The IkB kinases (IKKs) lie downstream of the NF-κB-inducing kinase (NIK) and activate NF-κB by phosphorylation of IκBα. This leads to IκBα degradation and release of NF-κB. In U937 monocyctic cells, interleukin (IL)-1β (1 ng/ml) and tumor necrosis factor (TNF)-α (10 ng/ml) induced κB-dependent transcription equally. However, IKK activity was strongly induced by TNF-α but not by IL-1β. This was consistent with IκBα phosphorylation and degradation, yet TNF-α-induced NF-κB DNA binding was only 30–40% greater than for IL-1β. This was not explained by degradation of IκBα, IκBe, or p105 nor nuclear translocation of NF-κB-IκBa complexes or degradation-independent release of NF-κB. Dominant negative (NIK) repressed TNF-α and IL-1β-induced κB-dependent transcription by ~60% and ~33%, respectively. These data reveal an imprecise relationship between IKK activation, IκBa degradation, and NF-κB DNA binding, suggesting the existence of additional mechanisms that regulate NF-κB activation. Finally, the lack of correlation between DNA binding and transcriptional activation plus the fact that PP1 and genistein both inhibited κB-dependent transcription without affecting DNA binding activity demonstrate the existence of regulatory steps downstream of NF-κB DNA binding. Therapeutically these data are important as inhibition of the NIK-IKK-IκBa cascade may not produce equivalent reductions in NF-κB-dependent gene expression.

The acute phase transcription factor nuclear factor κB (NF-κB) is an inducible enhancer of many inflammatory genes including cytokines, chemokines, and adhesion molecules as well as enzymes such as inducible nitric-oxide synthase and cyclooxygenase-2 (reviewed in Refs. 1 and 2). Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α rapidly induce NF-κB DNA binding and κB-dependent transcription in most cell types (1, 2). NF-κB DNA binding activation component comprises hom- and, more usually, heterodimers of Rel proteins such as RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) (2). NF-κB is held inactive in the cytoplasm by inhibitory IκB proteins including IκBa, IκBβ, IκBγ, and IκBe. Agonists such as TNF-α and IL-1β result in phosphorylation of IκBα at serines 32 and 36 (3, 4). These phosphorylation events lead to ubiquitination of IκBα followed by its rapid degradation by the 26 S proteasome (5). This releases active NF-κB, typically p50/p65 heterodimers, which translocates to the nucleus and activates transcription via κB enhancer elements. Recently a high molecular mass, ~700 kDa, complex has been described that contains kinase activity specific for serine 32 and 36 of IκBα (6–8). The two main IκB kinase (IKK) activities in this complex, termed the IKK signalsome, have been cloned and are called IKKe and IKKβ. In addition, the upstream kinase, where the IL-1β and TNF-α signaling pathways converge before IKK activation, has been identified as a mitogen-activated protein kinase kinase kinase kinase and named NF-κB-inducing kinase (NIK) (9). As NIK interacts with and stimulates IKK activity and can phosphorylate IKKe on serine 176, it is likely that NIK lies immediately upstream of the IKKs in the NF-κB activation cascade (10–12).

However, the degree to which these signaling events apply to different cell types and different inducing agents is presently unclear. In asthmatic individuals, elevated levels of activated NF-κB are found in sputum macrophage, suggesting that cells of the monocytic lineage may play a significant role in asthmatic inflammation (13). Human monocyctic U937 cells were therefore used to investigate the NIK-IKK-IκBα cascade. This leads to IκBa degradation, NF-κB DNA binding and κB-dependent transcription (data not shown). The two main IκB kinase (IKK) activities in this complex, termed the IKK signalsome, have been cloned and are called IKKe and IKKβ. Experimental Procedures

