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Tyrosine Phosphorylation of IκB-α Activates NF-κB without Proteolytic Degradation of IκB-α

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Summary

The transcription factor NF-κB regulates genes participating in immune and inflammatory responses. In T lymphocytes, NF-κB is sequestered in the cytosol by the inhibitor IκB-α and released after serine phosphorylation of IκB-α that regulates its ubiquitin-dependent degradation. We report an alternative mechanism of NF-κB activation. Stimulation of Jurkat T cells with the protein tyrosine phosphatase inhibitor and T cell activator pervanadate led to NF-κB activation through tyrosine phosphorylation but not degradation of IκB-α. Pervanadate-induced IκB-α phosphorylation and NF-κB activation required expression of the T cell tyrosine kinase p56^{lck}. Reoxygenation of hypoxic cells appeared as a physiological effector of IκB-α tyrosine phosphorylation. Tyrosine phosphorylation of IκB-α represents a proteolysis-independent mechanism of NF-κB activation that directly couples NF-κB to cellular tyrosine kinase.

Introduction

Signal transduction pathways are complex networks of biochemical reactions that ultimately culminate in specific patterns of nuclear gene expression mediated by transcription factors (TFs). Preexisting TFs that are involved in immediate-early cellular responses can be posttranslationally activated by a variety of mechanisms. A frequent strategy is the induction of DNA binding activity that may involve the dimerization of subunits. For instance, binding of steroid hormones or tyrosine phosphorylation with subsequent SH2 domain interaction can promote DNA binding via dimerization of subunits. A large number of TFs translocate from the cytoplasm to the nucleus as part of their activation process. Other TFs are already bound to DNA but require some covalent modification to unleash their transactivating potential.

Phosphorylation is one of the most frequent and important posttranslational modification of proteins. It was also shown to be an important regulator of TF activity (Karin, 1994). In the case of the NF-κB/Rel family of TFs (reviewed in Baeuerle and Henkel, 1994; Liou and Baltimore, 1993), serine phosphorylation triggers a proteolytic degradation of inhibitory subunits, called IκB proteins (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). The activation of TFs by protein kinases can directly couple events at cell surface receptors to nuclear gene expression. STAT factors require only one kinase in their signal transduction pathway whereas activation of the nuclear SRF/ets complex involves activation of an entire kinase cascade.

NF-κB/Rel TFs are composed of five distinct DNA-binding subunits, called p50, p52, p65/RelA, c-Rel, and Rel-B (Liou and Baltimore, 1993). The different family members can associate in various homo- or heterodimers through a highly conserved N-terminal sequence, called the NRD (NF-κB/rel/Dorsal) (Grimm and Baeuerle, 1993) or Rel homology domain. NF-κB complexes containing the p65 subunit seem to have a pivotal role in the generation of an immune response. NF-κB, which is activated by antigens, viruses, bacteria, prooxidants, and inflammatory lymphokines, participates in the transcriptional initiation of diverse genes whose products are important in immune and inflammatory responses. Examples are the genes encoding interleukins −1, −2, −6, and −8, IL-2 receptor α chain, various adhesion molecules, major histocompatibility class I molecules, and immunoglobulin κ light chain (Baeuerle and Henkel, 1994).

Inactive NF-κB is present in the cytosol associated with an inhibitory molecule of the IκB family (Baeuerle and Baltimore, 1988). All six known members of the IκB family (IκB-α, IκB-β, IκB-γ, Bcl-3, p100, p105, reviewed in Beg and Baldwin, 1993) contain an ankyrin (ANK) repeat domain that is composed of 5–7 closely adjacent repeats required for both association with NF-κB and inhibitory activity (Beg and Baldwin, 1993). Interaction of IκB-α with an NF-κB dimer prevents the nuclear uptake of the DNA-binding subunits through the masking of nuclear localization signals (Beg et al., 1992; Zabel et al., 1993). Uncomplexed IκB-α can enter the nucleus and may dissociate DNA-bound NF-κB–DNA complexes. Phosphorylation of IκB-α by various kinases has been shown to dissociate NF-κB–IκB-α complexes in vitro (Ghosh and Baltimore, 1990). The situation is rather different in vivo where phosphorylation of IκB-α on serine residues 32 and 36 does not lead to dissociation of the NF-κB–IκB-α complex but targets IκB-α for rapid degradation by the proteasome (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995).

Tyrosine phosphorylation of proteins by protein tyrosine kinases (PTKs) is a primary and important step in the initiation of various mitogenic signaling cascades (Ullrich and Schlessinger, 1990; Cantley et al., 1991). Engagement of receptor-associated PTKs stimulates the ras/raf-1/map kinase pathway (Johnson and Vaillancourt, 1994), which leads to activation of the c-*fos* and

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c-*jun* proto-oncogenes via phosphorylation of preexisting DNA-bound factors in their transactivation domain (Karin, 1994; Hill and Treisman, 1995). Moreover, PTKs have recently been shown to directly activate a new family of TFs, the STAT factors (Darnell et al., 1994).

A direct role of tyrosine phosphorylation in NF-κB activation is not well documented. Two reports have shown that c-rel and IκB proteins become tyrosine phosphorylated upon neutrophil activation and hypoxia, respectively, but the significance of the modifications remained unknown (Druker et al., 1994; Koong et al., 1994). One report showed that v-*src* overexpression in T cells caused NF-κB activation (Eicher et al., 1994) while another study showed that v-*abl* overexpression blocked NF-κB activity in pre-B cells (Klug et al., 1994).

In a previous study, we have shown that a protein tyrosine phosphatase (PTP) inhibitor, pervanadate, stimulates protein tyrosine phosphorylation and downstream events of the T-cell activation process including induction of NF-κB DNA-binding activity (Imbert et al., 1994). We demonstrate here that there is a direct connection between PTK stimulation and NF-κB activation through tyrosine phosphorylation of IκB-α. In contrast to other activators of NF-κB, tyrosine phosphorylation of IκB-α does not lead to degradation of the protein. Tyrosine phosphorylation of IκB-α induces dissociation from NF-κB and precedes the appearance of a DNA binding activity on κB sites in the nucleus. Lck-deficient mutants of Jurkat had a defect in both tyrosine phosphorylation of IκB-α and NF-κB activation. Reoxygenation of hypoxic cells was shown to induce tyrosine phosphorylation of IκB-α. Tyrosine phosphorylation of IκB-α thus represents a new mechanism of NF-κB activation that has the potential to directly couple NF-κB to surface receptor–associated tyrosine kinases.

Results

Pervanadate Activates the DNA Binding Activity of NF-κB

We have previously reported that pervanadate is a potent activator of Jurkat leukemic T cells (Imbert et al., 1994) through its ability to increase tyrosine phosphorylation of cellular proteins after inhibition of protein tyrosine phosphatases (PTPs). As determined by electrophoretic mobility shift assay (EMSA), a 1 hr stimulation with pervanadate induced a dose-dependent appearance of an NF-κB–DNA binding activity in the nuclei of stimulated Jurkat cells (Figure 1A, compare lanes 4–6 to lane 1). The effect of 250 μM pervanadate (Figure 1A, lane 4) was as strong as that of phorbol 12-myristate 13-acetate (PMA) (Figure 1A, lane 3). The two compounds showed an additive effect (Figure 1A, lanes 7–9) similar to that observed with the combination of PMA and a calcium ionophore (lane 2). When cells were stimulated with 200 μM H_2O_2 alone, (Figure 1A, lane 12), 200 μM vanadate alone (lane 14), or with H_2O_2 in the presence of catalase (control pV, lane 13) no induction of NF-κB-DNA binding activity could be detected, demonstrating that the observed activation was due to vanadate peroxide (pervanadate).

