Ultraviolet Light Activates NFκB through Translational Inhibition of IκBα Synthesis*

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UV light induces a delayed and prolonged (3–20 h) activation of NFκB when compared with the immediate and acute (10–90 min) activation of NFκB in response to tumor necrosis factor α treatment. In the early phase (3–12 h) of NFκB activation, UV light reduces inhibitor of NFκB (IκB) through an IκB kinase-independent, but polyubiquitin-dependent, pathway. However, the mechanism for the UV light-induced reduction of IκB and activation of NFκB is not known. In this report, we show that UV light down-regulates the total amount of IκB through decreasing IκB mRNA translation. Our data show that UV light inhibits translation of IκB in wild-type mouse embryo fibroblasts (MEFSS) and that this inhibition is prevented in MEFSSA cells in which the phosphorylation site, Ser-51 in the eukaryotic translation initiation factor 2 α (eIF2α)-subunit, is replaced with a non-phosphorylatable Ala (S51A). Our data also show that UV light-induced NFκB activation is delayed in MEFSSA cells and in an MCF-7 cell line that is stably transfected with a transdominant negative mutant protein kinase-like endoplasmic reticulum kinase (PERK). These results suggest that UV light-induced eukaryotic translation initiation factor 2 α-subunit phosphorylation translationally inhibits new IκB synthesis. Without a continuous supply of newly synthesized IκB, the existing IκB is degraded through a polyubiquitin-dependent proteasomal pathway leading to NFκB activation. Based upon our results, we propose a novel mechanism by which UV light regulates early phase NFκB activation by means of an ER-stress-induced translational inhibition pathway.

Ultraviolet light activates the nuclear factor κB (NFκB)1 (1, 2). One well established mechanism for NFκB activation is that, upon stimulation such as with tumor necrosis factor (TNFα) or lipopolysaccharide treatment, an inhibitor of NFκB (IκB) kinase (IKK) is activated and phosphorylates IκBα at Ser-32 and Ser-36 (3, 4). The phosphorylated IκBα dissociates from the NFκB and is rapidly degraded through the polyubiquitin-dependent proteasomal pathway. The free NFκB then translocates to the nucleus and activates target genes (5–8). However, this mechanism is not applicable to UV light-induced early phase (within 12 h) activation of NFκB (2, 9). Compared with TNFα, UV light activates NFκB in a delayed and prolonged manner. The UV light-induced early phase activation of NFκB is dependent upon the degradation of IκB through the polyubiquitin-degradation pathway (9); however, the mechanism is not understood. It was reported that UV light does not induce IKK activation nor N-terminal serine phosphorylation of IκBα during this time (2). It has also been reported that IKK activity is required and that the IKK-targeted serine phosphorylation sites on IκBα are critical for UV light-induced NFκB activation, even though IKK activation is not detected above the basal level after UV light irradiation (10). In this report, we elucidate the role of translational regulation in early phase activation of NFκB after UV light irradiation. Previously, we reported that UV light irradiation activates an RNA-dependent protein kinase-like endoplasmic reticulum (ER)-stress activated kinase (PERK) that phosphorylates eukaryotic translation initiation factor 2 α (eIF2α) and inhibits protein synthesis (11). Now we show that UV light-induced early phase activation of NFκB is due to the translational inhibition of new IκB synthesis through the phosphorylation of Ser-51 in eIF2α.

MATERIALS AND METHODS

UV Light Irradiation—Ultraviolet C light was generated from a 15 W ultraviolet C light source (UVP Inc., Upland, CA). The intensity of ultraviolet C light was standardized by using a UV light meter (UVP Inc., Upland, CA) set at 3 W/m². The media was withdrawn during the ultraviolet C irradiation. After UV light irradiation, fresh medium was added to each plate.

Analysis of Total IκB—Cells were harvested with Nonidet P-40 lysis buffer (2% Nonidet P-40, 80 mM NaCl, 100 mM Tris-HCl, and 0.1% SDS), and the protein concentrations were measured with the Bio-Rad protein assay kit (Bio-Rad). Equal amounts of protein samples were subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with either an anti-IκB antibody or a mouse monoclonal anti-β-actin antibody (Sigma). Two anti-IκB antibodies were used in the experiments. A rabbit polyclonal anti-IκB antibody (sc-371; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for mouse IκBα, and a mouse monoclonal anti-IκB antibody (sc-1643; Santa Cruz Biotechnology) was used for human IκBα. After extensively washing with Tris-buffered saline plus Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Signals were detected by using a SuperSignal™ chemiluminescent kit (Pierce).

