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Mechanism of UV-Induced I κ B α -Independent Activation of NF- κ B

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Abstract

Nuclear factor-kappa B (NF- κ B) plays an important role in UV-induced skin tumorigenesis. Activation of NF- κ B by UV-irradiation is composed of two phases. The early phase culminates with maximal levels of DNA binding ability at 4 h postirradiation and is dependent on translational inhibition. The late-phase activation of NF- κ B occurs between 16 and 48 h post-irradiation and the mechanism is not clear due to the fact that NF- κ B was activated in the presence of high level of I κ B α . In this report, we provide evidence that without translational inhibition, the transcription of I κ B α was induced by UV-irradiation. In the late-phase of UV-induced NF- κ B activation, the I κ B α depletion is the combined result of regulation at both transcriptional and translational levels. Neither ubiquitination nor proteasomal degradation have detectable attributions to I κ B α breakdown. We also demonstrate that UV only induced phosphorylation of p65(S276), while tumor necrosis factor- α induced phosphorylation at both Ser276 and 536 sites of p65. Based upon our results, we propose a novel mechanism for translation-regulated I κ B α depletion and MSK-mediated NF- κ B activation at 24 h post-UV-irradiation.

INTRODUCTION

Nuclear factor-kappa B (NF- κ B) activation upon UV-irradiation is composed of two phases. The early phase of activation culminates with maximal levels of DNA binding ability at 4 h postirradiation. During this time, activation of NF- κ B is dependent on translational inhibition (1,2) and is independent of induced activation of I κ B kinase (IKK) (3,4). The late-phase activation of NF- κ B occurs between 16 and 48 h postirradiation (5,6). The mechanism for the late-phase activation of NF- κ B was suggested to be dependent on DNA-damage signaling, IKK activation and ubiquitin-mediated I κ B degradation (4,7). However, our previous results demonstrated that NF- κ B is activated in the presence of high level of I κ B α in mouse embryonic fibroblast (MEF) cells with a Ser51 $\rightarrow$ Ala mutation at the phosphorylation site in the eukaryotic initiation factor (eIF2 α) (MEF^{A/A}) (2). In this report, we elucidate that I κ B α depletion in the late-phase of UV-irradiation is a result from a combined regulation at both transcription and translation levels. Neither the ubiquitination nor the proteasomal cleavages have detectable attributions to the late-phase I κ B α depletion. We also provide evidence that the late-phase activation of NF- κ B is independent of I κ B α depletion and is likely to be mediated through protein kinase A (PKA)/mitogen- and stress-activated protein kinase (MSK) pathway.

MATERIALS AND METHODS

Cell culture

Wild-type MEFs (MEF^{S/S}) and mutated ones (MEF^{A/A}), where Ser51 on the alpha subunit of the eIF2 α is mutated to a nonphosphorylatable Ala, were grown in 10% fetal bovine serum-

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enriched Dulbecco's Modified Eagle Medium (Cellgro) containing minimum essential medium essential and nonessential amino acids (Invitrogen). The cells were incubated at 37°C and 10% CO₂.

UV irradiation

UV was generated from a 15 W UVC lamp (UVP). The intensity of UV was standardized by a UV meter (UVP) set at 3 W m⁻². The media was withdrawn during the irradiation. After UV light irradiation, fresh medium was added to each plate.

Protein extraction

Nuclear and cytosolic proteins were extracted from the cells using NucBuster Protein Extraction Kit according to manufacturer's procedure (EMD Chemicals). Protein concentrations were measured with a Bio-Rad protein DC assay kit (Bio-Rad Laboratories). Total proteins were extracted from the cells using Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 80 mM NaCl, 100 mM Tris-HCl and 0.1% SDS). The cell debris was removed by centrifugation.

Analysis of total IκBα

Equal amounts of protein samples were subjected to SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were probed with either an anti-IκBα antibody or a mouse monoclonal anti-β-actin antibody (Sigma). A rabbit polyclonal anti-IκB antibody (sc-371; Santa Cruz Biotechnology) was used for mouse IκBα. After extensively washing with Tris-buffered saline plus Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected by using a SuperSignalTM chemiluminescent kit (Pierce).