Incubation of pervanadate-stimulated cell extracts

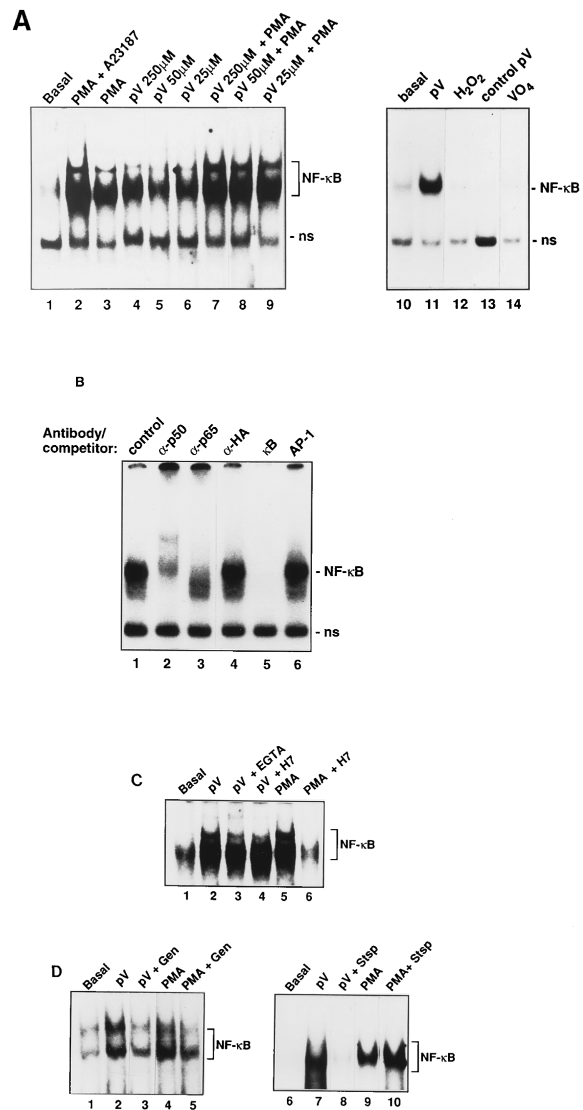

Figure 1. Pervanadate-Induced NF-κB-DNA Binding Activity

(A) Dose response effect of pervanadate (lanes 1–9). Jurkat cells were stimulated for 1 hr with the indicated doses of pervanadate, PMA (10 ng/ml), and calcium ionophore A23187 (2 μM). Nuclear extracts were incubated with a ^{32}P-labeled NF-κB probe corresponding to the κB site in the IL-2 gene promoter and analyzed by EMSA. NF-κB-DNA specific complex and nonspecific complex are indicated. Characterization of pervanadate effect (lanes 10–14). Cells were stimulated with 200 μM pervanadate, 200 μM H_2O_2 or 200 μM vanadate. Control pV represents a mixture containing only catalase and H_2O_2.

(B) Analysis of pervanadate-induced NF-κB complexes. Extracts of pervanadate-stimulated cells were incubated with control IgG (lane 1), anti-p50 (lane 2), p65 (lane 3), or HA (lane 4) antibodies prior to EMSA analysis. The specificity of the complex was analyzed by incubation with an excess of unlabeled κB (lane 5) or AP-1 (lane 6) oligonucleotides.

(C) Effect of EGTA and H7 on pervanadate- or PMA-induced NF-κB. Jurkat cells were incubated in a calcium-free medium containing 1 mM EGTA or preincubated for 20 min with H7 (100 μM) prior to stimulation with pervanadate (200 μM) or PMA (10 ng/ml) for 1h.

(D) Effect of tyrosine kinase inhibitors. Cells were preincubated with genistein (100 μM) or staurosporine (50 nM) 20 min before stimulation with pervanadate or PMA for 1 hr.

with anti-p50 (Figure 1B, lane 2) or anti-p65 antibodies (lane 3) resulted in the abrogation of NF-κB/DNA complexes, whereas control IgG (lane 1) or an irrelevant influenza hemagglutinin-specific antibody had no effect (lane 4). The specificity of the pervanadate-induced protein–DNA complex was further confirmed by the demonstration that an excess (100-fold) of unlabeled κB oligonucleotide (Figure 1B, lane 5) but not of an AP-1 oligonucleotide (lane 6) prevented its binding to the ^{32}P-labeled κB probe. These results show that pervanadate activated a bona fide p50–p65 NF-κB dimer in Jurkat cells.

Pervanadate-Induced NF-κB Activation Is Independent of Protein Kinase C- and Calcium-Regulated Events but Is Blocked by PTK Inhibitors

Preincubation of cells with the PKC inhibitor H7 did not detectably affect the activation of NF-κB by pervanadate (Figure 1C, compare lane 4 to lane 2) while it strongly suppressed NF-κB stimulation by PMA (lane 6). Moreover, we did not detect any stimulation of PKC by pervanadate (data not shown). While addition of EGTA to the medium blocked the calcium influx stimulated by pervanadate (Imbert et al., 1994), it had no effect on pervanadate-induced NF-κB activation (Figure 1C, compare lanes 2 and 3). The effect of pervanadate thus appeared to be independent of PKC- and calcium-dependent signals.

Preincubation of Jurkat cells with 100 μM of the PTK inhibitor genistein strongly reduced activation of NF-κB by pervanadate (Figure 1D, compare lanes 2 and 3). Genistein also affected the induction of NF-κB by PMA (compare lanes 4 and 5). Staurosporine in the nanomolar range has been described as a powerful PTK inhibitor, while higher concentrations will inhibit PKC in addition (Secrist et al., 1990). As shown in Figure 1D, 50 nM staurosporine completely blocked NF-κB induction by pervanadate (compare lanes 7 and 8) but had no effect on PMA induction (lane 10). These pharmacological data suggest that activation of one or several PTKs is a critical event in the activation of NF-κB by pervanadate.

Pervanadate Induces Phosphorylation of IκB-α on Tyrosine

We did not detect tyrosine phosphorylation of p50 or p65. However, analysis of αP-Tyr immunoprecipitates by Western blotting with a polyclonal serum directed against human IκB-α suggested that pervanadate stimulated tyrosine phosphorylation of IκB-α within 10 min (Figure 2A, compare lanes 2 and 1).

As shown on an anti-IκB-α Western blot of whole cell lysates, pervanadate stimulation did not alter the amount of IκB-α protein (Figure 2A, compare lanes 3 and 4; see also Figure 3) but induced a change in the mobility of IκB-α in SDS gels with an apparent molecular size of 37 kDa in unstimulated cells (Figure 2A, lane 3) to 39 kDa in pervanadate-treated cells (lane 4). Almost all IκB-α detected by Western blotting was affected by the mobility reduction. Maximal pervanadate stimulation occurred at 200 mM (data not shown). Cell lysates from pervanadate-treated or untreated Jurkat cells were then used for immunoprecipitation with control IgG (Figure 2B, lanes 1 and 2) or with the IκB-α-specific antibody (lanes 3 and 4) followed by Western blot analysis with the αP-Tyr mAb. A 10 min stimulation of Jurkat cells with pervanadate induced tyrosine phosphorylation of IκB-α (Figure 2B, lane 4). The immunoaffinity-purified tyrosine phosphorylated form of IκB-α migrated at the same position as the 39 kDa protein detected by Western blotting with IκB-α antibodies in pervanadate-treated cells. No tyrosine phosphorylated IκB-α could be detected in unstimulated cells (Figure 2B, lane 3). Maximal tyrosine phosphorylation of IκB-α occurred between 15 and 30 min of pervanadate stimulation (Figure 2C). These results show that pervanadate induces an apparently stochiometric de novo phosphorylation of human IκB-α on tyrosine that results in an apparent molecular size increase of 2 kDa.

The Appearance of the Pervanadate-Induced 39 kDa Form of IκB-α Is Blocked by PTK Inhibitors

Preincubation of Jurkat cells with 50 nM staurosporine for 20 min completely prevented the induction of the 39 kDa variant by pervanadate (Figure 2D, compare lanes 4 and 7 to lane 2). Genistein (100 μM) was less effective and only partially prevented tyrosine phosphorylation of IκB-α by 200 μM pervanadate (lane 3). When pervanadate was used at a suboptimal (50 μM) concentration, genistein completely blocked the appearance of the 39 kDa IκB-α variant (lane 6). By contrast, the PKC inhibitor H7 did not prevent IκB-α tyrosine phosphorylation in response to 200 μM pervanadate (compare lanes 10 and 9).

Protein Tyrosine Phosphatase Treatment Converts the 39 kDa Variant of IκB-α into the 37 kDa Variant

To verify that the mobility shift of IκB-α in response to pervanadate was entirely due to tyrosine phosphorylation, we treated the cell lysates with recombinant PTP1C fused to glutathione-S-transferase (GST) and coupled to agarose. To ensure that exogenously added pervanadate would not block GST-PTP1C, we removed the excess pervanadate by extensive washing of the cells after stimulation, expecting that the intracellular pervanadate is depleted through reaction. Moreover, vanadate was omitted from the lysis buffer. The 39 kDa variant of IκB-α in pervanadate-stimulated Jurkat cells (Figure 2E, lane 2) was still observed in vanadate-free lysis buffer (lane 6). Incubation in the presence of GST-PTP1C did not affect the migration of the 37 kDa IκB-α from normal cells (lane 5) but completely converted the 39 kDa variant from pervanadate-stimulated cells (lane 6) into a form precisely comigrating with the 37 kDa variant of nonstimulated cells (lane 7). The dephosphorylation activity of PTP1C on IκB-α was blocked by addition of vanadate to the lysis buffer (lane 3). To demonstrate the activity of PTP1C, cellular proteins from total lysates were analyzed by Western blotting with αP-Tyr mAb. Incubation with PTP1C markedly decreased pervanadate-induced tyrosine phosphorylation (compare lane 10 to lane 9), except when vanadate was present during lysis (lane 11).

