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Degradation of IκBα Is Limited by a Postphosphorylation/ Ubiquitination Event

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Regulation of IκBα during activation was examined using EGFP. Single cell analysis showed that both localisation- and cytokine-induced degradation of IκBα are dependent on expression levels. Cells expressing higher levels of the inhibitor demonstrated an increase in nuclear IκBαEGFP with a pronounced enhancement in the nuclear/cytoplasmic ratio. Enhancing the levels of the endogenous IκBα by relA transfection caused significant reduction in IL-1-mediated degradation of the fusion protein. Similarly, IκBαEGFP-transfected cells showed an inverse correlation between the level of the fusion protein and IL-1-mediated degradation. Comparing absolute levels demonstrated a biphasic response, with reduction in cells expressing over 15-fold that of endogenous levels. Further experiments using Western analysis showed a positive correlation between both phosphorylation and ubiquitination of IκBαEGFP, and the level the inhibitor. In contrast, and in agreement with the single cell analysis, while IL-1 stimulation caused the expected degradation at lower levels of the fusion protein, breakdown of IκBαEGFP was totally inhibited at the higher transfection levels. The data show that turnover of IκBα is saturable and suggest that limitation of the pathway by enhanced inhibitor expression is regulated through a post phosphorylation/ ubiquitination event, at the level of degradation.

Key Words: **transcription factors; signal transduction; NF-κB; IκBα; IL-1β.**

The NF-κB transcriptional factors play an important role in the regulation of the expression of a number of cellular genes involved in inflammation and immune responses (1–3). The activity of NF-κB is controlled by its inhibitor, IκB, which binds NF-κB in a complex and inhibits its activation (4–6). IκB proteins including IκBα, IκBβ, IκBγ, IκBε, p105, p100 and Bcl-3 are involved in the regulation of NF-κB. IκBα, a 37-kDa protein, is the first member of this family to be cloned (7, 8) and the best characterised IκB. It interacts with NF-κB through an 6-ankyrin like repeat domain in the central portion of the protein. Its N-terminal segment, referred to as the signal response domain, contains phosphorylation sites at serines 32 and 36 (9–14). While the C-terminal part of the protein contains a PEST domain, characteristic of mammalian proteins with high turnover (15).

NF-κB is activated by many agonists including cytokines (TNFα, IL-1, and IL-2), bacteria, virus, stress, and cell adhesion (3, 16–19). In response to IL-1 stimulation, IκB proteins become phosphorylated on serine 32 and 36 by IκB kinase (IKK), undergo ubiquitination and subsequently 26S proteasome-mediated degradation (20–25). This releases free NF-κB which is transported into the nucleus where it can activate genes bearing cognate binding motifs, initiating immune and inflammatory responses. Recent data demonstrate an increase in complexity of NF-κB regulation, which is in part dependent on localisation and nucleo-cytoplasmic shuttling of IκBα/NF-κB family members (26, 27).

In our present study, a fusion protein of IκBα with EGFP (IκBαEGFP) was used to assess the effect of IκBα expression on localisation and degradation of the inhibitor during IL-1β stimulation. We show that both localisation and turnover of IκBαEGFP during activation of the pathway, are dependent on the initial level of the inhibitor. Further, our data suggest that regulation induced through increased levels of the inhibitor occurs at or immediately upstream of degradation but subsequent to phosphorylation and ubiquitination, indicating the presence of a rate-limiting step at the level of proteosome activation.

Abbreviations used: IκB, inhibitor of NF-κB; NF-κB, nuclear factor κB; IL-1, interleukin 1; (E)GFP, (enhanced) green fluorescent protein; IKK, IκB kinase.

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MATERIALS AND METHODS

Materials. *pEGFP-N2* vector was from Clontech (Palo Alto, CA). Polyclonal antibodies to IκBα and phospho-IκBα were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-rabbit or mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody, anti-β-actin, tunicamycin and *N*-ethylmaleimide were purchased from Sigma. IL-1β was a gift Steve Pool at NIBSC.

Plasmids. The plasmid *pIκBαEGFP* encodes a red shifted variant of green fluorescent protein fused to the carboxyl terminus of IκBα. It was constructed by first cloning the IκBα cDNA from pIκBα ctag (28) digested with *Bam*HI plus *Eco*RI into *pEGFP-N2* digested with *Bgl*II plus *Eco*RI then infilling of a *Sma*I plus *Eco*RI digest to align the reading frames. The plasmid *pCMV:RelA* was constructed by removing the EGFP fragment (HindIII/Eco47III) from *pEGFPRelA* (29) and infilling using Klenow polymerase.

