Tumor Necrosis Factor and Interleukin-1 Lead to Phosphorylation and Loss of I κ B α : a Mechanism for NF- κ B Activation

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Nuclear factor κ B (NF- κ B) is a critical regulator of several genes which are involved in immune and inflammation responses. NF- κ B, consisting of a 50-kDa protein (p50) and a 65-kDa protein (p65), is bound to a cytoplasmic retention protein called I κ B. Stimulation of cells with a variety of inducers, including cytokines such as tumor necrosis factor and interleukin-1, leads to the activation and the translocation of p50/65 NF- κ B into the nucleus. However, the in vivo mechanism of the activation process remains unknown. Here, we provide the first evidence that the in vivo mechanism of NF- κ B activation is through the phosphorylation and subsequent loss of its inhibitor, I κ B α . We also show that both I κ B α loss and NF- κ B activation are inhibited in the presence of antioxidants, demonstrating that the loss of I κ B α is a prerequisite for NF- κ B activation. Finally, we demonstrate that I κ B α is rapidly resynthesized after loss, indicating that an autoregulatory mechanism is involved in the regulation of NF- κ B function. We propose a mechanism for the activation of NF- κ B through the modification and loss of I κ B α , thereby establishing its role as a mediator of NF- κ B activation.

The transcription factor NF- κ B has been identified as a critical component of several signal transduction pathways. Its widespread biological significance is demonstrated in part by its activation in response to several different agents in a variety of cell types (for a review of NF- κ B, see references 5 and 22). Activation of NF- κ B involves its release from a cytoplasmic inhibitor protein, I κ B, and subsequent translocation of active NF- κ B into the nucleus (3). Nuclear NF- κ B regulates a variety of genes involved in immune and inflammation responses such as those encoding interleukin-2 (IL-2), major histocompatibility complex class I, IL-6, and cellular adhesion molecules (5, 22). Numerous agents which can cause the release of NF- κ B from I κ B have been identified; they include inflammatory cytokines like tumor necrosis factor α (TNF- α) and IL-1 α ; the tumor promoter and activator of protein kinase C, phorbol myristate acetate (PMA); bacterial lipopolysaccharides (LPS); DNA-damaging agents; double-stranded RNA, and infection by viruses including human immunodeficiency virus type 1 (5, 22). Interestingly, recent studies have shown that antioxidants can block the activation of NF- κ B by various inducers (41, 42, 45), raising the intriguing possibility that the ability of diverse signaling pathways to activate NF- κ B could involve the production of reactive oxygen intermediates (ROI).

TNF and IL-1 are multifunctional cytokines which mediate various inflammation and cellular immune responses in part by their abilities to regulate the expression of other cytokine genes (for example, IL-6) (for a review, see references 14 and 50). In many cases, the transcriptional regulatory properties of TNF and IL-1 are mediated by their abilities to activate NF- κ B (16, 33, 37). Interestingly, it has been shown that the TNF- α and IL-1 α activation of NF- κ B occurs independently of protein kinase C (10, 18). Recently, it has been reported that TNF- α signaling involves a phos-

pholipid pathway which leads to the production of ceramide catalyzed by the enzyme sphingomyelinase (15, 43). It has been proposed that 1,2-diacylglycerol (DAG) can lead to the activation of both protein kinase C and sphingomyelinase and that both of these enzymes can eventually lead to the activation of NF- κ B (43). These observations demonstrate that distinct pathways can lead to the activation of NF- κ B. The in vivo mechanism of NF- κ B activation is unknown, although in vitro experiments have suggested that phosphorylated I κ B can no longer interact with NF- κ B (20, 30, 44).

The best-characterized member of the NF- κ B family (originally defined as NF- κ B) consists of a 50-kDa protein (NF- κ B1), which is derived from a 105-kDa precursor, and a 65-kDa protein (RelA) (4, 11, 17, 21, 31). Dimerization between NF- κ B family members is mediated through the highly conserved N-terminal region, which has been called the *rel* homology region (21, 31, 35, 36, 39). The p65 subunit also has a C-terminal transcriptional activation domain, making p50/65 NF- κ B a potent transcriptional activator (6, 40). Members of the NF- κ B family are typically localized in the cytoplasm through the masking of their nuclear localization sequence by the associated I κ B molecule (8, 9, 25). Several forms of I κ B, including I κ B α /MAD-3 and its avian homolog pp40, Bcl-3, I κ B γ , and the p105 precursor of the p50 subunit of NF- κ B, have now been described (13, 19, 23, 24, 27, 38). Purification and determination of peptide sequences of a major form of I κ B, called I κ B α (13), revealed that they were identical to the predicted protein sequence of the MAD-3 clone (23). We refer to this protein as I κ B α /MAD-3 in this article. All the I κ B forms identified to date contain multiple copies of the so-called ankyrin/SWI6 repeats, which have been shown to be involved in interactions with NF- κ B (28, 51). The presence of multiple forms of I κ B and NF- κ B subunits underscores the role of this transcription factor family in numerous cellular events and exemplifies the combinatorial complexity of their regulation.

The widespread biological importance of NF- κ B has di-

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rected considerable attention towards understanding its mechanism of activation. Here, we provide the first *in vivo* evidence that stimulation of cells with a variety of NF- κ B inducers can lead to the phosphorylation and the rapid loss of I κ B α /MAD-3. The time course of I κ B α /MAD-3 loss correlates with the appearance of nuclear NF- κ B. Interestingly, two different forms of NF- κ B associated with I κ B α /MAD-3 were found, with one form being the typical p50/65 heterodimer and the other form likely being a p65 homodimer. Stimulation of cells led to the release of both of these NF- κ B forms. Our data also demonstrate that I κ B α /MAD-3 is rapidly resynthesized after loss, thereby suggesting an autoregulatory mechanism for NF- κ B regulation.

