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CK2 Is a C-Terminal IκB Kinase Responsible for NF-κB Activation during the UV Response

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Summary

NF-κB is activated in response to proinflammatory stimuli, infections, and physical stress. While activation of NF-κB by many stimuli depends on the IκB kinase (IKK) complex, which phosphorylates IκBs at N-terminal sites, the mechanism of NF-κB activation by ultraviolet (UV) radiation remained enigmatic, as it is IKK independent. We now show that UV-induced NF-κB activation depends on phosphorylation of IκBα at a cluster of C-terminal sites that are recognized by CK2 (formerly casein kinase II). Furthermore, CK2 activity toward IκB is UV inducible through a mechanism that depends on activation of p38 MAP kinase. Inhibition of this pathway prevents UV-induced IκBα degradation and increases UV-induced cell death. Thus, the p38-CK2-NF-κB axis is an important component of the mammalian UV response.

Introduction

The mammalian UV response is a gene induction response that is elicited in response to UV exposure (Herrlich et al., 1997). The UV response is mediated through activation of several transcription factors such as AP-1, NF-κB, and p53 (Devary et al., 1991; Shaulian et al., 2000; Stein et al., 1989). Although UV exposure can result in extensive DNA damage, only p53 activation is dependent on nuclear signals that are generated by damaged DNA (Liu et al., 1996). By contrast, activation of AP-1 or NF-κB depends on cytoplasmic signals that are generated independently of DNA damage (Devary et al., 1993). The mechanism of AP-1 activation in response to UV radiation has been investigated in great detail and shown to depend on induction of c-Jun expression and its N-terminal phosphorylation (Devary et al., 1991). Both of these processes require activation of JNK, a member of the MAP kinase (MAPK) family (Dérijard et al., 1994; Hibi et al., 1993; Tournier et al., 2000). In addition to JNK, UV radiation results in activation of p38 MAPK, and both JNK and p38 activation contribute to induction of c-Fos expression in UV-irradiated cells (Cavigelli et al., 1995). By contrast, the mechanism of NF-κB activation in response to UV irradiation has remained enigmatic.

Normally, NF-κB transcription factors are regulated through interaction with inhibitory proteins, the IκBs (Ghosh et al., 1998). The binding of IκBs to NF-κB dimers masks the nuclear localization sequences (NLS) of the latter and traps them in the cytoplasm. Most stimuli that lead to NF-κB activation activate a protein kinase, the IκB kinase (IKK), that phosphorylates IκBs at two N-terminal serines (serines 32 and 36 in IκBα) and thereby triggers their ubiquitin-dependent degradation (Karin and Ben-Neriah, 2000). Although UV radiation does lead to ubiquitin-dependent degradation of IκBα, in most cell types this process does not depend on phosphorylation of the N-terminal serines or activation of IKK (Bender et al., 1998; Li and Karin, 1998). One possible exception is cells in which a mutant form of the β-TrCP E3 ubiquitin ligase involved in IκB degradation (Karin and Ben-Neriah, 2000) was overexpressed. Under these conditions, weak UV-induced N-terminal IκBα phosphorylation was observed, but UV-induced IKK activity could not be detected (Huang et al., 2002). Without further knowledge of a protein kinase responsible for UV-dependent IκB phosphorylation and degradation in other cell types (Bender et al., 1998; Li and Karin, 1998), the mechanism of UV-induced NF-κB activation had remained a mystery. Nonetheless, the ability of NF-κB to inhibit apoptosis in response to a variety of stimuli, including DNA damaging agents (Karin and Lin, 2002; Wang et al., 1996), suggests its activation is an important component of the mammalian UV response.

Here we describe the identification of a UV-activated IκB kinase whose activity depends on the β subunit of CK2. CK2 (formally known as casein kinase II) is a highly conserved and ubiquitous serine/threonine kinase that is composed of two catalytic subunits (αα, α′α′, or αα′) and two β regulatory subunits (Allende and Allende, 1995). The regulation and function of CK2 are not well understood and until recently it was thought to be a constitutive, nonregulated protein kinase (Allende and Allende, 1995). In fact, CK2 was previously shown to be responsible for constitutive phosphorylation of IκBα at C-terminal sites (Barroga et al., 1995; McElhinny et al., 1996). However, new evidence obtained in the past year or two suggests that CK2 is a stress-activated protein kinase that may participate in the transduction of survival signals (Ahmed et al., 2002; Litchfield, 2003). Consistent with this notion, we show that CK2-mediated IκBα phosphorylation has an important UV-protective function.

Results

Identification of a UV-Activated Kinase that Binds and Phosphorylates IκBα

Previous studies have shown that NF-κB activation in response to UV irradiation involves proteasome-dependent degradation of IκBα (Bender et al., 1998; Li and Karin, 1998). Unlike other stimuli, induction of IκBα deg-

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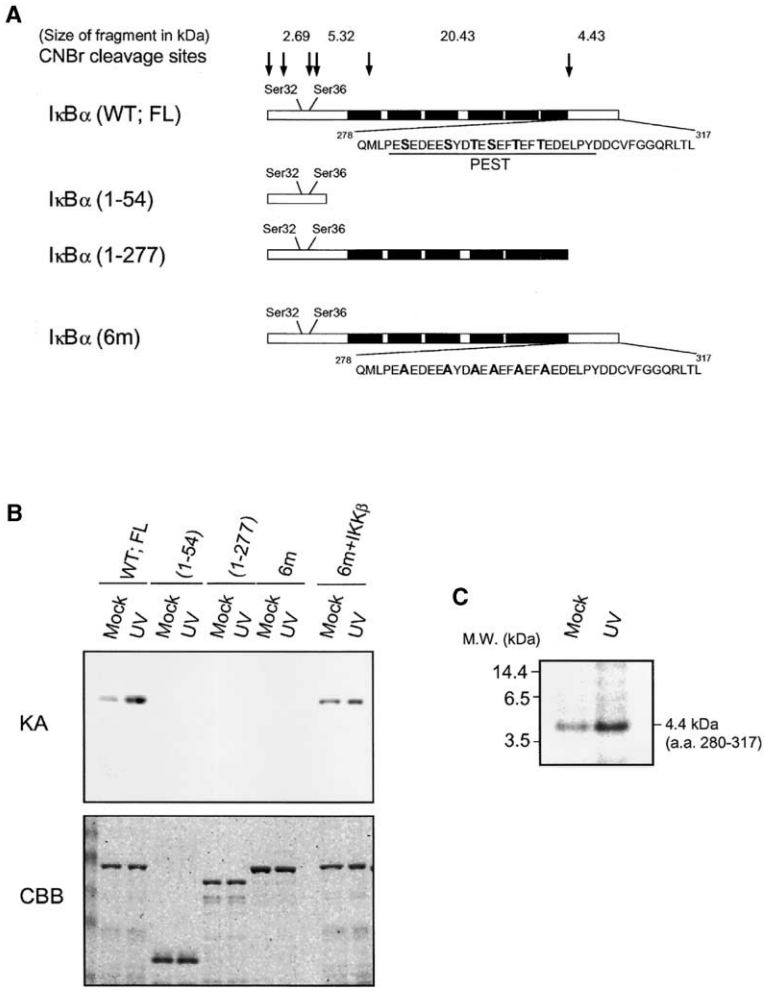