Tissue culture. Human gingival fibroblasts (two strains, transfer number: 10–19) and Hela cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% fetal calf serum (FCS), penicillin and streptomycin and kept at 37°C in a 5% CO_2 atmosphere. The cells were transiently transfected by calcium phosphate co-precipitation with glycerol shock (60 s, 15% glycerol in PBS) 4 h after transfection. For Fluorescence Microscopy, cells were plated on 8-chamber coverslips (NUNC) at 10,000 cells/chamber in 0.5 ml medium 48 h prior to transfection. Transfection was with 0.5 μg *pIκBαEGFP*/10^4 cells in a total volume of 50 μl. For Western blotting, transfection was performed with 1 or 2 μg *pIκBαEGFP*, or 2 μg *pCMV:RelA* in 4×10^5 cells/well seeded on 6-well plates. A total of 2 μg plasmid was used under all conditions by addition of appropriate amounts of empty vector (*pCMV*).

Microscopy. At various times after transfection (6, 24, 48, 72 h) EGFP fusion proteins were visualised using a Molecular Dynamics confocal laser scanning microscope fitted with a 37°C stage incubator. Laser power was set to 10 mW, band selection to 488 nm, PMT voltage to 750 and laser attenuation varied to maintain pixel density below 200 which is within the linear range of the instrument. Data were collected with a 60× Plan Apo oil immersion objective (NA 1.4) and a 50-μm aperture generating an optical section of 0.54 μm. To quantitate the cytoplasmic and the nuclear fusion protein level, transfected cells were scanned horizontally through the nucleus. Images were exported and analysed using NIH image. Relative fluorescence was calculated by measuring the mean intensity of representative areas of nucleus or cytoplasm and dividing by the attenuation, then dividing by 2.41 for consistency with previous results obtained with PMT at 666 V (29).

Western blotting. Cell extracts were prepared 24 h after transfection by lysis in 1× Laemmli sample buffer. For enhancing the levels of ubiquitinated IκBα proteins, cells were incubated with tunicamycin (5 μg/ml, 2 h), and subsequently lysed with 1× Laemmli sample buffer containing 5 mM *N*-ethylmaleimide (30). Proteins were separated on 12.5% SDS–PAGE and transferred to PVDF membrane (Amersham). Total and phosphorylated IκBα was detected using primary rabbit polyclonal anti-IκBα (sc-371, Santa Cruz Biotechnology, CA; or #9242, Cell Signaling Technology, Beverly, MA) and phospho-IκBα (#9241, Cell Signaling Technology, Beverly, MA), respectively, at 1:5000 dilution followed by HRP conjugated anti-rabbit IgG at 1:2000 dilution and visualised by ECL (Amersham) according to the manufacturer's instructions. Relative amount of total protein was assessed by determining the level of β-actin, used for correction of loading.

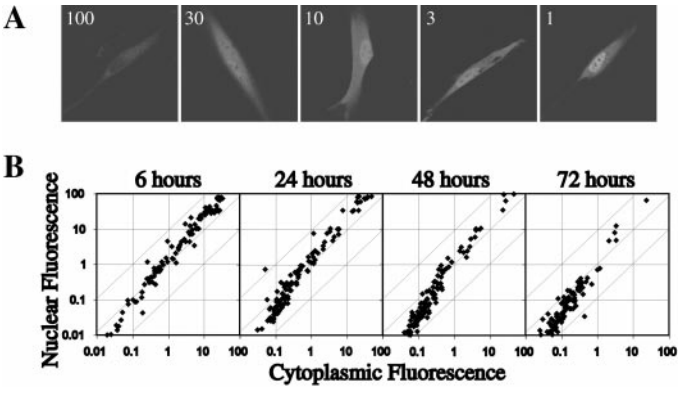

FIG. 1. Expression and intracellular distribution of IκBαEGFP in transfected fibroblasts. (A) Confocal micrographs of fibroblasts transfected with *pIκBαEGFP*. The images represent typical cells expressing lower (left) and successively higher (right) levels of the fusion protein. The incident laser power was adjusted as indicated to optimize the signal. (B) Scatter graphs showing the nuclear and cytoplasmic IκBαEGFP fluorescence in approximately 100 cells chosen at random at the indicated times after transfection. The diagonal lines mark equal nuclear and cytoplasmic fluorescence (center), 10:1 nuclear to cytoplasmic ratio (top) and 1:10 nuclear to cytoplasmic ratio (bottom).