Cell Culture—U937 cells (ECACC code 85011440) were cultured at 37°C in a humidified atmosphere with 5% CO2 in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.8 μg/ml amphotericin B (complete medium) and were maintained between 2–9 × 106 cells/ml. Cells were stimulated in RPMI medium as above but supplemented with 2% fetal calf serum at 5 × 106 cells/ml. IL-1β and TNF-α (R&D Systems, Abingdon, Oxon) were used at 1 ng/ml and 10 ng/ml, respectively. Where used, cycloheximide (10 μg/ml; Sigma) was added 5 min before stimulation, N-carbenoxoloy-Leu-Leu-γ-cysteic acid (MG-132) (10 μM; Sigma) was added 60 min before stimulation, and PP1 and genistein (Calbiochem) were added 30 min before stimulation at concentrations of 10 μM and 100 μM, respectively. Drugs were dissolved in dimethyl sulfoxide (Me2SO) and were diluted to final concentrations of less than 0.1% (v/v). At this level Me2SO had no effect on activation of NF-κB or κB-dependent transcription (data not shown). Plasmids—The NF-κB-dependent reporter, pGL3.6κB.BG.luc, contains two tandem repeats of the sequence 5′-GGG GAC TTT C CC TGG-3′.
Preparation of Cytoplasmic and Nuclear Extracts—Extracts were prepared essentially according to Osborn et al. (15). Cells were treated with ice-cold Hank’s balanced salt solution before resuspension in 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1% v/v Nonidet P40 (NP-40), 0.25% v/v SDS (radioimmunoprecipitation buffer) and incubated on ice for 60 min. After centrifugation at 1,000 × g for 2 min at 4°C, the supernatant (cytoplasmic extracts) was retained. For electrophoretic mobility shift assays (EMSA), nuclei were resuspended in 10 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 5% v/v glycerol, 0.5 mM PMSF, and 1 mM DTT (Buffer B) and incubated on ice for 60 min with vigorous mixing. Nuclear debris was removed by centrifugation, and supernatants were diluted 4-fold in 20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM DTT (Buffer B) and incubated on ice for 60 min with vigorous mixing. Nuclear debris was removed by centrifugation, and supernatants were diluted 4-fold in 20 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 1 mM DTT (Buffer B) and incubated on ice for 60 min with vigorous mixing.

Western Blot Analysis—Cytoplasmic or nuclear extract, 20 μg, was added to 125 mM Tris-HCl, pH 6.8, 6.5% glycerol, 0.1% w/v bromphenol blue, 2% v/v 2-mercaptoethanol (2 μg/ml before the addition of radiolabeled oligonucleotides). Reactions were separated on 6% nondenaturing acrylamide gels in 0.25× TBE buffer and visualized by autoradiography.

Results

Induction of NF-κB-dependent Transcription and DNA Binding by IL-1β and TNF-α—Transient transfection of the NF-κB-dependent reporter, pGL3.6x.BG.Luc, into U937 cells showed that IL-1β (1 ng/ml) and TNF-α (10 ng/ml) activate κB-dependent transcription to similar extents (8.3 ± 1.9-fold for IL-1β and 8.5 ± 1.6-fold for TNF-α) (Fig. 1A). These responses were κB-specific, as a reporter with mutated κB recognition sites, pGL3.6x.BM.BG.Luc, produced 10–100-fold lower luciferase expression, which was unaffected by TNF-α or IL-1β treatment (data not shown).

EMSA revealed that both IL-1β and TNF-α strongly induced NF-κB DNA binding (Fig. 1B). However, the TNF-α response occurred with faster kinetics than the IL-1β response and generally showed the highest overall activation (Fig. 1B). Thus TNF-α-induced NF-κB was near maximal by 15 min, whereas IL-1β-induced NF-κB was not maximal until 30 min post-stimulation. Supershift analysis was performed to identify the Rel proteins involved in these responses (Fig. 1C). In IL-1β and TNF-α-treated extracts, anti-p50 antisera and anti-p65 antisera resulted in reduced mobility of DNA binding complexes. The NF-κB complex was unaffected by anti-c-Rel, RelB, and p52 antisera. These data indicate that in U937 cells, IL-1β- and TNF-α-inducible NF-κB DNA binding complexes are made up of both p50 and p65 proteins and not c-Rel, RelB, or p52. The fact that heterogeneous bands were observed suggests that both homo- and heterodimers of p50 and p65 may be present.

