Characterization of NF\(\kappa\)B Activation by Detection of Green Fluorescent Protein-tagged I\(\kappa\)B Degradation in Living Cells*

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Activation of the transcription factor NF\(\kappa\)B requires rapid degradation of its inhibitor, I\(\kappa\)B\(\alpha\). To facilitate the study of I\(\kappa\)B\(\alpha\) degradation, we fused I\(\kappa\)B\(\alpha\) protein to enhanced green fluorescent protein to construct I\(\kappa\)B\(\alpha\)-enhanced green fluorescent protein (IG). We demonstrated by both flow cytometry and Western blot analysis that the half-life of IG in the presence of human tumor necrosis factor (TNF) \(\alpha\) is approximately 5 min, which is similar to the half-life of native I\(\kappa\)B\(\alpha\). The degradation coincided with NF\(\kappa\)B translocation from the cytoplasm to the nucleus and NF\(\kappa\)B-mediated induction of transcription. Phorbol 12-myristate 13-acetate (PMA), but not forskolin, also induces degradation of IG fusion protein. The half-life of IG in the presence of PMA is approximately 15 min, longer than when induced with TNF\(\alpha\). Co-treatment with TNF\(\alpha\) and PMA did not result in a synergistic effect on IG degradation, although they stimulate different kinases in two different signaling pathways. Degradation of IG was inhibited by mutations at serine residues 32 and 36, which are the target sites of the phosphorylation modification that initiates degradation of I\(\kappa\)B\(\alpha\). We also demonstrated that basal degradation of IG in the presence of cycloheximide is inhibited by such mutations, suggesting that basal degradation of I\(\kappa\)B\(\alpha\) also requires phosphorylation as the signal for degradation. Finally, we showed that the rate of TNF\(\alpha\)-induced degradation of IG remains almost constant throughout the cell cycle, except at the mitotic phase, in which IG degrades more slowly.

Regulated degradation of specific proteins is part of the intracellular biochemical changes that contribute to the regulation of signal transduction pathways, cell proliferation, growth arrest, and apoptosis. For instance, tumor necrosis factor (TNF) \(\alpha\)-mediated NF\(\kappa\)B activation requires the rapid degradation of I\(\kappa\)B\(\alpha\), the inhibitor of NF\(\kappa\)B (1–4). NF\(\kappa\)B is a transcription factor that regulates the expression of a number of genes whose products contribute to inflammation and immune responses (5, 6). Before activation, NF\(\kappa\)B is sequestered in the cytoplasm by forming a complex with I\(\kappa\)B\(\alpha\) (7–9). The nuclear translocation signal of NF\(\kappa\)B is masked by the inhibitor (10). NF\(\kappa\)B can be activated by a number of stimuli, including TNF\(\alpha\), interleukin 1\(\beta\), lipopolysaccharide, and phorbol esters (PMA). Initiated by TNF\(\alpha\) binding, a number of proteins including TRADD, TRAF2, and RIP aggregate around the TNF\(\alpha\) type 1 receptor (11–13), which triggers the phosphorylation of I\(\kappa\)B\(\alpha\) and leads to the rapid dissociation of NF\(\kappa\)B from I\(\kappa\)B\(\alpha\) (14–17). A protein kinase complex, which includes NIK, IKKa, and IKKB, is involved in the phosphorylation of I\(\kappa\)B\(\alpha\) (18–22). The phosphorylated I\(\kappa\)B\(\alpha\) is further modified by ubiquitination enzymes and degraded by the 26S proteasome (23). Serine residues 32 and 36 of I\(\kappa\)B\(\alpha\) have been identified to be the specific target sites of phosphorylation (16, 24–26). Mutations at these positions abolish both phosphorylation and degradation of I\(\kappa\)B\(\alpha\) (16, 24–26). Once released from the I\(\kappa\)B\(\alpha\) complex, NF\(\kappa\)B immediately translocates from the cytoplasm to the nucleus, where it mediates the transcriptional activation of genes, such as interleukin 2 and I\(\kappa\)B\(\alpha\). I\(\kappa\)B\(\alpha\) degradation is rapid and is completed within 5–40 min after stimulation by TNF\(\alpha\) (1, 2, 7). I\(\kappa\)B\(\alpha\) also degrades in the absence of TNF\(\alpha\), but this basal degradation is much slower than induced degradation. The half-life of the basal degradation is around 2 h (2, 29). It is unclear whether the basal degradation of I\(\kappa\)B\(\alpha\) also requires phosphorylation for initiation of the degradation process (30). Therefore, the relationship of basal degradation to induced degradation remains uncertain.