Figure 1. Identification of a UV-Activatable IκBα Kinase

(A) Schematic representation of human IκBα constructs used in this study. Solid boxes represent the ankyrin repeat motifs. The amino acid sequence of the C-terminal domain containing a PEST motif (underlined) is shown. CK2 phosphorylation sites and their alanine substitution mutations are indicated in bold letters. CNBr cleavage sites are indicated by the arrows, and the predicted sizes of cleavage products are shown at the top.

(B) A UV-activated kinase that phosphorylates IκBα. HeLa cells were mock treated or exposed to UVC (20 J/m^2) radiation. After 20 min, cells were harvested, whole-cell lysates were prepared, and samples containing 300 μg protein were incubated for 3 hr with the indicated GST-IκBα fusion proteins immobilized to GSH-agarose beads. After washing, a solid-phase kinase assay was performed. For GST-IκBα(6m), the kinase reaction was repeated in the presence of a small amount of recombinant IKKβ. The phosphorylated GST-IκBα proteins were resolved by SDS-PAGE. The gel was stained with Coomassie brilliant blue (CBB), dried, and subjected to autoradiography (KA).

(C) The UV-activated kinase phosphorylates IκBα at C-terminal sites. Phosphorylated GST-IκBα(WT) was separated by SDS-PAGE and transferred to a PVDF membrane. After autoradiography, a portion of the membrane containing the phosphorylated protein was cut, the phosphopeptide was eluted, digested with CNBr, and separated on a Tris-tricine gel. The phosphopeptides were detected by autoradiography. The 4.4 kDa band corresponds in size to the CNBr fragment from amino acid 280 to the C terminus (amino acid 317).

radation by UV does not involve phosphorylation of serines 32 and 36, nor IKK activation (Bender et al., 1998; Li and Karin, 1998). To identify a protein kinase that phosphorylates IκBα in response to UV irradiation and may therefore trigger its degradation, we employed the solid-phase kinase assay, previously used to identify JNK as the UV-activated kinase that targets c-Jun (Hibi et al., 1993). Full-length human IκBα coding region (Figure 1A) was fused to the bacterial glutathione S-transferase (GST) coding region, and the resultant fusion protein was produced in *E. coli* and immobilized on glutathione (GSH)-agarose beads to generate an affinity matrix. HeLa cells were either mock treated or UV irradiated with a dose of 20 J/m^2 and harvested 20 min later to prepare whole-cell lysates. The affinity matrix was incubated with these lysates, washed, and the kinase reaction was carried out on the beads. A UV-activated protein kinase that bound to and phosphorylated the full-length IκBα polypeptide was detected (Figure 1B). To obtain more information about this kinase, and identify the region of IκBα it interacts with, we constructed GST-fusion proteins containing residues 1–54 and 1–277 of IκBα. Neither GST-IκBα(1-54) nor GST-IκBα(1-277) were phosphorylated after incubation with lysates of either nonirradiated or UV-irradiated cells (Figure 1B). These results suggest that the region of IκBα between amino acids 278 and 317 may harbor both the phosphorylation site(s) and the docking site (see below) for the UV-activated kinase. This possibility is consistent with an earlier report that residues between 277 and 287 of IκBα are required for its UV-induced degradation (Bender et al., 1998).

The same region of IκBα was also reported to contain CK2 phosphorylation sites (Lin et al., 1996; McElhinny et al., 1996; Schwarz et al., 1996). To examine whether the CK2 phosphorylation sites are the ones recognized by the UV-responsive protein kinase, we generated a GST-IκBα fusion protein harboring alanine substitutions at all six possible CK2 target sites (designated as 6m) and used it as a substrate in the solid-phase kinase assay. The six alanine substitution mutation completely abolished UV-induced phosphorylation of IκBα but had no effect on its phosphorylation by IKKβ (Figure 1B). To confirm that the C-terminal region (amino acids 278–317) of IκBα does indeed contain the phosphoacceptor sites for the UV-activated kinase, we subjected full-length IκBα that was phosphorylated by the kinase present in the UV-irradiated cell extract to phosphopeptide mapping. The phosphorylated protein was digested with cyanogen bromide (CNBr), and the resultant peptides were separated on a Tris-tricine gel. A single phosphopeptide of about 4.4 kDa was detected (Figure 1C). The size of

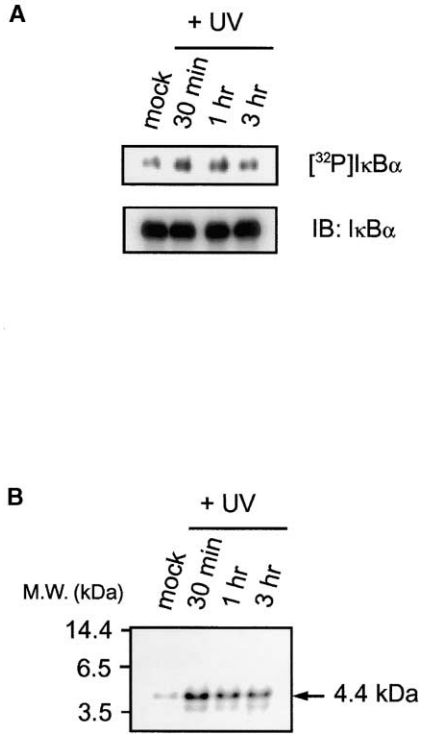

Figure 2. UV Irradiation Induces the Phosphorylation of IκBα at C-Terminal Sites, In Vivo

(A) UV irradiation increases IκBα phosphorylation. HeLa cells were metabolically labeled with [^{32}P] orthophosphate for 4 hr. After exposure to UVC radiation (20 J/m^2), the cells were harvested at the indicated time points and kept frozen on dry-ice until lysis. IκBα was immunoprecipitated from cell lysates, separated by SDS-PAGE, transferred to a PVDF membrane, and visualized by autoradiography. To monitor the amount of total IκBα, the membrane was immunoblotted with an anti-IκBα antibody.