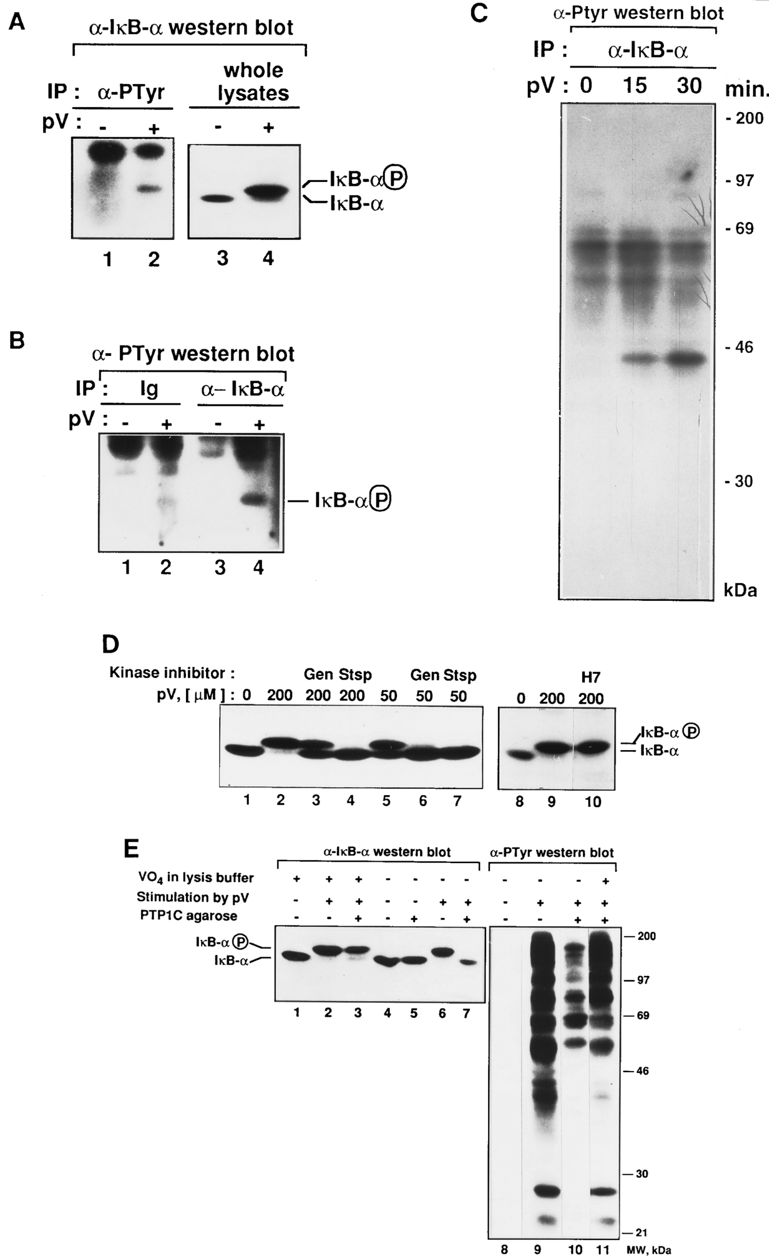

Figure 2. Pervanadate Induces Tyrosine Phosphorylation of IκB-α

(A) After cell stimulation, cell lysates were immunoprecipitated with anti-phosphotyrosine mAb (lanes 1 and 2) or left untreated (lanes 3 and 4) followed by a Western blot with anti-IκB-α antiserum.

(B) Lysates were incubated with control Ig (lanes 1 and 2) or anti-IκB-α mAb (lanes 3 and 4) before analysis of tyrosine phosphorylation by Western blotting with anti-phosphotyrosine mAb.

(C) Time course of IκB-α phosphorylation. Phosphorylation was analyzed by immunoprecipitation with anti-IκB-α mAb and Western blotting with anti-phosphotyrosine mAb.

(D) Effect of kinase inhibitors on pervanadate-induced IκB-α mobility shift. Cells were preincubated for 20 min with H7 (100 μM), genistein (100 μM) or staurosporine (50 nM) before stimulation with pervanadate (200 μM: lanes 2–4, 9, and 10 or 50 μM: lanes 5–7). Phosphorylation was analyzed by anti-IκB-α Western blotting.

(E) Dephosphorylation of IκB-α by PTP1C. Cell lysates were made in the presence (lanes 1–3 and 11) or absence (lanes 4–10) of vanadate and left untreated (lanes 1, 2, 4, 6, 8, and 9) or treated (lanes 3, 5, 7, 10, and 11) with PTP1C-agarose for 1 hr at 30°C. A Western blotting analysis with anti-IκB-α antiserum (lanes 1–7) or anti-phosphotyrosine mAb (lanes 8–11) was performed.

Pervanadate-Induced Tyrosine Phosphorylation Does Not Trigger Degradation of IκB-α

At various times after stimulation, whole cell lysates were analyzed by Western blotting with anti-IκB-α serum. After 30 min of pervanadate stimulation, all detectable IκB-α protein was in the tyrosine phosphorylated 39 kDa form (Figure 3A, upper panel). No variation in the amount of 39 kDa IκB-α was detected for up to 150 min. Newly synthetized IκB-α, which started to appear after ~60 min, was immediately converted into the tyrosine phosphorylated form. Next, Jurkat cells were preincubated with cycloheximide to prevent new IκB-α synthesis. Under these conditions, some degradation of 39 kDa IκB-α could be observed. After quantitation of IκB-α levels by scanning the autoradiograms (Figure 3B), a half-life of 120–140 min was determined for tyrosine phosphorylated IκB-α. This half life is very similar to the one measured for unmodified IκB-α from unstimulated cells (data not shown) demonstrating that tyrosine phosphorylation did not affect the half-life of IκB-α.

When cells were stimulated with a combination of a calcium ionophore and PMA, no shift in the migration of IκB-α was detected (Figure 3A, lower panel), but a rapid degradation of the molecule occurred that was enhanced by pretreatment with cycloheximide. In this case, the half life of IκB-α decreased to ~25 min (Figure 3B). Thus, in contrast to serine phosphorylation, tyrosine phosphorylation does not target IκB-α for rapid degradation. However, when cells were stimulated with pervanadate 15 min before or at the same time as the combination of A23187 + PMA, a sharp decrease in the amount of tyrosine-phosphorylated IκB-α was observed

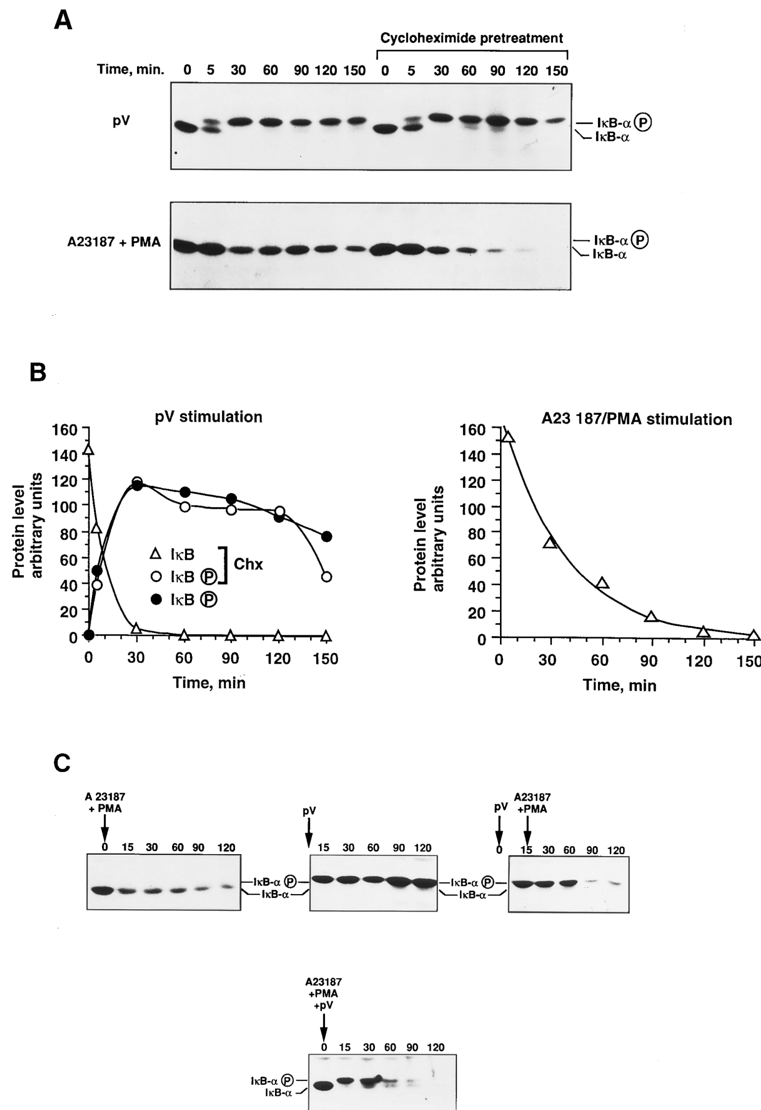

Figure 3. Pervanadate Does Not Induce Degradation of IκB-α

(A) Cells were left untreated or pretreated with cycloheximide (10 μg/ml for 30 min) before activation with pervanadate (200 μM) or a combination of A23187 (2 μM) and PMA (10 ng/ml). At the indicated times cell aliquots were harvested and analyzed by IκB-α Western blotting.

