RelA Control of IκBα Phosphorylation
A POSITIVE FEEDBACK LOOP FOR HIGH AFFINITY NF-κB COMPLEXES*

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Lin Yang‡‡, Kehinde Ross‡, and Eva E. Qwarnstrom‡¶
From the 3Cell Biology Unit, Functional Genomics, Division of Genomic Medicine, The Medical School, University of Sheffield, Sheffield S10 2JF, United Kingdom and the 4Department of Pathology, School of Medicine, University of Washington, Seattle, Washington 98195

NF-κB-IκB complex formation regulates the level and specificity of NF-κB activity. Quantitative analyses showed that RelA-NF-κB-induced IκBα binding is regulated through inhibitor retention and phosphorylation. RelA caused an increase in IκBα phosphorylation and in degradation, which was enhanced monotonically with inhibitor concentration. In vivo analysis demonstrated the RelA-induced IκBα/RelA interactions to be specific, saturable, and phosphorylation-dependent. In addition, it showed that phosphorylation regulates both the level and affinity of the complexes and demonstrated an increased average affinity to coincide with reduction in the level of complexes during cytokine-induced pathway activation. The data show that RelA regulation of NF-κB-IκBα complex formation is IκBα phosphorylation-dependent and that IκBα/NF-κB binding is dynamic and determined by concentration of the subunits. In addition, they suggest that regulation of both complex levels and affinities through phosphorylation, with effects on the system steady state, participate in selective activation of the NF-κB pathway.

NF-κB transcription factors are central to inflammatory and immune responses (1–3). They are composed of homo- and heterodimers of a family of at least five related subunits, characterized by a ~300-amino acid Rel homology domain (4). Activation is controlled by IκB (inhibitor of NF-κB) proteins, the degradation of which is a critical step in activation of NF-κB (5). The IκB family includes seven proteins containing multiple ankyrin-like repeats, which mediate interaction with the Rel homology regions of NF-κB dimers. IκBα, the most extensively studied protein in this family, interacts primarily with p50/RelA and p50/c-Rel heterodimers but also with c-Rel and RelA homodimers (4, 6). IκBα inhibits NF-κB activation by masking its nuclear localization signals and thereby sequestering the two proteins in the form of complexes in the cytoplasm (7).

Induction of the pathway is mediated by a significant number of stimuli, including cytokines, lipopolysaccharides, cellular stress, and cell adhesion (4, 8, 9). During activation, NF-κB is transported into the nucleus and activates transcription of genes bearing cognate binding motifs (4). Transport is made possible by dissociation of complexes following phosphorylation of IκB on serines 32 and 36 by IκB kinases, ubiquitination, and proteasome degradation (10–13). The significance of IκB/NF-κB interactions as a regulatory step in pathway activation has been extensively documented (6, 14–16).

Here we report the use of the fusion protein IκBαEGFP1 to examine regulation of the function of IκBα by NF-κB. RelA regulation of IκBα and of IκBα/NF-κB interactions were analyzed in single living cells, using confocal microscopy, showing that all aspects of IκBα function are critically dependent on that of NF-κB and that phosphorylation of the inhibitor constitutes the basis for interaction and selection during pathway activation.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid pIκBαEGFP encodes a red-shifted variant of green fluorescent protein fused to the carboxyl terminus of IκBα, constructed by cloning the IκBα cDNA from pIκBα cotg (kind gift from Prof. Ronald Hay) (17) digested with BglII plus EcoRI into pEGFP-N2 (Clontech) digested with BglII plus EcoRI and in-filling of a SmaI plus EcoRI digest for alignment of the reading frame. Plasmid pIκBαEYFP was constructed by subcloning the same IκBα fragment into pEYFP-N1 (Clontech), digested with BglII plus EcoRI as described (18). Plasmid pECFP-relA was constructed by cloning the relA cDNA from pBlue-script-relA (19), digested with HindIII plus BamHI, into similarly prepared pECFP-C1 (Clontech). Plasmid pECFP-p50 was constructed by cloning the p50 cDNA from pSV-NFκB1/p50 (19) digested with HindIII plus BglII into pECFP-C1 (Clontech) and in-filling of the Xhol site. Site-directed mutagenesis of pIκBαEGFP (Ser → Ala at positions 32 and 36) was performed using the Muta-Gen Phagemid kit (Bio-Rad). Mutagenesis of pECFP-relA to obtain nonfluorescent fusion proteins was performed by deletion of residues 65–67 (Thr, Trp, and Gly) within the ECFP N terminus, critical for fluorescence, using the QuikChange™ site-directed mutagenesis kit (Stratagene). Oligonucleotides were synthesized from Life Technologies. All constructs were confirmed by sequence analysis.