RESULTS AND DISCUSSION

To analyse the degradation of IκBα in single living cells, we generated a fusion construct of IκBα with enhanced fluorescent protein (EGFP) (*pIκBαEGFP*). On transient transfection of *pIκBαEGFP* in fibroblasts, the fusion protein showed a wide range of expression levels, varying up to 100-fold between cells (Fig. 1A). These experiments showed that subcellular localisation of IκBαEGFP was regulated by the level of expression. In cells with low expression levels, IκBαEGFP was preferentially localised in the cytoplasm. At higher expression levels, a disproportional amount of the fusion protein was present in the nucleus, resulting in an increase in the nuclear/cytoplasmic ratio (Fig. 1B). In addition, the confocal analysis demonstrated that the level of expression was time dependent, with the majority of the cells showing successively lower levels of IκBαEGFP at later times after transfection (Fig. 1B). As expected, reduction in total IκBαEGFP correlated with a decreased amount of fusion protein in the nucleus, with a significant amount present only in a few cells after 48 and 72 h. The change in localisation with time after transfection was however less prominent than observed with EGFPrelA, which showed very low nuclear staining except in a few cells at these times (29). This likely reflects an increase in endogenous IκBα induced through NF-κB dependent autoregulation (31–33). The increase in nuclear IκBαEGFP in cells with higher expression levels implies that endogenous NF-κB in the cytoplasm was titered out, and free IκBαEGFP imported into the nucleus. This is consistent with previous studies demonstrating predomi-

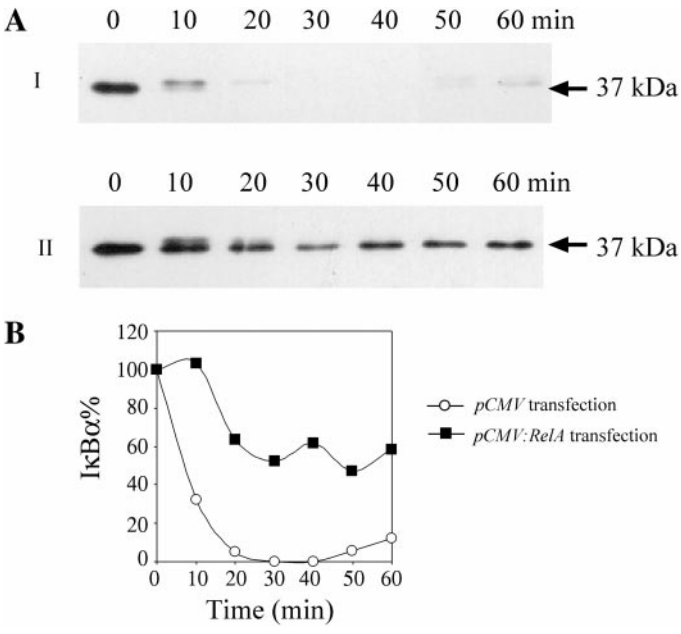

FIG. 2. Degradation of endogenous IκBα is dependent on initial cytoplasmic expression levels. (A) The level of endogenous IκBα demonstrated by Western blotting of nontransfected (I) and *pCMV:RelA* transfected (II) cells stimulated with IL-1 for various times as indicated. (B) Quantitation of Western blot as above using NIH image.

nantly cytoplasmic distribution of NF-κB-bound IκBα and nuclear translocalisation of free IκBα (34, 35).

Subsequent experiments assessed the effect of IκBα expression levels on cytokine induced degradation, initially by monitoring the impact of increasing levels of the endogenous protein. This was determined by comparing levels of total IκBα during IL-1 stimulation of cells expressing enhanced levels of the inhibitor, induced by transfection with *pCMV:RelA* (31), with levels in mock transfected cells (Fig. 2A). These experiments showed an inverse correlation between initial cytoplasmic levels and the rate of degradation. Thus, upon stimulation with IL-1, degradation of IκBα in mock transfected cells was observed initially at 5 min with a total degradation after 30 min of stimulation. In comparison, in cells with increased amount of the inhibitor, only 50% of IκBα was degraded after this time, and levels remained unchanged for up to 60 min (Figs. 2A and 2B).