(B) Half life of IκB-α after activation. The levels of the different forms of IκB-α, after stimulation with pervanadate or A23187 and PMA in the presence of cycloheximide, were evaluated by scanning the autoradiogram shown on (A).

(C) Tyrosine phosphorylated IκB-α is sensitive to A23187 + PMA–induced degradation. For all conditions, cells were pretreated for 30 min with cycloheximide (10 μg/ml); A23187 (2 μM) and PMA (10 ng/ml) were then added after or at the same time as pervanadate (200 μM).

and no IκB-α protein was detected 2 hr after stimulation (Figure 3C). These results show that tyrosine phosphorylation of IκB-α does not protect the molecule from degradation and support that pervanadate did not influence the proteolytic pathway responsible for IκB-α degradation.

Induction of Tyrosine Phosphorylation by Pervanadate Correlates with Dissociation of IκB-α/NF-κB Complexes

IκB-α immunoprecipitates from pervanadate-treated cells were tested for the presence of p65 NF-κB by Western blotting. In unstimulated cells, p65 was found associated with IκB-α (Figure 4A, lane 1). The amount of coprecipitating p65 decreased with time (lanes 2–4). No p65 could be detected in association with tyrosine-phosphorylated IκB-α after 1 hr (lane 4). A 15 min stimulation with a combination of PMA/ionophore also reduced the association (lane 5). To rule out that tyrosine-phosphorylated IκB-α was still attached to NF-κB in protein-DNA complexes, immunodepletion experiments were performed. Total cell extracts were incubated with anti-p65 or anti-IκB-α antibodies preabsorbed to protein A-sepharose, and supernatants were analyzed for the presence of NF-κB-DNA binding activity by EMSA. As shown in Figure 4B, incubation with anti-p65 antibodies depleted the NF-κB–DNA binding activity that was induced by either pervanadate (lane 8) or the combination of calcium ionophore and PMA (lane 9). By contrast, control IgG (lanes 1–3) or anti-IκB-α antibody (lanes 4–6) did not diminish the amount of NF-κB-DNA complex. These results demonstrate that tyrosine phosphorylation dissociates IκB-α from NF-κB.

No Tyrosine Phosphorylation of IκB-α in Lck-Deficient Jurkat Variants

Triggering the T cell receptor on JCaM1 cells that lack Lck activity because of a splicing defect failed to stimulate tyrosine phosphorylation of cellular proteins, calcium influx, and late activation events such as CD69 expression (Strauss and Weiss, 1992). Transfection of

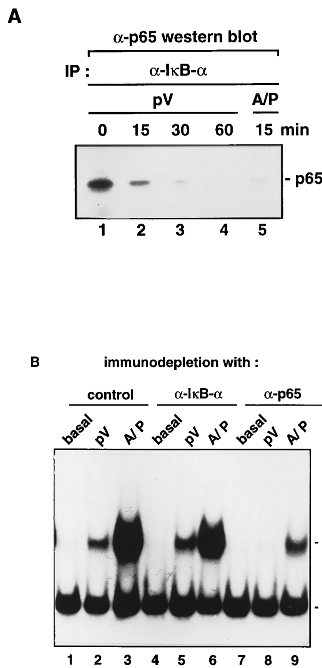

Figure 4. IκB-α Dissociates from NF-κB upon Tyrosine Phosphorylation

(A) Coprecipitation IκB-α/p65. Total cell extracts from unstimulated Jurkat cells (lane 1) or cells treated with pervanadate (200 μM) for various time periods (lanes 2–4) or with A23187 (2 μM) + PMA (10 ng/ml) for 15 min (lane 5) were incubated with anti-IκB-α antibodies preadsorbed to protein A-sepharose. Immune complexes were then analyzed by Western blotting with anti-p65 NF-κB antibodies.

(B) Immunodepletion. Total cell extracts from unstimulated (lanes 1, 4, and 7), pervanadate (200 μM) (lanes 2, 5, and 8) or A23187 (2 μM) + PMA (10 ng/ml) (lanes 3, 6, and 9) treated Jurkat cells were incubated with control (lanes 1–3), anti-IκB-α (lanes 4–6), or anti-p65 (lanes 7–9) antibodies preadsorbed to protein A-sepharose. After removal of immune complexes by centrifugation, supernatants were analyzed by EMSA.

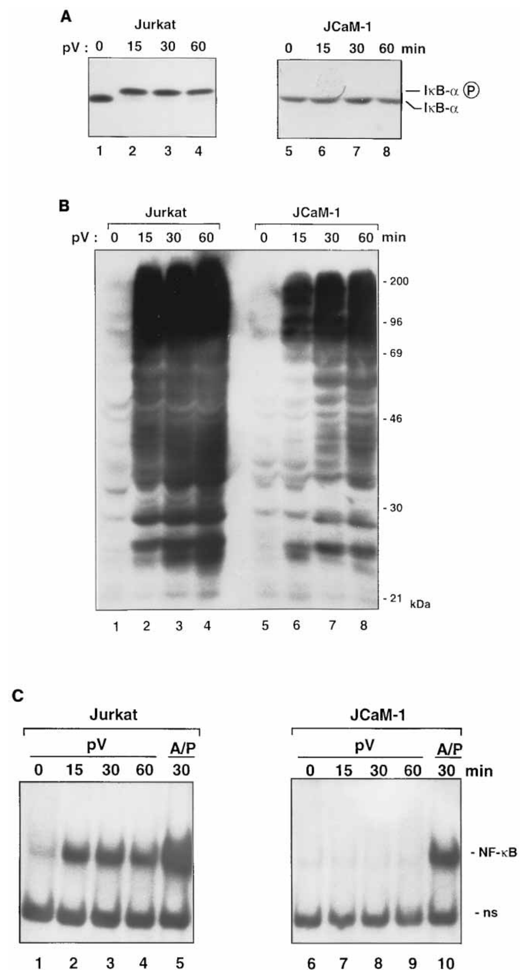

Figure 5. Analysis of IκB-α Phosphorylation and NF-κB Induction in Lck-Deficient Variants of Jurkat

(A) Pervanadate failed to induce a slowly migrating form of IκB-α in JCaM1 cells. Cell extracts were analyzed by anti-IκB-α Western blotting.

(B) Global tyrosine phosphorylation of cellular proteins. Cellular extracts from Jurkat (lanes 1–4) or JCaM1 cells (lanes 5–8) were analyzed by anti-phosphotyrosine Western blotting.

(C) Pervanadate failed to activate NF-κB in JCaM1 cells. NF-κB-DNA binding activity was analyzed as described in legend to Figure 1.

lck cDNA in JCaM1 restored full TcR-CD3 signaling (Strauss and Weiss, 1992). In a time course experiment (Figure 5A), no tyrosine phosphorylation-dependent mobility shift of IκB-α was observed in JCaM1 cells (lanes 5–8), in contrast to normal Jurkat cells (lanes 1–4). Tyrosine phosphorylation of some but not all cellular proteins induced by pervanadate was affected in JCaM1 cells (Figure 5B, lanes 5–8) when compared to Jurkat cells (lanes 1–4). Pervanadate also failed to activate NF-κB-DNA binding activity in JCaM1, even after 60 min of stimulation (Figure 5C, lanes 6–9; compare to parental Jurkat, lanes 1–4). However, a PMA/ionophore stimulation could activate NF-κB in JCaM1 cells (lane 10) as in normal cells (lane 5), demonstrating that JCaM1 cells did not have a general defect in NF-κB activation. The T cell–specific tyrosine kinase $p56^{lck}$ thus appears essential for pervanadate-induced NF-κB activation by IκB-α tyrosine phosphorylation.