(B) Phosphopeptide mapping. In vivo labeled IκBα isolated as described above was separated by SDS-PAGE and transferred to a PVDF membrane, and the band containing ^{32}P-IκBα was excised and subjected to phosphopeptide mapping using CNBr and Tris-tricine gels as described in Figure 1C. The 4.4 kDa band containing the C-terminal phosphorylation sites is the one whose phosphorylation increases after UV irradiation.

this band is fully consistent with the size of a predicted CNBr fragment spanning amino acids 280–317 (Figure 1A). It should be noted that this is the only predicted CNBr fragment similar in size to the CNBr-generated phosphopeptide shown above. The sizes of the other predicted CNBr cleavage products of IκBα are 20.4, 5.3, and 2.7 kDa and two fragments of less than 1 kDa. To confirm that the sites recognized by the UV-activated kinase are also phosphorylated more extensively in UV-irradiated cells, we conducted in vivo labeling experiments. HeLa cells were labeled with ^{32}P and IκBα was isolated either before or at different time points after UV irradiation. As shown in Figure 2A, UV irradiation increased the overall level of IκBα phosphorylation. To determine whether the sites responsible for the increased labeling of IκBα are indeed the C-terminal sites, the isolated ^{32}P-labeled IκBα polypeptides from nonirradiated and UV-irradiated cells were digested with CNBr and subjected to phosphopeptide mapping as described above. The only peptide whose phosphorylation was clearly increased in response to UV irradiation was the 4.4 kDa peptide that contains the C-terminal sites (Figure 2B).

The UV-Activated IκBα Kinase Contains the CK2β Subunit

The results described above strongly suggest that the UV-responsive kinase phosphorylates IκBα at the same C-terminal sites that are phosphorylated by CK2 or a subset thereof. To characterize the docking site for this kinase in the IκBα polypeptide in more detail, lysates prepared from mock-treated or UV-irradiated cells were incubated with different GST-IκBα fusion proteins immobilized on GSH-agarose beads, and the solid-phase kinase assays were repeated. This time we have also collected the supernatants in addition to the bead-bound fraction and subjected them to immunecomplex kinase assay with an anti-CK2β antibody (see below). As shown in Figure 3A, incubation with either GST beads or GST-IκBα(1-277) beads did not remove CK2 activity from the lysate, indicating that the docking site is not present in the first 277 amino acids of IκBα. On the other hand, incubation with either GST-IκBα(WT), GST-IκBα(6m), or GST-IκBα(241-317) beads resulted in nearly complete removal of UV-induced IκBα kinase activity from the cell lysates. Thus, the docking site for the UV-activated kinase that phosphorylates IκBα is located between amino acids 241 and 317, most likely C-terminal to residue 277. This is the same region of IκBα that contains the phosphoacceptor sites for the kinase.

The C-terminal phosphoacceptor sites of IκBα are proximal to acidic residues, thereby respresenting typical CK2 phosphorylation sites (Pinna, 1990). Furthermore, these sites were previously shown to be phosphorylated by purified CK2 (Lin et al., 1996; McElhinny et al., 1996; Schwarz et al., 1996). At the time, however, CK2 was believed to be a constitutive protein kinase. To examine whether the UV-activated IκBα kinase described above is related to CK2, we carried out immunodepletion experiments using antibodies to the CK2β subunit. Immunodepletion of CK2β removed the UV-stimulated IκBα kinase activity from cell lysates, whereas mock depletion with a control mouse IgG had no effect (Figure 3B). To further examine whether the UV-activated IκBα kinase is indeed CK2, we used an anti-CK2β antibody to immunoprecipitate CK2 from lysates of mock-irradiated and UV-irradiated HeLa cells and examined the ability of the immunecomplexes to phosphorylate GST-IκBα(WT). This experiment revealed that CK2 phosphorylated IκBα and that its specific activity was increased up to 5-fold within 15 min of UV exposure (Figure 3C). Furthermore, doses of UVC as low as 5 J/m^2 were sufficient for substantial stimulation of CK2 activity toward IκBα. We also examined CK2 kinase activity toward GST-IκBα(6m), which harbors alanine substitutions at the CK2 phosphoacceptor sites. As shown in Figure 3D, the mutant substrate was no longer phosphorylated by CK2 isolated from UV-irradiated cell lysates, but was still phosphorylated by recombinant IKKβ, which uses serines 32 and 36 as phosphoacceptor sites. In addition, we have shown that incubation of UV-irradiated cell lysates with GST-IκBα fusion proteins that

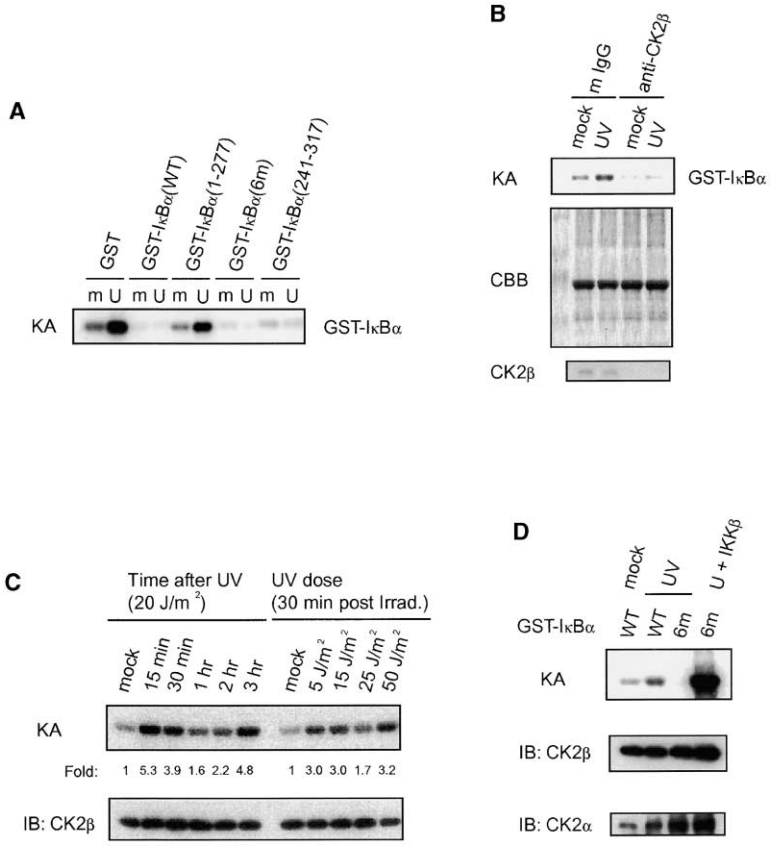

Figure 3. The IκBα Kinase Activity of CK2 Is Activated in Response to UV Irradiation

(A) The UV-activated kinase binds to the C-terminal region of IκBα. Cells were mock treated (m) or UVC irradiated (U) and whole-cell lysates were prepared. The lysates were incubated with the indicated GST-fusion proteins bound to GSH agarose beads. After incubation, the beads were spun down and the supernatants were collected. The supernatants were subjected to immunecomplex kinase assay with anti-CK2β antibody using GST-IκBα as a substrate.

