Mechanism for Biphasic Rel A·NF-κB1 Nuclear Translocation in Tumor Necrosis Factor α-stimulated Hepatocytes*

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The proinflammatory cytokine, tumor necrosis factor α (TNFα), is a potent activator of angiotensinogen gene transcription in hepatocytes by activation of latent nuclear factor-κB (NF-κB) DNA binding activity. In this study, we examined the kinetics of TNFα-activated translocation of the 65-kDa (Rel A) and 50-kDa (NF-κB1) NF-κB subunits mediated by inhibitor (IκB) proteolysis in HepG2 hepatoblastoma cells. HEP2 cells express the IκB members IκBα, IκBβ, and IκBγ. In response to TNFα, Rel A·NF-κB1 translocation and DNA binding activity follows a biphasic profile, with an “early” induction (15–30 min), followed by a nadir to control levels at 60 min. This biphasic profile is followed by a “late” induction (>120 min). The early phase of Rel A·NF-κB1 translocation depends on simultaneous proteolysis of both IκBα and IκBβ isoforms; IκBγ is inert to TNFα treatment. The 60-min nadir is due to a rapid IκBα resynthesis that reassociates with Rel A and completely inhibits its DNA binding activity; the 60-min nadir is not observed when IκBα resynthesis is prevented by cycloheximide treatment. By contrast, selective inhibition of IκBβ proteolysis by pretreatment of HepG2 cells with the peptide aldehyde N-acetyl-Leu-Leu-Nor-leucinal completely blocks the late phase of Rel A·NF-κB1 translocation. These studies indicate the presence of inducible and constitutive cytoplasmic NF-κB pools in hepatocytes. TNFα induces a coordinated proteolysis and resynthesis of IκB isoforms to produce dynamic changes in NF-κB nuclear abundance.

Multicellular organisms have evolved mechanisms for the coordinate expression of inducible genes through ligand-dependent receptors. Ligand binding to high affinity receptors located on the plasma membrane generate second messenger signals that can influence the activity or abundance of transcription factors through post-translational modifications including signal-induced phosphorylation and/or proteolysis. Hormone-activated gene transcription plays an important role in many homeostatic processes, including the cytokine cascade (1), and the change in expression of liver genes in response to systemic inflammation known as the acute-phase response (APR1; reviewed in Refs. 2–4).

The APR is the consequence of inducible transcriptional activation of hepatic genes required for blood pressure regulation, such as angiotensinogen (2, 5), and those involved in macrophase opsonization and wound repair (6) through the effects of macrophase-activated interleukins-1, interleukin-6, and tumor necrosis factor α (TNFα) (6). Hepatocyte-specific transactivators modified during the APR include AP-1 (7), signal transducers and activators (8), nuclear factor-interleukin 6 (9), and nuclear-factor-κB (NF-κB) (10). The angiotensinogen gene is transcriptionally activated during the APR by the effect of a single regulatory element, the acute-phase response element (APRE) (11, 12). The APRE is a target for intracellular signaling initiated by the liganded TNFα type I receptor that activates latent DNA binding activity of the potent NF-κB transcription factor family (3, 12, 13).

NF-κB is a family of homo- and heterodimieric proteins related by an NH2-terminal −300 amino acid Rel homology domain including the proteolytic processed NF-κB1 and NF-κB2 subunits, as well as the Rel A (p65), c-Rel, and Rel B subunits (reviewed in Ref. 10). Dimerization of various NF-κB subunits produce complexes with various intrinsic DNA-binding specificities (14), transactivation potentials (10, 15–18), and subcellular localization (19). For example, NF-κB1 homodimers are constitutively nuclear and bind DNA avidly, but lack significant transcriptional activity; by contrast, Rel A·NF-κB1, Rel Ac-Rel, and Rel A·NF-κB2 heterodimers are cytoplasmic and exhibit various degrees of transcriptional activator properties (reviewed in Refs. 10, 20). UV cross-linking (11), gel mobility shift assays with subunit-specific NF-κB antibodies (12), and transient overexpression assays (12, 13) indicate that Rel A·NF-κB1 heterodimers are the major species of hormone-inducible NF-κB subunits that bind the APRE in hepatocytes. The Rel A·NF-κB1 complex is sequestered in a latent cytoplasmic form by association with various inhibitor (IκB) proteins, including IκBα (pp40/MAD-3) (21–23), IκBβ (24), IκBγ (the COOH-terminal protein encoded by translation of the alternative splicing of the p105 NF-κB1 mRNA precursor (25), and p105 itself (26, 27) that associate with Rel A through a protein inter- active domain homologous to erythrocyte ankyrin. Dissociation of Rel A from IκB is prerequisite for Rel A nuclear translocation (23, 28–30); current evidence favors a two-step dissociation that first requires inducible NH2-terminal phosphorylation (IκBα is phosphorylated at serine residues 32 and 36) followed by proteolysis through the 26 S proteasome (30–32).