IkB Degradation by IL-1β and TNF-α—Western blot analysis was performed on cytoplasmic extracts to examine the effect of IL-1β and TNF-α on IkB degradation. Cells treated with vehicle showed no change in IkBα protein for the duration of the experiment (Fig. 2A, left panel). After IL-1β treatment, little or no change in IkBα levels was observed during the first 15 min of post-stimulation, and by 30 min a marked loss of only 40% loss of IkBα was observed (Fig. 2A, middle panel). By 1 h, IkBα protein levels had returned to above resting levels, which is consistent with NF-κB-dependent activation of the IkBα gene (17, 18). In marked contrast, TNF-α caused a rapid loss of IkBα within 15 min of stimulation, and again reconstitution was observed 1 h post-stimulation (Fig. 2A, right panel). These data

IKK Kinase Assay—IKK signalsomes were immunoprecipitated from cytoplasmic extracts, 200 μg, using 1 μg of IKKα antibody (H744) (Santa Cruz) and kinase assays performed as described previously (7). Briefly, one-half of each immunoprecipitate was resuspended in 25 μl of 20 mM HEPES pH 7.9, 2 mM MgCl₂, 2 mM MnCl₂, 10 μM adenosine triphosphate (ATP), 2 μg/ml of phosphatase inhibitor, 16 mM NaF, 10 mM 4-nitrophenyl phosphate, 0.5 mM Na₃VO₄, 1 mM benzamidine, 0.5 mM PMSF, aprotinin at 25 μg/ml, leupeptin at 10 μg/ml, pepstatin at 2 μg/ml, and 1 mM DTT (kinase buffer) in the presence of the substrates 1 μM GST-IκBα (1–54) or mutant GST-IκBβ (1–54) (S22A, S36A) (gifts from Sarah Crowe, Rhone Poulenc Rorer, Dagenham, Essex, UK) and kinase assays performed as described previously (7). To check loading and confirm the presence of IKKs, remaining immunoprecipitates were subject to SDS-PAGE and immunoblotted using 1,500 dilutions of IkKa and IkKβ (H740) antibodies (Santa Cruz).

Metabolic Labeling of p65—Cells were incubated in phosphate-free RPMI media for 2 h before the addition of [32P]orthophosphate (Amer shamPharmacia Biotech) at 0.2 mCi/ml and incubated for a further 4 h. Cells were then treated as indicated before harvesting in 200 μl of 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.5% SDS (radioimmunoprecipitation buffer) supplemented with 0.5% PMSF, 2 mM Na₃VO₄, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 50 mM NaF on ice for 15 min. Immunoprecipitation of p65 was performed as above except that radiomun precipitation buffer was used in place of Buffer A. Samples were run on 10% SDS-PAGE before autoradiography or Western blotting.

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agree with the fact that NF-κB DNA binding was induced more rapidly by TNF-α than by IL-1β. However, by 30 min, NF-κB DNA binding was essentially similar with both stimuli, yet loss of IκBα by TNF-α stimulation was almost total, whereas IL-1β resulted in only a 15% loss of IκBα yet caused over 60% relative DNA binding activity. One explanation for these discrepancies could be that IL-1β-dependent induction of IκBα resynthesis occurred more rapidly such that total disappearance of IκBα was prevented. This possibility was addressed by stimulation in the presence of cycloheximide to prevent new protein synthesis (Fig. 2B). In control cells, cycloheximide had little effect on IκBα protein levels (Fig. 2B, left panel). After IL-1β treatment in the presence of cycloheximide, loss of IκBα protein was less than 50% that of control at 30 min. This contrasts with the complete loss of IκBα within 15 min of TNF-α treatment in the presence of cycloheximide.

The above data raise the possibility that IκBα-dependent release of NF-κB may not fully account for the induction NF-κB DNA binding activity observed after IL-1β treatment in U937...
cells. We therefore focused on the potential role of other IκB proteins in activation of NF-κB. At 60 min, IκBβ showed a maximal loss of 30–40% with IL-1β and more than 50% loss after TNF-α treatment (Fig. 2C). The kinetics of TNF-α-induced loss of IκBβ were delayed with respect to loss of IκBα and the repression of IκBα protein levels, although modest, were more prolonged. The effects of IL-1β on loss of IκBβ were less pronounced than for TNF-α and only observed around 1–2 h post-stimulation. Consequently, IκBβ does not appear to contribute to levels of activated NF-κB observed before 1 h after IL-1β treatment.

