ± 0.2 (IL-2Rα promoter).

To evaluate the specificity of this interaction, titration studies were performed with the IκBαΔN effector plasmid in Jurkat T cells cotransfected with Tax and distinct Tax-responsive reporter constructs driven by various naturally occurring promoters (Fig. 2). Consistent with results obtained with the synthetic HIV-κB-CAT construct, IκBαΔN potently repressed the activity of the full-length HIV LTR in a dose-dependent manner (Fig. 2A). In contrast, IκBαΔN failed to significantly downregulate transcription directed from the HTLV-1 LTR, which responds to Tax by a CREB/ATF-dependent mechanism (83, 89). As shown in Fig. 2B, Tax-induced transcription from the κB-responsive IL-2Rα promoter was also attenuated (>90%) in cells expressing low doses of IκBαΔN. However, higher doses of this deletion mutant in Tax-expressing cells led to only modest inhibitory effects (≤15%) on the transcriptional activity of the c-*fos* promoter, a response mediated primarily through the indirect interaction of Tax with an intrinsic serum response factor-binding site (26). This pattern of promoter-specific repression confirmed that the N-terminal region of IκBα is selectively involved in the mechanism by which Tax deregulates the expression of NF-κB-dependent transcription units.

In vivo interactions between ectopic IκBα and endogenous RelA. The RelA (p65) transactivator subunit of NF-κB serves as the high-affinity receptor for IκBα (5, 11, 27, 86). This dynamic interplay between RelA and IκBα thus governs the subcellular distribution and transcriptional activity of NF-κB. To examine whether these deletion mutants of IκBα interacted functionally with RelA, immunoprecipitation and gel retardation analyses were performed on cytoplasmic and nuclear extracts from these T-cell transfectants. To distinguish ectopic from endogenous IκBα in these studies, we used IκBα cDNAs that were fused in frame with sequences encoding the FLAG epitope (13, 64). Results from CAT assays indicated that the presence of this epitope did not alter the functional phenotype of these IκBα constructs (data not shown). As shown in Fig. 3A, these epitope-tagged derivatives of IκBα were efficiently expressed in the cytoplasm (lanes 2 to 4). However, consistent with prior in vitro studies (34, 39), removal of the C terminus of IκBα completely disrupted its RelA-binding function (lane 8). In contrast, the N-terminal deletion mutant of IκBα, which blocked the Tax transcriptional response (Fig. 1), retained the capacity to form stable complexes with endogenous RelA in the cytoplasm (Fig. 3A, lane 7).

To assess whether these complexes were functionally latent but competent for induction, Jurkat T cells were cotransfected with expression vectors for RelA and graded doses of each IκBα deletion mutant. Nuclear extracts were then prepared from recipient cells and analyzed for κB-specific DNA binding activity in gel retardation assays. As shown in Fig. 3B (pCMV4 panel), two nucleoprotein complexes were detected with extracts from control cells expressing ectopic RelA. Results of

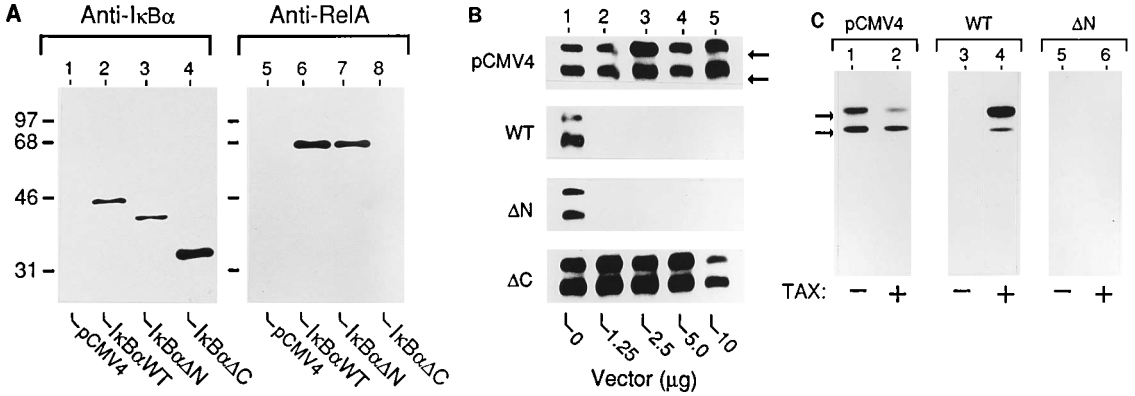

FIG. 3. Biochemical properties of IκBα deletion mutants. (A) Association with endogenous RelA. Jurkat T cells were transfected with epitope-tagged derivatives of the indicated IκBα cDNA expression vectors (20 μg) or with the unmodified parental vector (pCMV4; 20 μg). Cytoplasmic complexes containing ectopic IκBα were isolated by immunoaffinity chromatography, fractionated by SDS-PAGE, and analyzed by immunoblotting with a peptide-specific antiserum for either IκBα (amino acids 1 to 29 and 289 to 317) or RelA (amino acids 529 to 551). Sizes are indicated in kilodaltons. (B) Inhibition of nuclear RelA/NF-κB DNA binding. Jurkat T cells were cotransfected with a cDNA expression vector encoding human RelA (10 μg) and graded doses of the indicated IκBα expression vectors. Nuclear extracts (5 μg) from transfectants were added to DNA binding reaction mixtures containing a ^{32}P-labeled palindromic κB probe (κB-pd) (8) and analyzed on native 5% polyacrylamide gels. Nucleoprotein complexes containing RelA homodimers (upper arrow) and NF-κB p50/RelA heterodimers (lower arrow) are indicated. (C) Induction of nuclear NF-κB/RelA activity. Jurkat cells were cotransfected with cDNA expression vectors encoding RelA (10 μg), Tax (10 μg), and the indicated IκBα constructs (5 μg). After 48 h of growth, cultures were treated with cycloheximide (50 μg/ml) for 2 h in order to prevent the de novo synthesis of IκBα. Nuclear extracts were prepared and analyzed in gel retardation assays as described above. Nucleoprotein complexes containing RelA homodimers and p50/RelA (NF-κB) heterodimers are indicated with arrows.

DNA-protein cross-linking and antibody binding studies (data not shown) indicated that the upper complex contained RelA homodimers and the lower complex contained p50/RelA (NF-κB) heterodimers, which form as a result of RelA-mediated transactivation of the NF-κB1 gene encoding p50 (84). Consistent with its defect in RelA binding, IκBαΔC failed to inhibit these κB-specific activities when expressed over a wide concentration range (ΔC panel). However, even at low dosage, the wild-type and N-terminally deleted forms of IκBα completely blocked both of these DNA binding activities (WT and ΔN panels). As shown in Fig. 3C, coexpression with HTLV-1 Tax led to efficient inactivation of IκBαWT, as evidenced by the accumulation of functional RelA and NF-κB in the nuclear compartment (lanes 3 and 4). In contrast, Tax failed to activate latent complexes containing the N-terminally truncated inhibitor (lanes 5 and 6). Taken together, these results indicate that the C-terminal portion of IκBα is required for efficient formation of latent NF-κB complexes in the cytoplasm of T cells, whereas the N-terminal segment of IκBα (the SR domain) subserves a distinct regulatory function that is required for Tax-mediated induction of nuclear NF-κB.