Assay for IκB Synthesis—The mouse embryo fibroblasts (MEFs) were UV light-irradiated (30 J/m²). At the indicated time points after irradiation (Fig. 1), the cells were incubated with methionine/cysteine-free minimal essential medium (Invitrogen) for 15 min and then pulse-

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The abbreviations used are: NFκB, nuclear factor κB; TNFα, tumor necrosis factor α; IKK, IκB kinase; IκB, inhibitor of NFκB, ER, endoplasmic reticulum; PERK, RNA-dependent protein kinase-like endoplasmic reticulum-stress activated kinase; eIF2α, eukaryotic translation initiation factor 2 α; MEFs, mouse embryo fibroblasts; EMSA, electrophoretic mobility shift assay; MEFs, MEFs with wild-type eIF2α; MEFSSA, MEFs with an Ser-51 → Ala mutation at the phosphorylation site in eIF2α.
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labeled with Redivue pro mix ([35S]-Met/Cys (100 μCi/ml and 1,000 Ci/mmol, respectively; Amer sham Biosciences) for 30 min in Met/Cys-free minimal essential medium (Invitrogen). After washing with PBS, cell extracts were prepared in Nonidet P-40 lysis buffer. The protein concentration was measured with a Bio-Rad protein DC assay kit. IxB was then immunoprecipitated from equal amounts of proteins using anti-IxB antibody (sc-711, Santa Cruz Biotechnology) and protein A-agarose (Santa Cruz Biotechnology). The immunoprecipitates were subjected to SDS-PAGE. The gel was treated with En3Hance (PerkinElmer Life Sciences) and dried. The newly synthesized IxB presence was then analyzed by autoradiography.

Assay for Protein Synthesis—The UV light-irradiated MEFs were pulse-labeled with [35S]-Met/Cys as described above. The [35S]-incorporation was analyzed by SDS-PAGE or trichloroacetic acid precipitation. For electrophoresis, equal amounts of cell lysates were resolved on a SDS-12% PAGE. The gel was stained with Coomassie Blue R-250 and subjected to SDS-PAGE, which was followed by Western blotting analysis with antibody against phosphorylated eIF2β (Research Genetics, Inc., Huntsville, AL) (12). The levels of eIF2β phosphorylation were visualized by the SuperSignal™ chemiluminescent system (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—A pair of 23-bp synthetic oligonucleotides (5'-GATCCAGGGGACTTTCCGGA-3') containing the NFkB-binding site was annealed and labeled with [32P] using T4 polynucleotide kinase and [32P]-γ-ATP. A DNA-binding reaction mixture of 20 μl contained 20 mM Tris-HCl, pH 7.5, 4% Ficoll-400, 2 mM EDTA, 0.5 mM dithiothreitol, 0.5 μg of poly(dI-dC). [32P]-labeled oligonucleotide (20,000 cpm), and 8 μg of nuclear extract from MCF-7 cells or MEFs after UV light treatment. In the cases indicated, 0.2 μg of Rel A antibody or Rel B antibody and a 100-fold excess of mutated consensus-binding sequence (5'-GATCCACCGATGGATCGAAG-3') were also included. The mixture was incubated at room temperature for 45 min and analyzed onto a 5% non-denaturing polyacrylamide gel. The gel was run in 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 120 V, dried, and autoradiographed.

Immunofluorescence Staining—MCF-7 and MCF-7-PERKAC cells were UV light-irradiated and then, at the indicated time points (Fig. 4), were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin. The cells were incubated with a mouse anti-NFkB monoclonal antibody. After washing with PBS, the cells were incubated with a fluorescein-conjugated goat anti-mouse antibody. The localization of NFkB was visualized with an Olympus IX70 fluorescence microscope using a Kodak M290 camera with an exposure time of 0.25 s.

RESULTS

UV Light Inhibits Translation of IxB through Phosphorylation of Ser-51 Residue of eIF2α—Because the half-life of IxB is short (2), inhibition of new IxB synthesis can have a significant impact upon the total amount of intracellular IxB. Indeed, treating cells with a protein synthesis inhibitor such as cycloheximide can induce NFkB activation (13, 14). Because UV light irradiation inhibits protein synthesis through induction of eIF2α phosphorylation (11), we examined whether eIF2α phosphorylation plays a role in UV light-induced NFkB activation. MEFs with wild-type eIF2α (MEFSS) or with an Ser-51 → Ala mutation at the phosphorylation site in eIF2α (MEF51A) (15) were used in the experiments. We first measured the total amount of IxB in UV light-irradiated MEF cells. Western blot analysis demonstrated that UV light irradiation significantly reduced IxB in MEFSS cells by 40–97% within 4–24 h (Fig. 1A, lanes 2–4), whereas IxB remained at a high level in MEF51A cells (Fig. 1A, lanes 6–8). We then analyzed IxB translation in MEFSS and MEF51A cells. The cells were UV light-irradiated and then metabolically pulse-labeled for 30 min with [35S]-Met/Cys at the indicated time points (Fig. 1). The expression levels of IxB were normalized by the expression levels of β-actin and expressed as a percentage of IxB expression at 0 h post-UV light irradiation. The immunoprecipitation analysis of newly synthesized IxB in UV light-irradiated MEFSS and MEF51A cells. The cells were first UV light-irradiated and then pulse-labeled with [35S]-Met/Cys at the indicated time points. The total IxB was immunoprecipitated by using anti-IxB antibody, and the newly synthesized IxB was detected by autoradiography. The immunoprecipitated heavy chain of Ig (Ig-HC) was visualized by Coomassie Blue R-250 staining to ensure that equal amounts of antibody were used. The intensities of the immunoprecipitated IxB bands were quantified by ImageJ and were expressed as a percentage of IxB synthesis at 0 h post-UV light irradiation.