The Tyrosine Phosphorylation Site in IκB-α

To identify the tyrosine residue responsible for the mobility shift of IκB-α in response to pervanadate, C-terminal deletions were constructed in which the codons for tyrosine 289 (Δ289), 248 (Δ248), or 181 (Δ181) were replaced by STOP codons (Figure 6A). The different constructs were then transiently transfected in COS cells. Tyrosine phosphorylation of wild-type IκB-α was only induced by pervanadate when an expression vector for the tyrosine kinase lck was cotransfected (Figure 6B, lanes 3 and 4 compared to lanes 1 and 2). Tyrosine phosphorylation of the Δ181 mutant could also only be observed in the presence of lck (Figure 6B, lanes 7 and 8 compared to lanes 5 and 6). Identical results were obtained with Δ289 and Δ248 mutants (data not shown). This demonstrates the importance of lck in the control of IκB-α tyrosine phosphorylation in response to pervanadate and points at tyrosine 42 as the potential phosphorylation site. Tyrosine 42 was then mutated to phenylalanine both in full-length IκB-α and in the truncated

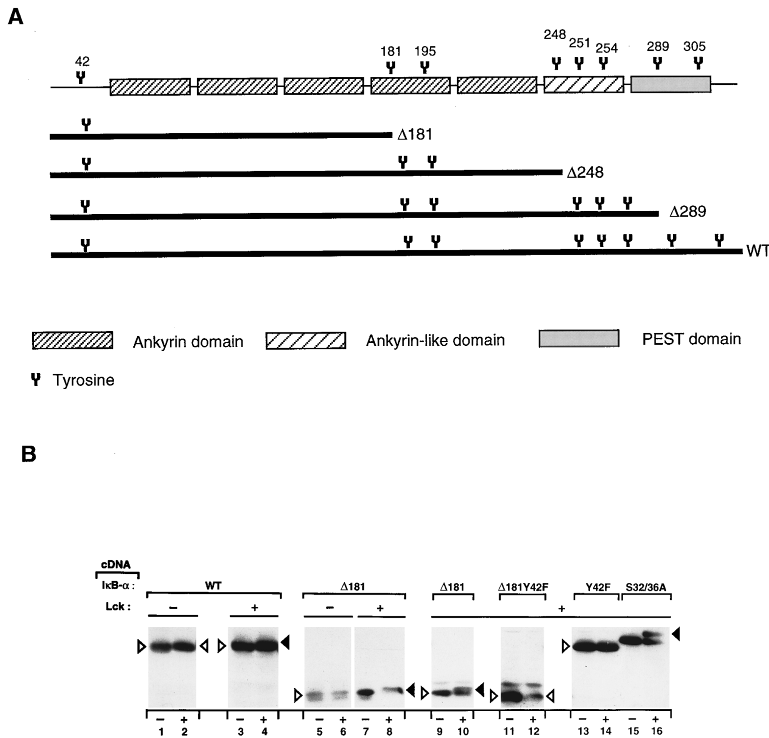

Figure 6. Localization of Tyrosine Phosphorylation Sites on IκB-α

(A) Physical map of IκB-α and schematic representation of deletion mutants.

(B) Cos cells were transfected with wild-type IκB-α (lanes 1–4) or different mutants: Δ181 mutant (lanes 5–10), Δ181Y42F (lanes 11 and 12), Y42F single mutant (lanes 13, 14), S32/36 double mutant (lanes 15, 16), and without (lanes 1, 2 5, 6) or with lck (lanes 3, 4, 7–16). After 48 hr cells were stimulated with pervanadate (250 μM for 20 min, even lanes). Cell extracts were analyzed by anti-IκB-α Western blotting. The open arrowheads point to the nonphosphorylated forms of IκB-α whereas the closed ones point to the tyrosine phosphorylated species.

mutant Δ181. Mutation of tyrosine 42 entirely blocked the appearance of the tyrosine phosphorylation–dependent migration shift on both full-length IκB-α (Figure 6B, lanes 13 and 14) and the truncated protein Δ181 (lanes 11 and 12). Mutations of serines 32 and 36 to alanines did not affect the generation of the slower migrating form of IκB-α in response to pervanadate (lanes 15 and 16). Altogether, these results strongly suggest that IκB-α is phosphorylated on tyrosine 42 after pervanadate treatment of intact cells.

Cell-Free Phosphorylation/ Dephosphorylation Experiments

We tested whether tyrosine phosphorylated IκB-α in cell extracts from pervanadate-treated cells can reinhibit NF-κB after treatment with the CD45 tyrosine phosphatase. Cell extracts from control or pervanadate-stimulated Jurkat cells were incubated with immunoprecipitated CD45 and the NF-κB–DNA binding activity analyzed by EMSA (Figure 7A). CD45 treatment strongly decreased the NF-κB activity in extracts from pervanadate-stimulated cells (lane 4). Blocking CD45 with vanadate reversed the effect of CD45 (lane 6). In contrast, NF-κB activity in extracts from PMA/ionophore-treated Jurkat cells did not decrease upon CD45 treatment (data not shown). In a second set of experiments, we tested whether a purified PTK can relieve the inhibitory effect of recombinant IκB-α. Activated NF-κB in extracts from pervanadate-stimulated Jurkat cells (Figures 7B and 7C, lanes 2) was inhibited upon addition of rIκB-α (lanes 4). This did not occur when either Lck tyrosine kinase (Figure 7B, lane 5) or insulin receptor tyrosine kinase (Figure 7C, lane 8) were added and incubated under phosphorylation conditions. The effect of the Insulin receptor tyrosine kinase required the presence of its ligand, insulin (Figure 7C, compare lane 8 to lane 6). Insulin was recently reported to activate NF-κB. Together, the results suggest that NF-κB activity can be reversibly regulated by tyrosine phosphorylation/dephosphorylation of IκB-α.

Reoxygenation Is a Physiological Effector of IκB-α Tyrosine Phosphorylation

A recent report by Koong et al. (1994) suggested that hypoxia induces tyrosine phosphorylation of IκB-α. As shown in Figure 8A (lane 5) hypoxia did not induce the appearance of a tyrosine phosphorylated 39 kDa IκB-α variant. However, as reported recently (Rupec and Baeuerle, 1995) reoxygenation of hypoxic cell cultures potently activated NF-κB (Figure 8B, lane 3). Reoxygenation induced the conversion of IκB-α into a 39 kDa variant that was not degraded during a 90 min reoxygenation period (Figure 8A, lanes 2–4) as seen upon pervanadate treatment. The effect was already maximal after 30 min (Figures 8A, lane 2). Immunoprecipitation with anti-PTyr mAb verified that reoxygenation induced phosphorylation of IκB-α on tyrosine (data not shown). In support of this notion, the PTK inhibitor staurosporine blocked NF-κB activation in response to reoxygenation of hypoxic Jurkat cell cultures (Figure 8B, lane 2). This identifies reoxygenation as a physiological stimulus of IκB-α tyrosine phosphorylation and subsequent NF-κB activation.

Discussion

Regulation of NF-κB Activation by Serine Phosphorylation

Phosphorylation is involved in the regulation of the activity of many TFs. It may regulate subcellular localization,

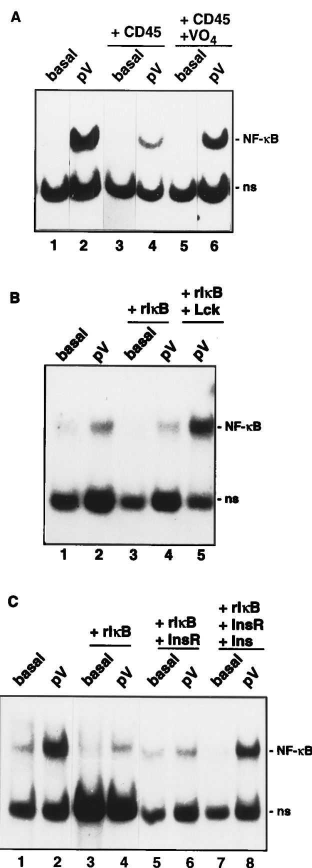

Figure 7. In Vitro Phosphorylation/Dephosphorylation Experiments

Cellular extracts were incubated for 1 hr at 30°C with phosphatase or kinases. NF-κB-DNA-binding activity was then visualized by EMSA.

(A) Dephosphorylation by CD45. Cellular extracts from untreated (lanes 1, 3, and 5) or 30 min pervanadate-activated Jurkat cells (lanes 2, 4, and 6) were mixed with immunoprecipitated CD45 in the absence (lanes 3 and 4) or the presence (lanes 5 and 6) of vanadate in the reaction mixture .