The observations that distinct IκB family members are expressed, and perhaps regulated, in a tissue-restricted fashion (24, 31) prompted us to investigate the kinetics of latent Rel A·NF-κB1 activation in hepatocytes. We report the unanticipated findings that TNFα produces a biphasic Rel A·NF-κB1 translocation in HepG2 hepatoblastoma cells. HepG2 cells express the IκB members IκBα, IκBβ, and IκBγ. In response to TNFα, Rel A·NF-κB1 translocation and DNA binding activity follows a biphasic profile, with an “early” induction (15–30 min), followed by a nadir to control levels at 60 min, and a “late” induction (>120 min). The early phase of Rel A·NF-κB1 translocation depends on simultaneous proteolysis of both IκBα and IκBβ isoforms; IκBγ is inert to TNFα treatment. The 60-min nadir is due to a rapid IκBα resynthesis that reassociates with Rel A and completely inhibits its DNA binding activity; the 60-min nadir is not observed when IκBα resynthesis is prevented by cycloheximide treatment. By contrast, selective inhibition of IκBβ proteolysis by pretreatment of HepG2 cells with the peptide aldehyde N-acetyl-Leu-Leu-Nor-leucinal completely blocks the late phase of Rel A·NF-κB1 translocation. These studies indicate the presence of inducible and constitutive cytoplasmic NF-κB pools in hepatocytes. TNFα induces a coordinated proteolysis and resynthesis of IκB isoforms to produce dynamic changes in NF-κB nuclear abundance.

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1 The abbreviations used are: APRE, acute-phase response element; EMSA, electrophoretic mobility shift assay; IκB, inhibitor of NF-κB;

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nuclear translocation, with an “early” peak at 15–30 min, return to control (1 h), and a later peak (>2 h induction). In hepatocytes, the IκB family members α, β, and γ, but not the NF-κB p105 precursor are expressed. Early Rel A/NF-κB translocation is due to simultaneous proteolysis of both IκBα and IκBβ. By 1 h, IκBα is rapidly synthesized and reassociates with Rel A; this reassociation (due to “overshoot”) results in complete inhibition of Rel A/NF-κB binding even in the continued absence of IκBα. As IκBα levels fall after 1 h, Rel A/NF-κB binding is again detectable in the nucleus. Inhibition of IκBα proteolysis by the peptide aldehyde N-acetyl-Leu-Leu-norleucinal completely prevents the second phase of Rel A/NF-κB binding, demonstrating the requirement of IκBα for prolonged Rel A/NF-κB nuclear action.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment

The human hepatoblastoma cell-line HepG2 was obtained from ATCC (Rockville, MD) and grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% dialyzed fetal bovine serum, 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics (penicillin/streptomycin/ fungizone) in a humidified atmosphere of 5% CO₂. Recombinant human TNFa (rTNFa, Genentech) was added to a final concentration of 30 ng/ml in culture medium and cells were incubated for the indicated time periods at 37 °C. For pretreatments, calpain inhibitor I (200 μM, CalBiochem, San Diego, CA) or cycloheximide (50 μg/ml, Sigma) were added in medium 1 h or 30 min prior to TNFa stimulation, respectively.

Preparation of Subcellular Extracts

Cytoplasmic Extracts—HepG2 cell pellets were washed two times with phosphate-buffered saline and then resuspended in Buffer A (50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μM/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml aprotinin, and 0.5% Nonidet P-40). After 10 min on ice, the lysates were centrifuged at 4,000 × g for 4 min at 4 °C, the supernatant constitutes the cytoplasmic extract.