A, Western

|                | MEF SS | MEF AA |
|----------------|--------|--------|
| Time (h):      | 0      | 0      |
| IxB            | 1.0    | 1.0    |
| β-actin        | 1.0    | 1.0    |
| Lane           | 1      | 1      |

B, [35S]-incorporation

|                | MEF SS | MEF AA |
|----------------|--------|--------|
| Time (h):      | 0      | 0      |
| IxB            | 1.0    | 1.0    |
| Ig-HC          | 1.0    | 1.0    |
| Lane           | 1      | 1      |

Fig. 1. UV light-induced eIF2α phosphorylation inhibits new IxB synthesis. A, immunoblot analysis of total amounts of IxB and β-actin in UV light-irradiated MEFSS and MEF51A cells. The intensities of the bands were quantified by ImageJ version 1.31 (Mac OS X, NIH). The expression levels of IxB were normalized by the expression levels of β-actin and expressed as a percentage of IxB expression at 0 h post-UV light irradiation. B, immunoprecipitation analysis of newly synthesized IxB in UV light-irradiated MEFSS and MEF51A cells. The cells were first UV light-irradiated and then pulse-labeled with [35S]-Met/Cys at the indicated time points. The total IxB was immunoprecipitated by using anti-IxB antibody, and the newly synthesized IxB was detected by autoradiography. The immunoprecipitated heavy chain of Ig (Ig-HC) was visualized by Coomassie Blue R-250 staining to ensure that equal amounts of antibody were used. The intensities of the immunoprecipitated IxB bands were quantified by ImageJ and were expressed as a percentage of IxB synthesis at 0 h post-UV light irradiation.
UV Light-induced NFκB Activation Requires Phosphorylation of eIF2α—To confirm that UV light induces early phase NFκB activation through a translational regulation pathway, we examined NFκB activation further in the MEFs using an electrophoretic mobility shift assay (EMSA). UV light treatment for 4–24 h increased the activation of NFκB in wild-type MEFs (Fig. 3A, lanes 2–4). In contrast, NFκB was not significantly activated in MEFΔΔ cells at 4 or 8 h (Fig. 3A, lanes 6 and 7). However, at 24 h post-UV light irradiation, there was an increase in the activation of the NFκB (Fig. 3A, lane 8). This delayed activation is consistent with a previous report showing that in the late stage of UV light irradiation, IKK is involved in UV light-induced activation of NFκB (2).

To identify the subunit of NFκB that is activated after UV light irradiation, we performed a super-gel-shift assay using antibodies against Rel A (p65) and Rel B of NFκB family. Only anti-Rel A was able to produce a supershift of the DNA-protein complex (Fig. 3B; Ref. 16), supporting the idea that UV light specifically activates Rel A. To further elucidate whether the translational inhibition mechanism is unique for UV light irradiation, we measured NFκB activation in the same cell lines after TNFα treatment. The results demonstrate that there is no difference in TNFα-induced activation of NFκB between MEFSS and MEFΔΔ cells (Fig. 3C, lanes 1–6 versus 7–12). These results suggest that translational inhibition of IκB is critical for UV light-induced early phase activation of NFκB.

PERK Mediates IκB Depletion in Response to UV Light—PERK is an ER-stress-induced eIF2α kinase (17, 18) that mediates UV light-induced eIF2α phosphorylation and protein synthesis inhibition (11). We examined whether PERK mediates UV light-induced down-regulation of IκB by studying a parental control MCF-7 cell line and a line that stably expresses a trans-dominant negative mutant PERK (PERKΔC) mutant that has a carboxyl-terminal truncation (11). Where IκB was reduced 20–40% within 4–24 h post-UV light irradiation in parental MCF-7 cells (Fig. 4A, lanes 1–4), IκB was
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To determine whether the deactivation of NFκB was due to the inhibition of NFκB translocation caused by the elevated IκB level in MCF-7-PERKΔC cells, we analyzed NFκB translocation in the cells. NFκB translocated into the nucleus at 9 h post-UV light irradiation in MCF-7 cells (Fig. 4C, top row). However, this translocation was delayed in MCF-7-PERKΔC cells (Fig. 4C, bottom row).