Tissue Culture and Transfection—Human gingival fibroblasts (HGFQ1 and HGFQ2, transfer 10–19) and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) and 5 mM sodium pyruvate, 100 μg/ml penicillin and streptomycin and kept at 37 °C in a 95% O2, 5% CO2 atmosphere. Cells were plated 24 h prior to transfection and transiently transfected using calcium phosphate co-precipitation with glycerol shock (60 s, 15% glycerol in phosphate-buffered saline) 4 h after transfection, as described previously (20, 21). Control experiments demonstrated that transfection of mediators of the NF-κB pathway, under these conditions, had no effect on endogenous cytokine production, which was significant for pathway activation (20–22). Stimulation with IL-1 (1 nM) kindly provided by Dr. Steve Poole (National Institute of...
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Biological Standards and Control was carried out 24 h after transfection for various times, as indicated.

Confocal Microscopy—EGFP fusion proteins were visualized using a confocal laser-scanning microscope (Molecular Dynamics, CLSM 2010) fitted with a 37 °C stage incubator and coupled to a Nikon Diaphot 300 microscope and a Silicon Graphics work station. Laser power was set to 10 mW, band selection to 488 nm, photo multiplier tube voltage to 750, and intensity of laser light to maximize density below 20% and within the linear range of the instrument. Emission scans were done with a × 60 Plan Apo oil immersion objective (numerical aperture of 1.4) and a 50-µm aperture generating an optical section of 0.54 µm and using a 530-nm band pass filter. To quantitate the cytoplasmic and the nuclear fusion protein level, transfected cells were scanned horizontally through the nucleus, and images were analyzed using NIH Image. Relative fluorescence was calculated by measuring mean pixel densities in representative areas of nucleus or cytoplasm and normalized by dividing by the attenuation and further by 2.41 for consistency with previous results obtained with a photo multiplier tube of 666 V (20).

Western Analysis—The relative levels of endogenous and exogenous IκBα were determined by Western analyses as described (20) using cells transfected with ctag-containing, or fluocrome-tagged (pEGFP, pEFYP, pECFP, plkBcEYPF, or pECFPSerA) NF-κB signaling components to make it possible to distinguish endogenous and exogenous proteins. Cell extracts were prepared by lysis in 10× Laemmli sample buffer (23), resolved on 12.5% SDS-PAGE, and transferred to polyvinylidine difluoride membrane (Amersham Biosciences) with β-actin used as an internal control. Total and phosphorylated IκBα were detected using primary antibodies (anti-IκBα and anti-IκBαSer53) and secondary antibodies (1:2000, Santa Cruz Biotechnology) and streptavidin/Texas Red (0.2 g/ml, 15 min; Amersham Biosciences). Data (red fluorescence) were acquired using 750 V (yellow fluorescence). All data were analyzed using MLAB for the Macintosh (Civilized Software, Silver Spring, MD).

Immunofluorescence—Immunofluorescent staining of transfected cells was carried out as described previously (20). Briefly, incubation with a rabbit polyclonal IκBα antiserum (1 µg/ml, 1 h, room temperature; Santa Cruz Biotechnology) of cells transfected with IκBαEGFP was carried out following fixation in methanol (−20 °C, 5 min) and incubation in blocking serum (5%, 1 h) and prior to incubation with biotinylated secondary antibody (2 µg/ml, overnight at 4 °C; Santa Cruz Biotechnology) and streptavidin/Texas Red (0.2 µg/ml, 15 min; Amersham Biosciences). Data (red fluorescence) were acquired using 750 V with excitation at 588 nm and emission at 590 nm. Excitation and emission of the NF-κB fusion protein was done at 488 and 530 nm, respectively, as above. Green and red fluorescence was plotted for individual cells over a range of transfection levels. The level of GFP fluorescence was corrected for minor reduction in intensity (15%) induced by methanol fixation.