Both the gene and cDNA of the green fluorescent protein (GFP) have been cloned from the jellyfish Aequorea victoria (31). GFP has been widely used to study gene expression and protein localization (32–35) because its fluorescence emission does not require substrates or cofactors (36), and fluorescence detection can be made in real time. The key sequence of Ser-Tyr-Gly (amino acids 65–67) within GFP undergoes spontaneous oxidation to form a cyclized chromophore that emits fluorescence (37). Mutation of Ser to Thr in the chromophore (S65T) leads to a higher fluorescence intensity of GFP. Enhanced GFP (EGFP) is one such mutant. It contains the mutations S65T and F64L and is encoded by a gene with human-optimized codons (38–40). Crystallographic structures of wild-type GFP and the mutant S65T reveal that the tertiary structure of GFP resembles a barrel (41, 42), and this compact structure makes GFP a very stable protein. Fusing EGFP to the degradation domain of mouse ornithine decarboxylase, we were able to generate a destabilized EGFP with a half-life of 2 h (43). This study indicates that EGFP is able to degrade in vivo when fused with a degradation domain and suggests that EGFP can be used as a general reporter to measure protein degradation in vivo when fused to a full-length rapid turnover protein.

In the present study, we used EGFP to monitor I\(\kappa\)B\(\alpha\) degradation by making a fusion protein of I\(\kappa\)B\(\alpha\) and EGFP. The fusion protein was found to be degraded as well as I\(\kappa\)B\(\alpha\) upon
TNFα treatment. Therefore, IκBα degradation can be analyzed simply by monitoring the change of the fluorescence intensity of the fusion protein without cell disruption. Using EGFP, we were able to analyze the degradation more efficiently and rapidly than possible with other methods. We showed that both TNFα and PMA, but not forskolin, induce degradation of the fusion protein. Degradation induced by PMA is slower than that induced by TNFα, and no synergistic effect was detected when cells were treated with both TNFα and PMA simultaneously. During the study, we also found that the basal degradation of IκBα, like the induced degradation, also requires phosphorylation at serines 32 and 36 for initiation of the degradation process. Finally, we found that IG degradation at the mitotic phase is slower than IG degradation at the other phases.

**EXPERIMENTAL PROCEDURES**

The cDNAs encoding IκBα and EGFP were amplified with Pyro DNA polymerase. IκBα was amplified with primers that incorporated a SacII recognition sequence at the 5’ end and a BamHI sequence at the 3’ end. The stop codon of IκBα was deleted during polymerase chain reaction amplification to make an open reading frame with EGFP. EGFP was amplified with primers that incorporated a BamHI recognition sequence at the 5’ end and an EcoRI sequence at the 3’ end. The amplified polymerase chain reaction products were ligated at the BamHI site, and the resulting fusion construct (IκBα-EGFP) was cloned into the SacII and EcoRI sites of the pTRE expression vector (CLONTECH, Palo Alto, CA) for use in the tetracycline-regulated expression system (44). The IκBα mutant S32A/S36A was made with overlap extension mutagenesis (45).

pTRE-IG and pTRE-IGS23A/S36A expression vectors were transfected into HeLa Tet-Off™ cells (CLONTECH) for degradation studies. HeLa Tet-Off™ cells stably express a fusion protein of the tet repressor and the herpes simplex virus VP16 (tTA) and thus can be used for tetracycline-regulated expression of a gene cloned into the pTRE vector (44). 24 h after transfection, the transfected cells were subject to functional analyses. To make stable cell lines, cell were co-transfected with pTK-Hyg and cultured in medium containing 200 μg/ml hygromycin. Drug-resistant colonies were then screened for green fluorescence under a Zeiss Axioskop Model 50 fluorescence microscope.