(B) Phosphorylation by Lck. Total extracts from pervanadate-treated Jurkat cells, containing NF-κB-DNA-binding activity (lane 2) were incubated with 1 ng of rIκB-α (lanes 3–5) without (lanes 3 and 4) or with Lck tyrosine kinase (lane 5).

(C) Phosphorylation by insulin receptor tyrosine kinase. Extracts from untreated (lanes 1, 3, 5, and 7) or 30 min pervanadate-activated Jurkat cells (lanes 2, 4, 6, and 8) were incubated alone (lanes 1 and 2) or with rIκB-α (lanes 3–8) in the presence of nonactivated (lanes 5 and 6) or insulin-activated insulin receptor (lanes 7 and 8).

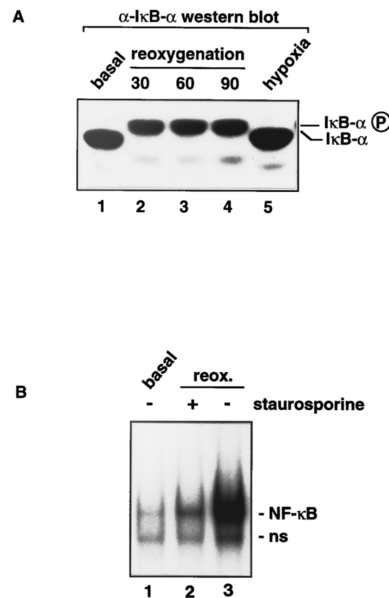

Figure 8. Reoxygenation Induces Tyrosine Phosphorylation of IκB-α and PTK-Dependent Activation of NF-κB

Jurkat cells were incubated in hypoxic conditions for 5 min, followed by re-exposure to atmospheric oxygen.

(A) IκB-α mobility shift. Reoxygenation time course (lanes 2–4). Hypoxic condition (lane 5). The extracts were analyzed by anti-IκB-α Western blotting.

(B) Preincubation with staurosporine (lane 2) prevented reoxygenation-induced NF-κB-DNA binding activity (lane 3).

dimerization, binding to DNA, or the transactivation potential. Phosphorylation is an important regulator of NF-κB activation at different levels. Both, the p50 and p65 subunits of NF-κB are phosphorylated (Naumann and Scheidereit, 1994). Although the sites and kinases have not been identified, phosphorylation appears to play a role in increasing the affinity of NF-κB for its target sequences (Naumann and Scheidereit, 1994). cAMP-dependent phosphorylation events were reported to down-modulate NF-κB activity in T cells (Serfling et al., 1989). Most importantly, phosphorylation regulates activity of the inhibitor IκB-α, which sequesters NF-κB in the cytosol. Several studies reported that IκB-α is constitutively phosphorylated (Davis et al., 1991; Kerr et al., 1991), presumably by casein kinase II (Barroga et al., 1995), but the role of this modification is unknown. Many if not all activators of NF-κB have been reported to induce degradation of IκB-α (Beg and Baldwin, 1993; Henkel et al., 1993). This degradation is preceded by activation of an as yet unidentified protein serine kinase that phosphorylates IκB-α on serines 32 and 36 within an N-terminal-regulatory domain (see pathway I on regulatory model in Figure 9) (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). Phosphorylation on these sites does not lead to dissociation of IκB-α from NF-κB (Finco et al., 1994; Miyamoto et al., 1994; Palombella et al., 1994; Alkalay et al., 1995; Brown et al., 1995; DiDonato et al., 1995; Lin et al., 1995; Traenckner et al., 1995) Mutation of serines 32 and 36 to alanine residues prevents inducible phosphorylation and degradation of IκB-α. Moreover, mutated IκB-α can act as a dominant negative mutant to attenuate κB-dependent transactivation (Brown et al., 1995; Traenckner et al., 1995).

Regulation of NF-κB Activation by Tyrosine Phosphorylation

Inhibition of tyrosine phosphatases by pervanadate in Jurkat cells leads to a rapid increase in tyrosine phosphorylation of cellular proteins followed by triggering of the T cell activation cascade (Secrist et al., 1993; Imbert et al., 1994). One of the events induced is a potent activation of NF-κB that appears to be entirely mediated by de novo phosphorylation of IκB-α on tyrosine. This

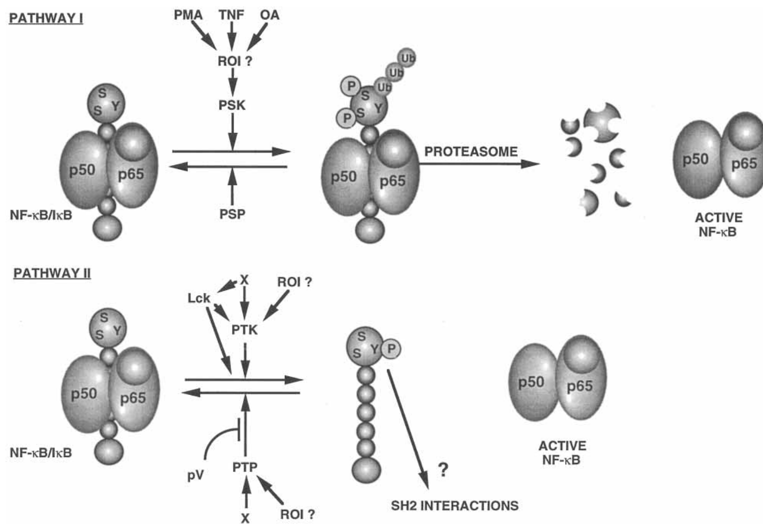

Figure 9. Model of NF-κB Activation

Pathway I: activation of NF-κB through degradation of IκB-α. PMA, phorbol ester; TNF, tumor necrosis factor; OA, okadaic acid; ROI, reactive oxygen intermediates; PSK, protein serine kinase; PSP, protein serine phosphatase; Ub, ubiquitin. SS, serines 32 and 36. Pathway II: NF-κB activation through tyrosine phosphorylation of IκB-α. PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; X, unidentified effector; pV, pervanadate. Y, tyrosine 42.

is evident from the absence of significant tyrosine phosphate from the DNA binding subunits of NF-κB and the stoichiometric modification of IκB-α. The latter is suggested by a stimulus-dependent complete conversion of IκB-α into the phosphoform (39 kDa) and its complete reversion into the dephospho form (37 kDa) upon PTP treatment. At present, we cannot completely rule out that the tyrosine phosphorylation of IκB-α was accompanied by some serine or threonine phosphorylation, but these could not have accounted for the change in IκB-α mobility, suggesting that they were substoichiometric. The tyrosine phosphorylation of IκB-α was an early event and closely coincided with the activation of NF-κB.

How May Tyrosine Phosphorylation Release IκB-α?

In contrast to serine phosphorylation, tyrosine phosphorylation of IκB-α did not lead to degradation of the molecule. The normal turnover of IκB-α of 2 hr was not detectably modified by tyrosine phosphorylation while it was decreased to 25 min by serine phosphorylation. Moreover, inhibition of proteasome proteolytic activity did not affect NF-κB induction by pervanadate (data not shown). In vitro phosphorylation/dephosphorylation experiments strongly suggested that tyrosine phosphorylation directly interfered with the interaction between IκB-α and NF-κB. IκB-α contains eight potential tyrosine phosphorylation sites (Haskill et al., 1991) that are highly conserved among species (de Martin et al., 1995). Mutational analysis hinted at phosphorylation of IκB-α on Tyr 42. Tyrosine 42 is located in the N-terminal regulatory region in close proximity to serines 32 and 36, which regulate degradation of IκB-α. Tyrosine-phosphorylated peptides can be specifically recognized by SH2 domains, which are structural motifs found in numerous cytoplasmic signaling proteins (Pawson, 1995). Phosphorylated tyrosine 42 in IκB-α may bind an SH2 domain-containing protein. It may be this interaction that ultimately removes the inhibitor from an equilibrium binding with NF-κB. This attractive possibility is currently under investigation. The presence of acidic residues from −1 to −3 to tyrosine 42 (DEEYEQM) makes it a potential phosphorylation site for tyrosine kinases. Amino acids at +1 and +3 create a potential binding site for the SH2 domain of the p85 subunit of PI3 kinase (Haskill et al., 1991; Songyang et al., 1993).

Which Tyrosine Kinase Activates NF-κB upon Pervanadate Stimulation?