To investigate the effect of inhibitor concentration on cytokine induced degradation in single cells, continued monitoring of cultures transfected with various levels of *pIκBαEGFP* and stimulated with IL-1 was carried out by confocal microscopy. Initial experiments showed a successive increase in degradation with enhanced concentrations of IL-1, reaching a plateau at 10^{-9} M (data not shown), corresponding to saturated levels both as regards IL-1 receptor binding (36) and activation of the NF-κB pathway (37). Using saturated concentrations of IL-1 these experiments showed a pronounced reduction in the level of IκBαEGFP degradation in cells with high expression levels of the inhibitor (Fig. 3A). Quantitation of such data over a wide range of expression levels showed an inverse correlation between cytoplasmic levels of IκBαEGFP and the relative level of degradation after IL-1 stimulation (Fig. 3B). In addition, correlation of absolute levels of degradation, over a range of concentrations of the inhibitor, demonstrated a biphasic response (Fig. 3C). Thus, a pronounced increase in degradation was seen in cells with concentrations up to 6 fluorescent units, corresponding to 14-fold of that of endogenous levels (38), while at higher levels, 15- to 30-fold of endogenous amounts, a pronounced reduction was observed. This biphasic response is similar to that observed for relA nuclear translocation (29) and suggests the presence of a limiting step upstream of IκBα degradation, regulated by inhibitor levels. This type of effect could be induced through a feedback mechanism, possibly involving NF-κB subunits and regulated by complex for-

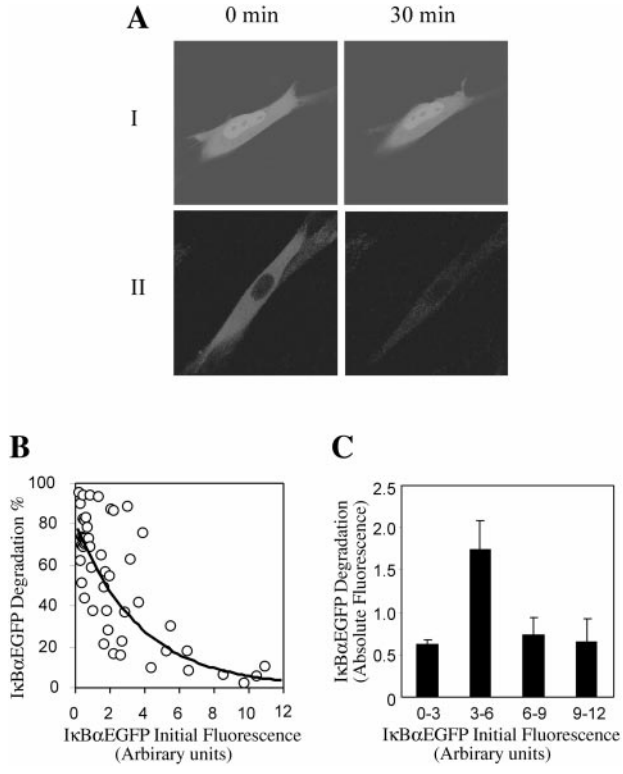

FIG. 3. Degradation of IκBαEGFP is dependent on initial cytoplasmic expression levels. (A) Confocal micrographs of fibroblasts with higher (I) and lower (II) initial levels of IκBαEGFP 24 h after transfection and at 0 and 30 min after IL-1 stimulation. The laser power was adjusted as in Fig. 1. (B) The percentage degradation of IκBαEGFP following transfection with *pIκBαEGFP* stimulation with IL-1 for 30 min was determined for individual cells and plotted against the initial fluorescence. Best-fit curve to the data is shown. (C) The average reduction in absolute IκBαEGFP fluorescence following 30 min stimulation with IL-1β for cells pooled according to increasing initial cytoplasmic levels of the fusion protein.