Assay for p65 phosphorylation

Equal amounts of nuclear and cytosolic proteins were loaded on a 10% SDS-PAGE, followed by electroblotting onto nitrocellulose membrane. The membrane was then probed with anti-p65 (06-418; Upstate Biotechnology), phospho-p65(536) (3033; Cell Signaling) or phospho-p65(276) (3037; Cell Signaling) antibodies followed by HRP-conjugated, secondary antibodies. To prove the equal loading of the protein, the membrane was stripped using western blot stripping solution (Pierce) and β-actin was probed with anti-β-actin antibody (Sigma).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from UV-treated MEFs by RNeasy Mini Kit (Qiagen). Complementary DNA was prepared from 1 μg of RNase-free DNase-treated RNA, using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time polymerase chain reactions (PCR) were prepared with iQ SYBR Green Super Mix (Bio-Rad Laboratories) and 0.2 μM concentration of the following primers:

IκBα, 5': TTGGTCAGGTGAAGGGAGAC and 3': AGGTCTGCGTCAAGACTGCT.

β-actin, 5': TATGGAATCCTGTGGCATCC and 3': GTACTTGCCTCAGGAGGAG.

The reaction was performed on an iCycler (Bio-Rad Laboratories) starting with incubation at 95°C for 2.5 min followed by 40 cycles of 60 s at 95°C, 30 s at 60°C and 30 s at 72°C. Data was analyzed with the comparative delta threshold cycle method. Relative amounts of IκBα transcripts were normalized to the levels of β-actin mRNA in each sample.

RESULTS

Phosphorylation of eIF2 α prevents UV-induced transcriptional activation of I κ B α

To access the regulatory mechanism of I κ B α at 24 h post-UV, we first determined transcription and translation of I κ B α in the irradiated MEF^{S/S} and MEF^{A/A} cells. Quantitative real-time RT-PCR was used to determine the levels of mRNA in UV treated and nontreated cells. At 24 h post-UV treatment, the mRNA levels of I κ B α were not significantly changed in MEF^{S/S} cells, but increased five-fold in MEF^{A/A} cells (Fig. 1A). The total amount of I κ B α protein was decreased in MEF^{S/S} cells and remained high in MEF^{A/A} cells (Fig. 1B), which agrees with our previous observation (2). These results suggest that phosphorylation of eIF2 α prevents transcriptional activation of I κ B α expression, while it inhibits translation in the late period post-UV-irradiation. The reduction of I κ B α in the late-phase UV-induced NF- κ B activation is the combined result of regulation at both transcriptional and translational levels.

UV-induced I κ B α reduction is ubiquitination and proteasomal degradation independent

Earlier reports indicated that UV activates IKK during the late-phase of NF- κ B activation (7). On the other hand, IKK phosphorylates I κ B α and promotes its depletion through ubiquitination and proteasomal degradation (8,9). Given that translational inhibition also plays a role in the regulation of I κ B α at 24 h after UV-irradiation (Fig. 1), we determined the contributions of ubiquitination and proteasomal degradation for maintaining the level of I κ B α upon UV-irradiation. In the present studies, a membrane-permeable proteasome inhibitor MG132 (Sigma-Aldrich) and an I κ B α ubiquitin ligase inhibitor Ro106-9920 (Sigma-Aldrich) were used to inhibit UV-induced I κ B α depletion. Surprisingly, both inhibitors failed to influence I κ B α levels in MEF^{S/S} and MEF^{A/A} cells at 24 h post-UV-irradiation (Fig. 2A and B), while tumor necrosis factor- α (TNF α)-induced I κ B α was totally rescued by Ro106-9920 (10 μ M) in MEF^{S/S} (Fig. 2C, lane 4). The results suggest that neither ubiquitination nor proteasomal degradation have detectable attributions to the late-phase I κ B α depletion upon UV treatment. These results also suggest that IKK may not be involved during this period of NF- κ B activation.