Sucrose Density-purified Nuclear Extracts—For the purification of nuclei (33, 34), nuclear pellets were resuspended in Buffer B (1.7 M sucrose, 50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μM/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotinin), and centrifuged at 15,000 × g for 30 min at 4 °C. The resultant nuclear pellets were incubated in Buffer C (10% glycerol, 50 mM HEPES (pH 7.4), 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μM/ml pepstatin A, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotinin) with frequent vortexing for 30 min at 4 °C. After centrifugation at 15,000 × g for 5 min at 4 °C, the supernatant is saved for nuclear extract. Both of cytoplasmic and nuclear extracts were normalized for protein amounts determined by the Bradford assay using bovine serum albumin as a standard (Bio-Rad).

Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were performed as described previously with minor modifications (12, 13). Nuclear extracts (10 μg) were incubated with 40,000 cpm of ²²P-labeled APRE WT duplex oligonucleotide probe and 2 μg of poly(dA-dT) in a buffer containing 8% glycerol, 100 mM NaCl, 5 mM MgCl₂, 5 mM dithiothreitol, and 0.1 mg/ml phenylmethylsulfonyl fluoride in a final volume of 20 μl, for 15 min at room temperature. The complexes were fractionated on 6% native polyacrylamide gels run in TBE buffer (25 mM Tris, 25 mM boric acid, and 0.5 mM EDTA), dried, and exposed to Kodak X-AR film at −70 °C. Competition was performed by the addition of 100-fold molar excess nonradioactive double-stranded oligonucleotide competitor at the time of addition of radioactive probe. The sequences of the APRE double-stranded oligonucleotides are as shown below.

APRE WT: GATCCACCAAGTGGGATTTCCCAAGCTGACCA GTGGTGGTACACCTAAAGGCCTGATCTGCTTAG

APRE M6: GATCCACCAAGTGGGATTTCCCAAGCTGACCA GTGGTGGTACACCTAAAGGCCTGATCTGCTTAG

APRE M2: GATCCACCAAGTGGGATTTCCCAAGCTGACCA GTGGTGGTACACCTAAAGGCCTGATCTGCTTAG

SEQUENCES 1–3

Antibody supershift assays were performed by adding to the binding reaction 1 μl of affinity-purified polyclonal antibodies and incubating for 1 h on ice. All of the antibodies used in these assays were obtained commercially (Santa Cruz Biotech, Santa Cruz, CA). For the NF-κB DNA-binding supershift assay, the nuclear extracts were incubated with the indicated amounts of bacterially-expressed full-length IκBα protein in binding reaction. Inactivated IκBα was prepared by boiling the recombinant IκBα protein for 30 min in phosphate-buffered saline.

Expression of Polyhistidine-tagged IκBα

The full-length cDNA encoding human IκBα was subcloned as an EcoRI fragment into the pRSETB expression plasmid under the control of the T7 promoter (Invitrogen, San Diego, CA). The plasmid was transformed into Escherichia coli BL21(DE3)pLyS8 and the 47-kDa protein of the T7 protein (In Vitrogen, San Diego, CA). The plasmid was transformed into Escherichia coli BL21(DE3)pLyS8 and the 47-kDa protein was induced with the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) during logarithmic growth and purified under native conditions on a nickel-agarose column (35). The protein was >90% pure as judged by SDS-PAGE and Coomassie Blue staining.

Western Blotting and Coimmunoprecipitation

For Western immunoblot, a constant amount of cytoplasmic or nuclear extracts (200–300 μg as indicated) from the above preparation were boiled in Laemmli buffer, separated on 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked in 8% milk and immunoblotted with the affinity-purified rabbit polyclonal antibodies (Santa Cruz Biotech) for IκBα (reactive with amino acids 297–317), IκBβ (reactive with amino acids 339–358), IκBγ (reactive with amino acids 471–490), Rel A (reactive with amino acids 3–19), or NF-κB-1 (reactive with amino acids 350–363). Immune complexes were detected by binding donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) followed by reaction in the enhanced chemiluminescence assay (ECL, Amersham) according to the manufacturer’s recommendations. For the coimmunoprecipitation assays, cytoplasmic extracts (1 mg) from either untreated or TNFα-stimulated HepG2 cells were incubated with Rel A antisera in a total volume of 1 ml of TST buffer (50 mM Tris-Cl, 5 mM EDTA, 150 mM NaCl, and 0.05% Triton X-100) for 2 h at 4 °C and collected on Protein A-agarose beads (Life Technologies, Inc.). After washing 5 times with TST buffer, the precipitates were boiled in Laemmli buffer and subjected to immunoblotting with anti-IκBα antibody following the procedure above. For the neutralization experiments, anti-Rel A and anti-IκBα antibodies were neutralized with a 20-fold excess of the relevant polypeptide overnight at 4 °C.