To examine the fluorescence intensity of EGFP or IG, the cells were cultured on coverslips. After transfection, the cells were treated with 0.1 g/ml recombinant human TNFα for 37 °C for 24 h and then fixed with 4% paraformaldehyde for 30 min. The cultured on coverslips. After transfection, the cells were incubated at 37 °C for 24 h and then fixed with 4% paraformaldehyde for 30 min. The coverslips were mounted on a glass slide for examining fluorescence under a fluorescence microscope. To determine protein turnover, the cells were treated with 0.1 μg/ml recombinant human TNFα (CLONTECH) for varying times before paraformaldehyde fixation. The transfected cells with/without TNFα treatment were collected by EDTA treatment, and the cell pellets were resuspended in 0.5 ml of PBS. Cell suspensions were analyzed for fluorescence intensity using a FACScan-Calibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA). EGFP was excited at 488 nm, and emission was detected using a 510/20 bandpass filter.

The transfected cells with/without CHX treatment were collected in PBS, and cell lysates were prepared by sonication. Proteins were resolved by SDS gel electrophoresis and transferred onto a membrane. IG fusion proteins were detected using a monoclonal antibody against GFP (CLONTECH). Bands were visualized with the Western Exposure chemiluminescent detection kit (CLONTECH).

To examine NFκB translocation, cells stably expressing IG were treated with TNFα. The treated cells were fixed with 4% formaldehyde in PBS (pH 7.4) for 30 min at room temperature and rinsed with PBS three times. The fixed cells were then permeabilized with a blocking solution for 1 h. The cells were then incubated with a 1:250 dilution of anti-Rel p65 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) in the blocking solution for 2 h. After washing with PBS three times, the cells were incubated with a 1:250 dilution of rhodamine-conjugated anti-rabbit IgG (Roche Molecular Biochemicals, Indianapolis, IN) for 45 min in PBS containing 4% bovine serum albumin. After rinsing three times with PBS, the stained cells were mounted in Citifluor (Ted Pella, Inc., Redding, CA). To determine the induction of SEAP mediated by NFκB, IG cells were transfected with pNFB-SEAP. TNF-mediated induction was determined by comparing SEAP levels within cells with and without TNFα treatment, using the Great EscA-Pv™ chemiluminescent detection kit (CLONTECH).

To establish stable expression of the IG fusion protein in HeLa cells, we co-transfected pTRE-IG with pTK-Hyg, selected drug-resistant colonies in the presence of hygromycin, and screened for fluorescence under a fluorescence microscope. The results suggest that the EGFP tag changes neither TNFα-mediated fluorescence decay of IG nor the protein’s half-life.

**RESULTS**

To investigate whether the biochemical degradation of IκBα can be monitored by fluorescence decay of the EGFP fusion protein, we appended EGFP to the C terminus of IκBα. The fusion was designated IG. The fusion IG was transiently expressed in HeLa Tet-Off™ or 293 Tet-Off™ cells by transfecting the expression vector pTRE-IG. The transfected cells were treated with TNFα for 1 h and collected for analysis by flow cytometry. As shown in Fig. 1, TNFα treatment resulted in decreased fluorescence of transfected cells, indicating sensitivity of IG fluorescence to TNFα treatment. To examine whether TNFα-mediated fluorescence decay of IG requires the phosphorylation of IκBα, we mutated serines 32 and 36 to Ala to make IG-S32A/S36A. The results from flow cytometry analysis demonstrated no decrease in the green fluorescence of IG-S32A/S36A after TNFα treatment in both HeLa and 293 cells (Fig. 1). Therefore, we concluded that TNFα-mediated fluorescence decay of IG, like IκBα degradation, needs the specific phosphorylation modification at serines 32 and 36.