Again, relatively minor decreases were observed for IκBε after IL-1β and TNF-α treatments, and in each case, the kinetics were similar, suggesting that IκBε does not play a major role in release of active NF-κB (Fig. 2D). In addition, degradation of the p50 precursor, p105, via the ubiquitin pathway may release active p50/p65 heterodimers (19), whereas in some cell types, including lymphoid cells, the C-terminal part of p105 can be independently transcribed to produce IκBγ (20, 21). Western blot analysis with an antibody for IκBγ, which also reacts with p105, revealed only p105 in U937 cells. Loss of p105 after treatment with IL-1β or TNF-α occurred with similar kinetics and in each case was no more than 50% (Fig. 2E). In addition, a p50 antibody that cross-reacts with IκBε, or TNF-α, was readily detected at 15, 30, and 60 min after both IL-1β and TNF-α stimulation, although modest, were more prolonged. The effects of IL-1β on loss of IκBε were less pronounced than for TNF-α and only observed around 1–2 h post-stimulation. Consequently, IκBε does not appear to contribute to levels of activated NF-κB observed before 1 h after IL-1β treatment.

For IL-1β, in resting cells, p65 was not detected in nuclear extracts. However, nuclear translocation of p65 was readily detected with the pan-IκB antibody (data not shown). These data suggest that IκBα, IκBε, or p105 may not be the major sources of active NF-κB observed on EMSA after IL-1β or TNF-α stimulation in U937 cells.

IκBα Remains Bound to p65 and Is Localized in the Cytoplasm after IL-1β Stimulation—In Jurkat T-cells, pervanadate causes release of NF-κB without degradation of IκBα, and this event involves tyrosine phosphorylation rather than serine phosphorylation of IκBα (22). We therefore used immunoprecipitation of p65 followed by Western blotting for IκBα to investigate whether IL-1β-stimulated release of NF-κB occurred without degradation of IκBα. After 15 min of IL-1β stimulation, but not TNF-α stimulation, IκBα was found to co-precipitate with p65 (Fig. 3A). By 90 min post-stimulation, co-precipitation of IκBα with p65 was observed for both stimuli, consistent with reappearance of newly synthesized IκBα. The specificity of p65 immunoprecipitation was confirmed by competition with specific blocking peptide (Santa Cruz). These data show that after IL-1β treatment, IκBα is still bound to p65, whereas after TNF-α treatment, there is no IκBα bound to p65.

Some studies have suggested that IκBα may translocate to the nucleus (23). Consequently, we have used Western blot analysis of nuclear extracts to explore the possibility that after IL-1β treatment, p65 translocates to the nucleus although still bound to IκBα. In resting cells, p65 was not detected in nuclear extracts. However, nuclear translocation of p65 was readily detected at 15, 30, and 60 min after both IL-1β and TNF-α treatments (Fig. 3B). In either case nuclear IκBα was not detected (even after prolonged overexposure of film), indicating that translocation of p65 was not accompanied by IκBα.

Immunoblotting of nuclear and cytoplasmic protein for p65 suggested that the level of cytoplasmic p65 in untreated cells was substantially greater than the nuclear level after either IL-1β or TNF-α treatment (Fig. 3B). Indeed, immunoblot analysis of cytoplasmic extracts after IL-1β or TNF-α treatments revealed only modest decreases in p65 immunoreactivity, indicating that only a fraction of the total cytoplasmic p65 was involved in nuclear translocation (Fig. 3C).

**Differential Activation of the NIK-IKK Pathway by IL-1β and TNF-α—**Various studies have shown that IL-1β and TNF-α stimulation converge at the level of NIK and cause IKK activation leading to phosphorylation of IκBα on serines 32 and 36 and its rapid degradation (6–12, 24). However in U937 cells, rapid agonist-dependent degradation of IκBα was not observed after IL-1β treatment, suggesting that there may be a defect in the signaling pathway leading to IκBα degradation.