**DISCUSSION**

Cells respond to environmental stimuli through rapid and reversible covalent modification of translation initiation factors to mediate immediate increases or decreases in protein synthesis (19, 20). Stress conditions that inhibit initiation of protein synthesis decrease the activity of eIF2α through phosphorylation of its α-subunit at Ser-51 (21). Four protein kinases are known to phosphorylate Ser-51 in eIF2α in response to different stress stimuli as follows: (i) the dsRNA-activated protein kinase PKR that is activated by dsRNA produced during viral infection (20); (ii) the general control of nitrogen metabolism kinase GCN2 that responds to amino acid depletion (22); (iii) the heme-regulated inhibitor kinase that responds to heme deprivation (23); and (iv) PERK that responds to the accumulation of unfolded proteins in the endoplasmic reticulum as well as to glucose depletion (24, 25). Previous studies demonstrated that PERK and GCN2 can mediate translational inhibition through the phosphorylation of eIF2α in response to UV light irradiation (11, 26, 27). However, the roles of PERK and GCN2 in UV light-induced translation inhibition are a source of controversy. This may be because of the different cell lines, assay conditions, and times used in the experiments. We now demonstrate that UV light irradiation signals through PERK-mediated translational inhibition of IκB, depletion of IκB, and activation of NFκB upon UV light irradiation.

The dependence of eIF2α phosphorylation upon IκB reduction was studied by using MEFαSS or MEFαVA (15). Our results show that UV light irradiation reduced the total amount of IκB in the MEFαSS cells but not in the MEFαVA cells (Fig. 1A). The total amount of IκB correlated with the rates of IκB translation in the cells (Fig. 1B). In addition, a reduced amount and a lower synthesis rate of IκB were observed in the MEFαVA cells (Fig. 1, A and B). This may be because of the lower background activity of NFκB in the MEFαVA cells (Fig. 3A), which regulates the expression of IκB (28). The requirement for PERK was studied in MCF-7 and MCF-7-PERKΔC cell lines (11). Our data show that NFκB was activated when IκB levels were reduced to ~60% in MCF-7 cells (Fig. 4, A and B). This result agrees with the results from the MEFαSS cells (Figs. 1 and 3). In MCF-7-PERKΔC cell lines, however, UV light-induced IκB reduction and NFκB activation were significantly inhibited (Fig. 4, A and B). These results suggest that PERK mediates UV light-induced IκB reduction and NFκB activation. Interestingly, whereas UV light-induced IκB reduction and NFκB activation correlated very well at all time points in both wild-type and mutant cell lines, they did not correlate in the late-phase (24 h) post-UV light irradiation in the mutant cell lines. In MEFαVA cells, NFκB activation was independent of IκB reduction at 24 h post-UV light irradiation (Figs. 1A and 3A). However, in MCF-7-PERKΔC, although the IκB level was high and NFκB was not activated at 24 h post-UV light irradiation, NFκB significantly translocated to the nucleus, in a manner similar to the parental cells (Fig. 4C). These results suggest that eIF2α phosphorylation and PERK activation may also play other roles in UV light-induced IκB degradation and NFκB activation. It will be interesting to identify the factors and signaling pathways that affect IκB degradation and NFκB activation in these mutated cell lines.
Depletion of IκB and the polyubiquitin-dependent proteasomal pathway.

In summary, our data show that UV light-induced early phase IκB degradation and NFκB activation can be prevented by the expression of a non-phosphorylatable eIF2α (S51A) mutant or a trans-dominant negative mutant PERK. Our data also show that UV light inhibits IκB translation, which can be reversed by expression of an eIF2α (S51A) mutant. Based on these results, we propose a novel mechanism for the UV light-induced early phase activation of NFκB (Fig. 5). We propose that UV light irradiation activates PERK, which phosphorylates eIF2α and inhibits new IκB synthesis. The existing IκB is depleted by natural degradation through the phosphorylation of Ser-32/Ser-36 by background IKK activity (10) and the polyubiquitin-dependent proteasomal pathway. Depletion of IκB leads to NFκB activation. These studies have identified a novel mechanism by which translational control can regulate gene transcription.

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Fig. 5. Model for UV light-induced activation of NFκB. Upon UV light irradiation, eIF2α is phosphorylated by PERK and inhibits IκB mRNA translation. The existing IκB is depleted by natural degradation through the polyubiquitin pathway. Subsequently, NFκB is released from IκB to translocate to the nucleus.

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