Pervanadate is a powerful inducer of tyrosine phosphorylation in many cell types. In Jurkat cells, pervanadate triggers several signaling pathways through lck, fyn, zap-70 (Imbert et al., 1996), and Jak-2 (V. I. and J.-F. P., unpublished data). We could so far not identify which PTK catalyzed tyrosine phosphorylation of IκB-α. Interestingly, tyrosine phosphorylation of IκB-α and NF-κB activation were both impaired in Lck-deficient Jurkat variants, suggesting that IκB-α could be phosphorylated by Lck or by an Lck-activated PTK that remains to be identified. Reconstitution experiments in COS cells also demonstrate the requirement for lck for induction of IκB-α tyrosine phosphorylation.

Expression of a constitutive PTK, v-src, in T-cells has been reported to induce a constitutive activation of NF-κB in the nucleus (Eicher et al., 1994). In addition, v-src expression could transactivate the HIV LTR in a κB-dependent manner, suggesting that increase in tyrosine phosphorylation could influence NF-κB activation. However, since these cells also displayed constitutive NFAT activation, it was not clear whether these events were directely regulated by tyrosine phosphorylation or whether they represented downstream events of a tyrosine phosphorylation-induced cascade (Eicher et al., 1994).

Reoxygenation Induces Tyrosine Phosphorylation of IκB-α

NF-κB has been reported to respond to changes in the cellular redox status and to be activated by prooxidant conditions (reviewed in Baeuerle and Henkel, 1994). One study has suggested that IκB-α can undergo tyrosine phosphorylation during hypoxia (Koong et al., 1994). While we were unable to detect NF-κB activation upon hypoxia we have observed an induction of IκB-α tyrosine

phosphorylation after reoxygenation of hypoxic Jurkat cells (Rupec and Baeuerle, 1995). The discrepancy with the results of Koong et al. (1994) could be due to a reoxygenation artifact in their experimental conditions. The activation of NF-κB by reoxygenation appears to be a very rapid event, since a maximal induction could be reached within 15 min (data not shown). Our results suggest that reactive oxygen intermediates (ROI) could be inducers of tyrosine phosphorylation of IκB-α. For instance, H_2O_2 is a potent inhibitor of tyrosine phosphatases leading to the activation of PTKs (Heffetz et al., 1992). In T cells, H_2O_2 activates lck, fyn, zap-70, and syk (Schieven et al., 1993; Schieven et al., 1994; and our unpublished observations). It remains to be understood why most of the stimuli that have been reported to activate NF-κB through an ROI-dependent mechanism appear to use the regular pathway of NF-κB activation via degradation of IκB-α. The ROI species (H_2O_2, •O_2-, or •OH) or their site of production may be important regulatory parameters. Our observations and hypotheses are summarized in Figure 9 (pathway II).

Tyrosine Phosphorylation of IκB-α and Oncogenesis

IκB-α has been proposed as a possible tumor suppressor since overexpression of IκB-α anti-sense RNA in 3T3 cells induced malignant transformation (Beauparlant et al., 1994). Constitutive tyrosine phosphorylation of IκB-α may participate in the transforming activity of oncogenic PTKs by inducing a constitutive activation of the genes controlled by NF-κB.

Experimental Procedures

Antibodies and Reagents

Antibodies used in this study are: anti-IκB-α rabbit polyclonal, anti-p50 rabbit polyclonal, anti-p65 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine 4G10 (UBI, Lake Placid, NY). A polyclonal affinity-purified antibody to human IκB-α was prepared and used as described (Henkel et al., 1993). The polyclonal anti-p65 serum and the anti-HA antibody were kindly given by J. Imbert and Profs. Klenck and Garten, respectively. Partially purified Lck and recombinant glutathione-S-transferase (GST) fused PTP1-C were from Upstate Biotechnology, Inc. (Lake Placid, NY). Pervanadate was prepared by mixing vanadate in incubation buffer (30 mM HEPES [pH 7.5], 150 mM NaCl, 4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM glucose) with 1 mM H_2O_2 for 15 min at 22°C. Catalase (200 μg/ml) was then added to remove residual H_2O_2. The concentration of pervanadate generated is denoted by the vanadate concentration added to the mixture.

Cell Culture

Cells of the human leukemic T cell line Jurkat were maintained in RPMI 1640 medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, and 5% fetal calf serum (GIBCO, Cergy Pontoise, France). JCaM1 cells were obtained from ATCC (Rockville, MD).

Immunoprecipitation—Immunoblotting

Cell stimulation, preparation of lysates, immunoprecipitation, and Western blot analysis were performed as previously described (Imbert et al., 1994).

Electrophoretic Mobility Shift Assay

Nuclear fractions were prepared as described (Imbert et al., 1994). When indicated, NF-κB-DNA binding activity was analyzed in total cellular extracts made in Totex lysis buffer (HEPES 20 mM [pH 7.9], NaCl 350 mM, glycerol 20%, NP-40 1%, $MgCl_2$ 1 mM, EDTA 0.5 mM, EGTA 0.1 mM, DTT 1 mM, PMSF 0.1%, Aprotinin 0.1%) (Baeuerle and Baltimore, 1988). Supernatants from a 15,000 g, 15 min centrifugation were collected.

For mobility shift assay the NF-κB probe was a synthetic double-stranded oligonucleotide containing the NF-κB binding site of the IL-2 gene promoter (5′-GATCCAAGGGACTTTCCATG-3′). The end-labeled probe was incubated with nuclear or totex extract samples (10 μg) for 20 min at 37°C. Complexes were separated by electrophoresis on a 5% nondenaturing polyacrylamide gel in 0.5× TBE.

For in vitro phosphorylation, partially purified Lck or insulin receptor were used. InsR was prepared from NIH 3T3 fibroblasts stably transfected with the insulin receptor cDNA. Insulin receptor was activated by incubation with 10^{-7} M insulin for 50 min at room temperature. Phosphorylation were performed in HEPES 10 mM (pH 7.4), NaCl 150 mM, ATP 15 mM, $MnCl_2$ 8 mM, $MgCl_2$ 4 μM for 30 min at 25°C.

Mutant Construction and Transfection

The IκB-α cDNA plasmid (Zabel et al., 1993) was used as a template for further polymerase chain reaction amplification. The full length sequence was amplified using sense primer A (5′-CGGAATTCCAGGCGGCCGAGCGCCCC-3′) containing a EcoRI site and reverse primer (5′-GGGGTACCTCATAACGTCAGACGCTG-3′) containing a KpnI site and further subcloned in the EcoRI/KpnI sites of pcDSRα mammalian expression vector. C-terminal deletions were constructed by polymerase chain reaction amplification using sense primer A and reverse Δ181 primer in which the tyr181 codon has been replaced by a stop codon (bold) and that included a KpnI site (5′-GGGGTACC**TCA**TTGGTAGCCTTCAGGATG-3′). Mutation of tyrosine 42 has been performed with a Transformer site-directed mutagenesis kit from Clontech (Palo Alto, CA) using a primer where the codon for tyrosine 42 has been replaced by a codon for phenylalanine (5′-AAAGACGAGGAGTTCGAGCAGATGGTC-3′). The S32/36A mutant has been previously described (Traenckner et al., 1995). Lck cDNA in pEFBOS vector was a kind gift of Dr. A. Carrera.

COS-7 cells were seeded at 25% confluency on 6-well plates 24 hr before transfection. After washing with phosphate-buffered saline, transfection was carried out in Dulbecco's modified Eagle's medium (DMEM) 10% Nu-serum (CBP, Bedford, MA) supplemented with 200 μg DEAE-dextran (Pharmacia), 100 μM chloroquine, and 1.5 μg of each vector for 4.5 hr. Dimethyl sulfoxide 10% was then added for 2 min. Cells were then washed twice in phosphate-buffered saline and incubated in DMEM 10% fetal calf serum for 48 hr before harvest and further analysis.

Reoxygenation Experiments

These experiments were performed as described (Rupec and Baeuerle, 1995): DMEM medium with the bicarbonate buffer replaced by 25 mM HEPES (pH 7.2) was placed in a chamber and equilibrated overnight with nitrogen. The hypoxic medium was added to the cells that were immediately placed into the chamber, which was subsequently flushed with nitrogen at 2 bars for 5 min. No significant increase in the oxygen pressure in the cell culture medium was measured with this regimen. For reoxygenation, the lid of the chamber was simply removed. Control experiments at normal oxygen pressure were identically performed but in the opened chamber.

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References

Alkalay, I., Yaron, A., Hatzubai, A., Jung, S., Avraham, A., Gerlitz, O., Pashut-Lavon, I., and Ben-Neriah, Y. (1995). In vivo stimulation of IκB phosphorylation is not sufficient to activate NF-κB. Mol. Cell. Biol. *15*, 1294–1301.