Western blot analysis using an antibody specific for the Ser32-phosphorylated form of IκBα showed phosphorylated IκBα at 5 min and at 60 min after TNF-α stimulation (Fig. 4A). This is consistent with a rapid IKK-dependent phosphorylation of IκBα before its degradation and the continued phosphorylation and turnover of newly synthesized IκBα 60 min after TNF-α stimulation (Fig. 4A). The IκBα species detected by the phospho-Ser32-specific antibody coincides exactly with the reduced mobility band observed with the pan-IκBα antibody confirming that this reduced mobility was indeed the result of serine 32 (and presumably 36) phosphorylation. By contrast, the phospho-Ser32-specific antibody failed to detect IκBα at any time point after IL-1β stimulation (Fig. 4A). Furthermore, IκBα phosphorylation at other sites, for example tyrosine 42 or other C-terminal serine residues (22, 25, 26), seems unlikely as no evidence of a mobility shift was observed after IL-1β treatment. These data strongly suggest that the IκBα serine 32 and 36 specific IKK kinase activity is active in TNF-α-treated but not in IL-1β-treated cells.

To examine this possibility, IKK signalosomes were immunoprecipitated using an anti-IKKα antibody and IκBα kinase

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**Fig. 3. Effect of IL-1β and TNF-α on p65/IκBα binding and cellular localization.** A, immunoprecipitation (IP) of p65 was performed using cytoplasmic extracts (100 μg) from cells that were either not stimulated or treated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml), as indicated. Immunoprecipitates were subjected to Western blot analysis for IκBα (lower panel) before stripping, and immunodetection was performed for p65 (upper panel). Specificity was confirmed by incorporation of p65 antibody blocking peptide (BP) in the immunoprecipitation. Heavy chain IgG is indicated. B, cells were either not stimulated or treated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) for the times indicated. Nuclear extracts were prepared, and 20 μg of protein was subjected to 10% SDS-PAGE. After immunoblotting for p65 (top panel), membranes were stripped and reprobed with IκBα (lower panel). Cytoplasmic protein (20 μg) from unstimulated cells was used as positive control for IκBα (cyt). C, cells were treated as in B, and cytoplasmic extracts, 20 μg, were used for p65 immunoblotting. In each case (A, B, and C) blots representative of three such experiments are shown.
activity assayed by phosphorylation of the substrate GST-\(\kappa B\alpha\) (1–54) (Fig. 4B). TNF-\(\alpha\) stimulation dramatically increased IKK activity between 0 and 10 min with maximal activity at around 2 min. Although this rapid increase in IKK activity was not observed after IL-1\(\beta\), a gradual increase in IKK activity was observed, reaching around 30% of the maximal response obtained with TNF-\(\alpha\) (Fig. 4B). The presence of both IKK\(\alpha\) and IKK\(\beta\) in immunoprecipitates was confirmed by Western blot analysis (Fig. 4B). The IKK\(\beta\) antibody detected a specific band at 87 kDa, whereas the IKK\(\alpha\) antibody detected a major band at 85 kDa and a minor band at 87 kDa corresponding to IKK\(\alpha\) and cross-reactivity with IKK\(\beta\), respectively. The specificity of immunoprecipitation was confirmed using control rabbit IgG antisera, which failed to immunoprecipitate either IKK\(\alpha\) or IKK\(\beta\) (Fig. 4C). Furthermore, no phosphorylation of \(\kappa B\alpha\) was observed when the substrate, GST-\(\kappa B\alpha\) (1–54), was either not present or replaced with the mutant GST-\(\kappa B\alpha\) (1–54; S32A, S36A) (Fig. 4C). The substrate specificity was further confirmed using partially activated IKK signalosome that had been biochemically purified from okadaic acid-stimulated HeLa cells (27). These results demonstrate that the rapid and transient IKK activation, after TNF-\(\alpha\) treatment in U937, does not occur after IL-1\(\beta\) stimulation of U937 cells.