MATERIALS AND METHODS

Cell culture and reagents. HeLa S3 cells were grown in Dulbecco modified Eagle medium H supplemented with 10% horse serum in a spinner flask. Jurkat T cells and 70Z/3 pre-B cells were grown in RPMI 1640 plus 10% fetal calf serum. TNF- α was obtained from Promega and used at a concentration of 10 ng/ml. PMA, phytohemagglutinin (PHA), and LPS were obtained from Sigma and were used at concentrations of 100 ng/ml, 1 μ g/ml, and 10 μ g/ml, respectively. IL-1 α was obtained from Promega and was used at a concentration of 5 ng/ml. Pyrrolidinedithiocarbamate (PDTC) and *N*-acetyl-L-cysteine (NAC) were both obtained from Sigma and were used at concentrations of 100 μ M and 30 mM, respectively, for 30 or 60 min before stimulation of cells with NF- κ B inducers.

Antibodies. The I κ B α /MAD-3 antibody (Ab 9) has been previously described (8). The p65 N-terminal antibody (p65-NAb) was generated by injecting rabbits with a peptide containing amino acids 1 to 21 of the predicted human p65 amino acid sequence (39). The p65 C-terminal antibody (p65-CAb) was a gift from W. Greene and was generated against a peptide containing amino acids 528 to 550 of human p65. The I κ B α /MAD-3 C-terminal antibody (I κ B α /MAD-3-CAb) and the c-Rel antibody 265 were gifts from N. Rice. I κ B α /MAD-3-CAb was generated against the 17 C-terminal amino acids of the human I κ B α /MAD-3 clone (38), while the c-Rel antibody was against amino acids 573 to 587 of human c-Rel (12). The p50 Ab 2 was a gift from A. Israël and has been previously described (31).

Immunoprecipitations. Coimmunoprecipitation of the two deoxycholate (DOC)-activated NF- κ B forms (Fig. 1A) with I κ B α /MAD-3-Ab 9 were carried out by the addition of either 2 μ l of I κ B α /MAD-3-Ab 9 alone or 2 μ l of I κ B α /MAD-3-Ab 9 plus 2 μ g of the competing peptide to 20 μ l of HeLa cytoplasmic extract, followed by a 10-min incubation at room temperature. The cytoplasmic extracts were then added to 20 μ l (1:1) of protein A-Sepharose (Sigma), incubated for another 10 min with frequent mixing, then washed twice with Tris-buffered saline, and finally resuspended in 20 μ l of Tris-buffered saline. DOC was then added to a final concentration of 0.8% and incubated for another 5 min, after which Nonidet P-40 (NP-40) was added to a final concentration of 1.2%. Two microliters of the resulting cytoplasmic extract was then analyzed by a gel shift assay (Fig. 1A). In a different experiment, 2 μ l of Ab 2 against the p50 subunit of NF- κ B or preimmune serum was added to 20 μ l of HeLa cytoplasmic extract, and then protein A-Sepharose was added as described above. The supernatant was then removed and analyzed by a gel shift assay (Fig. 1B).

³⁵S-labeled extracts were made by first growing 10⁷ cells in 1 ml of medium without methionine and cysteine for 1 h,

then adding ³⁵S-labeled methionine and cysteine (Translabel; ICN) to a concentration of 0.5 mCi/ml, and incubating for another 2 h in a 37°C incubator. The cells were then lysed in 1 ml of RIP buffer (25 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, and a protease inhibitor cocktail) for 10 min on ice. The lysates were then spun in a microcentrifuge for 10 min, after which the supernatants were removed. One hundred microliters of the supernatant was then diluted with 200 μ l of RIP buffer, and the appropriate immune serum alone or immune serum plus peptide was added. The antibody binding reactions were allowed to proceed for 30 min on a rotating wheel at 4°C, after which the lysates were briefly spun, and the supernatant was added to 40 μ l of protein A-Sepharose (1:1) and incubated for another 60 min. The lysate supernatants were then discarded, and the protein A-Sepharose beads were washed twice in each of the following buffers as previously described (47): (i) 1 M NaCl–20 mM Tris-HCl (pH 7.6)–0.1% NP-40, (ii) 0.2 M NaCl–20 mM Tris-HCl–1% NP-40–0.1% sodium dodecyl sulfate (SDS)–1 mM EDTA, and (iii) 20 mM Tris-HCl–0.1% NP-40. The beads were then boiled for 5 min in an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and spun, and the supernatants were loaded onto SDS-denaturing gels. The gels were then analyzed by fluorography.

For phosphate labeling, 10⁷ cells were grown in media without phosphate for 3 h, and then [³²P]H₃PO₄ was added to 1 mCi/ml for 15 min. The cells were then induced with various agents for the periods indicated elsewhere in the text. The immunoprecipitations were carried out as described above except that phosphatase inhibitors (50 mM NaF, 10 mM Na₃VO₄, and 50 mM β -glycerophosphate) were also included in the RIP buffer. The immunoprecipitates were then analyzed by SDS-PAGE followed by autoradiography.

Nuclear and cytoplasmic extracts. Cells were fractionated by a combination of two previously described procedures (10, 46). Briefly, cells were washed in phosphate-buffered saline, pelleted, and resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, protease inhibitors, and NP-40). NP-40 concentrations used were 0.3% for HeLa cells, 0.1% for 70Z/3 cells, and 0.075% for Jurkat cells. After 5 min on ice, the lysates were spun at 2,500 rpm in a microcentrifuge at 4°C for 4 min. The supernatant was then removed and respun at 14,000 rpm for 5 min. The supernatants were used as cytoplasmic extracts. The pelleted nuclei from the first spin were briefly washed in lysis buffer without NP-40. The nuclear pellet was then resuspended in an equal-volume nuclear extract buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol), and then 5 M NaCl was added to achieve a final salt concentration of 400 mM. After a 10-min incubation at 4°C, the nuclei were briefly vortexed and spun at 14,000 rpm for 5 min. The supernatant was then removed and used as a nuclear extract.