RESULTS

Biphasic Induction of Rel A/NF-κB DNA-binding in Hepatocyte Nuclei—Exposure of cultured HepG2 human hepatocytes to 20 ng/ml rTNFα for 6 h results in an induction of NF-κB DNA binding activity and transcriptional activity (12, 13). To resolve the induction kinetics of various NF-κB family members in the rTNFα response, we examined a 6-h time course of APRE DNA binding activity using extracts of sucrose cushion-purified nuclei using EMSAs. In EMSAs performed under conditions that resolve the individual heterodimeric NF-κB species binding to the radiolabeled APRE, four nucleoprotein complexes could be resolved (C1-C4, Fig. 1A). The C1 complex was weakly and variable inducible at 15 min. By contrast, the C2 complex was strongly inducible in a biphasic manner with the first peak occurring at 15 min (a 16-fold induction relative to control), declining by 30 min, and was undetectable at 60 min (the early binding phase). At 120, the C2 complex reappeared (4.1-fold relative to control) and persisted as long as 360 min (the “late” binding phase). Binding specificity of the complexes was demonstrated using site-specific competitors of the APRE in the EMSA (Fig. 1B). Complexes C1 and C2 both competed with homologous APRE WT but not APRE M2 or APRE M6.
Gel mobility supershift assays using subunit-specific NF-κB antibodies was used to demonstrate the composition of the strongly inducible APRE-binding C2 complex (Fig. 1C). Addition of Rel A, but not preimmune antibody resulted in the selective diminution of the C2 complex with the simultaneous appearance of a supershifted band. Similarly, addition of NF-κB1 antibody also diminished the intensity of the C2 nucleoprotein complex. Taken together, these data indicate that the DNA binding activity of the C2 is biphasic upon rTNFα treatment, C2 binds to the APRE with NF-κB binding specificity and is composed of the Rel A-NF-κB1 heterodimer.

Nuclear Translocation of Rel A and NF-κB1 Parallel the Biphasic Changes in DNA Binding of the C2 Complex—HepG2 cells fractionated into cytoplasmic and highly purified nuclear extracts (by sucrose cushion centrifugation) were assayed in Western immunoblots for changes in relative abundance of Rel A and NF-κB1. The Rel A antibody recognized a single ~65-kDa antigen (Fig. 2, arrow) that could be specifically blocked by preadsorption using recombinant Rel A protein (not shown). In unstimulated cells, the majority of Rel A was located in the cytoplasmic fraction. By 15 min of rTNFα treatment, a slight depletion in the cytoplasmic fraction was noted with a concomitant 3.7-fold increase in nuclear Rel A. At 60 min, nuclear Rel A has diminished to levels approximating control followed by a second increase in Rel A abundance at 120 and 360 min. Changes in nuclear NF-κB1 abundance, detected by a specific polyclonal antibody as a 50-kDa band, paralleled those observed for nuclear Rel A (Fig. 2), and for DNA binding of the C2 complex (Fig. 1). These data indicate that rTNFα controls cytoplasmic:nuclear positioning of Rel A and NF-κB1 in hepatocytes in a biphasic pattern.

Dynamic Expression and Differential Regulation of IκB Isoforms in Response to rTNFα Treatment—Cytoplasmic extracts of control and rTNFα-treated HepG2 cells were assayed for the expression and relative changes in IκB abundance using antibodies that recognized specific epitopes of IκBa, IκBβ, and IκBγ as determined by the appropriate molecular weight and ability of peptide preadsorption to compete for the immunostaining (Fig. 3A). In control cells, 37-kDa IκBa was abundantly detected, as was 46-kDa IκBβ and 70-kDa IκBγ (Fig. 3B). With rTNFα treatment, both IκBa and IκBβ, but not IκBγ, disappeared within 15 min of treatment. Abundance of IκBa returned to a 2-fold greater than control levels at 60 min producing an “overshoot” in its synthesis; by 120 min, IκBa returned to control levels. In contrast, although 46-kDa IκBβ disappeared simultaneously with IκBa after TNFα treatment, no resynthesis of IκBβ was observed. These data indicate the abundance of IκBa and β is regulated by rTNFα treatment, whereas the abundance of IκBγ is not. Moreover the robust IκBa reassembly at 1 h corresponds to the “nadir” of Rel A-NF-κB1 DNA binding activity and nuclear abundance (cf. Figs. 1A and 2).