The SR domain of IκBα specifies determinants for targeted phosphorylation and degradation. The transient induction of NF-κB during T-cell activation is dependent on the prior degradation of IκBα in the cytoplasm (10, 19, 80). In this context, we reasoned that the SR domain of IκBα, which lacks determinants for NF-κB binding, might regulate the stability of this inhibitor in the presence of HTLV-1 Tax. To test this hypothesis, the steady-state levels of epitope-tagged IκBα protein and its N-terminally deleted counterpart were examined in Tax-expressing T cells. To provide stringent control for these experiments, we also used expression vectors for Tax containing missense mutations that selectively disrupt its ability to access either the CREB/ATF (Tax-M47) or the NF-κB/Rel (Tax-M22) transcription factor pathway (77). Thus, Tax-M22 fails to induce the nuclear expression of NF-κB in Jurkat T lymphocytes, whereas the Tax-M47 mutant is fully competent to execute this function (77). As demonstrated by immunoblotting (Fig. 4A), coexpression of IκBα with wild-type Tax led to a significant reduction in the steady-state level of cytoplasmic IκBα protein relative to that observed in Tax-deficient cells (lanes 1 and 2). Consistent with their differing capacities to induce NF-κB, the degradative loss of ectopic IκBα was also evident in cells expressing the Tax-M47 mutant (lane 4) but not in cells expressing the Tax-M22 mutant (lane 3). In contrast to the destabilizing effects of Tax on wild-type IκBα, Tax and Tax-M47 both failed to mediate a decline in the steady-state level of the IκBαΔN protein (lanes 6 and 8). These results indicate that the SR domain of IκBα contains requisite regulatory determinants not only for NF-κB activation by Tax but also for Tax-induced degradation of IκBα.

Recent studies have shown that IκBα is phosphorylated prior to its degradative loss during normal T-cell activation (10, 19, 52, 57, 81). This labile phosphorylated form of IκBα is readily detected as a more slowly migrating electrophoretic variant that accumulates in cells treated with inhibitors of the proteasome degradation pathway (62). To determine whether the increased stability of IκBαΔN was associated with an altered pattern of phosphorylation, we used the previously described proteasome inhibitor MG132 (62) in an attempt to prevent Tax-induced turnover of ectopic IκBα in transfected T cells. As shown in Fig. 4B, a modified form of IκBαWT was weakly detected in cells treated with MG132 alone (compare lanes 1 and 2), presumably as a result of arrest of basal turnover in the absence of NF-κB inducer. Expression of this electrophoretic variant of IκBα was substantially augmented in

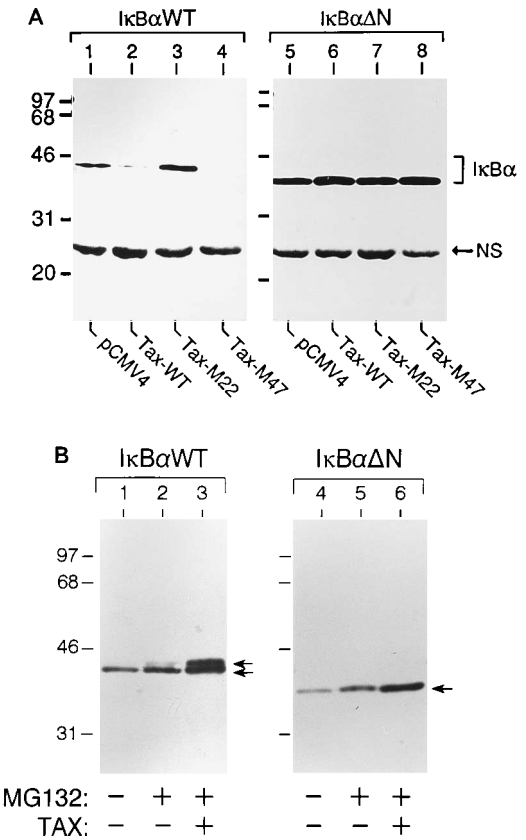

FIG. 4. The SR domain of IκBα contains determinants for Tax-induced phosphorylation and degradation. (A) Jurkat T cells were cotransfected with expression vectors for the indicated epitope-tagged derivatives of IκBα (1 μg), wild-type Tax (Tax-WT; 10 μg), or Tax mutants (77) defective in either NF-κB (Tax-M22) or CREB/ATF (Tax-M47) activating function (10 μg). Cytoplasmic extracts were fractionated by immunoaffinity chromatography, resolved by SDS-PAGE, and subjected to immunoblotting with an IκBα-specific antiserum. NS, nonspecific. (B) Jurkat T cells were cotransfected with plasmids encoding the indicated epitope-tagged derivatives of IκBα (5 μg) in the presence or absence of a Tax expression vector (10 μg). After 24 h of culture, samples were treated with the proteosome inhibitor MG132 (100 μM, 4 h). Cytoplasmic extracts were prepared in the presence of a cocktail of phosphatase inhibitors (50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μM sodium molybdate, 50 μM zinc chloride, and 20 mM β-glycerol phosphate) and subjected to immunoprecipitation with anti-FLAG antibody. Immune complexes were fractionated by SDS-PAGE and probed on immunoblots with an IκBα-specific antiserum. Major immunoreactive species are indicated with arrows. Sizes are indicated in kilodaltons.

cells cotransfected with HTLV-1 Tax (lane 3), despite the ability of Tax to stimulate the breakdown of IκBα in MG132-deficient cells (Fig. 4A). On the basis of its selective sensitivity to calf intestinal phosphatase (data not shown), this modified form of IκBα likely derived from an altered pattern of phosphorylation relative to the cytoplasmic pool of IκBα present in unstimulated cells. Unlike IκBαWT, Tax apparently failed to mediate the induced modification of IκBαΔN, as evidenced by the conspicuous absence of a characteristic electrophoretic doublet (lanes 4 to 6). These results suggest that the SR domain of IκBα is essential for both signal-dependent phosphorylation and degradation of this cytoplasmic inhibitor. Furthermore, the finding that MG132 prevents the breakdown of IκBαWT in Tax-expressing cells implicates the proteasome degradation pathway in the IκBα-dependent mechanism by which this retroviral gene product activates NF-κB.