Gel shift assays. Gel shift assays were carried out as previously described (23). DOC reactions were performed by the addition of sodium DOC to a concentration of 0.8% for 5 min, after which NP-40 was added to a concentration of 1.2%. Radiolabeled probe and poly(dI-dC) · (dI-dC) were then added, and the binding reaction mixtures were incubated for 15 min before being loaded onto nondenaturing gels.

Western blots. Western blots (immunoblots) were carried out either with the Western blot AP system (Promega) or by ECL Western blotting (Amersham) according to the manufacturer's recommendations. I κ B α /MAD-3 was dephosphor-

FIG. 1. Two forms of NF- κ B are associated with I κ B α /MAD-3. (A) A gel shift assay analyzing the NF- κ B forms that are immunoprecipitated with an antibody against I κ B α /MAD-3 (I κ B α /MAD-3-Ab 9) using HeLa cytoplasmic extracts. Lanes 1 and 2, binding activity before (-) and after (+) treatment with DOC, respectively; lanes 3 and 4, binding activity immunoprecipitated in the presence of I κ B α /MAD-3-Ab 9 alone or I κ B α /MAD-3-Ab 9 plus the competing peptide (P), respectively. The two DOC-activated forms that bind to a major histocompatibility complex class I enhancer probe are indicated (arrows). IP, immunoprecipitate. (B) A gel shift assay analyzing the effects of various antibodies on the two forms of NF- κ B. Lanes 1 and 2, extracts incubated with a p50 antibody (Ab 2) and protein A-Sepharose or with preimmune (PI) serum and protein A-Sepharose, respectively. After the incubations, the extracts were treated with DOC and analyzed by a gel shift assay. Lanes 3 and 4, effect of the absence (-) or presence, respectively, of a p65 antibody (p65-CAb) on the DOC-activated NF- κ B forms. Lanes 5 and 6, two DOC-activated NF- κ B forms from HeLa cytoplasmic (C) extracts or the nuclear (N) NF- κ B complexes induced after TNF- α treatment of HeLa cells, respectively. Lanes 7 to 9, effects of various antibodies on the TNF- α -induced nuclear NF- κ B complexes. The various NF- κ B complexes are indicated (arrows). ns, nonspecific band. (C) Results of an SDS-10% PAGE analyzing the coimmunoprecipitation of I κ B α /MAD-3 with p65 antibodies (p65-NAb) using metabolically labeled (with [35 S]methionine and [35 S]cysteine) HeLa extracts. Lanes 1 and 2, results of immunoprecipitating with p65-NAb alone or p65-NAb plus the competing peptide, respectively. The inhibited bands are indicated (arrows). Lanes 3 and 4, results of a second immunoprecipitation with I κ B α /MAD-3-Ab 9 alone or with I κ B α /MAD-3-Ab 9 plus the competing peptide, respectively, when the first immunoprecipitation is done with p65-NAb. Lanes 5 and 6, results of a second immunoprecipitation with I κ B α /MAD-3-Ab 9 alone or with I κ B α /MAD-3-Ab 9 plus the competing peptide, respectively, when the first immunoprecipitation is done with p65-NAb plus the competing peptide.

ylated in dephosphorylation buffer plus calf intestinal phosphatase (both from Boehringer Mannheim) in the presence or absence of phosphatase inhibitors (NaF, Na₃VO₄, and β -glycerophosphate) and then subjected to Western blot analysis.

Northern blots. HeLa S3 cells were stimulated with 10 ng of TNF- α per ml for the periods indicated above. RNA extraction and Northern (RNA) blot analysis were carried out by a previously described procedure (1) using a radiolabeled I κ B α /MAD-3 probe.

RESULTS

Two forms of NF- κ B are associated with I κ B α /MAD-3 in vivo. Cytoplasmic extracts generally contain little or no free NF- κ B. However, NF- κ B is readily detectable when cytoplasmic extracts are treated with a disrupting agent like sodium DOC (2). Interestingly, upon DOC treatment of HeLa cytoplasmic extracts, two complexes were detected by using an NF- κ B major histocompatibility complex class I enhancer probe (Fig. 1A, lanes 1 and 2). The complex present in these extracts that approximately comigrates with

the DOC-activated complexes may represent a small amount of free NF- κ B. To determine whether the DOC-activated complexes were associated with I κ B α /MAD-3, an immunoprecipitation experiment was carried out by incubating cytoplasmic extracts with either a previously described I κ B α /MAD-3 antibody, I κ B α /MAD-3-Ab 9 (8), alone or I κ B α /MAD-3-Ab 9 plus the peptide against which it was generated. The immune complexes were then precipitated with protein A-Sepharose and washed. I κ B α /MAD-3-NF- κ B complexes were disrupted by the addition of DOC, after which the supernatants were analyzed by a gel shift assay. Both protein complexes were immunoprecipitated with I κ B α /MAD-3-Ab 9 but not when the competing peptide was present (Fig. 1A; compare lanes 3 and 4). These results demonstrate that I κ B α /MAD-3 is bound to at least two forms of cytoplasmic NF- κ B. Similar results were obtained with the mouse pre-B-cell line 70Z/3, the human B-cell line Raji, and human fibroblasts (data not shown).