To establish stable expression of the IG fusion protein in HeLa cells, we co-transfected pTRE-IG with pTK-Hyg, selected drug-resistant colonies in the presence of hygromycin, and screened for fluorescence under a fluorescence microscope. One of green fluorescent clones, designated as HeLa-Off/IG, is shown in Fig. 2. HeLa-Off/IG cells were treated with TNFα for 0, 5, 10, 15, 20, and 25 min and subjected to fluorescence microscopy. Green fluorescence was not detectable within 20 min after treatment with TNFα (Fig. 2A). Next we used flow cytometry to quantitatively analyze the half-life of IG fluorescence. Both total fluorescence and the percentage of fluorescent cells declined to a basal level after 15 min of treatment. Fluorescence declined to 50% of uninduced levels 5 min after induction. This half-life is similar to that of endogenous IκBα protein in HeLa cells (1, 2, 7). The results suggest that the EGFP tag changes neither TNFα-mediated regulation of IκBα turnover nor the protein’s half-life.

To confirm that the decay in fluorescence correlates with a decrease in IG protein levels, we measured the amount of the fusion protein by Western blot analysis with a monoclonal...
antibody against GFP. At 0 min, we saw a band at 65 kDa, corresponding to the predicted size of the IG protein (Fig. 2C). The intensity of this band declined with increasing length of TNFα treatment. The band’s intensity was reduced to about 50% at approximately 5–10 min of treatment, confirming the IG protein half-life of 5–10 min. Therefore, the intracellular fluorescence intensity of the IG fusion protein correlates directly to the IG protein level.

To examine whether IG degradation leads to NFκB translocation, we detected NFκB protein in IG cells with polyclonal antibodies against p65 as the primary antibody and rhodamine-conjugated secondary antibody. Before TNFα treatment, both NFκB and IκBα are localized to the cytoplasm. Degradation of IG was induced by adding TNFα, which leads to immediate translocation of NFκB from the cytoplasm to the nucleus (Fig. 3A). No difference in translocation of NFκB was found in IG cells and control HeLa cells. NFκB-mediated transcription was measured by the induction of the NFκB response elements, κB4, located on a reporter vector transiently transfected into the cells. The TNF-induced induction of the reporter protein SEAP in the IG cells is the same as that in control HeLa cells (Fig. 3B). These results indicated that degradation of the IG fusion protein is associated with NFκB translocation and transcription induction. Therefore, the EGFP tag on IκBα does not change normal NFκB function.

It has been shown that protein kinase A in vitro (46) and protein kinase C both in vitro (46) and in vivo (1, 2) are able to activate NFκB. We first examined whether protein kinase A can induce degradation of IG by treatment of HeLa-Off/IG cells with forskolin. Analysis by flow cytometry showed that fluorescence of the treated cells remained the same during the 3-h period of treatment (Fig. 4A). This result indicates the insensitivity of IκB degradation to protein kinase A, which does not agree with the in vitro study in which protein kinase A activates NFκB. We also tested whether protein kinase C can activate NFκB by treatment of HeLa-Off/IG cells with PMA. As shown in Fig. 4B, the fluorescence of IG rapidly decayed after PMA treatment, and the half-life of IG fluorescence is about 15 min. The sensitivity of IG degradation to protein kinase C is similar to that seen in the previous studies. The degradation rate of IG fluorescence induced by PMA is slower that induced by TNFα (Figs. 2B and 4B). To examine the difference more carefully, we did a side-by-side comparison of the degradation of IG fluorescence induced by TNFα and PMA. Treatment of HeLa-Off/IG cells with TNFα resulted in rapid degradation of IG, but treatment with PMA produced a slightly delayed degradation response (Fig. 5). This delay is approximately 5 min.