Identification of two regulatory serines in the SR domain of IκBα. Inspection of the deduced N-terminal sequences for the

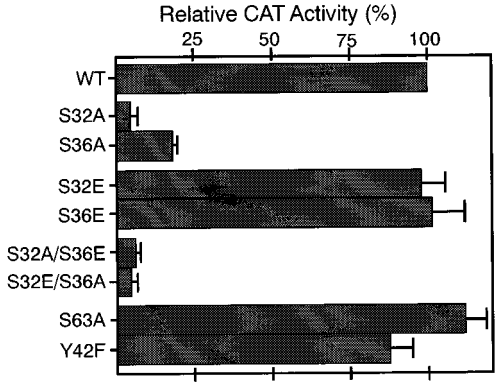

FIG. 5. The SR domain of IκBα contains regulatory serine sites. Jurkat T cells were cotransfected with HIV-κB-CAT (5 μg) along with cDNA expression vectors encoding Tax (5 μg) and the indicated site-directed mutants of IκBα (2.5 μg). After 48 h of culture, cells were harvested and assayed for CAT activity. Tax-dependent increases in reporter gene activity are expressed as a percentage of the activity induced in cells transfected with the IκBαWT (WT) effector (fold induction = 29.9 ± 2.6). Each bar represents the mean ± standard error of the mean from at least three independent transfections.

rat (85), avian (22), porcine (23), and human (34) homologs of IκBα revealed the presence of two strictly conserved serines within the SR domain (Ser-32 and Ser-36 in human IκBα) that were absent in the IκBαΔN construct. These two serines are positioned within consensus phosphorylation sites for casein kinase II (33, 63). To explore whether either Ser-32 or Ser-36 constitutes a site for regulatory phosphorylation events that permit Tax-induced activation of NF-κB, a series of specific amino acid substitutions were introduced into the full-length IκBα protein by site-directed mutagenesis. As shown in Fig. 5, substitution of alanine for either Ser-32 or Ser-36 in ectopic IκBα (mutant S32A or S36A) markedly attenuated Tax-mediated transactivation of HIV-κB-CAT in Jurkat T-cell transfectants. These dominant-negative effects were highly selective, because IκBα constructs containing point mutations at two other potential phosphorylation sites in the N-terminal region of IκBα, including an alanine substitution at Ser-63 (mutant S63A) or phenylalanine for Tyr-42 (mutant Y42F), failed to inhibit the Tax response.

To extend these findings, we attempted to reconstitute the signaling activity of IκBα mutants S32A and S36A by replacing alanine with charged amino acids that can function as phosphoserine mimetics (1, 17). As shown in Fig. 5, the ability of Tax to transactivate HIV-κB-CAT in the presence of ectopic IκBα was significantly reconstituted in vivo (>90% relative to the wild type) by introducing glutamic acid at either site (mutants S32E and S36E). However, introduction of alanine at the adjacent unmodified serine site (Fig. 5; mutants S32A/S36E and S32E/S36A) converted these reconstituted IκBα mutants to a signaling-defective phenotype. These results indicate that Ser-32 and Ser-36 function as independent regulatory sites within the SR domain of IκBα, both of which are critical for Tax-mediated induction of NF-κB.

Biochemical analyses of site-directed IκBα mutants. To confirm that the observed dominant-negative phenotypes were due to specific defects in the signaling function of IκBα, we next performed studies with extracts from T cells transfected with select site-directed mutants. As demonstrated by immunoblotting with RelA-specific antibodies (Fig. 6A), variants of IκBα containing either an alanine or glutamic acid substitution at Ser-32 (mutants S32A and S32E) retained the capacity to associate with endogenous RelA complexes in the absence of Tax (lanes 7 and 8), indicating that these mutations did not perturb the formation of latent NF-κB/IκBα complexes. However, in keeping with their distinct functional phenotypes (Fig. 5), only IκBα mutant S32A failed to permit Tax-induced expression of NF-κB DNA binding activity in the nuclear compartment (Fig. 6B, lanes 5 and 6). These data indicate that the S32A mutant of IκBα is a constitutive repressor of NF-κB with a specific defect in its ability to transduce Tax-dependent signals that uncouple NF-κB/IκBα complexes. In contrast to this dominant-negative phenotype, introduction of glutamic acid rather than alanine at Ser-32 was sufficient to rescue the signal-transducing function of IκBα in the presence of HTLV-1 Tax (Fig. 6B, lanes 7 and 8).

On the basis of the finding that deletion of IκBα sequences encompassing Ser-32 rendered IκBα refractile to Tax-induced degradation (Fig. 4A), we next examined whether these two functionally distinct classes of point mutations differentially affected the steady-state levels of cytoplasmic IκBα in cells expressing wild-type or mutated forms of Tax. As shown in Fig. 6C (lanes 1 to 4), expression of the S32E mutant of IκBα was selectively repressed in T cells transfected with wild-type Tax and Tax-M47, both of which induce the NF-κB signaling pathway. These results were fully consistent with that observed in cells expressing IκBαWT (Fig. 4A). However, replacement of Ser-32 with alanine rather than glutamic acid (mutant S32A) was associated with the escape of IκBα from Tax-induced breakdown (Fig. 6C, lanes 5 to 8), thus recapitulating the stability profile of the IκBαΔN mutant in Tax-expressing cells (Fig. 4A). Taken together, these functional and biochemical results suggest that the phosphorylation status of Ser-32 and/or Ser-36 may influence Tax-induced entry of IκBα into its degradative pathway.

Constitutive repression of NF-κB-directed transcription in activated T cells. In addition to HTLV-1 Tax, expression of the active nuclear form of NF-κB in human T lymphocytes is posttranslationally induced by a number of cellular signaling pathways, including those mediated by cell surface receptors for antigen (41) and the cytokine TNF-α (19, 61, 74). Prior studies with Jurkat T cells have clearly demonstrated that activation of these pathways leads to the rapid degradation of endogenous IκBα (10, 19, 25, 80).

To examine these Tax-independent pathways for NF-κB activation, Jurkat T cells were transfected with cDNA expression vectors encoding either IκBαWT or a mutant lacking the SR domain (IκBαΔN) and were then treated with TNF-α. Alternatively, transfectants were treated with PMA and ionomycin, a combination that simulates the effects of antigen receptor stimulation on protein kinase C activity and on release of ionized calcium from intracellular stores (24, 50, 88). As shown in Fig. 7, both of these NF-κB agonists induced the rapid degradation of ectopic IκBαWT in transfected T cells (lanes 1 to 5). However, forms of IκBα lacking the SR domain were resistant to degradation under either stimulatory condition (lanes 6 to 10). Similar results were obtained with the S32A mutant of IκBα in T cells treated with PMA plus ionomycin (data not shown), a finding that is fully consistent with the stabilizing effects of this point mutation on IκBα when introduced into Tax-expressing cells (Fig. 6C).