IκBa Rapidly Reassociates with Rel A: The Role of IκBa

Fig. 1.A, rTNFα induces a biphasic pattern of Rel A-NF-κB1 binding in HepG2 nuclei. Autoradiogram of EMSA using 10 μg of nuclear protein prepared from cultured HepG2 hepatoblastoma cells stimulated for the indicated times (in min) with 30 ng/ml rTNFα binding to radiolabeled APRE WT DNA. Migration of various complexes (C1–C4) is shown at the left. Complexes C3 and C4 are constitutive. Complex C2 exhibits a biphasic induction pattern with a return to control values at 60 min. C1 is weakly and variably inducible. After quantitation, the C1 complex increases (values given in fold increase relative to controls): 5.7 (15 min), 1.8 (30 min), 1.1 (60 min), 1.6 (120 min), and 1.1 (360 min); for the C2 complex: 16.5 (15 min), 6.1 (30 min), 0.3 (60 min), 4.1 (120 min), and 4.9 (360 min). B, and C2 bind with NF-κB binding specificity. Autoradiogram of competition EMSA using 10 μg of 15-min stimulated HepG2 nuclear extract binding to radiolabeled APRE WT in the absence (−) or presence of 100-fold molar excess of APRE site mutations (“Experimental Procedures”). Location of complexes is located at left. Complexes C1 and C2 compete with wild type APRE, but not mutants oligonucleotides, indicating sequence-specific recognition of the NF-κB contact points on the APRE (5).
Overshoot in Inhibiting Nuclear Rel A Abundance—We noted that the disappearance of nuclear Rel A occurred simultaneously with enhanced IκBα abundance, indicating that enhanced IκBα synthesis (overshoot) and reassociation with Rel A may underlie the phenomenon of the 60-min nadir. To directly measure association of Rel A with IκBα, we performed a two-step immunoprecipitation-Western immunoblot using cytoplasmic extracts from HepG2 hepatocytes. In this assay, Rel A complexes are captured and washed under nondenaturing conditions, the latter representing the Rel A:NF-κB1 heterodimer, whereas the nonspecific C4 complex was unaffected. These data demonstrate that Rel A:NF-κB1 complex associated with IκBα is unable to bind DNA at any phase of its induction.

Our data indicated that enhanced IκBα synthesis results in inhibition of Rel A:NF-κB1 binding at 60 min of rTNFα stimulation. To confirm this model, EMSA was used to determine the pattern of Rel A:NF-κB1 binding after IκBα resynthesis was inhibited using the protein synthesis inhibitor cycloheximide. In the presence of 50 μg/ml cycloheximide, the biphasic Rel A:NF-κB1 binding pattern was abolished and converted into a single monotonic profile as shown in EMSA (Fig. 5). In this same experiment, cytoplasmic IκBα disappeared at 15 min and was undetectable at 1 h of rTNFα treatment assayed by Western immunoblot (data not shown), indicating requirement of new protein synthesis for the nadir in Rel A:NF-κB1 binding. These data indicate: 1) IκBα reassociated with the Rel A:NF-κB1 complex at 60 min; 2) IκBα is capable of inhibiting DNA-binding of Rel A:NF-κB1; and 3) IκBα appearance at 60 min requires new protein synthesis. We conclude that the enhanced IκBα resynthesis at 60 min (IκBα overshoot) underlies the biphasic pattern of Rel A:NF-κB1 translocation, by producing the nadir in C2 binding.

IκBβ Proteolysis Is Required for the Late Phase of Rel A:NF-κB1 Translocation—Previous work has indicated that chymotrypsin-like enzyme(s) mediate IκB proteolysis (36), prompting us to examine whether inhibitors could selectively interfere with IκBβ proteolysis so that we could determine its role in biphasic NF-κB activation in hepatocytes. The effect of pretreatment with the peptide aldehyde N-acetyl-Leu-Leu-norleucinal (calpain inhibitor I) was determined using Western immunoblot assays of cytoplasmic extracts (Fig. 6). In response to rTNFα, IκBα proteolysis was clearly evident at 15 and 30 min. By contrast, IκBβ abundance was similar, or exceeded, control values from 15 to 360 min (compare with Fig. 3B). IκBβ was unaffected throughout the time course of the experiment. We conclude that calpain inhibitor I preferentially blocks IκBβ, but not IκBα proteolysis in hepatocellular cells.