Baeuerle, P.A., and Baltimore, D. (1988). IκB: a specific inhibitor of the NF-κB transcription factor. Science *242*, 540–546.

Baeuerle, P., and Henkel, T. (1994). Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. *12*, 141–179.

Barroga, C.F., Stevenson, J.K., Schwarz, E.M., and Verma, I.M. (1995). Constitutive phosphorylation of IκBα by casein kinase II. Proc. Natl. Acad. Sci. USA *92*, 7637–7641.

Beauparlant, P., Kwan, I., Bitar, R., Chou, P., Koromilas, A.E., Sonenberg, N., and Hiscott, J. (1994). Disruption of IκBα regulation by antisense RNA expression leads to malignant transformation. Oncogene *9*, 3189–3197.

Beg, A.A., and Baldwin, A.S., Jr. (1993). The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. *7*, 2064–2070.

Beg, A.A., Ruben, S.M., Scheinman, R.I., Haskill, S., Rosen, C.A., and Baldwin, A.S., Jr. (1992). IκB interacts with the nuclear localization sequences of the subunits of NF-κB: a mechanism for cytoplasmic retention. Genes Dev. *6*, 1899–1913.

Brockman, J.A., Scherer, D.C., McKinsey, T.A., Hall, S.M., Qi, X., Young Lee, W., and Ballard, D.W. (1995). Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. Mol. Cell. Biol. *15*, 2809–2818.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science *267*, 1485–1488.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapelier, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281–302.

Darnell, J.E.J., Kerr, I.A., and Stark, G.R. (1994). Jak-Stat pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science *264*, 1415–1421.

Davis, N., Ghosh, S., Simmons, D.L., Tempst, P., Liou, H., Baltimore, D., and Bose, H.R., Jr. (1991). Rel-associated pp40: an inhibitor of the rel family of transcription factors. Science *253*, 1268–1271.

de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H., and Bach, F.H. (1995). Cytokine-inducible expression in endothelial cells of an IκB-α-like gene is regulated by NF-κB. EMBO. J. *12*, 2773–2779.

DiDonato, J.A., Mercurio, F., and Karin, M. (1995). Phosphorylation of IκBα precedes but is not sufficient for its dissociation from NF-κB. Mol. Cell. Biol. *15*, 1302–1311.

Druker, B.J., Neumann, M., Okuda, K., Franza, B.R., Jr., and Griffi, J.D. (1994). Rel is rapidly tyrosine phosphorylated following granulocyte-colony stimulating factor treatment of human neutrophils. J. Biol. Chem. *269*, 5387–5390.

Eicher, D.M., Tan, T.-H., Rice, N.T., O'Shea, J.J., and Kennedy, I.C.S. (1994). Expression of v-*src* in T cells correlates with nuclear expression of NF-κB. J. Immunol. *152*, 2710–2719.

Finco, T.S., Beg, A.A., and Baldwin, A.S., Jr. (1994). Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors. Proc. Natl. Acad. Sci. USA *91*, 11884–11888.

Ghosh, S., and Baltimore, D. (1990). Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature *344*, 678–682.

Grimm, S., and Baeuerle, P.A. (1993). The inducible transcription factor NF-κB: structure–function relationship of its protein subunits. Biochem. J. *290*, 297–308.

Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko, A.D., Sampson-Johannes, A., Mondal, K., Ralph, K., and Baldwin, A.S.J. (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell *65*, 1281–1289.

Heffetz, D., Rutter, W.J., and Zick, Y. (1992). The insulinomimetic agents H_2O_2 and vanadate stimulate tyrosine phosphorylation of potential target proteins for the insulin receptor kinase in intact cells. Biochem. J. *288*, 631–635.

Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., and Baeuerle, P.A. (1993). Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature *365*, 182–185.

Hill, C.S., and Treisman, R. (1995). Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell *80*, 199–211.

Imbert, V., Peyron, J.-F., Farahi Far, D., Mari, B., Auberger, P., and Rossi, B. (1994). Induction of tyrosine phosphorylation and T-cell activation by vanadate peroxide, an inhibitor of protein tyrosine phosphatases. Biochem. J. *297*, 163–173.

Imbert, V., Farahifar, D., Auberger, P., Mary, M., Rossi, B., and Peyron, J.-F. (1996). Stimulation of the TcR-CD3 signaling pathway by the tyrosine phosphatase inhibitor pervanadate is mediated by inhibition of CD45. Evidence for two interconnected lck/fyn- and zap-70-dependent signaling pathways. J. Inflammation *46*, 65–77.

Johnson, G.L., and Vaillancourt, R.R. (1994). Sequential protein kinase reactions controlling cell growth and differentiation. Curr. Opin. Cell. Biol. *6*, 230–238.

Karin, M. (1994). Signal transduction from the cell surface to the nucleus trough the phosphorylation of transcription factors. Curr. Opin. Cell Biol. *6*, 415–424.

Kerr, L.D., Inoue, J.I., Davis, N., Link, E., Baeuerle, P.A., Bose, H.J. and Verma, I.M. (1991). The rel-associated pp40 protein prevents DNA binding of Rel and NF-κB: relationship with IκBβ and regulation by phosphorylation. Genes Dev. *5*, 1464–1476.

Klug, C.A., Gerety, S.J., Shah, P.C., Chen, Y.Y., Rice, N.R., Rosenberg, N., and Singh, H. (1994). The v-abl tyrosine kinase negatively regulates NF-κB/Rel factors and blocks κ-gene transcription in pre-B-lymphocytes. Genes Dev. *8*, 678–687.

Koong, A.C., Chen, E.Y., and Giacca, A.J. (1994). Hypoxia causes the activation of nuclear factor κB through the phosphorylation of IκBα on tyrosine residues. Cancer Res. *54*, 1425–1430.

Lin, Y.-C., Bown, K., and Siebenlist, U. (1995). Activation of NF-κB requires proteolysis of the inhibitor IκB-α: signal-induced phosphorylation of IκB-α alone does not release active NF-κB. Proc. Natl. Acad. Sci. USA *92*, 552–556.

Liou, H.-C., and Baltimore, D. (1993). Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. *5*, 477–487.

Miyamoto, S., Maki, M., Schmitt, M.J., Hatanaka, M., and Verma, I.M. (1994). Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB. Proc. Natl. Acad. Sci. USA *91*, 12740–12744.

Naumann, M., and Scheidereit, C. (1994). Activation of NF-κB in vivo is regulated by multiple phosphorylations. EMBO J. *13*, 4597–4607.

Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-KB1 precursor protein and the activation of NF-κB. Cell *78*, 773–785.

Pawson, T. (1995). Protein modules and signaling networks. Nature *373*, 573–580.

Rupec, R.A., and Baeuerle, P.A. (1995). The genomic response of tumor cells to hypoxia and reoxygenation. Differential activation of transcription factors AP-1 and NF-κB. Eur. J. Biochem. *234*, 632–640.

Schieven, G.L., Kirihara, J.M., Myers, D.E., Ledbetter, J.A., and Uckun, F.M. (1993). Reactive oxygen intermediates activate NF-κB in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases in human lymphocytes. Blood *82*, 1212–1220.

Schieven, G.L., Mittler, R.S., Nadler, S.G., Kirihara, J.M., Bolen, J.B., Kanner, S.B., and Ledbetter, J.A. (1994). ZAP-70 tyrosine kinase, CD45, and T cell receptor involvement in UV- and H_2O_2-induced T cell signal transduction. J. Biol. Chem. *269*, 20718–20726.

Secrist, J.P., Sehgal, I., Powis, G., and Abraham, R.T. (1990). Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. J. Biol. Chem. *265*, 20394–20400.

Secrist, J.P., Burns, L.A., Karnitz, L., Koretzky, G.A., and Abraham, R. (1993). Stimulatory effects of the protein tyrosine phosphatase inhibitor, pervanadate, on T-cell activation events. J. Biol. Chem. *268*, 5886–5893.

Serfling, E., Balthelmas, R., Peuffer, I., Schenk, B., Zarius, S., Swoboda, R., Mercurio, F., and Karin, M. (1989). Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. EMBO J. *8*, 465–473.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, S.E., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767–778.

Strauss, D.B., and Weiss, A. (1992). Genetic evidence for the involvement of lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell *70*, 585–593.

Traenckner, E.B.-M., Pahl, H.L., Henkel, T., Schmidt, K.N., Wilk, S., and Baeuerle, P.A. (1995). Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. *14*, 2876–2883.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203–212.

Zabel, U., Henkel, T., dos santos Silva, M., and Baeuerle, P.A. (1993). Nuclear uptake control of NF-κB by MAD-3, an IκB protein present in the nucleus. EMBO J. *12*, 201–211.