(B) Immunodepletion of CK2 abolishes UV-activated IκBα kinase activity. Cells were mock treated or UVC irradiated and whole-cell lysates were prepared. The lysates were incubated with either purified mouse IgG or anti-CK2β antibody (mouse IgG) followed by immunoprecipitation to remove immunoreactive materials. The depleted lysates were subjected to solid-phase kinase assay as shown in Figure 1B using GST-IκBα(WT) as an affinity ligand and substrate. To confirm that CK2 was depleted, aliquots of the supernatant were immunoblotted with anti-CK2β antibody. The presence of IκBα was monitored by straining with Coomassie brilliant blue.

(C) UV irradiation activates CK2. For the time course experiment, HeLa cells were exposed to 20 J/m² of UVC and harvested at the indicated time points. For the dose-response experiment, cells were exposed to the indicated doses of UVC and lysates were prepared 30 min later. CK2 was immunoprecipitated with anti-CK2β antibody, and its kinase activity was analyzed by immunecomplex kinase assay (KA) with IκBα as a substrate. Gel loading was monitored by immunoblotting (IB) with anti-CK2β antibody. Relative kinase activity was determined by phosphoimaging.

(D) CK2 phosphorylates IκBα at C-terminal sites. HeLa cells were mock treated or UVC (20 J/m²) irradiated. After 30 min, CK2 was isolated by immunoprecipitation with anti-CK2β antibody and its kinase activity was measured with either WT IκBα or IκBα(6m) as substrates. Where indicated, recombinant IKKβ was added to the kinase reaction.

contain the C-terminal region of IκBα (its PEST domain) results in depletion of CK2 activity (Figure 3A). Collectively, these results strongly suggest that the UV-activated kinase responsible for IκBα phosphorylation is CK2.

Knockdown of CK2β Abolishes UV-Induced IκBα Degradation

To confirm that CK2 is indeed the UV-responsive IκBα kinase, we "knocked down" expression of its CK2β subunit by RNA interference (Elbashir et al., 2001). Transfection of HeLa cells with a specific siRNA directed against CK2β subunit mRNA resulted in substantial reduction in CK2β expression at 24 hr posttransfection (Figure 4). At that time point, induction of IκBα degradation in response to UV irradiation was almost completely inhibited. As a control, transfection of a specific siRNA directed against κB-Ras2 (Fenwick et al., 2000) mRNA did not result in such an effect (Figure 4). The CK2β siRNA had no effect on expression of CK2α (Figure 4) or on TNFα-induced IκBα degradation (data not shown).

The CK2 Phosphorylation Sites Are Required for UV-Induced IκBα Degradation

To further investigate whether IκBα phosphorylation by CK2 is essential for its UV-induced degradation, we reconstituted fibroblasts derived from IκBα knockout mice (Beg et al., 1995) with WT mouse IκBα and a mutant in which all of the CK2 phosphorylation sites (only five sites in the mouse polypeptide versus six sites in the human polypeptide) were replaced with alanines (5m). Several independent pools of reconstituted cells were obtained, and the UV-induced degradation of IκBα was assessed by immunoblotting. While epitope (HA)-tagged IκBα (WT) was properly degraded in response to UV irradiation, no UV-induced degradation of the HA-tagged IκBα(5m) mutant was observed (Figure 5A). In fact, UV irradiation resulted in increased accumulation of the mutant protein, probably due to its increased synthesis. By contrast, mutation of the CK2 phosphoacceptor sites did not affect TNFα-induced IκBα degradation.

To obtain more quantitative information regarding the stability of IκBα, and its CK2 phosphorylation site mutant (5m) in UV-irradiated cells, we determined the half-lives of both proteins. Knockout cells reconstituted with each version of IκBα were UV irradiated (20 J/m²), and incubated for up to 4 hr in the presence of cycloheximide to inhibit de novo protein synthesis. Cells were harvested at the indicated time points and lysates were prepared. We examined the level of IκBα by immunoblotting, and quantitated it by densitometry. As shown in Figure 5B, WT IκBα was considerably less stable than

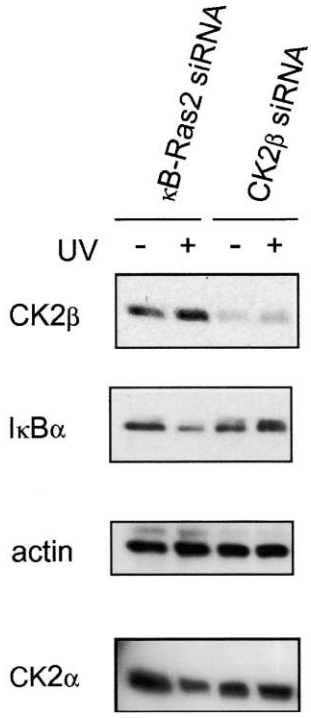

Figure 4. Knockdown of CK2β Abrogates UV-Induced IκBα Degradation

HeLa cells were transfected with 20 nmol of siRNA for either CK2β or κB-Ras2 (used as a control) mRNAs. After 24 hr, cells were either mock treated or UV irradiated (20 J/m^2), and lysates were prepared 5 hr later. Expression levels of CK2β, CK2α, and IκBα were examined by immunoblotting. Actin levels were used to monitor loading.

the 5m mutant ($t_{1/2}$ of 15.6 and 40.8 min for the WT and 5m IκBα proteins, respectively) in UV-irradiated cells. The results strongly suggest that the CK2 phosphorylation sites are required for induction of rapid IκBα turnover after UV irradiation.