Immunoprecipitation—Biochemical analysis of complex formation was carried out by immunoprecipitation using an anti-ReA antibody (Santa Cruz Biotechnology), followed by Western analysis. A total of 106 cells transfected with wild type and mutant plkBcEYPF, alone or together with pCMV:relA, before and after IL-1 stimulation. Thus, HeLa cells seeded at 3 × 106 cells/10-cm plate were transfected with the indicated cDNAs, totaling 4.4 µg/plate, and after 24 h they were left unstimulated or treated with IL-1B for 8 and 40 min and lysed. Immunoprecipitation of IκBα was carried out as above, followed by incubation with 5 µg of anti-ReA antibody and protein G conjugated to Sepharose beads (overnight, 4 °C, Sigma). Following resuspension in lysis buffer, precipitates were separated by gel electrophoresis (20% SDS, Tris-HCl) and transferred to polyvinylidine difluoride membranes for Western analysis, as above.

FRET Analysis—Images of ECFP, EYFP, and FRET were obtained through a × 60 Plan Apo oil immersion objective (numerical aperture of 1.4) using a Nikon Diaphot 300 microscope and recorded by a 12-bit Hamamatsu digital camera C4742-95 driven by OpenLab software (Improvision). Complex formation was determined through measuring close association of fluorophores ECFP and EYFP by excitation of the donor, resulting in an increase in acceptor emission fluorescence and/or a decrease in donor fluorescence due to FRET (24–26) using a series of filter sets (Omega Optical): XF114 for ECFP (440DF21 excitation, 455DRLP dichroic, 480DF30 emission), XF104 for EYFP (500DF25 excitation, 525DRLP dichroic, 545DF35 emission), and XF88 for FRET (440DF21 excitation, 455DRLP dichroic, 545DF35 emission). Relative fluorescence was calculated for the various emission settings by measuring mean intensity in representative areas of nucleus or cytoplasm and normalized by dividing by the attenuation, as above. All images were corrected for background, and FRET images were further processed by subtracting overspill of ECFP (50.7%) and EYFP (30.4%) fluorescence assessed using vectors containing ECFP or EYFP only. The accuracy of the FRET signal was further confirmed by inhibition of emission at 545 nm after photobleaching the acceptor-EYFP at 550-nm excitation. In addition, control experiments included demonstration of a correlation between the reduction in yellow fluorescence and increase in cyan intensity.

Binding Analyses—The level of IκBα/RelA binding was measured by FRET following co-transfection with IκBαEYFP (yellow fluorescence) and ECFPrelA (cyan fluorescence), before and after IL-1 stimulation (30 min) in four independent experiments. The FRET signal from single transfected cells expressing a range of IκBα or S23A/S36A-phospho-deficient IκBα mutant (ligand) concentrations was quantitated, as reported for assessment of protein/protein interactions in a variety of systems in live cells (27–30). Analysis was limited to including cells expressing levels of fusion protein of up to 8–10 times that of the endogenous levels, corresponding to fluorescence of 2.5–3, to ascertain reliable cytokine responses (20, 21). Readings were ranked according to yellow fluorescence (IκBαEYFP), and to increase accuracy, they were pooled in consecutive groups of three for data analysis. The specificity of the interaction was demonstrated by competition of the IκBαEYFP binding carried out by co-transfection of larger size pools (pCMV:relA or pCMV:50), or using a mutant pECFPrelA lacking the cyan fluorescent site. Saturated binding and apparent dissociation constants were estimated by nonlinear least squares fitting of a noncooperative one-site equilibrium-binding model of the FRET versus IκBα (yellow fluorescence). All data were analyzed using MLAB for the Macintosh (Civilized Software, Silver Spring, MD).

Cellular concentrations of the various fusion proteins were determined by Western analysis of transfected cells as above and were included in calculations of binding affinity (Kd) using the equation

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\text{Bound (molecules/cell)} = \frac{\text{Receptor} \times K_a \times [\text{Ligand}]}{1 + K_a \times [\text{Ligand}]}
\]

where Bound (molecules/cell) represents the level of bound IκBα/cell determined by FRET, Receptors/cell or Binding protein is RelA concentration (molecules/cell); and [Ligand] is IκBα concentration (molecules/cell).

Controls included calculations using various size pools for data analysis (groups of 3–6), as well as using larger data sets allowing up to 12 individual cell readings for each IκBα concentration, all demonstrating the same results as regards specific binding sites and affinity values. In addition, the single cell analysis was assessed by determining the R2 values. These were found to be the same or higher than those obtained from conventional binding analysis, varying by 15 ± 5%, demonstrating a high level of accuracy, and further revealing an underlying Gaussian distribution as regards cell/cell variation.