TNFα-induced degradation of IκB proceeds via the activation of the IKK complex, whereas PMA-induced degradation of IκB requires activation of the 90-kDa ribosomal S6 kinase (pp90rsk) (27, 28). A dominant negative mutant of pp90rsk inhibits PMA-induced but not TNFα-induced degradation of IκB, further
sug\textsuperscript{suggesting} that these two pathways are different. To examine whether PMA has any additive effect on TNF\textsubscript{a}-induced degradation, we co-treated HeLa-Off/IG cells with TNF\textsubscript{a} and PMA. The resultant degradation rate is identical to that induced by TNF\textsubscript{a} (Fig. 5). Therefore, there is no synergistic effect of these two inducers on the degradation of IG.

\textsuperscript{IxB}a is subject to both TNF\textsubscript{a}-induced and basal degradation. The basal degradation of \textsuperscript{IxB}a has been characterized in the presence of CHX. In the absence of de novo protein synthesis, \textsuperscript{IxB}a exhibits a baseline half-life of 2 h in HeLa cells (2) and 2.5 h in the 70Z/3 murine pre-B cell line (29). To determine whether basal degradation of IG has a half-life similar to that of \textsuperscript{IxB}a, we treated HeLa-Off/IG cells with CHX for 0, 1, 2, 3, and 4 h and collected the cells for analysis by flow cytometry. The time-course fluorescence decay was plotted and is shown in Fig. 6A. The half-life of the fusion in the constitutive degradation is about 2 h, which is similar to that of \textsuperscript{IxB}a. Pretreatment of IG cells with CHX for 1 h decreased the total fluorescence (initial fluorescence intensity of 80) but did not change the degradation patterns of TNF\textsubscript{a} and PMA (Figs. 5 and 6B), suggesting that TNF\textsubscript{a}- or PMA-induced degradation of IG is not affected by the basal degradation.

It was unclear whether the basal degradation of \textsuperscript{IxB}a requires the same phosphorylation events as the TNF\textsubscript{a}-induced degradation (30). To examine this, we transiently expressed \textsuperscript{IxB}a and \textsuperscript{IxB}a phosphorylation sites (Fig. 6C). Therefore, both basal degradation and induced degradation of \textsuperscript{IxB}a require the same phosphorylation event to initiate degradation.

To examine TNF\textsubscript{a}-mediated degradation of \textsuperscript{IxB}a at the different stages of cell cycle, we pretreated HeLa/IG cells with the following compounds: (a) mimosine that arrests cells at the G\textsubscript{1} phase, (b) excess thymidine at the G\textsubscript{1}-S phase, and (c) nocodazole at the mitotic phase. Induced HeLa/IG cells at the G\textsubscript{1} phase arrested by mimosine did not change the sensitivity of \textsuperscript{IxB}a to TNF\textsubscript{a}, although the fluorescence intensity of the cells at the G\textsubscript{1} phase (initial fluorescence intensity of 111) is lower than that of dividing cells (initial fluorescence intensity of 197) (Fig. 7). Treatment of HeLa/IG cells with excess thymidine did not change the fluorescence intensity, as well as TNF\textsubscript{a}-induced degradation of \textsuperscript{IxB}a (Fig. 7). As at the G\textsubscript{1} phase, HeLa/IG cells at the mitotic phase induced by treatment with nocodazole showed a lower initial fluorescence intensity of 106, but TNF\textsubscript{a}-induced degradation of \textsuperscript{IxB}a was slower than that of the dividing cells or the other arrested cells. The half-life of \textsuperscript{IxB}a is extended to be a few minutes longer. These results suggested that the degradation rate of \textsuperscript{IxB}a varies at different phases of the cell cycle.