UV-induced NF- κ B activation is *via* an I κ B α independent pathway

As UV activates the 65 kDa NF- κ B (p65) in the presence of high level of I κ B α in MEF^{A/A} cells at 24 h post-UV (2), we further determine the mechanism that is involved in the late-phase NF- κ B activation by analyzing the UV-induced translocation of p65 in the nucleus of UV-treated and nontreated MEF^{S/S} and MEF^{A/A} cells. At 24 h post-UV-irradiation nuclear p65 levels increased 3.3- and 3.7-fold in MEF^{S/S} and MEF^{A/A} cells, respectively (Fig. 3A and Table 1), which agrees with our previous observation (2). This result confirms that UV-induced late-phase activation of NF- κ B is independent of translational inhibition of I κ B α . The UV-induced reduction of I κ B α correlates but does not cause NF- κ B activation in MEF^{S/S} cells.

To assess whether IKK is involved in UV-induced late-phase activation of NF- κ B, we analyzed phosphorylation of p65 at Ser536, which is known to be achieved through the IKK signaling pathway (10,11). The cells were UV-irradiated and the phosphorylation of p65 at Ser536 was monitored by immunoblot analysis of nuclear proteins using specific antibody against phosphorylated p65(S536). At 24 h post-UV-irradiation, the phosphorylation of p65(S536) is not changed (Fig. 3A, lanes 1 vs. 2; Table 1) in wild-type MEF^{S/S} cells, while it is dramatically diminished in the mutated MEF^{A/A} cells (Fig. 3A, lanes 3 vs. 4; Table 1). The results suggest that UV-induced late-phase activation of NF- κ B is independent of IKK activation.

After ruling out the IKK signaling pathway, we determined whether the NF- κ B activation occurs *via* PKA and MSK-mediated signaling. Both PKA and MSK phosphorylate p65 at Ser276, which is located in the Rel homology domain (RHD) (12). Specific antibody against phosphorylated p65(S276) was used to probe the phosphorylation of nuclear p65 in MEF^{S/S}

and MEF^{A/A} cells at 24 h post-UV-irradiation. While not detected in untreated cells, the phosphorylation of p65(S276) was significantly increased in both cell lines after UV-irradiation (Fig. 3A, lanes 1 vs. 2 and 3 vs. 4; Table 1). These results indicate that UV-induced late-phase activation of NF- κ B is correlated with phosphorylation of p65(S276).

To determine the relationship of p65 phosphorylation and nuclear translocation, we measured the p65 and phospho-p65 in the cytosol. The total amount of p65 and phospho-p65 was not changed in the cytosol after UV-irradiation (Fig. 3B). However, while the phospho-p65(S276) was not detected, the p65(S536) was highly phosphorylated in the cytosol (Fig. 3B). These results suggest that p65(S276) is phosphorylated directly in the nucleus. As the p65(S276) is phosphorylated by PKA in the cytosol (13–15) and MSK in the nucleus (16), the results also suggest that UV-induced NF- κ B activation occurs *via* MSK-mediated phosphorylation of p65 (S276).

To further elucidate whether the phosphorylation pattern of nuclear p65 is unique for UV-irradiation, we measured the phosphorylation of nuclear p65(S536) and p65(S276) in the same cell lines after TNF α treatment. The results demonstrate that TNF α induces phosphorylation of p65 at both S536 and S276 sites in MEF^{S/S} cells (Fig. 3C, lanes 1 vs. 2), which agrees with previous reports stating that TNF α activates both IKK and PKA/MSK signaling pathways (17). These results suggest that UV-induced late-phase activation of NF- κ B is truly independent of IKK signaling pathway.

DISCUSSION

Activation of NF- κ B is a highly regulated multilevel process. NF- κ B forms a complex with I κ B, which masks NF- κ B's nuclear localization sequence and retains it in the cytoplasm of cells. One well-established mechanism for NF- κ B activation is that IKK is activated and phosphorylates I κ B at the N-terminal serines (Ser32 and 36 for I κ B α), which leads to the dissociation of I κ B from NF- κ B and is rapidly degraded through the proteasomal pathway. The free NF- κ B then translocates to the nucleus and activates the target genes (18–20). Although the most important regulatory step is the cytoplasmic to nuclear transport (21,22), the transactivation potential needed to promote transcription is conferred by additional posttranslational modifications, which mainly consist of serine phosphorylations in the transactivation domain and RHD (23).