RESULTS

Kinetic analysis of single cell data obtained by confocal microscopy demonstrated a pronounced enhancement of IκBαEGFP degradation by co-transfection with RelA alone or together with p50, increasing IL-1-induced turnover from 60 to 90–95% (Fig. 1, A and B). In contrast, levels of a serine 32/36 phosphorylation-deficient IκBα mutant (31–33) were unaffected by IL-1 stimulation, either when transfected alone or following co-transfection with NF-κB subunits. In addition, there was a positive correlation between the magnitude of the effect and the initial cytoplasmic levels of IκBα (Fig. 1C).
the average endogenous levels (Fig. 3B). Quantitation by Western analysis of cells transfected with pIxBαEGFP and pECFP-PreLA showed average concentrations of 400,000 and 370,000 molecules/cell for IxBαEGFP and ECFPPrelA (Fig. 3C), respectively, and revealed endogenous IxBα levels to be about 135,000 molecules/cell, 2.5-fold higher than that estimated for the average levels of endogenous RelA, as expected (20, 22), with all values close to the ranges demonstrated for the endogenous proteins (22).

Confocal microscopy further demonstrated retention of IxBα in the cytoplasm by RelA (Fig. 4). Thus, an increase in nuclear localization of tagged (Fig. 4A, 1–4) or untagged (Fig. 5A, 5 and 6) inhibitor at higher expression levels (Fig. 4A, 3 and 5) was not observed in the presence of a comparable amount of the NF-κB subunit (Fig. 4A, 4 and 6). In addition, quantitation over a wide range of IxBαEGFP expression levels showed the effect to correlate with increasing levels of (pCMV) relA in a concentration-dependent manner (Fig. 4, B and C).

The RelA/IxBα interdependence was further analyzed by simultaneous observations of RelA and IxBα using cyan (ECFP) and yellow (EYFP) variants of GFP, respectively (pECFPrelA and pIxBαEYFP) (Fig. 5A). These experiments demonstrated that in the unstimulated state, both subunits were located in the cytoplasm. Quantitation showed that, similarly to experiments using EGFP containing constructs (20), IL-1 stimulation caused a successive increase in nuclear RelA but had no appreciable effect on cytoplasmic levels (Fig. 5B).

Similarly, the rate of reduction of cytoplasmic inhibitor levels during activation was the same as that using the green fluorescent protein (Fig. 5B and 2B) and coincided with a reduction in the much lower levels of nuclear IxBα. The levels of RelA/IxBα complexes were assessed by measuring FRET using ECFPrelA as a donor and IxBαEYFP as the acceptor (34). FRET was observed in cells expressing both fusion proteins demonstrating in vivo association of IxBαEYFP and ECFPrelA (Fig. 5A, 6 and 9), not seen in control cells, transfected with constructs containing EYFP and ECFP only (Fig. 5A, 3). A reduction in FRET signal (compare 6 and 9) following cytokine stimulation correlated with nuclear translocation of RelA (compare 4 and 7) and a decrease in cytoplasmic IxBα (compare 5 and 8). Both the level of FRET and of IxBα showed a steady state at about 50% after 30 min of IL-1 stimulation (Fig. 5B). The addition of RelA caused a concentration-dependent increase in the level of complexes over a range of inhibitor concentrations, reaching successively higher levels of saturation (Fig. 5C).

Analysis by immunoprecipitation of transfected cells demonstrated that RelA caused an increase in IxBα-containing complexes, both in control and IL-1-stimulated cells. Using a phosphospecific antibody, the experiments similarly demonstrated a significant increase in the level of complexed phosphorylated inhibitor in the presence of RelA (Fig. 6A). Transfecting the Ser32/Ser36-phosphodeficient mutant, however, resulted in a significant reduction in IxBα/RelA interactions, in both control and IL-1-stimulated cultures (Fig. 6B). Quantitation further demonstrated a correlation between effects on complex levels (1.96 ± 0.4) and phosphorylation (2.35 ± 0.58) using tagged or untagged RelA, before and after stimulation, with unaffected low levels using the phosphodeficient IxBαEGFP mutant (Fig. 6C).