To determine the identities of these two complexes, we tested antibodies against the p50 and p65 subunits for possible reactivity against these complexes (Fig. 1B). A previously described p50 antibody (Ab 2) (31) did not super-

FIG. 2. Treatment of cells with various inducers results in the loss of I κ B α /MAD-3 and concomitant appearance of nuclear NF- κ B. (A) A Western blot analysis of cytoplasmic extracts following treatment of cells with different inducers. Equal numbers of cells were either left untreated (-) or stimulated with the inducer(s) indicated on the left. At the time points (in minutes) shown above each panel, cytoplasmic and nuclear extracts were prepared. 0', samples processed immediately after addition of the inducer. Equivalent amounts of cytoplasmic extracts were then analyzed by a Western blot as indicated in Materials and Methods by using I κ B α /MAD-3 antibodies. I κ B α /MAD-3-Ab 9 was used for the TNF- α , PMA, and PMA-PHA Western blots, while I κ B α /MAD-3-CAb was used following LPS and IL-1 α treatments. I κ B α /MAD-3 (arrow) and a nonspecific (ns) band are indicated. TNF- α and PMA treatments were carried out with HeLa cells, PMA-PHA treatment was carried out with Jurkat cells, and LPS and IL-1 α treatments were carried out with 70Z/3 cells. (B) Gel mobility shift analysis of nuclear extracts following treatment of cells with different inducers. Equal amounts of nuclear extract were analyzed by a gel shift assay as described in Materials and Methods. Inducers are shown on the left, and the time points (in minutes) are indicated above each panel. The various forms of NF- κ B which are induced following stimulation are indicated (arrows).

shift either complex, most likely as a consequence of the presence of DOC in the binding reaction (data not shown). Using an alternate approach, we preincubated the extract with p50-Ab 2 or with preimmune serum in addition to protein A-Sepharose. The supernatant was then removed and treated with DOC and then subjected to a gel shift analysis (Fig. 1B). Interestingly, the faster-migrating complex was almost completely depleted by p50-Ab 2 but not by preimmune serum (Fig. 1B; compare lanes 1 and 2). The more slowly migrating complex was not affected by p50-Ab 2 or by preimmune serum. However, an antibody against p65 (p65-CAb) supershifted both complexes (Fig. 1B, lane 4). We then tested whether activation of NF- κ B by an agent such as TNF- α can lead to the appearance of both of these complexes. Analysis of nuclear extracts from TNF- α -stimulated HeLa cells showed the appearance of two complexes with the same mobilities as the DOC-activated complexes (Fig. 1B; compare lanes 5 and 6, upper and middle arrows). In addition, the previously described p50 homodimers (31) were also detected in these nuclear extracts (Fig. 1B, lanes 6 to 9, lower arrow). Furthermore, the middle and lower complexes were supershifted with p50-Ab 2 while the middle and upper complexes were supershifted with p65-CAb (Fig. 1B, lanes 7 and 8, respectively). None of the complexes were affected by c-Rel antibodies, suggesting that c-Rel is unlikely to be a component of these complexes (Fig. 1B, lane 9). The c-Rel antibody has previously been shown to be capable of supershifting c-Rel present in crude extracts (48).

We have generated an N-terminal p65 antiserum (p65-NAb) which showed exclusive reactivity to the upper p65-containing DOC-activated complex in the earlier bleeds. Even in later bleeds, the antibody shows much higher affinity for the upper complex than for the lower one (data not shown). An immunoprecipitation with an early bleed of p65-NAb using [35 S]methionine- and [35 S]cysteine-labeled HeLa cell lysates (Fig. 1C) precipitated a protein of 65 kDa (upper arrow) along with another protein, of 36 kDa (lower arrow), both of which were inhibited by the peptide against which p65-NAb was generated (Fig. 1C; compare lanes 1 and 2). The 36-kDa protein was shown to be I κ B α /MAD-3 by boiling the p65-NAb immunoprecipitates and performing a second immunoprecipitation with I κ B α /MAD-3-Ab 9 (Fig.

FIG. 3. In vivo phosphorylation of I κ B α /MAD-3 following stimulation with TNF- α . (A) A Western blot analysis demonstrating that the modified form of I κ B α /MAD-3 is phosphorylated. Equal amounts of HeLa cytoplasmic extracts prepared 2.5 min after TNF- α stimulation were either left untreated (lane 1) or treated with calf intestinal phosphatase (CIP) alone (lane 2) or with CIP in the presence (+) of phosphatase inhibitors (lane 3), as indicated at the top. Samples were then analyzed by a Western blot with I κ B α /MAD-3-Ab 9. The modified and unmodified forms of I κ B α /MAD-3 are indicated (arrows). (B) Immunoprecipitation of I κ B α /MAD-3 after phosphate labeling of HeLa cells. HeLa cells were depleted of phosphate, and then [32 P]H $_3$ PO $_4$ was added. The samples in lanes 1 and 2 were left untreated, while the samples in lanes 3 and 4 were stimulated for 2.5 min with TNF- α . Cells were subsequently lysed, I κ B α /MAD-3 was immunoprecipitated with I κ B α /MAD-3-Ab 9 alone (lanes 1 and 3) or I κ B α /MAD-3-Ab 9 preincubated with the peptide against which it was generated (lanes 2 and 4) and the immunoprecipitate was analyzed by SDS-PAGE followed by autoradiography. I κ B α /MAD-3 is indicated (arrow).

1C, lanes 3 to 6). No other specifically precipitated proteins were detected. As expected, p65-CAB, which supershifts both of the DOC complexes, also immunoprecipitated p50 along with p65 and I κ B α /MAD-3 (data not shown). These results suggest that p65 homodimers might also be present in cells and that the upper DOC-induced complex could be a p65 homodimer. Taken together, our results demonstrate that I κ B α /MAD-3 is associated in vivo with both a p50/p65 heterodimer (NF- κ B) and another complex that contains p65 but not p50 and is likely to be a p65 homodimer (also, see Discussion).