To extend these biochemical results, we next assessed the functional properties of each IκBα mutant in T cells cotransfected with the HIV-κB-CAT reporter. Both TNF (Fig. 8A) and PMA-ionomycin (Fig. 8B) stimulated κB-directed transcription from the HIV-κB enhancer in the presence of transfected IκBαWT. In contrast, induction of HIV-κB-CAT by these NF-κB agonists was prevented in T cells expressing a deletion mutant of IκBα that lacked the Tax-responsive SR

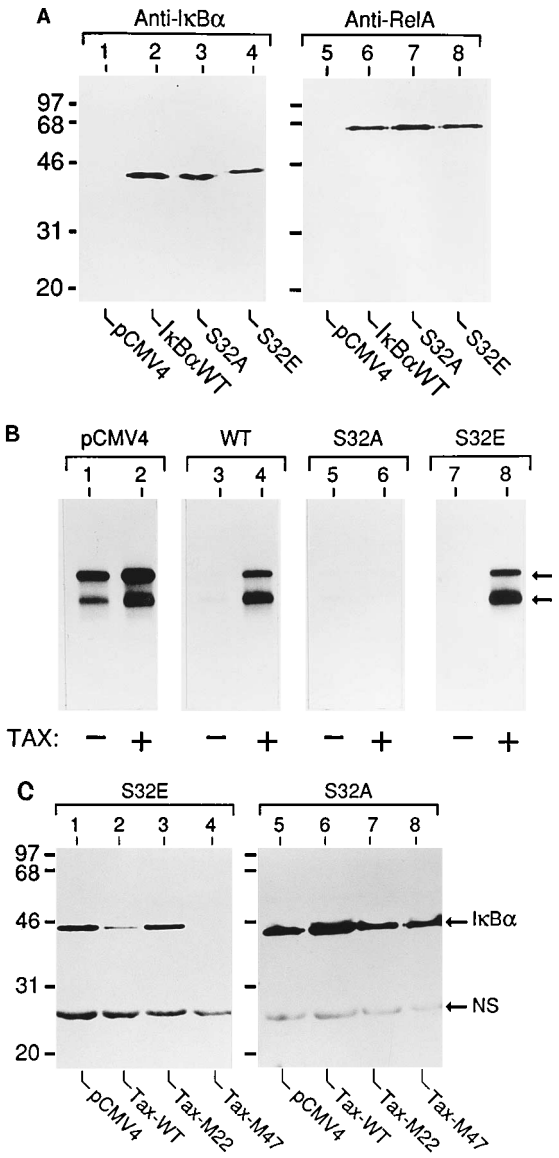

FIG. 6. Biochemical properties of site-directed IκBα mutants. (A) Association with endogenous RelA. Jurkat T cells were transfected with FLAG epitope-tagged derivatives of the indicated pCMV4-based IκBα expression vectors (20 μg). Cytoplasmic complexes containing ectopic IκBα were immunoprecipitated with a monoclonal anti-FLAG antibody, fractionated by SDS-PAGE, and analyzed by Western blotting with a peptide-specific antiserum for either IκBα (lanes 1 to 4) or RelA (lanes 5 to 8). (B) Induction of nuclear NF-κB/Rel activity. Jurkat T cells were cotransfected with pCMV4-based expression vectors encoding RelA (10 μg), Tax (10 μg), and the indicated IκBα constructs (5 μg). After 48 h of culture, cells were treated with cycloheximide (50 μg/ml) for 2 h and then harvested. Gel retardation assays were performed with nuclear extracts as described for Fig. 3B. Arrows indicate the positions of nucleoprotein complexes containing RelA homodimers (upper) and RelA/p50 heterodimers (lower). (C) Steady-state levels of IκBα. Jurkat cells were cotransfected with expression vectors for the indicated epitope-tagged mutants of IκBα (1 μg) and either control vector (pCMV4; lanes 1 and 5) or the indicated forms of Tax (10 μg). Mutants of the wild-type Tax protein (Tax-WT) that are defective in either NF-κB (Tax-M22) or CREB/ATF (Tax-M47) activating function have been previously described (77). Cytoplasmic extracts were prepared after 48 h of culture, immunoprecipitated with a monoclonal anti-FLAG antibody, fractionated by SDS-PAGE, and analyzed by Western blotting with an IκBα-specific antiserum (amino acids 289 to 317). The positions and sizes (in kilodaltons) of molecular weight markers are indicated at the left in panels A and C. NS, nonspecific.

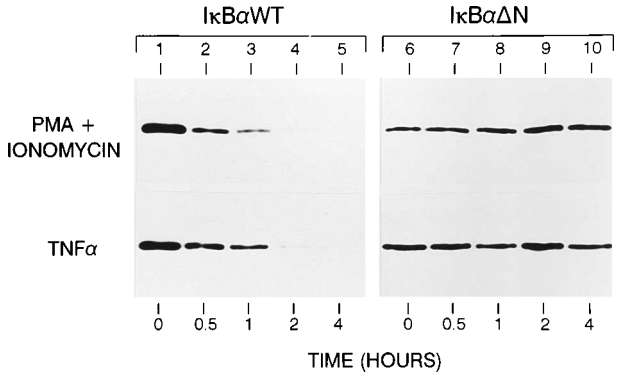

FIG. 7. The SR domain regulates the induced degradation of IκBα in response to T-cell activation signals. Jurkat T cells were transfected with expression vectors (5 μg) encoding FLAG epitope-tagged derivatives of either IκBαWT (lanes 1 to 5) or IκBαΔN (lanes 6 to 10). After 48 h of culture, transfected cells were pretreated with cycloheximide (50 μg/ml, 1 h) and then stimulated for the indicated time with either TNF-α (300 U/ml) or combinations of PMA (50 ng/ml) and ionomycin (1 μM). Tagged proteins were immunoprecipitated with a monoclonal anti-FLAG antibody, fractioned by SDS-PAGE, and detected by Western blotting using an IκBα-specific antiserum. Under these conditions, no significant degradation of IκBα was observed in control cells treated with cycloheximide in the absence of NF-κB agonist.

domain (IκBαΔN). These signal-dependent transcriptional responses were also attenuated in T cells expressing a mutant of IκBα containing an alanine substitution at either Ser-32 or Ser-36. However, both signal transduction pathways were significantly reconstituted in the presence of IκBα constructs containing glutamic acid rather than alanine at these two regulatory sites. These results with TNF-α- and PMA-ionomycin-