The NF-κB/IxBα interaction was further characterized using FRET by measuring signals from transfected cells expressing a range of IxBα levels and modeling the data by noncooperative binding of ligand (IxBα) to a single class of sites (RelA). Specificity of the interaction was demonstrated by competition by increasing amounts of pCMVrelA or pCMV p50, or of a non-

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**Fig. 1. NF-κB enhances IL-1-stimulated inhibitor degradation at high levels of IxBα.** A, series of confocal images of fibroblasts, co-transfected with equal amounts of pIxBαEGFP and pCMVrelA, obtained at various times after the addition of IL-1β as indicated. Bar, 20 μm. B, average IxBαEGFP levels were determined at various times during IL-1β stimulation and transfected with pIxBαEGFP or a mutant pIxBαEGFP, lacking phosphorylation sites at serines 32 and 36, alone or co-transfected with pCMVrelA or with pCMVrelA and pCMVp50. C, the percentage degradation of the IxBαEGFP fusion protein following transfection with pIxBαEGFP alone or together with pCMVrelA or together with pCMVrelA and pCMVp50, following 15- or 60-min stimulation with IL-1β, was determined for individual cells and plotted against initial fluorescence. A best fit logarithmic curve to the data is shown.

The RelA-mediated effect on turnover was confirmed by Western analysis, which, in addition, demonstrated an enhanced phosphorylation of IxBαEGFP in IL-1-stimulated cells following co-transfection (Fig. 2, A and B). The RelA-mediated effect on phosphorylation of the inhibitor protein resulted in a 2-fold increase in non-IL-1-stimulated levels and caused a 50–100% enhancement of the peak value at 5–10 min of IL-1 stimulation. In addition, these experiments showed similar but less pronounced effects on the endogenous protein.

The dependence of the RelA-induced effects on IxBα concentration, demonstrated by single cell analysis, prompted quantitation of inhibitor levels. Immunocytochemical staining of transfected cells and monitoring of red and green fluorescence demonstrated a linear relationship between total and transfected levels of the protein over a wide range of concentrations (Fig. 3, A and B). A mean transfection level of 1 fluorescent unit corresponded to an increase in cytoplasmic IxBα to about 3-fold
fluorescing mutant of ECFPrelA (ECFPrelAmut) (Fig. 7A). This showed a dose-dependent inhibition of the FRET signal, which corresponded to about 70% at 4-fold overexpression of either of the RelA constructs, whereas co-transfection with p50 had no effect. Further, in agreement with biochemical data, shown in Fig. 6C, using the phosphorylation site-deficient IκBα-mutant resulted in about a 50% reduction in specific RelA binding (Fig. 7A).

In addition, these experiments showed that IκBα/RelA interaction (FRET) is dynamic and dependent on IκBα concentration (EYFP fluorescence), demonstrating saturation before and after incubation with IL-1 (Fig. 7B). A pronounced fall in the FRET signal after 30 min of IL-1 stimulation corresponded to a reduction in complex concentration to 38 ± 8%, correlating with the decrease determined by immunoprecipitation (see Fig. 6). In comparison, using the phosphonegative mutant resulted in a much decreased level of complexes, which were unaffected by IL-1 stimulation (Fig. 7B). Characterization of the interaction by Scatchard analysis demonstrated a pronounced (4.2 ± 1.0-fold) increase in affinity ($K_a$) of RelA binding to the wild type inhibitor following stimulation with the cytokine, relative to that calculated for control cultures (Fig. 7C). Quantitation of fusion protein levels, as in Fig. 3C, gave estimated affinity constants ($K_a$) for these interactions in the range of 10$^7$ to 5 × 10$^7$ M$^{-1}$ in unstimulated cells and between 5 × 10$^7$ and 5 × 10$^8$ M$^{-1}$ following IL-1 stimulation, respectively. In comparison, the affinity of RelA for the phosphonegative mutant was only 25% of that for the wild type and, in addition, was unaffected by cytokine activation (Fig. 7C).

**DISCUSSION**

The data presented in this report are in broad agreement with the widely accepted model based on NF-κB/IκB interdependence and regulation through IκB phosphorylation. The real time GFP measurements on single cells, in addition, show that the IκBα/NF-κB interaction is specific, saturable, and dynamic; that the RelA-induced increase in binding relies on trafficking; and that regulation of complex formation and of the affinity of the interaction are IκBα phosphorylation-dependent.