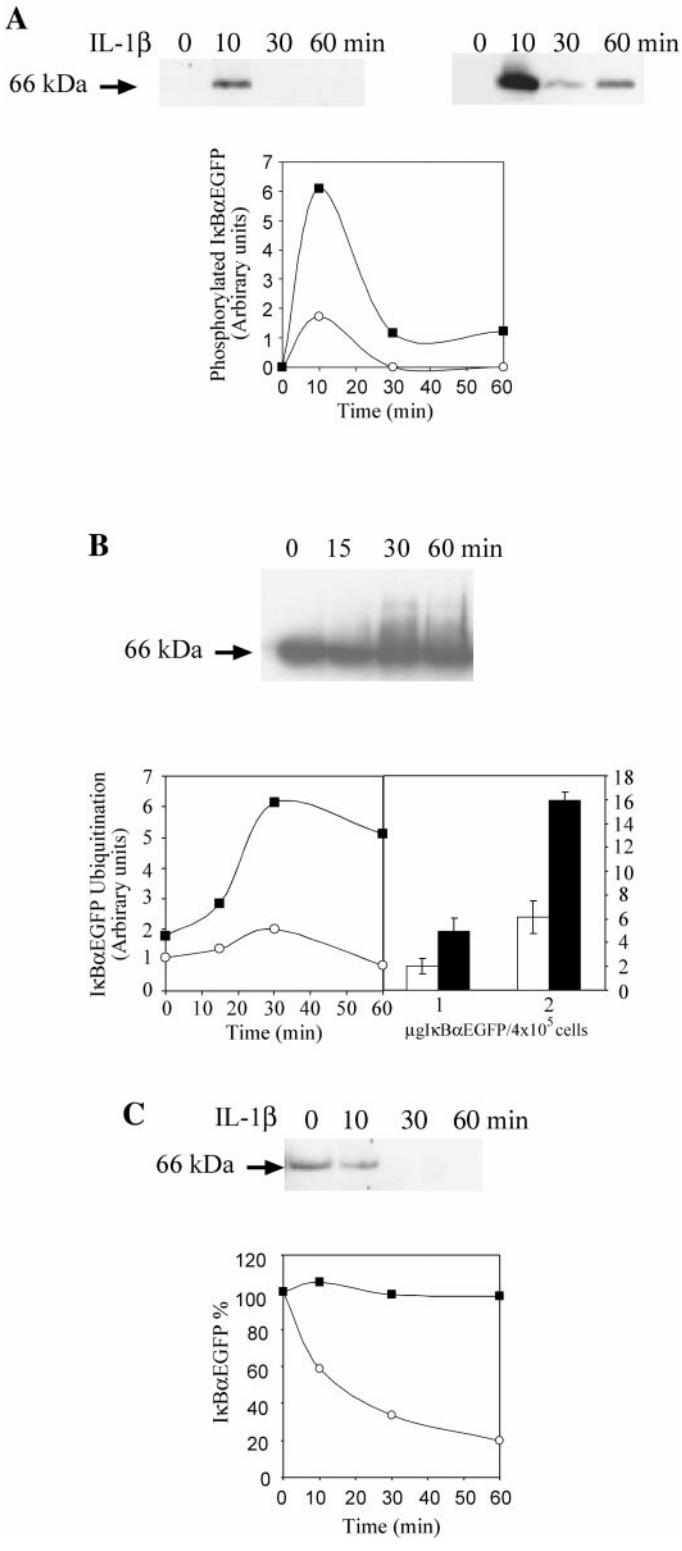

FIG. 4. Degradation of IκBαEGFP is limited by a post-phosphorylation/ubiquitination event. Hela cells transfected with 1 μg or 2 μg/4 $\times$ 10^5 cells of *pIκBαEGFP* were stimulated with 1 nM IL-1β. At indicated times, whole cell extracts were collected and the levels of phosphorylated (A), ubiquitinated (B), and total (C) IκBαEGFP protein were determined using Western blotting. (A) Phosphorylation of IκBαEGFP: Western analysis of cells transfected with 1 μg/4 $\times$ 10^5 cells (left) and 2 μg/4 $\times$ 10^5 cells (right) of *pIκBαEGFP* and stimulated with IL-1 as above, was carried out using a phospho-IκBα antibody as described under Materials and Methods. Bottom graph: Quantitation of Western-blots, as above of cells with 1 μg (○) and 2 μg (■) *pIκBαEGFP*/4 $\times$ 10^5 cells respectively, using NIH image. Data are expressed as signal above background measured at 0 min for each condition and after correction for variations in loading, determined by β-actin. Shown is one representative experiment from a total of three. (B) Ubiquitination of IκBαEGFP: Cells transfected with various levels of *pIκBαEGFP* and incubated in the presence or absence of tunicamycin, were stimulated with 1 nM IL-1β. Upper panel: Western analysis were carried out on whole cell extracts collected after addition of IL-1β for the times indicated, and the levels of ubiquitinated IκBαEGFP protein determined using an anti IκBα antibody. Bottom graph (Left): Quantitation of the level of ubiquitination from Western blots as above in cells transfected with 1 or 2 μg/4 $\times$ 10^5 cells of *pIκBαEGFP*, incubated in the absence of tunicamycin and stimulated with IL-1 for the times indicated. Data, analysed by NIH image, are expressed relative to the level at 0 min in cells transfected with the lower amount *pIκBαEGFP* and after correction for variations in loading determined by β-actin. Graph represents the average of 3 experiments. Bottom graph (right): Peak levels of ubiquitinated IκBα in cells transfected with indicated amounts of *pIκBαEGFP* and incubated in the absence (open bars) or presence (filled bars) of tunicamycin. Graph represents the mean $\pm$ SEM of data from three experiments analysed by NIH image and expressed as above. (C) Upper panel: IκBαEGFP levels in cells transfected with 1 μg/4 $\times$ 10^5 cells *pIκBαEGFP* and stimulated with IL-1 for the times indicated. Bottom graph: Quantitation of Western blots of cell extract from cultures transfected with 1 μg (○) or 2 μg/4 $\times$ 10^5 cells (■) of *pIκBαEGFP* using NIH image. Data are expressed relative to the level at 0 min of IL-1β stimulation for each condition, after correction for variations in loading determined by β-actin. Graph represents the average of two experiments.