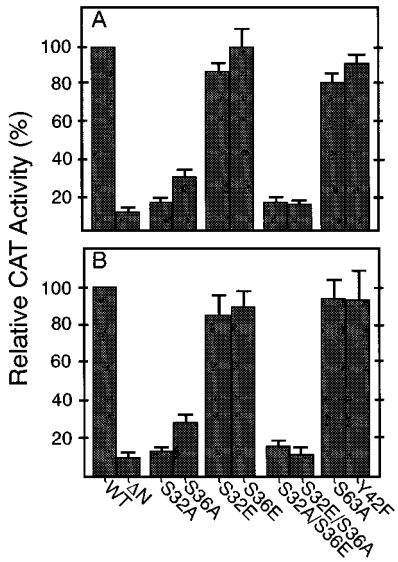

FIG. 8. Constitutive repression of cytokine and T-cell activation pathways by dominant-negative mutants of IκBα. Jurkat T cells were cotransfected with HIV-κB-CAT (5 μg) and expression vectors encoding the indicated site-directed mutants of human IκBα (2.5 μg). After 24 h of growth, half of each culture was stimulated for 20 h with either TNF-α (300 U/ml) (A) or a combination of PMA (50 ng/ml) and ionomycin (1 μM) (B). CAT activities were measured in normalized extracts as described previously (59). For each mutant, induction of reporter gene activity over basal levels (fold induction) is expressed as a percentage of the activity induced in cells transfected with the IκBαWT cDNA (mean fold induction = 9.4 ± 0.9 for TNF-α and 17.1 ± 1.7 for PMA-ionomycin). Error bars depict standard errors of the mean percentage value derived from at least three independent transfections.

stimulated T cells correlated precisely with the functional phenotypes of all IκBα mutants introduced into Tax-expressing cells (Fig. 5).

DISCUSSION

The amino terminus of IκBα specifies an SR domain. Since its initial discovery in B lymphocytes (75), NF-κB has emerged as an essential component of the inducible transcriptional machinery that mediates T-cell activation and growth (31, 32, 46). In addition, NF-κB is coopted by HIV and HTLV-1 to subserve distinct retroviral functions in infected $CD4^+$ cells (30, 31). A hallmark feature of NF-κB is its extraordinary capacity to respond to a diverse range of physiologic and pathologic cues, including T-cell receptor ligands, cytokines, and viral proteins (32). It is now clear that the transcriptional activity of NF-κB is regulated from the cytoplasmic compartment by IκBα, which is rapidly degraded during cellular activation (9, 10, 19, 20, 25, 35, 80, 81). However, a unifying explanation for how disparate viral and cellular stimuli converge on this enzymatic checkpoint has remained elusive.

To examine this signaling mechanism in detail, we devised an approach to reconstitute human T lymphocytes with ectopic forms of IκBα that interface with the endogenous NF-κB signaling pathway. When the wild-type IκBα cDNA was expressed in this in vivo system, the resultant ectopic protein fully recapitulated the inhibitory and signal-transducing functions of endogenous IκBα. In contrast, a deletion mutant of IκBα lacking amino acids 1 to 36 (IκBαΔN) failed to support NF-κB-directed transcription from the HIV enhancer in cells stimulated with potent NF-κB inducers, including HTLV-1 Tax, TNF-α, and combinations of phorbol ester and calcium ionophore. Based on coimmunoprecipitation and gel retardation studies, however, this dominant-negative form of IκBα retained both its NF-κB binding and inhibitory functions in unstimulated cells.

These in vivo results thus define N-terminal regulatory sequences in IκBα that integrate proximal biochemical signals leading to the induced nuclear translocation of NF-κB. This SR domain of IκBα represents a key target for antigen- and cytokine receptor-mediated pathways that converge on the set of T-cell activation genes under NF-κB control. The SR domain of IκBα is also inextricably linked to the pathophysiologic mechanism by which HTLV-1 Tax deregulates the function of NF-κB, resulting in the aberrant expression of a subset of these growth-related cellular genes. Moreover, preliminary experiments conducted with stably transfected pre-B cells indicate that this region of IκBα is required for the induction of NF-κB in response to bacterial lipopolysaccharide (data not shown).

These findings do not exclude the possibility that the SR domain collaborates with other distal regulatory sequences in IκBα in order to govern its biologic activity. For example, acquisition of this pleiotropic signaling function may also involve the C-terminal region of IκBα (amino acids 243 to 317), which contains PEST sequences characteristic of many proteins that undergo rapid turnover (33, 66). In this regard, we have found that removal of the entire PEST domain of IκBα disrupts its RelA binding function in vivo. However, preliminary studies with an IκBα mutant retaining a portion of the PEST domain indicate the presence of additional determinants in this C-terminal region that may act in concert with the N-terminal SR domain of IκBα to mediate its signal-transducing function.

Role of IκBα in NF-κB induction by HTLV-1 Tax. Recent studies with mice transgenic for the Tax gene suggest that the constitutive pattern of NF-κB expression in HTLV-1-infected cells may be required to maintain a neoplastic state of deregulated growth control (45). Insights into the precise mechanism underlying this pathogenic virus-host interaction have been complicated by the pleiotropic effects of HTLV-1 Tax on the T-cell activation program and its ability to assemble with several components of the NF-κB pathway that are structurally and functionally related to IκBα. These Tax-interactive components include the Rel-related precursors of the p50 and p52 NF-κB/Rel subunits (p105 and p100, respectively) (12, 36, 87), as well as IκBγ (37), which corresponds to the C-terminal half of p105 (38). Like IκBα, all three of these polypeptides contain multiple ankyrin motifs and inhibit the nuclear expression and DNA binding activity of select Rel polypeptides (9, 38, 53, 58, 65). However, evidence is currently lacking for either the presence of IκBγ in human T cells or for Tax-induced processing of p100 and p105 to their functionally active subunits (42, 82).

This study provides four new lines of experimental evidence demonstrating that the SR domain of IκBα couples Tax to the host NF-κB signaling pathway. First, Tax failed to stimulate the transcriptional activity of NF-κB-responsive promoters in cells expressing a deletion mutant of IκBα (IκBαΔN) that lacked this N-terminal regulatory region. These inhibitory effects were selective, because the ability of Tax to transactivate promoters that function independently of NF-κB (e.g., HTLV-1 LTR) was unimpeded in cells coexpressing IκBαΔN. Second, in vivo expression of IκBαΔN but not its wild-type counterpart prevented Tax-induced nuclear translocation of NF-κB. Third, whereas Tax stimulated the turnover of wild-type IκBα, removal of the SR domain rendered this inhibitor completely resistant to Tax-induced degradation. Taken together, these latter two findings establish a direct mechanistic link between the ability of Tax to stimulate the degradation of IκBα and to trigger the induction of nuclear NF-κB expression. Fourth, point mutations in Tax that ablate its NF-κB-inducing function (77) also block the ability of Tax to stimulate IκBα breakdown, thus providing further evidence for the specificity and functional relevance of this virus-host interaction. These findings extend two recent studies indicating that Tax induces the proteolytic breakdown of endogenous IκBα in both stable transfectants and HTLV-1-infected cells (42, 82). Furthermore, the finding that IκBα accumulates in Tax-expressing cells following treatment with an inhibitor of the proteasome (Fig. 4B) implicates this specific degradation pathway in the mechanism by which Tax inactivates IκBα.