The UV-induced activation of NF- κ B occurs through two pathways: a membrane dependent pathway in the early phase, and a nuclear DNA damage dependent pathway in the late-phase of activation (7). We previously provided evidence that UV does not reduce the half-life of I κ B α within 12 h post-irradiation; instead, UV inhibits nascent protein synthesis, which leads to a reduction in the net amount of I κ B α due to the short half-life of I κ B α (7). We also demonstrated that expression of a nonphosphorylatable mutant of eIF2 α (S51A) protects the UV-induced reduction of I κ B α , which indicated that the reduction of I κ B α is translationally regulated. As a result, we concluded that for the early period, UV light induction of NF- κ B is owed to translational inhibition of I κ B α synthesis through eIF2 α Ser51 phosphorylation. However, due to the coexistence of high I κ B α levels and still activated NF- κ B in MEF^{A/A} cells at 24 h postirradiation the results were inconclusive for the late period activation. To further elucidate the mechanism for UV-induced late-phase activation of NF- κ B, we first determine transcriptional and translational regulation of I κ B α expression upon UV-irradiation. Our results show that mRNA levels of I κ B α increased five-fold in MEF^{A/A} cells (Fig. 1A), while remained the same in MEF^{S/S} cells. This is the first time to demonstrate that phosphorylation of eIF2 α prevents transcriptional activation of I κ B α expression in the late-phase of UV-irradiation. While the transcripts of I κ B α were increased, the total amount of I κ B α protein is unchanged in MEF^{A/A} cells. This is probably due to the fact that translation efficiency is partially reduced

in the UV-irradiated MEF^{A/A} cells at 24 h postirradiation as we previously reported (2,24). Indeed, the amount of newly synthesized I κ B α protein remained the same in MEF^{A/A} cells after UV-irradiation (2). These results demonstrate that I κ B synthesis after UV-irradiation is regulated at both transcriptional and translational levels.

After analyzing the synthesis of I κ B α , we determined the role of ubiquitination and proteasomal degradation for maintaining the level of I κ B α upon UV-irradiation. Our results demonstrated that neither ubiquitination nor proteasomal degradation have detectable attributions to the late-phase UV-induced I κ B α depletion (Fig. 2A and B). In addition, the phosphorylation on sites Ser32/36 of I κ B α could not be detected by western blot using specific phospho-I κ B- α antibody (5A5; Cell Signaling) (data not shown), which suggest that IKK is not significantly activated after UV-irradiation. To further determine whether UV activates IKK at 24 h post-irradiation, we measured p65 phosphorylation at Ser536, which is a direct target for activated IKK (12, 25). Our results indicate that UV did not induce phosphorylation of p65(S536) in MEF^{S/S} cells (Fig. 3A, lanes 1 vs. 2; Table 1). As the downstream effects of IKK are undetected at both the proteasomal degradation pathway or at the NF- κ B Ser536 phosphorylation, the results suggest that IKK activity is not significant during the late-phase of UV-irradiation. Interestingly, while p65(S536) is highly phosphorylated in the cytosol even without UV treatment (Fig. 3B), it is dramatically diminished in the mutated MEF^{A/A} cells upon UV-irradiation (Fig. 3A, lanes 3 vs. 4; Table 1). The increased phosphorylation of p65(S536) in the nucleus could not be due to a contamination of cytosolic protein as there was no α -tubulin detected by western blot analysis in these samples (data not shown). As the same samples were used for each blot, the lack of cross contamination was also evident from the comparison of phosphorylation of p65 (S536) (Fig. 3A and B, row 2, lane 4) and p65(S276) (Fig. 3A and B, row 3, lanes 2 and 4).