Under conditions where IκBβ proteolysis was preferentially inhibited, we next determined the kinetics of Rel A:NF-κB1 translocation. The effect of calpain inhibitor I pretreatment was to block completely the second late phase of Rel A:NF-κB1 induction (Fig. 7A). The early appearance of C2 at 15 and 30 min was attenuated, but not abolished, indicating the contribution of IκBβ proteolysis to the early phase of translocation. Western immunoblots for nuclear Rel A and NF-κB1 abundance confirmed that Rel A and NF-κB1 were induced corre-
**DISCUSSION**

An important mechanism in the transcriptional activation of the angiotensinogen gene during the APR is the TNFα-mediated activation of latent NF-κB subunits that bind to the APRE (3, 12, 13). In this study, we have identified mechanistically distinct phases of Rel A/NF-κB1 nuclear translocation that depend on dynamic changes in individual IκB subunit abundance. In this report, we describe the unanticipated and novel observation that TNFα produces a biphasic pattern of Rel A/NF-κB1 binding. Previous studies have only identified a monophasic induction pattern using 70Z/3 pre-B lymphocytes (24), Jurkat T-cells (38), and U937 macrophages (36) of varying duration. In hepatocytes, the biphasic pattern consists of an

**Biphasic NF-κB Translocation**

**Fig. 3. A**, expression of IκBa, IκBβ, and IκBγ isoforms in HepG2 cells. Autoradiograms of Western immunoblots using HepG2 cytoplasmic extracts from unstimulated cells probed with preimmune rabbit serum, anti-IκBa, IκBβ, and IκBγ primary antibodies, or the same antibodies preadsorbed with respective peptides (PreAd-IκBa, PreAd-IκBβ, and PreAd-IκBγ). The molecular weight (in kDa) of the protein standards is indicated at the left. IκBa staining produces a single specific 37-kDa band; IκBβ appears as a 46-kDa band (small arrow); IκBγ is identified as a 76-kDa band (arrow). No NF-κB1 precursor is recognized by IκBγ antibody (this antibody detects epitope shared by both isoforms). B, changes in steady-state IκB levels in response to rTNFα treatment. Western immunoblots from HepG2 cytoplasmic extracts taken from cells treated for indicated times with rTNFα (top) and preadsorbed with anti-IκBa, IκBβ, and IκBγ primary antibodies (left). The abundance of IκBa is rapidly diminished at 15 and 30 min, followed by enhanced levels (2.1-fold relative to control values) at 60 min, and return to control levels after 120 min. IκBβ staining is detectable in unstimulated cytoplasm and vanishes after 15 min of rTNFα treatment. IκBγ abundance is not affected by rTNFα.

**Fig. 4. A**, rapid reassociation of IκBa with Rel A during rTNFα treatment. Western immunoblot of immunoprecipitates from control and rTNFα-treated HepG2 cytoplasmic lysates. Antibody used in immunoprecipitation (IP), indicated in top panel, includes Rel A COOH-terminal antibody (raised to amino acids 434–551), or the same antibody preadsorbed with recombinant Rel A (Pre Ad-Rel A). Antibodies used in Western immunoblot (IB) includes Rel A and either IκBa or IκBβ preadsorbed with recombinant IκBa (PreAd-IκBa). The locations of Rel A, IgG, and IκB are indicated on left. IκBa staining is dependent on the use of both anti-Rel A in the immunoprecipitation and anti-IκBa in the immunoblot. IκBα association with Rel A is lost at 15 min and rapidly returns at 30 and 60 min. Note the maximal amount of IκBa associated with Rel A occurs at 60 min, the nadir of both Rel A and IκBα binding taken from early and late phases of induction. Autoradiogram of EMSA using nuclear extracts from rTNFα-treated HepG2 for 15 min (Early Induction) and 120 min (Late Induction) binding radiolabeled APRE WT. Homogenous recombinant polyhistidine-tagged human IκBa was added to extracts in the indicated amount for 15 min prior to fractionation. Rel A/NF-κB1 (C2) binding is completely inhibited with 200 ng of rhIκBa. C1 binding (Rel Ac-Rel) is weakly affected. Heat inactivated rhIκBa (Inact) has no effect.
shown direct association with Rel A regulator of Rel A translocation because other studies have indicated that selectively blocks IκBα proteolysis, allows us to demonstrate that the second phase of RelA-NF-κB1 translocation is solely dependent on signal-induced hydrolysis of IκBβ.