Our data underscore the reciprocal nature of NF-κB/IκB regulation, coupled with a system in which free components successively accumulate in the nucleus with increasing levels, and bound complexes largely remain in the cytoplasm, in the resting state (20). This is reflected in the inverse correlation between the inhibitor cytoplasmic concentration and the ratio of cytoplasmic to nuclear levels and by the cytoplasmic accumulation of IκBα in the presence of excess RelA. Regulation is probably influenced by the level of the endogenous proteins and of de novo synthesis through autoregulation (35), since the low transfection levels of the GFP fusions, corresponding to 2–3-
fold that of the endogenous protein, are well within the physiological range of variation (22). Thus, the successive increase in nuclear levels at higher concentrations of IkB-EGFP probably reflects titering out of endogenous NF-kB. Further, competition for the endogenous pool of NF-kB could account for the enhanced level of breakdown of IkB-EGFP and the limited effect of co-transfection at low levels of exogenous inhibitor (22).

Enhanced cytoplasmic retention of IkB-α, such as that induced by RelA, has significant effects on pathway regulation (36–41). This is supported by mathematical modeling, which shows that the NF-κB system is dissipative and far from equilibrium and is at a steady state determined by dissociation of complexes, intercompartmental trafficking, and synthesis and breakdown of components (21). Further, simple kinetics (Briggs-Haldane) show that saturable NF-κB/IkBα binding, under steady state conditions, can occur with apparent affinity constants much lower than those observed with pure components in vitro, in agreement with the lower affinities measured in our study, compared with those observed using Biorex (42).

The involvement of basal cycling in regulation (21), indicated by the slightly delayed decrease in FRET (47%) compared with that of the inhibitor fluorescence (56%) (significant at 5%), suggests a reassociation of released ECFFrelA with free IkBαEYFP prior to nuclear entry. The biological significance of RelA regulates localization of IkBα. A, confocal micrographs of fibroblasts transfected with lower (1 and 2) and higher (3 and 4) levels of pIκBαEGFP or higher levels of pCMV:IkBα (5 and 6), alone (1, 3, and 5) or co-transfected with pCMV:relA (2, 4, and 6), demonstrating increased nuclear levels of the fusion protein in cells with high levels of transfection (3 and 5), which are reduced in the presence of RelA (4 and 6). Bar, 20 μm. B, quantitation of nuclear and cytoplasmic levels of cells transfected with pIκBαEGFP alone or co-transfected with increasing amounts of pCMV:relA (as indicated), over a range of concentrations. C, quantitation of average nuclear and cytoplasmic fluorescence after transfection with pIκBαEGFP alone and together with increasing concentrations of pCMV:relA.
increased levels of RelA, can have marked effects on both the extent and mechanism of NF-κB-induced gene transcription.

Enhanced inhibitor phosphorylation in the presence of exogenous RelA is probably to some extent due to an increase in NF-κB-IκB complexes and reflects preferential phosphorylation of complexed inhibitor by IκB kinases (48). Conversely, the pronounced reduction in RelA/IκB binding at steady state, observed with the 32/36 mutant, demonstrated dependence of complex formation and affinity on IκB phosphorylation, as found for interactions through the PEST domain of the protein (49). In the absence of a direct involvement of the IκBα signal-receiving domain in NF-κB interaction (42), the effects on specific binding, observed using the mutants, could be explained by changes in inhibitor conformation in vivo. A decrease in binding of IκBα proteins lacking the phosphorylation sites necessary for proteosome degradation (6, 31, 32), is consistent with a critical role for RelA in controlling IκBα turnover. Of the three components of the classical κB complex, RelA is present at the lowest concentration (22). Linking IκBα turnover to RelA levels will therefore optimize signals through the IκB. In addition, ongoing studies suggest that further amplification through positive feedback is induced by increased activation of upstream kinases in the presence of excess RelA. This is in agreement with a system based on reutilizing a limited amount of RelA through repeated cycles of IκBα binding, phosphorylation, and degradation with continuous enhanced effects with increasing RelA concentrations.

Alterations in the level and affinity of IκBα-NF-κB complexes correlated with the rate of inhibitor degradation. With only 20% of NF-κB free of IκBα (20), a 4-fold change in complex affinity is likely to have a significant role in regulation of inhibitor turnover, subsequent to effects on basal shuttling and reduction in free NF-κB. The identical results obtained in the blocking experiments, using tagged and untagged RelA, show that the lower affinities we observe in vivo compared with that measured with purified proteins (41) are not artifacts of GFP tagging (50). The increase in NF-κB/IκBα affinity correlates with the enhanced cytoplasmic FRET/IκBαEYFP ratio following IL-1 stimulation, suggesting a selective process. This is also supported by the lack of a simple inverse correlation between the affinity and the level of complexes and indicates that low affinity complexes are specifically targeted for degradation during cytokine activation. The biological significance of this selection could be to facilitate a rapid release in response to a transient signal through the low affinity complexes, characterized by a high dissociation rate. The heterogeneity of NF-κB-IκBα complex affinity may thus constitute a buffer to incoming signals.