Several stimuli, including TNF- α and IL-1 α , cause the loss of I κ B α /MAD-3 and the concomitant activation of NF- κ B. After establishing an in vivo interaction between NF- κ B and I κ B α /MAD-3, we tested whether the levels of I κ B α /MAD-3 were affected by treatment of cells with various agents that are known to induce NF- κ B DNA-binding activity. The inflammatory cytokine TNF- α was first tested on HeLa cells which were previously shown to be responsive to this agent (29). I κ B α /MAD-3-Ab 9 readily detected a protein of 36 kDa in the cytoplasm of these cells by a Western blot analysis (data not shown). This protein was not detected when the antiserum was first incubated with the peptide against which I κ B α /MAD-3-Ab 9 was raised, thereby establishing its identity as I κ B α /MAD-3. HeLa cells were treated with TNF- α and, subsequently, fractionated into cytoplasmic and nuclear extracts at various times after treatment. The cytoplasmic extracts from the different time points were then analyzed by a Western blot using I κ B α /MAD-3-Ab 9 (Fig. 2A). Interestingly, the I κ B α /MAD-3 signal completely disappeared after 15 min of treatment. A second band, which was also inhibited by the I κ B α /MAD-3-Ab 9 peptide, appeared above I κ B α /MAD-3 at the 2.5- and 5-min time points. This band is likely to be a modified form of I κ B α /MAD-3 (see below). These results demonstrate that TNF- α stimulation of HeLa cells causes the modification and eventual disappearance of I κ B α /MAD-3. Nuclear extracts from the TNF- α -stimulated cells were then analyzed by a gel shift assay using

the major histocompatibility complex class I NF- κ B DNA-binding site (Fig. 2B). Elevated levels of NF- κ B were readily detectable after 2.5 min of TNF- α treatment. The two NF- κ B complexes bound to I κ B α /MAD-3 in uninduced cytoplasmic extracts (see above; Fig. 1B). No detectable nuclear form of I κ B α /MAD-3 was noticed following its loss from the cytoplasm, suggesting that its disappearance is not a consequence of its translocation into the nucleus (data not shown). Moreover, a C-terminal antibody against I κ B α /MAD-3 (I κ B α /MAD-3-CAB) also demonstrated the loss of the inhibitor from the cytoplasm, suggesting that the antigenic site for the N-terminal I κ B α /MAD-3-Ab 9 was not simply being masked following stimulation with TNF- α (data not shown). We, therefore, surmise that the mechanism of activation of NF- κ B after stimulation of cells with TNF- α is through the loss of I κ B α /MAD-3, resulting in the translocation of free NF- κ B into the nucleus.

We then tested whether other inducers of NF- κ B can also lead to the loss of I κ B α /MAD-3. The tumor promoter and activator of protein kinase C, PMA, is an established inducer of NF- κ B. HeLa cells were, therefore, treated with PMA, and cytoplasmic extracts were tested for I κ B α /MAD-3 as described above (Fig. 2A). PMA stimulation also led to the loss of I κ B α /MAD-3 and to the appearance of NF- κ B in nuclear extracts (Fig. 2B). However, the rate of I κ B α /MAD-3 loss was slower than that seen after TNF- α treatment, and no modified form was noticed. Moreover, PMA did not cause the complete loss of I κ B α /MAD-3. We then tested three other NF- κ B inducers: PMA-PHA, which mimics certain steps in T-cell activation, was tested on Jurkat T cells (Fig. 2A), and the B-cell mitogen LPS and the inflammatory cytokine IL-1 α were tested on 70Z/3 pre-B cells (Fig. 2A). Once again, I κ B α /MAD-3 disappeared after treatment in all cases and the two p65-containing NF- κ B complexes concomitantly appeared in the nucleus (Fig. 2B, upper and middle arrows in each panel). No modified form of I κ B was detected after PMA-PHA or LPS treatment, while a modified form was detected after IL-1 α treatment (Fig. 2A). Interestingly, a small increase in the levels of p50 homodimers was also detected, especially after PMA and LPS treatments (Fig. 2B, lower arrow in each panel). Taken together, these results suggest that the loss of I κ B α /MAD-3 is a general mechanism for the activation of NF- κ B.

To determine whether the modification of I κ B α /MAD-3 was caused by phosphorylation, we tested HeLa cytoplasmic extracts made 2.5 min after TNF- α treatment which contain both the modified and the unmodified forms of I κ B α /MAD-3. Treatment of these extracts with calf intestinal phosphatase followed by a Western blot analysis showed that the modified form was converted to the unmodified form (Fig. 3A; compare lanes 1 and 2). Furthermore, this effect of calf intestinal phosphatase could be blocked if phosphatase

FIG. 4. Both the loss of I κ B α /MAD-3 and the activation of NF- κ B are inhibited in the presence of antioxidants. Jurkat cells were stimulated with PMA-PHA with or without prior treatment with the antioxidant PDTC (100 μ M) as indicated, and then cytoplasmic and nuclear extracts were made. Western blotting was performed on cytoplasmic extracts (A), while gel shift assays were performed on nuclear extracts (B). I κ B α /MAD-3 and NF- κ B are indicated (arrows). Similar experiments were carried out by pre-treating 70Z/3 cells or HeLa cells with the antioxidant NAC (30 mM), then treating the cells with LPS or TNF- α , respectively, and analyzing them as described above.

inhibitors were included (Fig. 3A, lane 3). These results suggest that the modified form of I κ B α /MAD-3 is the result of phosphorylation. Using a different approach, we grew HeLa cells in the absence of phosphate for 3 h, and then radioactive 32 P-labeled H_3PO_4 was added. Subsequently, the cells were stimulated with TNF- α for 2.5 min, after which the cells were lysed and I κ B α /MAD-3 was immunoprecipitated with I κ B α /MAD-3-Ab 9. Analysis of the immunoprecipitate by SDS-PAGE revealed a dramatic increase in I κ B α /MAD-3 phosphorylation after TNF- α treatment (Fig. 3B; compare lanes 1 and 3). The identity of the phosphoprotein was confirmed by blocking the antiserum with the peptide against which I κ B α /MAD-3-Ab 9 was raised (Fig. 3B, lane 4). Moreover, these results also suggest that I κ B α /MAD-3 is not phosphorylated within the period of the experiment when TNF- α is not present (Fig. 3B, lane 1). No phosphorylated I κ B α /MAD-3 was detected after 30 min of TNF- α treatment, a result consistent with our previous observations on the loss of I κ B α /MAD-3 following stimulation (data not shown).