**DISCUSSION**

The intracellular biochemical changes that mediate NF-\textsubscript{x}B activation are well documented. The key element in the NF-\textsubscript{x}B activation pathway that determines NF-\textsubscript{x}B activation is \textsuperscript{IxB}a phosphorylation and degradation. Recent studies indicate that the kinases that mediate \textsuperscript{IxB}a phosphorylation are a large complex (18–22). Analysis of NF-\textsubscript{x}B activation is often monitored by its transcriptional induction of a reporter in vitro, but the time required for the induction is as long as 4–6 h. Because \textsuperscript{IxB} degrades very quickly after stimulation, it would be an ideal target to be monitored for analysis of NF-\textsubscript{x}B signaling pathways. We have demonstrated in our previous study (43) that EGFP can be used as a reporter for protein degradation.
the present study, we demonstrated that EGFP can be used for monitoring IκBα degradation and demonstrated that the IG fusion protein is functionally identical to IκBα. Like IκBα, the IG fusion protein responds to TNFα very quickly and specifically. The half-life of IG is 5 min in the presence of TNFα and 2 h in the presence of CHX, which agrees with previously described rates of degradation for IκB (1, 2, 7, 29). Furthermore, appending EGFP to IκB did not change the specific requirement of phosphorylation for initiation of TNFα-induced degradation. TNFα-induced degradation of the IG fusion protein is also prevented by the mutations at serine residues 32 and 36 of IκBα. Lastly, both NFκB translocation and NFκB-mediated transcription in IG-expressing cells are the same as those in control HeLa cells, suggesting that the addition of the EGFP tag and the expression of IG in the cells do not change the downstream events in NFκB signaling. Therefore, IκBα degradation can be analyzed simply by monitoring the change...
in fluorescence of the fusion protein. The simple detection of fluorescence will facilitate studies of IkBa degradation as well as NF-kB activation.

NFkB can be activated by a number of stimuli, and the activation requires phosphorylation and degradation of IkBa. By monitoring the degradation of Ig by flow cytometric analysis, we showed that the degradation cannot be induced by forskolin, indicating that protein kinase A is not involved in the activation of NFkB. Our conclusion is different from that of an in vitro study (46) in which translocation of NFkB in HeLa-Off/IG cells was untreated (1) or treated with 100 μM mimosine (2), 2 μM thymidine (3), or 50 ng/ml nocodazole (4) for 16 h. The cells were further treated with 0.1 μg/ml TNFα for 0, 5, and 10 min before harvesting for flow cytometric analysis.

Figure 7. Ig degradation at different phases of the cell cycle. HeLa-Off/IG cells were untreated (1) or treated with 100 μM mimosine (2), 2 μM thymidine (3), or 50 ng/ml nocodazole (4) for 16 h. The cells were further treated with 0.1 μg/ml TNFα for 0, 5, and 10 min before harvesting for flow cytometric analysis.

For degradation to occur, IkBa must be phosphorylated at serines 32 and 36. Only this phosphorylated protein can be degraded by the 26S protease. During M phase, the phosphorylation cascade may be altered or impeded, resulting in the impaired degradation of IkBa. Secondly, the slower degradation could be due to inactivation of the degradation machinery. The second scenario is unlikely, because some proteins, such as cyclin B1, use the same mechanism and degrade well in the same phase. The third possibility can be due to the existence of an inhibitory protein that is released from nucleus in the mitotic phase and prevents IkBa degradation. Additional studies need to be done to distinguish the possibilities.

Many regulatory proteins are short-lived. Rapid turnover provides a possible means to regulate their protein levels. Degradation of these proteins can be detected by the methods described here, which avoid complicated biochemical analysis, such as pulse-chase immunoprecipitation, and substitute a simple fluorescence assay. Because emission of EGFP does not require any cofactors or substrates, detection can be made in real time, and the data can be collected at multiple time points. Therefore, the method provides simple and quick detection of protein degradation in living cells. It can facilitate the analysis of a large number of samples, such as in high throughput screening.

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