The above result indicates that the signaling pathway leading to activation of IKK may be impaired in response to IL-1\(\beta\). As the upstream kinase, NIK, is thought to directly phosphorylate IKKs (11, 12), the role of NIK in IL-1\(\beta\)- and TNF-\(\alpha\)-dependent NF-\(\kappa B\) activation was examined by co-transfecting U937 cells with a \(\kappa B\)-dependent reporter and plasmids expressing either wild type or dominant negative versions of NIK (9). Wild type NIK strongly induced \(\kappa B\)-dependent transcriptional activity, which is consistent with a role for NIK in the NF-\(\kappa B\)-inducing pathway (Fig. 5A). Overexpression of dominant negative NIK inhibited TNF-\(\alpha\)-induced \(\kappa B\)-dependent transcriptional activity by 60.3 ± 3.2% but only inhibited IL-1\(\beta\)-induced activity by 33.5 ± 9.2% (Fig. 5, B). This discrepancy suggests that NIK may be less important in the signaling pathway leading to \(\kappa B\)-dependent transcription induced by IL-1\(\beta\) than by TNF-\(\alpha\).

**Effect of Proteasome, Src, and Tyrosine Kinase Inhibitors on NF-\(\kappa B\) DNA Binding and Transcriptional Activation—**The above results raise the possibility that in U937 cells, IL-1\(\beta\)-induced NF-\(\kappa B\) is released from a pool of molecules that is distinct from the pool associated with \(\kappa B\)α. As the ubiquitin-proteasome system is not only required for proteolysis of \(\kappa B\)α but also the proteolysis of p105 and most probably other \(\kappa B\) molecules such as I\(\kappa B\)β or \(\epsilon\), in U937 cells, the inhibitor, MG-132, was used to test for proteasome involvement in IL-1\(\beta\)-dependent activation of NF-\(\kappa B\) in U937 cells. This class of compound is potent inhibitors of the 26 S proteasome, and MG-132 has previously been shown to inhibit activation of NF-\(\kappa B\) and IL-8 production (30–32). First, the effect of MG-132 was confirmed by its ability to block \(\kappa B\)α degradation induced by TNF-\(\alpha\) (data not shown). In addition this compound blocked induction of NF-\(\kappa B\) DNA binding and \(\kappa B\)-dependent transcriptional activity by both IL-1\(\beta\) and TNF-\(\alpha\), suggesting that 26 S proteasome activity is necessary for both TNF-\(\alpha\)- and IL-1\(\beta\)-dependent NF-\(\kappa B\) activation (Fig. 6, A and B).

Recently, phosphorylation of \(\kappa B\)α at tyrosine 42 via the nonreceptor tyrosine kinase, c-Src, was shown to mobilize NF-\(\kappa B\) to the nucleus via a mechanism that does not involve \(\kappa B\)α degradation (22, 33). To further exclude this possibility, the selective Src family inhibitor, PP1 (34), and the protein-tyrosine kinase inhibitor, genistein, which inhibits induction of
NF-κB-dependent transcription by lipopolysaccharide in THP-1 cells (35), were tested. Neither PP1 nor genistein had any effect on IL-1β- or TNF-α-induced NF-κB DNA binding, indicating that these signaling pathways are not involved in the activation of NF-κB DNA binding (Fig. 7A). However, both PP1 and genistein inhibited κB-dependent transcription by IL-1β and TNF-α to similar extents, suggesting that tyrosine kinase activity, possibly of a Src family kinase, is necessary for NF-κB-dependent transcriptional activity.

Analysis of p65 Phosphorylation—As p65 phosphorylation has been shown to positively modulate the transcriptional activity of NF-κB (36–38), the effect of TNF-α and IL-1β on p65 phosphorylation was investigated (Fig. 8). As in these previous studies we found p65 to be present as a phospho protein. However, treatment with either IL-1β or TNF-α for up to 1 h failed to produce any substantial changes in p65 phosphorylation, suggesting that differences in the regulation of NF-κB activity are not mediated at this level.

Discussion

NF-κB is widely accepted as playing a key role in inflammation via involvement in both effector and target gene regulation. Consequently, the NIK-IKK-IκB signaling pathway now represents a prime candidate for therapeutic intervention (1, 2). However, numerous other signaling pathways including protein kinase C, phosphatidylycholine-specific phospholipase, sphingomyelinase, protein kinase A, tyrosine kinases, and mitogen-activated protein kinases are all implicated in the regulation of NF-κB (22, 33, 35, 37, 39–43). Clearly, numerous unresolved questions remain in respect of the events leading to NF-κB-dependent transcription. We report that in U937 monocytes, TNF-α and IL-1β, both, produced identical κB-dependent transcriptional responses, yet the magnitudes and kinetics of IKK activation and IκBα degradation were totally different. This result essentially leads to two possibilities.