CK2-Induced IκBα Phosphorylation and NF-κB Activation Have a UV-Protective Function

Next we examined the effect of CK2-mediated IκBα phosphorylation and degradation on NF-κB activation. While UV exposure of *IκBα*$^{-/-}$ cells reconstituted with WT IκBα resulted in RelA (p65) nuclear translocation, no UV-induced nuclear translocation of p65 could be detected in cells reconstituted with the IκBα(5m) mutant (Figure 6A, upper panels). Correspondingly, UV irradiation of cells expressing WT IκBα resulted in induction of NF-κB DNA binding activity, but no UV-induced NF-κB DNA binding activity could be detected in cells expressing the IκBα(5m) mutant (Figure 6A, lower panels). By contrast, and consistent with its effect on IκBα degradation, treatment with TNFα led to p65 nuclear translocation and NF-κB activation in both WT IκBα and IκBα(5m) expressing cells (Figure 6A).

We next investigated the physiological consequences of CK2-induced IκBα phosphorylation. As UV-induced IκBα degradation leads to NF-κB activation and NF-κB is an antiapoptotic transcription factor (Karin and Lin, 2002), we measured cell death as an end point. First, we examined the short-term effect of UVC irradiation on *IκBα*$^{-/-}$ cells reconstituted with either WT IκBα or the 5m mutant thereof. As shown in Figure 6B, knockout cells reconstituted with IκBα(5m) were more sensitive to UV than those with reconstituted with the WT protein. To further examine the UV-protective function of CK2-induced IκBα degradation, we performed long-term clonogenic survival assays, in which the number of colonies formed by UV-irradiated cells is determined 2–3 weeks after UV exposure. Again, knockout cells reconstituted with IκBα(5m) were found to be more sensitive to UV irradiation than those reconstituted with WT IκBα (Figure 6B). Thus, UV-induced IκBα degradation, which depends on its phosphorylation by CK2, protects cells against UV-induced cell death. It should be noted, however, that the protective effect of NF-κB was diminished at higher doses of UVC radiation (above 20 J/m^2), which cause much more extensive cell death (data not shown).

UV-Induced CK2 Activation Is Dependent on p38α MAPK

Recently, it was described that activation of CK2 by various stresses other than UV irradiation depends on activation of p38 MAPK, which acts as an allosteric regulator of CK2 (Sayed et al., 2000). We investigated whether UV-induced CK2 activation involves a similar mechanism. Indeed, we found that incubation of HeLa cells with the specific p38 inhibitor, SB203580, inhibited activation of CK2 by UV irradiation (Figure 7A). In addition, in fibroblasts derived from *p38α*$^{-/-}$ mice (Tamura et al., 2000), UV-induced CK2 activity was abrogated (Figure 7B). Furthermore, no delayed activation of CK2 could be detected in UV-irradiated *p38α*$^{-/-}$ cells followed for up to 9 hr postirradiation. Immunoblotting of the immunoprecipitated material from WT and *p38α*$^{-/-}$ cells revealed that there was no significant reduction in the amount of both catalytic (α) and regulatory (β) CK2 subunits in the knockout cells. As UV-induced CK2 activity is abrogated, we expected that IκBα degradation in these cells is also defective. Indeed, IκBα was no longer degraded in response to UV radiation in *p38α*$^{-/-}$ cells (Figure 7B). Correspondingly, *p38α*$^{-/-}$ cells exhibit defective induction of NF-κB DNA binding activity in response to UV radiation but retain a normal response to TNFα (Figure 7C). These results strongly suggest that UV-induced activation of CK2 is dependent on UV-induced p38 MAPK activity. In turn, CK2 activation is required for UV-induced IκBα degradation and NF-κB activation. It is also noteworthy that *p38α*$^{-/-}$ *cells* are more sensitive to UVC radiation than WT cells based on clonogenic survival assays (data not shown). Thus, the p38α-CK2 axis is required for IκBα degradation and NF-κB activation, which exerts a protective function in UV-irradiated cells.

Discussion

Compared to most other activators of NF-κB, short wavelength UV radiation has unique features. First, it does not cause IKK activation, and second, its ability to activate NF-κB, although still dependent on IκB degradation, does not require N-terminal phosphorylation of IκBs (Bender et al., 1998; Li and Karin, 1998). Thus,