Function of the SR domain. Prior biochemical studies have shown that IκBα is rapidly and transiently phosphorylated in response to T-cell activation signals before entering its degradative pathway (10, 19, 52, 57, 81). This inducibly phosphorylated form of IκBα accumulates in cells treated with proteasome inhibitors that completely block the activation of NF-κB, thus suggesting that phosphorylation is not sufficient to trigger the dissociation of intact IκBα from NF-κB (62). In this context, we have identified two regulatory serines within the SR domain of IκBα that influence its biologic activity in a conditional manner. Specifically, substitution of either Ser-32 or Ser-36 with alanine largely disrupted the signal-transducing function of IκBα in response to either Tax, TNF-α, or PMA-ionomycin. In contrast, introduction of a phosphoserine mimetic at these positions restored the in vivo signaling activity of IκBα. Although direct proof that Ser-32 and Ser-36 are sites for either basal or induced phosphorylation is lacking, these mutational studies are consistent with this possibility. Furthermore, phosphoamino acid analyses indicate that endogenous IκBα is basally phosphorylated primarily on serine and threonine residues (data not shown).

Two findings reported in this study indicate that the SR

domain of IκBα plays an essential role in the targeting mechanism that facilitates recognition of IκBα as a proteolytic substrate. First, we have observed that IκBα mutants lacking this domain escape from degradation in the presence of either Tax, TNF-α, or PMA-ionomycin, thereby blocking the nuclear translocation of NF-κB. Second, deletion of the SR domain prevented the induction of a labile phosphorylated form of IκBα that has been previously detected in cells stimulated with a variety of NF-κB-inducing agents (10, 19, 20, 52, 57, 81). These results demonstrate that the SR domain contains determinants that are critical for both signal-dependent phosphorylation and proteolysis of IκBα. One potential explanation for these findings is that disruption of the N-terminal region of the inhibitor interferes with a regulated phosphorylation step(s) that serves to brand IκBα for rapid breakdown. For example, our amino acid replacement studies suggest that agonist-induced changes in the phosphorylation status of the SR domain at Ser-32 and Ser-36 may influence the proteolytic stability of IκBα. However, in light of emerging evidence for involvement of the proteasome in this process (62), the possibility that the SR domain of IκBα functions in the acquisition of other distinct degradation signals cannot be excluded.

Conclusions. In summary, these studies reveal an SR domain within IκBα that is coupled to both viral and immune activation pathways for NF-κB induction. Our findings support a phosphorylation-dependent mechanism for functional release of NF-κB from IκBα control involving determinants within the SR domain that direct the induced entry of IκBα into its degradative pathway. In particular, site-directed mutations at two regulatory serines that are positioned within the SR domain of IκBα (Ser-32 and Ser-36) produce constitutive repressors of NF-κB-directed transcription with potent activity in human T lymphocytes. Such constitutive repressors, in combination with an appropriate delivery system, could prove applicable to the design of novel immunosuppressive and anti-inflammatory drugs.

ACKNOWLEDGMENTS

We thank Tom Maniatis, David Kranz, Earl Ruley, Jacek Hawiger, and John Exton for critical comments; Craig Rosen, Warner Greene, and Albert Baldwin for cDNA clones; and Emily Vance for manuscript preparation.

These studies were supported by the Howard Hughes Medical Institute and grant R01 AI33839 from the National Institute of Allergy and Infectious Diseases to D.W.B.

ADDENDUM IN PROOF

We have recently assembled evidence that simultaneous replacement of Ser-32 and Ser-36 with alanine prevents hyperphosphorylation of IκBα in T cells treated with PMA-ionomycin. These results provide further support for the proposed functional role of Ser-32 and Ser-36 in signal-dependent phosphorylation events that render IκBα susceptible to targeted degradation.

REFERENCES

1. **Alessi, D. R., Y. Saito, D. G. Campbell, P. Cohen, G. Sithanandam, U. Rapp, A. Ashworth, C. J. Marshall, and S. Cowley.** 1994. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO J. **13:**1610–1619.
2. **Andersson, S., D. L. Davis, H. Dahlback, H. Jörnvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264:**8222–8229.
3. **Baeuerle, P., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
4. **Baeuerle, P., and D. Baltimore.** 1988. IκB: a specific inhibitor of the NF-κB transcription factor. Science **242:**540–546.
5. **Baeuerle, P., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3:**1689–1698.
6. **Ballard, D. W., E. Böhnlein, J. W. Lowenthal, Y. Wano, B. R. Franza, and W. C. Greene.** 1988. HTLV-I Tax induces cellular proteins that activate the κB element in the IL-2 receptor α gene. Science **241:**1652–1655.
7. **Ballard, D. W., E. P. Dixon, N. J. Peffer, H. Bogerd, S. Doerre, B. Stein, and W. C. Greene.** 1992. The 65-kDa subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. USA **89:**1875–1879.
8. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hannink, and W. C. Greene.** 1990. The v-rel oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63:**803–814.
9. **Beg, A. A., and A. S. Baldwin, Jr.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
10. **Beg, A. A., T. S. Finco, P. V. Nantermet, and A. S. Baldwin, Jr.** 1993. Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα: a mechanism for NF-κB activation. Mol. Cell. Biol. **13:**3301–3310.
11. **Beg, A. A., S. M. Ruben, R. I. Scheinman, S. Haskill, C. A. Rosen, and A. S. Baldwin, Jr.** 1992. IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasm retention. Genes Dev. **6:**1899–1913.
12. **Béraud, C., S.-C. Sun, P. Ganchi, D. W. Ballard, and W. C. Greene.** 1994. Human T-cell leukemia virus type 1 tax associates with and is negatively regulated by the NF-κB2 p100 gene product: implications for viral latency. Mol. Cell. Biol. **14:**1374–1382.
13. **Blanar, M., and W. J. Rutter.** 1992. Interaction cloning: identification of a helix-loop-helix zipper protein that interacts with c-fos. Science **256:**1014–1018.
14. **Böhnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. C. Greene.** 1988. The same inducible nuclear protein(s) regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type I HIV. Cell **53:**827–836.
15. **Böhnlein, E., M. Siekevitz, D. W. Ballard, J. W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B. R. Franza, and W. C. Greene.** 1989. Stimulation of human immunodeficiency virus type I enhancer by the human T-cell leukemia virus type I *tax* gene product involves the activation of inducible cellular proteins. J. Virol. **63:**1578–1586.
16. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the *rel* oncogene and to cell-cycle motifs. Nature (London) **348:**76–80.
17. **Boyle, W., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–149.
18. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
19. **Brown, K., S. Park, T. Kanno, G. Franzoso, and U. Siebenlist.** 1993. Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκBα. Proc. Natl. Acad. Sci. USA **90:**2532–2536.
20. **Cordle, S. R., R. Donald, M. A. Read, and J. Hawiger.** 1993. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1. J. Biol. Chem. **268:**11803–11810.
21. **Cross, S. L., M. B., Feinberg, J. B. Wolf, N. J. Holbrook, F. Wong-Staal, and W. J. Leonard.** 1987. Regulation of the human interleukin-2 receptor α chain promoter: activation of a nonfunctional promoter by the transactivator gene of HTLV-I. Cell **49:**47–56.
22. **Davis, N., S. Ghosh, D. L. Simmons, P. Tempst, H. C. Liou, D. Baltimore, and H. R. Bose, Jr.** 1991. Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science **253:**1268–1271.
23. **deMartin, R., B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, and F. H. Bach.** 1993. Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB. EMBO J. **12:**2773–2779.
24. **Durand, D. B., M. R. Bush, J. G. Morgan, A. Weiss, and G. R. Crabtree.** 1987. A 275 basepair fragment at the 5′ end of the interleukin 2 gene enhances expression from a heterologous promoter in response to signals from the T cell antigen receptor. J. Exp. Med. **165:**395–407.
25. **Frantz, B., E. C. Nordby, G. Bren, N. Steffan, C. V. Paya, R. L. Kincaid, M. J. Tocci, S. J. O'Keefe, and E. A. O'Neill.** 1994. Calcineurin acts in synergy with PMA to inactivate IκB/MAD3, an inhibitor of NF-κB. EMBO J. **13:**861–870.
26. **Fujii, H., H. Tsuchiya, T. Chuhjo, T. Akizawa, and M. Seiki.** 1992. Interaction of HTLV-1 Tax with $p67^{SRF}$ causes the aberrant induction of cellular immediate early genes through CArG boxes. Genes Dev. **6:**2066–2076.
27. **Ganchi, P., S.-C. Sun, W. C. Greene, and D. W. Ballard.** 1992. IκB/MAD-3 masks the nuclear localization signal of NF-κB p65 and requires the transactivation domain to inhibit NF-κB p65 DNA binding. Mol. Biol. Cell **3:**1339–1352.
28. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D.**