In summary, the data show that translocation, phosphorylation, and complex formation are all critical for regulation of IκBα steady state levels by NF-κB. They demonstrate that
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Fig. 6. RelA induces phosphorylation-dependent complex formation. A, cells transfected with IκBαEYFP alone or co-transfected with an ECFPRelA-containing construct were stimulated with IL-1 for 0 or 8 min. Extracts were subjected to immunoprecipitation using an anti-RelA antibody and separated on a 12.5% SDS gel, and the levels of RelA, IκBα, and phospho-IκBα for the various conditions were determined by Western analysis, as described under “Experimental Procedures.” Similarly, the level of IκBαEYFP in the total lysate was assessed by Western analysis. One of two experiments is shown. B, cells were transfected with wild type IκBαEYFP or with a mutant lacking phosphorylation sites Ser23 and Ser36, together with ECFPRelA, and stimulated with IL-1 for 0 or 8 min, as above. Complexes were immunoprecipitated following extraction by using an anti-RelA antibody, separated on a 12.5% gel, and subjected to Western analysis, as in A. One of three experiments is shown. C, levels of total and phosphorylated complexes were determined for cells transfected with untagged or fluorescence-tagged IκBα or with a fluorescence-tagged mutant IκBα lacking phosphorylation sites serine 32 and 36, together with untagged or tagged RelA as indicated, and stimulated with IL-1 for 0, 8, or 40 min. Quantitation was carried out following immunoprecipitation, gel electrophoresis, and Western analysis, as above, by scanning autoradiograms. Left panel, the level of phosphorylated complexes at 0 and 8 min. Data represent the average of three experiments and are expressed relative to total IκBα in the lysate. Right panel, the total level of complexes at 0 and 40 min. One representative experiment of three is shown, with data expressed relative to total RelA.

IκBα/NF-κB binding is dynamic, saturable at steady state, and phosphorylation-dependent. They also show that both the extent and the affinity of the IκBα/NF-κB interaction play a role in system regulation, affecting both basal levels and those induced in response to incoming signals. The multifaceted effects of RelA on the concentration-dependent limitation of inhibitor turnover thus constitute a complex feedback loop, with effects on the system steady state and with expected significance for the specificity and level of NF-κB activity.