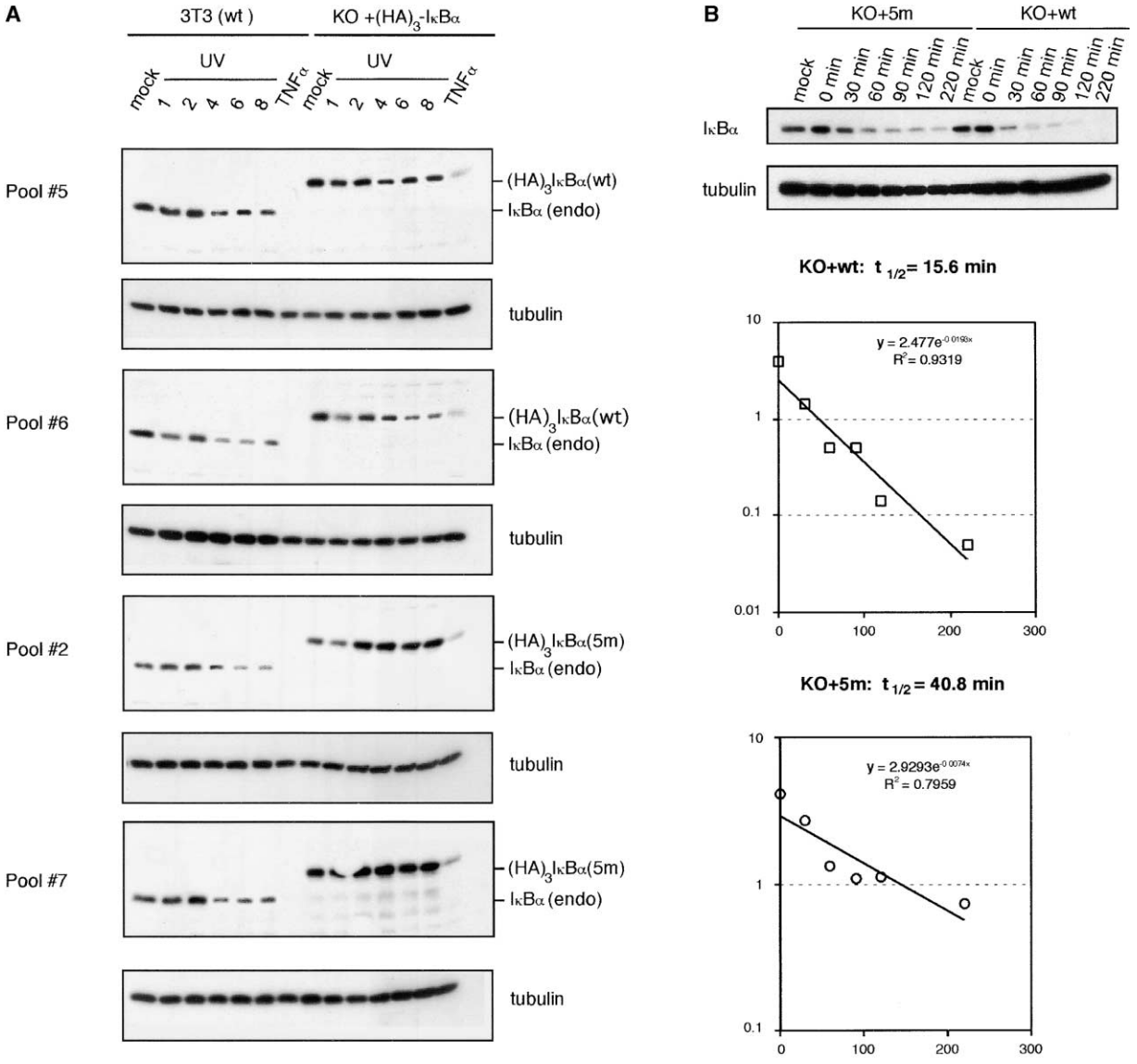

Figure 5. UV-Induced IκBα Degradation Requires the CK2 Phosphorylation Sites

(A) Fibroblasts derived from IκBα knockout mice were infected with recombinant retroviral vectors encoding HA-tagged WT or a mutant (5m) version of murine IκBα in which the CK2 phosphorylation sites were converted to alanines. Pools of infected cells were isolated and subjected to either mock treatment, UV irradiation (40 J/m^2), or incubation with TNFα (10 ng/ml). At the indicated time points (in hours; 15 min for TNFα or mock treatment), cell lysates were prepared and the levels of endogenous IκBα and HA-IκBα were examined by immunoblotting. The levels of tubulin were used to monitor loading.

(B) The half-lives of WT IκBα and IκBα(5m) were determined by adding cycloheximide to UV-irradiated cells at time 0. At the indicated time points, the cells were lysed and the relative levels of the two HA-tagged IκBα proteins were measured by immunoblotting and densitometry.

despite the discovery of IKK and elucidation of its role in NF-κB activation, the mechanism by which UV radiation results in NF-κB activation remained enigmatic. Here we show that UVC radiation leads to activation of a signaling pathway, distinct from the IKK pathway, which induces IκBα degradation in a manner dependent on phosphorylation of a cluster of C-terminal sites. The kinase that phosphorylates these sites is CK2. The evidence implicating CK2 as the UV-activated kinase responsible for NF-κB activation rests on several findings. First, the C-terminal phosphorylation sites recognized by CK2 are required for UV-induced IκBα degradation and NF-κB activation, but are dispensable for TNFα responsiveness. Second, CK2 interacts with a docking site present within the C-terminal portion of IκBα, and it is possible to use this interaction to isolate the UV activated IκBα kinase. Third, CK2's ability to phosphorylate IκBα at these C-terminal sites is strongly enhanced in response to UV radiation. Fourth, both the UV-induced degradation of IκBα and the activation of CK2 by UV are dependent on p38 activity. Fifth, a knockdown of the CK2β subunit abrogates UV-induced IκBα degradation.

For many years, CK2 was thought to be a constitutively active protein kinase in search of a specific physio-

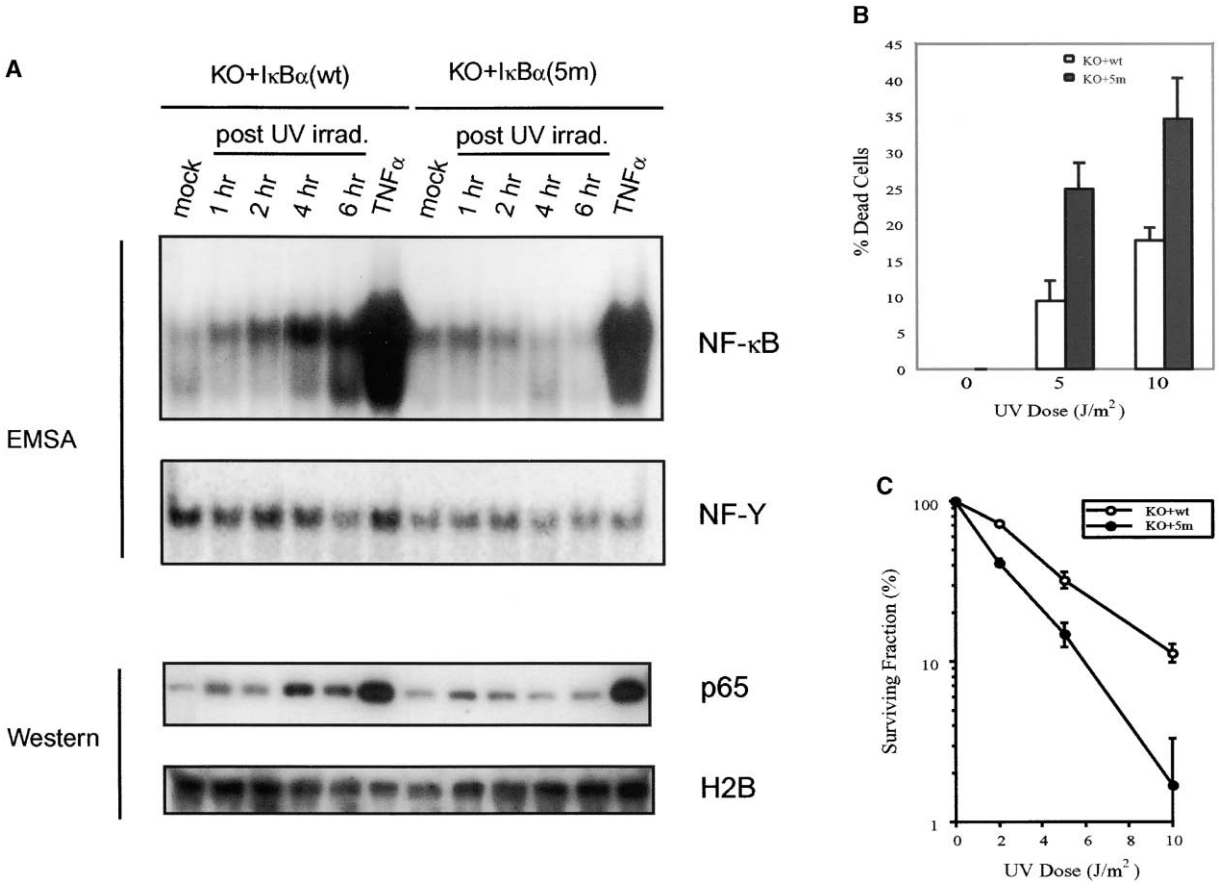

Figure 6. CK2-Mediated IκBα Phosphorylation Is Required for NF-κB Activation and Has a Protective Function