Baltimore. 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to *rel* and *dorsal*. Cell **62:**1019–1029.
29. **Gilman, M. Z., R. N. Wilson, and R. A. Weinberg.** 1986. Multiple protein-binding sites in the 5′-flanking region regulate c-*fos* expression. Mol. Cell. Biol. **6:**4305–4316.
30. **Green, P. L., and I. S. Y. Chen.** 1994. Molecular features of the human T-cell leukemia virus, p. 277–311. *In* J. A. Levy (ed.), The Retroviridae, vol. 3. Plenum Press, New York.
31. **Greene, W. C., E. Böhnlein, and D. W. Ballard.** 1989. HIV-1, HTLV-1 and normal T-cell growth: transcriptional strategies and surprises. Immunol. Today **10:**272–277.
32. **Grilli, M., J. Chiu, and M. Lenardo.** 1993. NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
33. **Haskill, S., A. A. Beg, S. M. Tompkins, J. S. Morris, A. D. Yurochko, A. Sampson-Johannes, K. Mondal, P. Ralph, and A. S. Baldwin, Jr.** 1991. Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell **65:**1281–1289.
34. **Hatada, E., M. Naumann, and C. Scheidereit.** 1993. Common structural constituents confer IκB activity to NF-κB p105 and IκB/MAD-3. EMBO J. **12:**2781–2788.
35. **Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary in the activation of transcription factor NF-κB. Nature (London) **365:**182–185.
36. **Hirai, H., J. Fujisawa, T. Suzuki, K. Ueda, M. Muramatsu, A. Tsuboi, N. Arai, and M. Yoshida.** 1992. Transcriptional activator Tax of HTLV-I binds to the NF-κB precursor p105. Oncogene **7:**1737–1742.
37. **Hirai, H., T. Suzuki, J. Fujisawa, J. Inoue, M. Yoshida.** 1994. Tax protein of human T-cell leukemia virus type I binds to the ankyrin motifs of inhibitory factor κB and induces nuclear translocation of transcription factor NF-κB proteins for transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**3584–3588.
38. **Inoue, J.-I., L. D. Kerr, A. Kakizuka, and I. M. Verma.** 1992. IκBγ, a 70 kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell **68:**1109–1120.
39. **Inoue, J.-I., L. D. Kerr, D. Rashid, N. Bose, Jr., and I. M. Verma.** 1992. Direct association of pp40/IκBβ with rel/NF-κB transcription factors: role of ankyrin repeats in the inhibition of DNA binding activity. Proc. Natl. Acad. Sci. USA **89:**4333–4337.
40. **Inoue, J.-I., M. Seiki, T. Taniguchi, S. Tsuru, and M. Yoshida.** 1986. Induction of interleukin-2 receptor gene expression by $p40^x$ encoded by human T-cell leukemia virus type I. EMBO J. **5:**2883–2888.
41. **Kang, S.-M., A. Chen-Tran, M. Grilli, and M. Leonardo.** 1992. NF-κB subunit regulation in nontransformed $CD4^+$ T lymphocytes. Science **256:** 1452–1456.
42. **Kanno, T., K. Brown, G. Franzoso, and U. Siebenlist.** 1994. Kinetic analysis of human T-cell leukemia virus type I Tax-mediated activation of NF-κB. Mol. Cell. Biol. **14:**6443–6451.
43. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. LeBail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israël.** 1990. The DNA binding subunit of NF-κB is identified to factor KBF1 and homologous to the *rel* oncogene product. Cell **62:**1007–1018.
44. **Kimata, J. T., and L. Ratner.** 1991. Temporal regulation of viral and cellular gene expression during human T-lymphotropic virus type I-mediated lymphocyte immortalization. J. Virol. **65:**3134–3141.
45. **Kitajima, I., T. Shinohara, J. Bilakovics, D. A. Brown, X. Xu, and M. Nerenberg.** 1992. Ablation of transplanted HTLV-1 Tax-transformed tumors in mice by antisense inhibition of NF-κB. Science **258:**1792–1795.
46. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
47. **Leung, K., and G. Nabel.** 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-kB-like factor. Nature (London) **333:** 776–778.
48. **Lowenthal, J., D. W. Ballard, E. Böhnlein, and W. C. Greene.** 1989. Tumor necrosis factor alpha induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor alpha-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA **86:**2331–2335.
49. **Lowenthal, J. W., E. Böhnlein, D. W. Ballard, and W. C. Greene.** 1988. Regulation of interleukin-2 receptor α subunit (Tac or CD25 antigen) gene expression: binding of inducible nuclear proteins to discrete promoter sequences correlates with transcriptional activation. Proc. Natl. Acad. Sci. USA **85:**4468–4472.
50. **Manger, B., A. Weiss, C. Weyand, J. Goronzy, and J. Stobo.** 1985. T cell activation: differences in the signals required for IL-2 production by non-activated and activated T cells. J. Immunol. **135:**3669–3673.
51. **Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J.-I. Inoue, M. Yoshida, and T. Taniguchi.** 1987. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-I-encoded $p40^x$ and T3/Ti complex triggering. Cell **48:**343–350.
52. **Mellits, K. H., R. T. Hay, and S. Goodbourn.** 1993. Proteolytic degradation of MAD3 (IκBα) and enhanced processing of the NF-κB precursor p105 are obligatory steps in the activation of NF-κB. Nucleic Acids Res. **21:**5059–5066.
53. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
54. **Meyer, R., E. N. Hatada, H.-P. Hohmann, M. Haiker, C. Bartsch, U. Rothlisberger, H.-W. Lahm, E. J. Schlaeger, A. P. G. M. van Loon, and C. Scheidereit.** 1991. Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **88:**966–970.
55. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
56. **Nakamaye, K., and F. Eckstein.** 1986. Inhibition of restriction endonuclease Nci I cleavage by phosphothioate groups and its application to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **14:**9679–9698.
57. **Naumann, M., and C. Scheidereit.** 1994. Activation of NF-κB *in vivo* is regulated by multiple phosphorylations. EMBO J. **13:**4597–4607.
58. **Naumann, M., F. G. Wulczyn, and C. Sheidereit.** 1993. The NF-κB precursor p105 and the proto-oncogene product Bcl-3 are IκB molecules and control nuclear translocation of NF-KB. EMBO J. **12:**213–222.
59. **Neumann, J., C. Morency, and K. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
60. **Nolan, G. P., S. Ghosh, H.-C. Lion, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a *rel*-related polypeptide. Cell **64:**961–969.
61. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor-kappa B. Proc. Natl. Acad. Sci. USA **86:** 2336–2340.
62. **Palombella, V. J., O. J. Rando, A. L. Goldberg, and T. Maniatis.** 1994. Ubiquitin and the proteasome are required for processing the NF-κB1 precursor and the activation of NF-κB. Cell **78:**773–785.
63. **Pearson, R. B., and B. E. Kemp.** 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol. **200:**62–81.
64. **Prickett, K. S., D. C. Amberg, and T. P. Hopp.** 1989. A calcium-dependent antibody for identification and purification of recombinant proteins. BioTechniques **7:**580–589.
65. **Rice, N. R., M. L. MacKichan, and A. Israël.** 1992. The precursor of NF-κB p50 has IκB-like functions. Cell **71:**243–253.
66. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:** 364–368.
67. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C.-H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a *rel*-related human cDNA that potentially encodes the p65-kD subunit of NF-κB. Science **251:**1490–1493.
68. **Ruben, S. M., R. Narayanan, J. Klement, C.-H. Chen, and C. Rosen.** 1992. Functional characterization of the NF-κB p65 transcriptional activator and an alternatively sliced derivative. Mol. Cell. Biol. **12:**444–454.
69. **Ruben, S. M., H. Poteat, T. H. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C. A. Rosen.** 1988. Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-1 tax gene product. Science **241:**89–92.
70. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
71. **Schreck, R., R. Grassman, B. Fleckenstein, and P. A. Baeuerle.** 1992. Antioxidants selectively suppress activation of NF-κB by human T-cell leukemia virus type I Tax protein. J. Virol. **66:**6288–6293.
72. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
73. **Schreiber, E., P. Matthias, M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
74. **Schütze, S., K. Potthoff, T. Machleidt, D. Berkovic, K. Wiegmann, and M. Kronke.** 1992. TNF activates NF-κB by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell **71:**765–776.
75. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705–716.
76. **Siekevitz, M., M. B. Feinberg, N. Holbrook, F. Wong-Staal, and W. C. Greene.** 1987. Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the transactivator (tat) gene product of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **84:**5389–5393.
77. **Smith, M. R., and W. C. Greene.** 1990. Identification of HTLV-1 *tax trans*-activator mutants exhibiting novel transcriptional phenotypes. Genes Dev. **4:**1875–1885.
78. **Sodroski, J., C. Rosen, W. C. Goh, and W. Haseltine.** 1985. A transcriptional activator protein encoded by the x-lor region of human T cell leukemia virus. Science **228:**1430–1434.
79. **Stein, B., H. Rahmsdorf, A. Steffen, M. Litfin, and P. Herrlich.** 1989. UV-induced DNA damage is an intermediate step in UV-induced expression of