To verify whether the impact of translational regulation on p65(S536) phosphorylation is unique for UV-irradiation, we analyzed TNF α -induced p65(S536) phosphorylation in the same cells. Our results show that p65(S536) phosphorylation is induced by TNF α in MEF^{S/S} cells, but reduced in MEF^{A/A} cells. These results demonstrate for the first time that translation regulates the phosphorylation of p65(S536). Another common posttranslation modification site on p65 is at Ser276, which is phosphorylated by PKA in the cytosol (13–15) and MSK in the nucleus (16). The phosphorylation of p65(S276) was dramatically increased in the nucleus in both cell lines after UV-irradiation (Fig. 3A, lanes 1 vs. 2 and 3 vs. 4; Table 1), while it was not significantly changed in cytosol (Fig. 3B, lanes 1 vs. 2 and 3 vs. 4). As I κ B α is required to break down for PKA activation (12) and we detected no p65(S276) phosphorylation in the cytosol, our results suggest that UV-induced late-phase activation of NF- κ B is likely to be mediated by the MSK signaling pathway.

In summary, I κ B α depletion during the late-phase NF- κ B activation by UV-irradiation is the combined result of regulation at both transcriptional and translational levels. Neither ubiquitination nor proteasomal degradation have detectable attributions to the I κ B α depletion. The UV-induced late-phase activation of NF- κ B is not a result of lower I κ B α levels, but this depletion correlates with the pattern of inducing a most probably MSK-mediated p65(S276) phosphorylation. Based on our results, we proposed a novel mechanism for UV-induced late-phase activation of NF- κ B (Fig. 4).

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A: mRNA levels of $\text{I}\kappa\text{B}\alpha$

B: Western blot analysis of $\text{I}\kappa\text{B}\alpha$

Figure 1.

Inability to block translation allows for transcriptional activation of $\text{I}\kappa\text{B}\alpha$. (A) Quantitative real-time PCR of $\text{I}\kappa\text{B}\alpha$ mRNA expression in non-treated and UV-irradiated MEFs. Relative amounts of $\text{I}\kappa\text{B}\alpha$ transcripts were normalized to the levels of β -actin housekeeping gene in each sample. (B) Western blot analysis of total $\text{I}\kappa\text{B}\alpha$ at 24 h post UV-C irradiation (30 J m^{-2}) of MEF^{S/S} and MEF^{A/A} cells. The intensities of the bands were quantified by ImageJ (v 1.31; NIH). Expression levels were normalized by the levels of β -actin and shown as a percentage of $\text{I}\kappa\text{B}\alpha$ expression at 0 h after induction.

A:

B:

C:

Figure 2.

The ubiquitination and proteasome inhibitors failed in rescuing UV-induced IκBα depletion. Total amounts of IκB and β-actin in UV-irradiated or TNFα-treated MEF^{S/S} and MEF^{A/A} cells were determined by western blot analysis. The cells were treated or not treated with UV-irradiation (30 J m⁻², panels A and B) for 24 h or TNFα (10 ng mL⁻¹, panel C) for 1 h in the presence or absence of MG132 (5 mM, panel A) or Ro106 (5 or 10 μM, panels B and C) as indicated. The intensities of the bands were quantified by ImageJ (v 1.31; NIH). The expression levels of IκB were normalized by the expression levels of β-actin and expressed as a percentage of IκB expression at 0 h post-UV light irradiation.

Figure 3.

The localization and phosphorylation of NF- κ B at 24 h post-UV-irradiation. The amounts of nuclear (panels A and C) or cytosolic (panel B) p65, p65-P(536), p65-P(276) and β -actin in UV-irradiated or TNF α -treated MEF^{S/S} and MEF^{A/A} cells were determined by western blot analysis. The cells were treated or not treated with UV-irradiation (30 J m⁻², panels A and B) for 24 h or TNF α (10 ng mL⁻¹, panel C) for 1 h.

Figure 4.
Proposed model for UV light-induced late-phase activation of NF-κB.

Table 1

Quantitative estimation of p65 and phospho-p65 in the nucleus.

	MEF^{S/S}		MEF^{A/A}	
UVC(J m ⁻²)	0	30	0	30
p65	1.0	3.3 ± 0.8	1.0	3.7 ± 0.6
p65-P(536)	1.0	1.1 ± 0.1	1.0	–
p65-P(276)	–	++	–	++

The band intensities of the nuclear p65, p65-P(536), p65-P(276) and β -actin (Fig. 1A) were quantified by ImageJ (v 1.31; NIH). The expression levels were normalized by the expression levels of β -actin and expressed as a percentage of their respective expressions at 0 h post-UV light irradiation. – = undetected and ++ = detected.