First, IKK activation and IκBα degradation are not exclusively required for NF-κB activation or second, some other step (or steps) that is required for NF-κB transcriptional activation is in fact rate-limiting.

In HeLa cells, IKK activation by TNF-α and IL-1β occurs very rapidly and is compatible with the TNF-α response in U937 cells (8). In contrast, IKK activation by IL-1β in U937 cells was substantially slower and only reached a peak of less than 30% that of the TNF-α-induced level. This resembled the effect observed in THP-1 cells after LPS stimulation in which IKK activation was not maximal until 1 h post-stimulation (44). We also examined IKK activity at up to 1 h post-IL-1β or TNF-α treatment, but no further increases in activity were observed (data not shown). In the above experiments the extent and kinetics of IKK activation and IκBα degradation correlated very closely. Despite this, no phosphorylated IκBα was detected in IL-1β-treated cells, suggesting that this level of IKK activation may not be physiologically relevant and that the loss of IκBα was via some other mechanism. However, after IL-1β treatment in the presence of the proteasome inhibitor MG-132, phosphorylated IκBα was detected by mobility shift and using a serine 32 phospho-specific antibody (data not shown). Thus, IL-1β causes phosphorylation of IκBα, and this is consistent with the low level of IKK activity. Reasons for the difference between the TNF-α and the IL-1β responses are presently unclear, as in other cell types these cytokines both result in rapid degradation of IκBα and NF-κB activation (4, 45). However, these reports along with our data and that of O’Connell et al. (44) highlight the existence of two kinetically, and presumably mechanistically, distinct pathways of IKK activation.

In view of the dramatically different kinetics and extents of IKK activation and IκBα degradation induced by IL-1β and TNF-α, we were surprised to find that TNF-α only resulted in around 30–40% more peak DNA binding activity than IL-1β. This and other discrepancies showed that loss of IκBα does not necessarily equate with NF-κB DNA binding. It was therefore possible that other IκBα proteins may also take part in activation of NF-κB. The kinetics of IκBβ degradation were consistent with the peak of TNF-α-induced NF-κB DNA binding activity observed around 1–2 h and with previous reports implicating IκBβ in the late or delayed NF-κB response (46, 47). With IL-1β there was little change in IκBβ levels by 30 min, suggesting that this protein does not contribute appreciably to DNA binding observed at 30 min. A number of reports have shown that serines 19 and 23 in IκBβ are substrates for the IKK complex, and their phosphorylation is required for proteolytic degradation (7, 27, 28). However, IκBβ is a considerably poorer IKK substrate compared with the equivalent IκBα reaction (7, 44). Despite this, it is difficult to explain the fact that, unlike IκBα, the kinetics of IκBβ degradation do not coincide with or follow closely behind the kinetics of IKK activation. Instead there was a considerable delay, suggesting that either phosphorylation of IκBβ by IKK occurs rapidly and concordantly with IKK activity but that the subsequent degradation occurs by a less rapid mechanism or that a IκBβ kinase exists that shows slower activation kinetics compared with the IκBα kinases. Likewise degradation of both IκBα and p105 was limited and similar for both treatments, whereas IκBγ was not detected in these cells. Consequently, these IκBs also fail to account for the substantial increase in DNA binding induced by IL-1β.