IκB Forms Expressed in Hepatocytes Include Regulated IκBα and β as well as Constitutive IκBγ—In non-B lymphocytes, NF-κB proteins are sequestered in an inactivated form in the cytoplasm by binding various members of the inhibitory IκB protein family. IκBα, IκBβ, IκBγ and NF-κB1 p105 are all candidate regulators of Rel A translocation because other studies have shown direct association with Rel A-NF-κB1 in cellulo and their ability to inhibit RelA-NF-κB1 DNA binding activity in vitro (23–25). The pattern of IκB expression and modification by signal transduction systems has not been systematically studied in liver cells. Relative levels of mRNA encoding the IκB isoforms have been shown to be expressed in a tissue-restricted fashion. For example, IκBβ mRNA is expressed highly in testes (where no IκBα mRNA is detectable) and IκBα is expressed more abundantly in spleen and lung than IκBβ (24). Although we have not measured transcript levels, we observe that IκBα protein appears to be more abundant than IκBβ in hepatocytes. IκBγ is a 70-kDa translation product of a unique mRNA that represents either an alternative splice product or a cryptic promoter from the nf-kb1 gene (25), and is reported to have the most highly restricted pattern of expression previously detected only in mouse lymphoid cell lines (25). Although we have not assayed for expression of the unique IκBγ transcript, we can identify IκBγ expression on the basis of antibody specificity and its appropriate 70,000 molecular weight in hepatocellular cells. In marked contrast to the behavior of IκBα and IκBβ, the steady state abundance of IκBγ appears not to be regulated by rTNFα treatment. The presence of constitutive IκBγ may account for the presence of residual Rel A in the cytoplasm of rTNFα-treated cells at times (15 min) when nearly complete IκBα and IκBβ proteolysis have occurred, and underscores the concept that there may be pools of Rel A in complex with IκB proteins whose abundance are themselves differentially responsive to distinct second messenger pathways. Finally, it is important to emphasize that our observations are made on a cell population and hence represent a statistical average of individual cellular responses to rTNFα. Based on this experimental design, we therefore cannot differentiate between the following interpretations: 1) two subsets of cells are present in...
the HepG2 culture, one group expresses IκBα and β and a second group expresses IκBγ only, with only the former population being rTNFα-responsive; versus 2) a homogenous cell population is present in the HepG2 culture that expresses all three IκB isoforms. Although this cell population is rTNFα-responsive, the abundance of individual IκB isoforms are controlled by different intracellular signaling pathways. Additional studies, at individual cell resolution, will be required to differentiate between these possibilities.

Mechanisms of TNFα-induced Changes in IκB Abundance—TNFα activates Rel A translocation by signal-induced modifications of the IκB inhibitor. TNFα-activated intracellular signals induces phosphorylation of IκBα at NH2-terminal serine residues (amino acids 32 and 36 (28, 30)), by a ubiquitination-dependent kinase, a process that subsequently targets IκBα for proteolysis (31, 32). Phospho-IκBα migrates at a distinct position on SDS-PAGE gels (28, 30), allowing for its identification (23, 28, 30, 32). Although we have not directly demonstrated inducible phosphorylation of IκBα in HepG2 cells, indirect evidence for IκBα phosphorylation is seen in Western immunoblots of cytoplasmic extracts from rTNFα-treated cells where the slower phospho-IκBα migrating species is seen with longer exposures (data not shown). Other studies have shown that phospho-IκBα is itself rapidly polyubiquitinated (Ubα) at lysine residues 21 and 22 (31) and subsequently proteolyzed through the 26 S proteasome pathway (29, 32). The 26 S proteasome, a 700-kDa protease complex, is known to degrade Ubα-conjugated proteins in an ATP-dependent fashion, but the protease(s) involved and their specificity are incompletely characterized (37).