Both the loss of I κ B α /MAD-3 and the activation of NF- κ B are inhibited in the presence of antioxidants. Recent studies have shown that oxidizing agents like hydrogen peroxide (H_2O_2) can lead to the activation of NF- κ B (42). The oxidizing properties of H_2O_2 are due to its breakdown into ROI inside cells. Most interestingly, it has been reported that antioxidants can block the activation of NF- κ B by not only H_2O_2 but also other, seemingly unrelated inducers like TNF- α , PMA-PHA, and LPS (41, 42, 45). These results suggest that different signaling pathways could involve the release of ROI as a signaling moiety. However, no conclusive evidence regarding the stage at which the blockage of the activation of NF- κ B occurs has been generated. In light of these observations, we tested whether the loss of I κ B α /MAD-3 could take place in the presence of antioxidants. We therefore tested the effects of the antioxidants PDTC and NAC on the abilities of different inducers to cause the loss of I κ B α /MAD-3 (Fig. 4). A Western blot analysis showed that the ability of PMA-PHA to cause the loss of I κ B α /MAD-3 in

Jurkat T cells was almost completely abolished in the presence of PDTC (Fig. 4A). A gel shift analysis showed that PDTC also inhibited the activation of NF- κ B by PMA-PHA (Fig. 4B). Moreover, LPS could not cause the complete loss of I κ B α /MAD-3 when 70Z/3 cells were pretreated with NAC (Fig. 4A). A gel shift analysis also showed that NF- κ B was only partially activated by LPS stimulation in the presence of NAC (Fig. 4B). Similar results were obtained when HeLa cells were treated with TNF- α in the presence of NAC (Fig. 4). PDTC or NAC had no effect on the viability of cells during the course of these treatments. We have also found that the treatment of 70Z/3 cells with H_2O_2 resulted in a gradual decrease in I κ B α /MAD-3 levels and a corresponding translocation of NF- κ B into the nucleus (data not shown). Interestingly, PDTC seemed more effective than NAC in preventing the loss of I κ B α /MAD-3 caused by PMA-PHA while NAC was more effective than PDTC after TNF- α and LPS stimulation (data not shown). These results could be due to the difference in the abilities of these two antioxidants to penetrate different cell types or their abilities to neutralize ROI once inside the cells. In any case, our results demonstrate that both the activation of NF- κ B and the loss of I κ B α /MAD-3 are inhibited in the presence of antioxidants. They also demonstrate that the loss of I κ B α is required for NF- κ B activation.

I κ B α /MAD-3 is rapidly resynthesized after loss, indicating an autoregulatory mechanism. The continued absence of I κ B α /MAD-3 after the stimulation of cells represents a situation in which newly synthesized NF- κ B may be rapidly translocated into the nucleus. This would lead to a long-term transcriptional activation of NF- κ B-responsive genes and to possible deleterious effects on cells. However, renewed synthesis of I κ B α /MAD-3 would make this response transient by binding to the newly synthesized NF- κ B. We therefore tested whether I κ B α /MAD-3 is resynthesized after its loss (Fig. 5A). A Western blot analysis revealed that, after only 1 h of TNF- α treatment, the levels of I κ B α /MAD-3 returned to approximately half the original level (Fig. 5A; compare lanes 1 and 3). This response was blocked by cycloheximide, demonstrating that new synthesis is responsible for the reappearance of I κ B α /MAD-3 (Fig. 5A, lane 4). Cycloheximide alone resulted in only a small decrease in I κ B α /MAD-3 levels (data not shown). Similar results were obtained after stimulation of cells with IL-1 α , PMA, PMA-PHA, and LPS (data not shown). Interestingly, a small amount of the newly synthesized I κ B α /MAD-3 was modified (Fig. 5A, lane 3), indicating that a TNF- α -induced kinase remains partially active for at least 1 h after treatment. Additional proof for renewed synthesis of I κ B α /MAD-3 was obtained by Northern blot analysis (Fig. 5B). TNF- α treatment strongly increased the levels of I κ B α /MAD-3 mRNA (Fig. 5B; compare lanes 1 and 2), and these levels remained elevated for at least 2 h (Fig. 5B, lanes 2 to 4). On the basis of these results, we propose that the loss and resynthesis of I κ B α /MAD-3 is part of an autoregulatory mechanism that ensures that NF- κ B activation is essentially transient. It also raises the possibility that NF- κ B regulates the I κ B α /MAD-3 promoter in a manner similar to the regulation of the NF- κ B p50/105 promoter (49).

DISCUSSION

Loss of I κ B α /MAD-3: a general mechanism for the activation of NF- κ B. Numerous studies have shown that the transcription factor NF- κ B is an important regulator of genes involved in a variety of cellular activities. These studies have

FIG. 5. I κ B α /MAD-3 loss is transient. (A) Western blot analysis of HeLa cells after stimulation with TNF- α . Equal numbers of cells were either left untreated (-) (lane 1) or treated with TNF- α for the indicated times (in minutes). The sample in lane 4 was incubated for 30 min with cycloheximide (Cyclo) prior to TNF- α treatment. Cytoplasmic extracts were then prepared and analyzed by Western blot using I κ B α /MAD-3-Ab 9. I κ B α /MAD-3 (arrow) and a nonspecific (ns) band are indicated. (B) Northern blot analysis of HeLa cells after stimulation with TNF- α . Equal numbers of cells were either left untreated (-) or stimulated with TNF- α for the indicated times (lanes 2 to 4). Poly(A) RNA was isolated, and Northern blot analysis was performed as described in Materials and Methods, by using a probe specific for the I κ B α /MAD-3 mRNA. The same blot, stripped and reprobed with a probe specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is shown at the bottom.