The observation that tyrosine phosphorylation of IκBα, possibly mediated by c-Src, may cause release of NF-κB from IκBα without proteolytic degradation could explain the above results (22, 33). However, our data exclude involvement of tyrosine phosphorylation and degradation-independent release of
NF-κB from IκBα in the induction of NF-κB DNA binding in U937 cells. Likewise, possible nuclear translocation of NF-κB, although bound to IκBα, was also excluded. Additionally, Western analysis revealed that only a fraction of the total p65 was mobilized to the nucleus on stimulation. Thus, complete loss of IκBα corresponded with a partial reduction in cytoplasmic p65, indicating that additional mechanisms must exist for retaining p65/NF-κB in the cytoplasm. Clearly IκBβ, p105, and IκBε may contribute to this effect. However, as these IκB proteins along with IκBα do not appear to fully account for the induction of NF-κB DNA binding, there could be a role for additional IκB genes, such as IκBδ and IκBf (48, 49). In this respect, the findings of Baueerle and Baltimore appear to be particularly salient (50). These authors describe a protein of 60–70 kDa in pre-B cells, which is the predominant IκB activity in these cells. As IκBβ (70 kDa) generally seems to be lowly expressed, if at all, and this protein is unlikely to correspond to IκBα (37 kDa), IκBβ (45 kDa), or IκBε (45 kDa), it may represent an as yet uncloned IκB molecule and could account for the effects observed here. Based on the data presented, we therefore predict that release of NF-κB from a number of cytoplasmic pools or stores is necessary to produce the overall response. These data are consistent with a role for IKK activation and IκBα in the immediate TNF-α-dependent NF-κB response and IκBβ in the delayed response. However, there appears to a role for additional IκB activities and activating pathways in mediating the intermediate NF-κB response. In this case it seems that proteasome activity is required for NF-κB activity, as the MG-132 totally prevented IL-1β- and TNF-α-dependent NF-κB DNA binding. Candidate molecules for this effect may include p105, IκBε, the putative IκBδ and IκBf genes, and the IκB protein identified by Baueerle and Baltimore (50).

The difference between IκBα degradation induced by IL-1β and TNF-α suggested a major difference in the signaling events leading to IκBα degradation. The greater inhibitory effect of dominant negative NIK on TNF-α-dependent reporter activity compared with the IL-1β effect implies a greater role for NIK in the TNF-α response. As NIK is thought to directly activate IKK (10–12), this hypothesis is in accord with the IKK activity data presented. However, despite testing a range of expression vector concentrations, dominant negative NIK failed to completely block reporter activation by either cytokine, implying that other signaling pathways that are not blocked by dominant negative NIK may also exist. Similar data are also found in respect of both IKKα and β, as dominant negative versions of these kinases often fail to repress or only partially repress κB-dependent responses (7, 44). Although these data may be explained by phenomena associated with overexpression, the existence of alternative NF-κB-activating pathways also remains a possibility.

Finally, this study also highlights one further area of discrepancy. This involves the lack of correlation between NF-κB DNA binding and κB-dependent transcription. In this study, IL-1β produced lower levels of DNA binding than TNF-α. However, the transcriptional responses to these cytokines were indistinguishable. One possibility is that TNF-α and IL-1β differentially modulate the transactivation potential of p65 via different degrees of p65 phosphorylation (36, 37, 51). However analysis of p65 phosphorylation showed no differences between TNF-α and IL-1β treatments, suggesting that downstream steps may be rate-limiting. This possibility is supported by the fact in monocytes that genistein had no effect on DNA binding yet prevented NF-κB transcriptional activation while enhancing AP-1-dependent transcription (35). Likewise in U937 cells, genistein and PP1 both showed little effect on NF-κB DNA binding but markedly attenuated transcriptional activation. Furthermore, we and others have previously reported similar effects in other cell types indicating that the control of transcriptional activation per se remains to be explored (35, 40).

In conclusion, we have presented a body of data that identifies a number of inconsistencies in the NF-κB transcriptional activation cascade. We therefore hypothesize the existence of parallel activation pathways that are distinct from the classical NIK-IKK-IκBα activation pathway. Indeed, because the completion of this study, a number of reports have shown that in addition to NIK and MEKK1, the mitogen-activated protein kinase kinase kinases, TPL-2, TAK1, MEKK2, and MEKK3, are also able to activate NF-κB further raising the possibility of parallel activation cascades (52–54). In addition, our data also point to the existence of signaling pathways which may impose rate-limiting steps on κB-dependent transcription. These findings have a number of important implications for anti-inflammatory strategies aimed at inhibiting the NIK-IKK-IκBα pathway. Drugs that cause relatively high degrees of inhibition of IKK or NIK may in fact only produce relatively modest changes in NF-κB activation at the DNA binding level. These changes may be further atten-
uated by downstream rate-limiting steps that are involved in transcriptional activation itself. The generation of highly selective NF-κB transcriptional activation complexes of the NF-κB and IKK inhibitors along with the continued cloning of NF-κB activation cascade will be required to answer these questions.

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