In epithelial cells and lymphocytes, serine protease inhibitors (1-tosylamido-2-phenylethyl chloromethyl ketone) and the peptide aldehyde (calpain inhibitor I) are effective in blocking IκBα proteolysis at concentrations that interfere with proteasome activity (31, 37). Based on this inhibitor sensitivity profile, the enzyme(s) in the 26 S proteasome complex mediating IκBα hydrolysis has been characterized as chymotrypsin-like. In this regard, we are surprised to find in hepatocytes, that calpain inhibitor I blocks IκBβ proteolysis relatively selectively. These data indicate a potential involvement of cell-type specific factors in the inducible proteolysis of IκBα.

Overshoot of IκBα Synthesis: Evidence for Exaggerated Rel A/IκBα Autoregulatory Pathway in Hepatocytes—Upon Rel A translocation into the nucleus, the synthesis of IκBα is activated (38, 39). Newly resynthesized IκBα reassociates with Rel A, inactivating its nuclear location and transcriptional response resulting in an autoregulatory feedback pathway. The important role of IκBα in terminating Rel A/NF-κB1 activation is illustrated in the persistent NF-κB1 activation upon rTNFα stimulation in fibroblasts cultured from mice with homozygous deletion of ikbα (40). The surprising observation in our studies is that, although IκBα is involved in terminating NF-κB action, it does so only transiently by overshoot resynthesis. That IκBα resynthesis results in a transient inhibition of Rel A/NF-κB1 DNA binding activity is supported by the following observations: 1) increased IkBα abundance by Western immunoblot (Fig. 3) is coincident with inhibition of Rel A/NF-κB1 binding at 60 min (Fig. 1A). 2) Increased IkBα is associated with Rel A by coimmunoprecipitation assays (Fig. 4A). 3) Rel A/NF-κB1 DNA binding activity from both early and late phases of induction is completely inhibited by rIκBα (Fig. 4B). 4) Rel A/NF-κB1 inhibition at 60 min is dependent on new protein synthesis (Fig. 5). At present, we are unable to explain why the increased IkBα levels at 60 min return to control levels at 120 min; this phenomenon is probably the result of accelerated turnover of IkBα in the presence of TNFα (as for LPS activation in 70Z/3 cells (23)).

Late Phase Translocation of Rel A/NF-κB1 Is Dependent on IκBβ Proteolysis—In Jurkat T-lymphocytes (24) and in mouse embryo fibroblasts (4), rTNFα produces only IκBα, but not IκBβ proteolysis, associated with a transient monotonic induction of Rel A/NF-κB1 binding. In hepatocytes, by contrast, rTNFα produces a biphasic, prolonged induction of Rel A/NF-κB1. A requirement for IκBβ proteolysis in producing the late phase of Rel A/NF-κB1 translocation is seen under conditions where IκBβ proteolysis is abolished. Earlier reports in other cell lines have shown that IκBβ is not subject to the Rel A-induced autoregulatory pathway, and once proteolyzed, is not resynthesized during the time course of these experiments (24); our data are consistent with this phenomenon. IκBβ proteolysis apparently allows for a prolonged nuclear response to the actions of TNFα in a cell restricted pattern. Based on their similar domain organizations and similar rapid proteolytic response to TNFα, it might be expected that IκBα and IκBβ would be proteolyzed through similar pathways. However, several features in HepG2 cells indicate that the mechanism for signal-induced proteolysis of IκBβ is distinct from that used for IκBα. Phosphorylated IκBα isoforms migrate more slowly than nonphosphorylated forms (28, 30), allowing their identification by Western immunoblot. In calpain inhibitor I-treated cells, rTNFα-inducible slower migrating species of IκBα corresponding to phosphorylated forms can be detected upon longer exposure. No such forms are seen with IκBβ (Fig. 6, middle panel), even under conditions when complete inhibition of proteolysis is observed. Second, and more importantly, proteolysis of IκBα is insensitive to 200 μM calpain inhibitor I whereas proteolysis of IκBβ is completely blocked by this concentration. These data probably indicate that rTNFα activates IκBβ proteolysis through two distinct pathways.

In summary, our studies have focused on the mechanisms for the novel observation of a biphasic induction of Rel A/NF-κB1 binding in hepatocytes. Rel A/NF-κB1 is associated with three IκB isoforms that are differentially regulated by rTNFα, producing pools of constitutive and inducible NF-κB complexes. We provide evidence for how dynamic changes in IκBα abundance influences the two distinct phases of Rel A/NF-κB1 translocation. Moreover, we report that separate proteolytic pathways are involved in IκBα and IκBβ degradation in hepatocytes producing unanticipated complexity in signal-induced regulation of IκBα abundance.

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