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REFERENCES
1. Kopp, E. B., and Ghosh, S. (1995) Adv. Immunol. 58, 1–27
2. Raserer, P. A., and Baltimore, D. (1996) Cell 87, 13–20
3. Christman, J. W., Lancaster, L. H., and Blackwell, T. S. (1998) Intensive Care Med. 24, 1131–1138
4. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
5. Israel, A. (1995) Trends Genet. 11, 203–205
6. Beg, A. A., and Baltimore, D. S. (1995) Genes Dev. 9, 2064–2070
7. Beg, A. A., Ruben, S. M., Scheinman, R. I., Haskell, S., Rosan, C. A., and Baldwin, A. S., Jr. (1999) Genes Dev. 6, 1899–1913; Correction (1999) Genes Dev. 6, 2064–2065
8. Beg, A. A., and Baltimore, D. (1996) Science 274, 782–784
9. Qwarnstrom, E. E., Ostberg, C. O., Turk, G. L., Richardson, C. A., and Bomszyk, K. (1994) J. Biol. Chem. 269, 30765–30768
10. Dilhara, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S., and Karin, M. (1996) Mol. Cell. Biol. 16, 1295–1304
11. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) Science 278, 860–866
12. Woronicz, J. D., Gao, X., Cao, Z., Rothe, M., and Goeddel, D. V. (1997) J. Biol. Chem. 272, 866–869
13. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252
14. Karin, M. (1999) J. Biol. Chem. 274, 27339–27342
15. Van Antwerp, D. J., and Verma, I. M. (1996) Mol. Cell. Biol. 16, 6037–6045
16. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Vanantwerp, D., and Miyamoto, S. (1996) Genes Dev. 9, 2725–2735
17. Rodriguez, M. S., Michalopoulos, J., Arenzana-Seisdedos, F., and Hay, R. T. (1995) Mol. Cell. Biol. 15, 2413–2419
18. Yang, L., Chen, H., and Qwarnstrom. E. (2001) Biochem. Biophys. Res. Commun. 285, 603–608
19. Ducket, C. S., Perkins, N. D., Kowalik, T. F., Schmid, R. M., Huang, E. S., Baldwin, A. S., Jr., and Nabel. G. J. (1993) Mol. Cell. Biol. 13, 1315–1322
20. Carlotti, F., Chapman, B., Dower, S. K., and Qwarnstrom, E. E. (1999) J. Biol. Chem. 274, 37941–37949
21. Carlotti, F., Dower, S. K., and Qwarnstrom, E. E. (2000) J. Biol. Chem. 275, 41028–41034
22. Schleser, K. Z., Dower, S. K., and Qwarnstrom, E. E. (2003) Biochem. J. 369, 331–339
23. Lassen, M. (1975) Nature 252, 680–685
24. Clegg, B. M. (1995) Curr. Opin. Biotechnol. 6, 103–110
25. Herman, B. (1989) Methods Cell Biol. 30, 219–243
26. Szollosi, J., Damjanovich, S., and Matyus, L. (1988) Cytometry 14, 159–179
27. Adams, B., Haroutunian, A. T., Buescher, Y. J., Taylor, S. S., and Tsien, R. Y. (1991) Nature 349, 694–697
28. Damelin, M., and Silver, P. A. (2000) Mol. Cell 5, 133–140
29. Mochizuki, N., Yamashita, S., Urykawa, K., Obha, Y., Naga, T., Miyawaki, A., and Matsuda, M. (2001) Nature 411, 1065–1067
30. Zuccolo, M., and Puzzo, T. (2000) IUBMB Life 49, 375–379
31. Brown, R., Gerstberger, S., Carlsson, L., Franzoso, G., and Siebenlist, U. (1995) Science 267, 1485–1491
32. Chen, Z., Hagler, J., Palombella, V. J., Malsandi, F., Scherer, D., Ballard, D., and Manuzzi, T. (1995) Genes Dev. 9, 1586–1597
33. Li, X., Fang, Y., Zhao, X., Jiang, X., Duong, T., and Rain, S. R. (1999) J. Biol. Chem. 274, 21244–21250
34. Mahajan, N. P., Linder, K., Berry, G., Gordon, G. W., Heim, R., and Herman, B. (1998) Nat. Biotechnol. 16, 547–552
35. Chiao, P. J., Miyamoto, S., and Verma, I. M. (1994) Proc. Natl. Acad. Sci. U. S. A 91, 28–32
36. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) EMBO J. 18, 6682–6693
37. Huang, T. T., Kudo, N, Yoshida, M., and Miyamotot, S. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1014–1019
38. Tam, W. F., Lee, L. H., Davis, L., and Sen, R. (2000) Mol. Cell. Biol. 20, 2269–2284
39. Turpin, P., Hay, R. T., and Dargemont, C. (1999) J. Biol. Chem. 274, 6804–6812
40. Pringent, M., Barlat, I., Langen, H., and Dargemont, C. (2000) J. Biol. Chem. 275, 36441–36449
41. Caunt, C. J., Kiss-Toth, E., Carletti, F., Chapman, R., and Qwarnstrom, E. E. (2001) J. Biol. Chem. 276, 6280–6288
42. Malek, S., Huxford, T., and Ghosh, G. (1997) J. Biol. Chem. 272, 25427–25435
43. Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1999) Mol. Cell. Biol. 15, 2689–2696
44. Jefferies, C., Bowie, A., Brady, G., Cooke, E. L., Li, X., and O’Neill, L. A. (2001) Mol. Cell. Biol. 21, 4544–4553
45. Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999) J. Biol. Chem. 274, 9108–9115
46. Zhang, H., Vuk, R. E., and Ghosh, S. (1998) Mol. Cell 1, 661–671
47. Hoffman, A., Levchenko, A., Scott, M., and Baltimore, D. (2002) Science 298, 1241–1245
48. Zandi, E., Chen, Y., and Karin, M. (1999) Science 281, 1360–1363
49. Phelps, C. B., Sengbazhalangney, L, Huxford, T., and Ghosh, G. (2000) J. Biol. Chem. 275, 29846–29846
50. Schmidt, J. A., Birbach, A, Hofer-Warinke, R., Pengg, M., Burner, U., Furtmüller, P. G., Binder, B. R., and de Martin, R. (2000) J. Biol. Chem. 275, 17035–17042