typically involved the stimulation of cells with agents that can lead to the appearance of NF- κ B in the nucleus or by *in vivo* transactivation assays using reporter plasmids containing NF- κ B-responsive sites derived from the promoter-enhancer regions of a variety of different genes. However, there is no information available on the general mechanism of NF- κ B activation. We have previously reported that the presence of I κ B α /MAD-3 is sufficient to retain both the p50 and the p65 subunits of NF- κ B in the cytoplasm while the absence of I κ B α /MAD-3 allows these subunits to be transported into the nucleus (8). These results prompted us to investigate the status of I κ B α /MAD-3 after stimulation of cells with different NF- κ B inducers. Here, we demonstrate that stimulation of cells with five different inducers of NF- κ B leads to the loss of I κ B α /MAD-3 and to the concomitant appearance of NF- κ B in the nucleus (Fig. 2). The kinetics of this process varied for different inducers; for example, in pre-B cells IL-1 α could cause the complete loss of I κ B α /MAD-3 in only 5 min while approximately 30 min was required for this process by LPS. The difference in kinetics of I κ B α /MAD-3 loss among different inducers could be at any number of different steps which precede the loss of I κ B α /MAD-3. In any case, the kinetics of I κ B α /MAD-3 loss closely parallel the nuclear appearance of NF- κ B, indicating that the rate-limiting step in the activation of NF- κ B is the loss of I κ B α /MAD-3. It would be interesting to see whether other I κ B forms like Bcl-3 and p105 are also targeted by the various inducers of NF- κ B.

Interestingly, we found that two major forms of NF- κ B are associated with I κ B α /MAD-3 and that both forms were liberated in an active DNA-binding form following stimulation of cells with NF- κ B inducers (Fig. 1B). We have found that the faster-migrating form is p50/65 NF- κ B while the more slowly migrating form contains p65 but not p50 and is likely to be a p65 homodimer (Fig. 1B). UV cross-linking of these complexes to a radiolabeled DNA fragment has confirmed these results (7a). Since the p50 and p65 subunits of NF- κ B have different DNA-binding site specificities as well as different transcriptional activation properties (32, 35, 40), it is possible that these two complexes provide diverse

functions in the regulation of NF- κ B-responsive genes. In general, only p50/65 NF- κ B has been shown to be activated in different cell lines after stimulation with different agents, although several κ B-binding complexes have been shown to be activated after PMA-PHA treatment of Jurkat T cells (7). However, our results indicate that two major κ B-binding complexes are activated after stimulation of cells with a variety of different agents (Fig. 2B). The more slowly migrating p65-containing form might have previously gone undetected because of differences in the DNA-binding sites used or because of binding reaction conditions that were different from those that were used here. For the same reason, it is possible that we did not detect other proteins bound to I κ B α /MAD-3. This might also explain why c-Rel, which has previously been shown to be associated with I κ B α /MAD-3 (38), was not detected in nuclear extracts of stimulated cells (Fig. 1B). On the other hand, only a very small proportion of c-Rel may actually be associated with I κ B α /MAD-3. A slight increase in p50 homodimer DNA binding was also noticed after stimulation of cells (Fig. 2B). This could be due to the association of a small amount of p50 homodimers with I κ B α /MAD-3, consistent with previous observations on the abilities of these proteins to associate *in vitro* (8).

Here, we have provided the first *in vivo* evidence that I κ B α /MAD-3 is phosphorylated after stimulation of cells with NF- κ B inducers (Fig. 3). These results may provide important insights into the first steps involved in the mechanism of NF- κ B activation. However, at present it is unclear what the exact role of I κ B α /MAD-3 phosphorylation is in its release from NF- κ B. Most likely and consistent with the results of previous *in vitro* experiments, phosphorylation of I κ B α /MAD-3 dissociates it from NF- κ B (20, 30). This would be followed by the translocation of free NF- κ B into the nucleus while the unbound I κ B α /MAD-3 becomes susceptible to proteolysis because of the exposure of a previously masked proteolytic cleavage site. However, it is also possible that phosphorylation makes I κ B α /MAD-3 susceptible to proteolysis but does not cause its release from NF- κ B. In this situation, it would actually be the proteolysis of I κ B α /MAD-3 which would be involved in the release of NF- κ B, even though phosphorylation would be a prerequisite for it to occur.

During TNF- α stimulation, unmodified I κ B α /MAD-3 first seemed to be phosphorylated and then was lost, indicating that the phosphoprotein is extremely short-lived (Fig. 2A). Our results indicate that, although phosphorylation of I κ B α /MAD-3 is evident in the case of stimulation of cells with fast-acting inducers like TNF- α and IL-1 α , the slowly acting inducers PMA, PMA-PHA, and LPS do not show a modified

FIG. 6. A mechanism for the autoregulation of NF- κ B (see text for details).

form. Since the phosphoprotein is extremely labile, it is possible that for slowly acting inducers the rate of conversion of the unmodified I κ B α /MAD-3 to the modified form is slower than the rate of disappearance of the modified form by proteolysis. In this instance, phosphorylated I κ B α /MAD-3 would never exist in a concentration large enough to be detectable at any given time. Therefore, the inability to detect phosphorylated I κ B α /MAD-3 by stimulation of cells with inducers like PMA, PMA-PHA, and LPS does not indicate that phosphorylation is not involved.