(A) CK2-mediated IκBα phosphorylation is required for NF-κB activation. *IkBα*$^{-/-}$ cells reconstituted to express either WT IκBα or the IκBα(5m) mutant were UVC irradiated (20 J/m²) or treated with TNFα (10 ng/ml for 15 min). At the indicated time points, the cells were collected and nuclear extracts were prepared. The extracts were used to examine p65 nuclear translocation by immunoblotting and NF-κB DNA binding activity by EMSA.

(B) Short-term survival assay. *IκBα*$^{-/-}$ cells reconstituted with either WT IκBα or the IκBα(5m) mutant were exposed to the to the indicated doses of UVC. After 24 hr, cell survival was measured using the MTT assay and the average fractions of dead cells (based on triplicates) were determined. KO + WT, open bars; KO + 5m, closed bars.

(C) Long-term clonogenic survival. *IκBα*$^{-/-}$ cells, reconstituted as above, were plated and, after 24 hr, exposed to the indicated doses of UVC. The fraction of surviving cells was determined by counting the number of colonies 2–3 weeks later. KO + WT, open circles; KO + 5m, closed circles.

logical function (Litchfield et al., 1994). In fact, CK2 was identified as a protein kinase responsible for constitutive phosphorylation of IκBα at C-terminal sites, which are part of a PEST motif (Barroga et al., 1995; McElhinny et al., 1996). It was even shown that phosphorylation of IκBα by CK2 regulates its intrinsic stability in nonstimulated cells but does not affect its TNFα-induced degradation (Lin et al., 1996). While our results confirm these earlier findings, they unexpectedly demonstrate that both CK2 activity and the C-terminal phosphorylation of IκBα are critical components of the signaling pathway triggered by UVC radiation that leads to NF-κB activation. Recently, CK2 was identified as a component of a protein complex responsible for UV-induced p53 phosphorylation (Keller et al., 2001). Furthermore, it was shown to be activated by a variety of other stressors, such as anisomycin and arsenite, a response found to be inhibited by p38 inhibitors (Sayed et al., 2000). Our results extend these findings and establish p38α and CK2 as critical components of the signaling cascade responsible for UV-induced NF-κB activation. While the phosphorylation of p53 by CK2 increases p53 activity (Keller et al., 2001), which could lead to either increased apoptosis or cell cycle arrest, the phosphorylation of IκBα by CK2 results in NF-κB activation and attenuation of apoptosis. By antagonizing p53-mediated apoptosis (Tergaonkar et al., 2002), the activation of NF-κB by UV radiation may promote p53-mediated cell cycle arrest. Thus, the identification of the role played by CK2 in NF-κB activation provides a much better explanation for its known antiapoptotic function (Ahmed et al., 2002; Litchfield, 2003), a function that could not be easily explained by the previously established targets for this enzyme. Interestingly, the antiapoptotic function of CK2 that is mediated through NF-κB activation is most obvious at low, physiologically relevant doses of UV radiation. At moderate to high doses of UV radiation, which cause extensive DNA damage, this protective function