human immunodeficiency virus type 1, collagenase, c-Fos, and metallothionein. Mol. Cell. Biol. **9:**5169–5181.

80. **Sun, S.-C., P. Ganchi, D. W. Ballard, and W. C. Greene.** 1993. NF-κB controls expression of inhibitor IκBα: evidence for an inducible autoregulatory pathway. Science **259:**1912–1915.
81. **Sun, S.-C., P. Ganchi, C. Béraud, D. W. Ballard, and W. C. Greene.** 1994. Autoregulation of the NF-κB transactivator RelA (p65) by multiple cytoplasmic inhibitors containing ankyrin motifs. Proc. Natl. Acad. Sci. USA **91:**1346–1350.
82. **Sun, S.-C., J. Elwood, C. Béraud, and W. C. Greene.** 1994. Human T-cell leukemia virus type I tax activation of NF-κB/Rel involves phosphorylation and degradation of IκBα and RelA (p65)-mediated induction of the c-*rel* gene. Mol. Cell. Biol. **14:**7377–7384.
83. **Suzuki, T., J. I. Fujisawa, M. Toita, and M. Yoshida.** 1993. The transactivator *tax* of human T-cell leukemia virus type 1 (HTLV-1) interacts with cAMP-responsive element (CRE) binding and CRE modulator proteins that bind to the 21-base-pair enhancer of HTLV-1. Proc. Natl. Acad. Sci. USA **90:**610–614.
84. **Ten, R. M., C. V. Paya, N. Israel, O. LeBail, M. G. Mattei, J. L. Virelizier, P. Kourilsky, and A. Israël.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-κB indicates that it participates in its own regulation. EMBO J. **11:**195–203.
85. **Tewari, M., P. Dobrzanski, K. L. Mohn, D. E. Cressman, J. C. Hsu, R. Bravo, and R. Taub.** 1992. Rapid induction in regenerating liver of RL/IF-1 (an IκB that inhibits NF-κB, RelB-p50, and c-Rel-p50) and PHF, a novel κB site binding complex. Mol. Cell. Biol. **12:**2898–2908.
86. **Urban, M., and P. Baeuerle.** 1990. The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. Genes Dev. **4:**1975–1984.
87. **Watanabe, M., M.-A. Muramatsu, H. Hirai, T. Suzuki, J. Fujisawa, M. Yoshida, K.-I. Arai, and N. Arai.** 1993. HTLV-I encoded Tax in association with NF-κB precursor p105 enhances nuclear localization of NF-κB p50 and p65 in transfected cells. Oncogene **8:**2949–2958.
88. **Weiss, A., and D. Littman.** 1994. Signal transduction by lymphocyte antigen receptors. Cell **76:**263–274.
89. **Zhao, L. J., and C. Z. Giam.** 1992. Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to HTLV-I 21-base-pair repeats by protein-protein interactions. Proc. Natl. Acad. Sci. USA **89:**7070–7074.