Implications of I κ B α /MAD-3 phosphorylation for understanding cytokine signal transduction pathways. Recent studies have identified a pathway for TNF- α signaling which is distinct from the protein kinase C pathway and involves the production of ceramide by a sphingomyelinase (15, 43). Sphingomyelinase activation has been linked to the production of DAG by a phosphatidylcholine-specific phospholipase C. Interestingly, a ceramide-activated protein kinase which appears to phosphorylate a minimum sequence of X-Ser/Thr-Pro-X, distinct from the consensus substrate sequences of other serine/threonine protein kinases has been recently identified (15, 34). Intriguingly, several such proline-containing sequences can be found in the ankyrin repeat domains and in the C-terminal end of the predicted I κ B α /MAD-3 amino acid sequence (23).

The abilities of the cytokines TNF- α and IL-1 α to influence numerous biological processes are largely dependent on their abilities to regulate gene expression. However, the failure to identify physiologically relevant substrates important for the regulation of gene expression has prevented an understanding of the signaling mechanisms of these cytokines. We believe that I κ B α /MAD-3 represents an important functional target for these cytokines. Since NF- κ B is a known regulator of a number of immune and inflammation response genes, its activation through the phosphorylation of I κ B α /MAD-3 provides important insights into the mechanisms of cytokine signal transduction.

Both the loss of I κ B α /MAD-3 and the activation of NF- κ B are inhibited in the presence of antioxidants. Considerable attention has been focused recently on the ability of antioxidants to block the activation of NF- κ B by virtually all inducers tested (41, 42, 45). These studies have indicated that ROI may be a component of several signal transduction pathways since the presence of antioxidants like PDTC and NAC can block these pathways. Here, we have shown that the effect of these agents manifests itself in the prevention of I κ B α /MAD-3 loss caused by NF- κ B inducers (Fig. 4). The abilities of three different inducers, PMA-PHA, LPS, and TNF- α , to cause the loss of I κ B α /MAD-3 were partially or completely blocked in the presence of either NAC or PDTC.

The inability to cause the complete loss of I κ B α /MAD-3 in the presence of antioxidants was clearly reflected in the partial or complete inhibition of the activation of NF- κ B after stimulation with these agents (Fig. 4). Interestingly, we have noticed that inducers which cause a relatively slow loss of I κ B α /MAD-3 (PMA-PHA and LPS) were almost completely blocked by antioxidants while those which cause a rapid loss of I κ B α /MAD-3 were either partially affected, as in the case of TNF- α (Fig. 4), or not affected at all, as in the case of IL-1 α (data not shown). It is possible that PMA-PHA and LPS lead to the slow release of low concentrations of ROI, which can be effectively neutralized by antioxidants, while TNF- α and IL-1 α cause the rapid release of large amounts of ROI, which are inefficiently neutralized by antioxidants under the experimental conditions used. The exact role of ROI in signal transduction is still unclear, although on the basis of our results for the *in vivo* phosphorylation of I κ B α /MAD-3, we propose that the mechanism could involve the activation of a kinase.

The resynthesis of I κ B α /MAD-3 following loss provides evidence for an autoregulatory mechanism. The results presented here demonstrate that the loss of I κ B α /MAD-3 following treatment of cells with NF- κ B inducers is essentially transient (Fig. 5). In the case of TNF- α , a significant amount of I κ B α /MAD-3 has already been resynthesized after 1 h of treatment (Fig. 5A). Northern blot analysis of I κ B α /MAD-3 after TNF- α treatment showed that the new synthesis of I κ B α /MAD-3 protein is likely due to the transcriptional induction of the I κ B α /MAD-3 mRNA (Fig. 5B). Similar results were also obtained with other inducers of NF- κ B used in this study (data not shown), raising the possibility that NF- κ B is itself involved in the transcriptional induction of I κ B α /MAD-3 mRNA. However, formal proof for this must await the identification of functional NF- κ B-binding sites in the I κ B α promoter. Since nuclear NF- κ B has a short half-life, it would eventually get depleted if new NF- κ B were not transported from the cytoplasm (26). The resynthesis of I κ B α /MAD-3 would ensure that the newly synthesized NF- κ B is, in fact, sequestered in the cytoplasm. This could be the basis for an autoregulatory mechanism which would ensure that the induction of NF- κ B is transient and that the cell returns to an uninduced state within a relatively short period. This mechanism would ensure that NF- κ B-regulated genes are only transiently induced and would therefore avoid any deleterious effects due to the continued activation of these NF- κ B-responsive genes.

On the basis of this study and previous results, we propose a model (Fig. 6) in which the engagement of a specific cell surface receptor leads to the activation of a kinase by a pathway that may involve the production of ROI (step 1 in Fig. 6). The activated kinase phosphorylates I κ B α /MAD-3, leading to its dissociation from NF- κ B (step 2). The free NF- κ B complex is then translocated into the nucleus (step 3) because of the exposure of its nuclear localization sequence previously masked by I κ B α /MAD-3 (8). Nuclear NF- κ B can transcriptionally activate various immune and inflammation response genes as well as those encoding I κ B α /MAD-3 and p50/105 (step 4). These mRNA species then give rise to functional proteins which reestablish the inactive cytoplasmic NF- κ B complexes (step 5). The p50 protein is derived from the cleavage of the p105 precursor (21, 31), while the p65 protein is synthesized from a constitutively produced mRNA (29a). This model proposes a negative-feedback mechanism which ensures an essentially transient NF- κ B activation following cell stimulation.

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ADDENDUM IN PROOF

Two recent articles (K. Brown, S. Park, T. Kanno, G. Franzoso, and U. Siebenlist, *Proc. Natl. Acad. Sci. USA* **90**:2532–2536, 1993; S.-C. Sun, P. A. Ganchi, D. W. Ballard, and W. C. Greene, *Science* **259**:1912–1915, 1993) also show the loss of I κ B α following treatment of cells with NF- κ B inducers as well as the autoregulation of NF- κ B through renewed synthesis of I κ B α .

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