mation (38), or be due to effects at the level of the IκB kinases (IKKs) and thus indirectly inhibit phosphorylation-dependent proteosome function.

The step responsible for limiting IκBα turnover during stimulation was determined by Western analysis. The involvement of the IKK induced phosphorylation was assessed by determining the levels of phosphorylated IκBαEGFP following IL-1 stimulation, using a phospho-specific antibody recognizing serine 32 (Fig. 4A). The experiments demonstrated an IκBαEGFP fusion protein with the expected size of 66 kDa. In addition, they showed a positive correlation between IκBαEGFP levels and phosphorylation over a range of transfections, leading to a pronounced increase in the 10-min peak in cells with higher levels of the exogenous protein. Similarly, the level of ubiquitination, determined at the same transfection conditions was shown to increase with enhanced levels of exogenous IκBαEGFP fusion protein (Fig. 4B). Quantitation of this type of experiments demonstrated a three fold increase in the peak level of ubiquitinated IκBα, measured 15–30 min after start of IL-1 stimulation, in cells transfected with twice the amount of cDNA (Fig. 4B, left graph). In addition, treatment with tunicamycin, which has been demonstrated to increase ubiquitina-

tion in a number of proteins (30), was shown to significantly enhance the levels of ubiquitinated IκBα. Incubation lead to between a two- and threefold enhancement in the peak level, in cultures transfected with either amount of the fusion protein (Fig. 4B, right graph), thus, showing no limitation in ubiquitination under these conditions. Finally, kinetics of turnover and the level of degradation of IκBαEGFP was assessed by Western analysis in cells transfected with different amounts of the *IκBαEGFP* construct (Fig. 4C). Quantitation of these data showed a level of degradation of *pIκBαEGFP* corresponding to between 60 and 80% in cells transfected with lower levels (1 μg/4 $\times$ 10^5 cells) of the fusion protein and following 30 to 60 min, respectively, of stimulation with saturated IL-1. In contrast, no reduction in IκBαEGFP levels was detected in cells transfected with twice this amount (2 μg/4 $\times$ 10^5 cells) and stimulated with IL-1 for up to one hour. Further, additional experiments showed that the turnover of IκBα was unaffected by enhancement of the level of ubiquitination at either transfection level, demonstrating identical profiles in the presence and absence of tunicamycin (data not shown). Taken together, this data implies that regulation of the pathway by inhibitor levels occurs subsequent to phosphorylation and ubiquitination and likely involve proteosome activity. The underlying mechanism is unlikely to constitute simple titering out of endogenous levels since phosphorylation, also dependent on complex formation, showed a positive correlation with IκBα levels. Instead, this data together with the pronounced increase in nuclear levels of the fusion protein at higher transfection levels could reflect a regulation of degradation involving trafficking. This could for example be mediated through inhibition or blocking of the pathway by nuclear IκBα/NF-κB complexes subsequent to separate, independent translocation of inhibitor and transcription factor (26).

In conclusion, our results show that both localisation and turnover of IκBα is dependent on the level of cytoplasmic expression. They show that while the absolute level of degradation demonstrates a biphasic response in relation to expression, IKK induced phosphorylation and subsequent ubiquitination show a positive correlation with the level of the inhibitor protein. The data thus suggest the presence of a limiting step in the NF-κB pathway at the level of proteosome activation which is regulated through cytoplasmic IκBα concentrations.

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