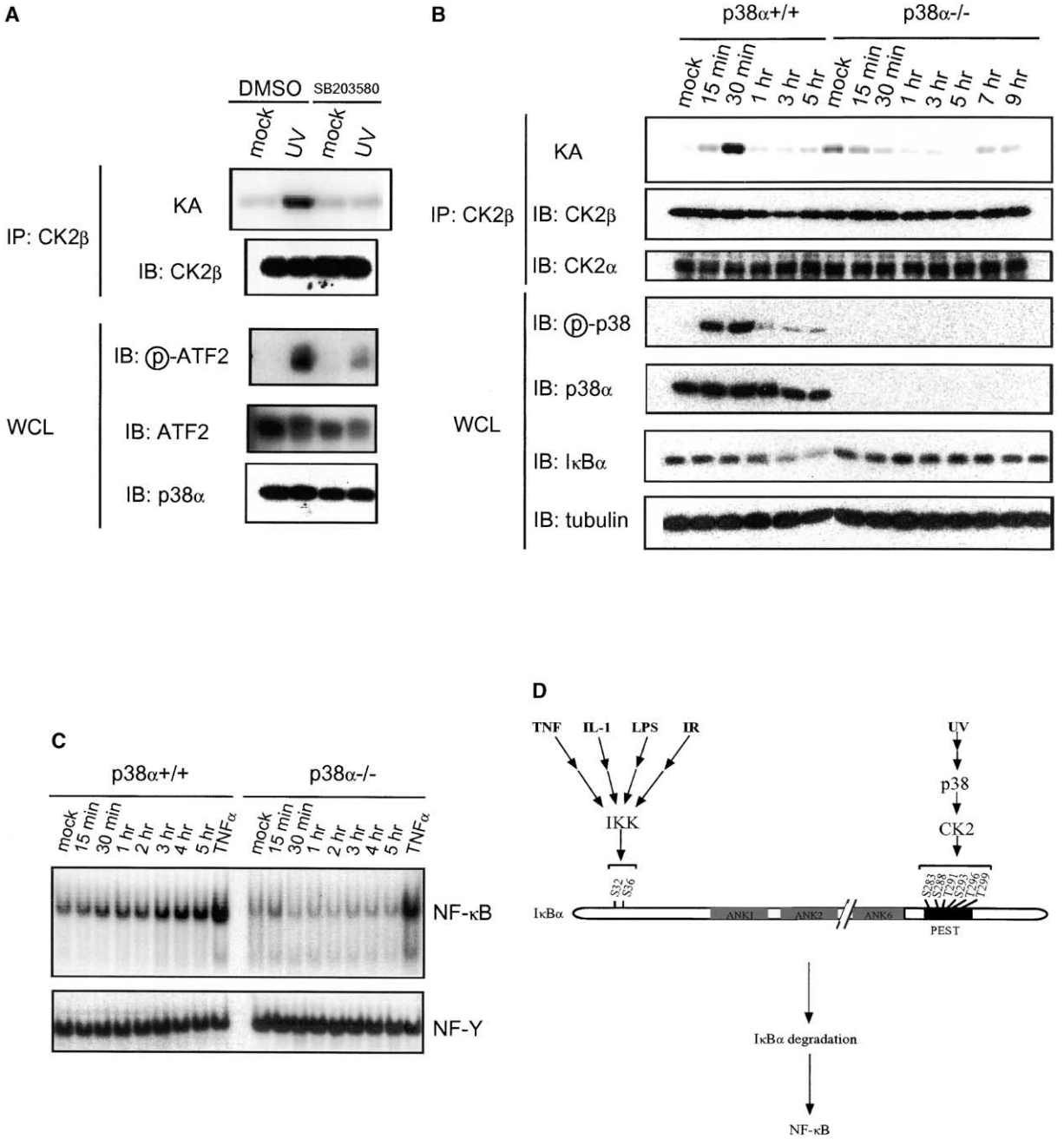

Figure 7. Activation of CK2 and UV-Induced IκBα Degradation and NF-κB Activation Require p38 MAPK Activity

(A) Inhibition of p38 blocks UV-induced CK2 activation. HeLa cells were preincubated with either DMSO or SB203580 (20 μM) and then mocktreated or exposed to UVC (20 J/m^2). After 30 min, lysates were prepared, CK2 was isolated by immunoprecepitation with anti-CK2β antibody, and its kinase activity toward IκBα was determined. Whole-cell lysates (WCL) were also immunoblotted with anti-phospho ATF2, anti-ATF2, and anti-p38α antibodies.

(B) p38 is required for UV-induced CK2 activation. Fibroblasts from WT (*p38*$\alpha^{+/+}$) and p38α knockout (*p38*$\alpha^{-/-}$) mouse embryos were mock treated or exposed to UVC (20 J/m^2) and harvested at the indicated time points. Cell lysates were prepared and subjected to immunecomplex kinase assays to determine CK2 activity and immunoblot analysis to examine the levels of the indicated proteins.

(C) NF-κB activation by UV requires p38α activity. Fibroblasts from *p38*$\alpha^{+/+}$ and *p38*$\alpha^{-/-}$ mouse embryos were UVC irradiated (20 J/m^2). At the indicated times, the cells were collected, nuclear extracts were prepared, and NF-κB and NF-Y DNA binding activities were determined by EMSA.

(D) Two different signaling pathways can lead to IκBα degradation and activation of NF-κB. While TNFα, IL-1, LPS, or ionizing radiation (IR) lead to IκBα degradation through the activation of IKK, which phosphorylates the N-terminal sites, UV radiation induces IκBα degradation by activating CK2, which phosphorylates the C-terminal sites.

is diminished, probably due to activation of proapoptotic pathways, such as those mediated by JNK or p53 (Shaulian et al., 2000).

Although the activation of CK2 by UV radiation is quite rapid, reaching a maximum within 15 min or less, the activation of NF-κB by UV is relatively slow in comparison to other NF-κB activators, such as TNFα. As the kinetics of UV-induced IκBα phosphorylation are similar to those of CK2 activation, the delayed activation of NF-κB is most likely caused by the slow kinetics of UV-induced IκBα degradation. The latter is most likely dependent on the stoichiometry of IκBα C-terminal phosphorylation, which seems lower than the stoichiometry of IKK-dependent N-terminal phosphorylation seen after treatment with potent IKK activators, such as TNFα (DiDonato et al., 1996). If only a small portion of the IκBα pool is phosphorylated at any given time, then only a small amount of IκBα is degraded via the proteasome at any given moment, resulting in slow NF-κB activation kinetics. As IκBα is resynthesized in response to NF-κB activation (Ghosh et al., 1998), the slow degradation kinetics also limit the extent of NF-κB activation, explaining why UV radiation does not result in robust NF-κB activation as seen after exposure to a saturating dose of TNFα.

The identification of CK2 as the protein kinase responsible for UV-induced NF-κB activation is also consistent with previous results which illustrated the ability of CK2β to confer UV resistance on cells derived from a patient suffering from xeroderma pigmentosum (XP), a human hereditary disorder characterized by high UV sensitivity and increased susceptibility to UV-induced skin cancer (Teitz et al., 1990). The genes responsible for this disorder have been identified (Friedberg, 1995), and none of their products is obviously related to CK2. Thus, it is possible that the overexpression of CK2β may simply promote activation of NF-κB and thereby confer UV resistance to XP cells, which are far more susceptible to UV-induced DNA damage than normal cells. The final outcome in any cell exposed to UV radiation would depend on the overall level of UV-induced DNA damage, which may determine the extent of p53 activation and the activities of antiapoptotic and proapoptotic signaling pathways. While NF-κB activation is antiapoptotic, the activation of JNK and c-Jun by UV radiation serve a proapoptotic function (Shaulian et al., 2000). By attenuating the antiproliferative activity of p53, which depends on p21 activation, c-Jun can promote p53-induced apoptosis (Shaulian et al., 2000).

Our results indicate that IκBα degradation can be induced not only through N-terminal phosphorylation but also through C-terminal phosphorylation (Figure 7D). While the C-terminal phosphorylation sites control both the basal turnover rate of IκBα and its accelerated degradation after UV irradiation, they are not required for TNFα-induced IκBα degradation which depends on the N-terminal sites. Thus, IκBα to our knowledge may represent the first example of a protein that can be targeted to ubiquitin-dependent proteasomal degradation through two distinct signaling pathways impinging on different sets of phosphorylation sites. This type of arrangement lends greater versatility to the regulatory function of this important protein.

Experimental Procedures

Cells and UV Radiation

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Immortalized fibroblasts (3T3 fibroblasts) derived from either *IκBα*$^{-/-}$ or *p38α*$^{-/-}$ mouse embryos were cultured in the same medium. UV irradiation was performed with an UVC lamp (G8T5) (General Electric). Dose rates were measured with UVX Radiometer (UVP Inc., Upland, CA). For UV irradiation as well as for mock treatment, the growth medium was aspirated and the cell layer covered with a small amount of PBS. After treatment was completed, growth medium was replenished. Cell viability assays were conducted as described (Shaulian et al., 2000).

Plasmids, Antibodies, and Production of Recombinant Proteins

Human IκBα cDNA and fragments thereof were amplified using the polymerase chain reaction (PCR). The PCR products were subcloned between the BamHI and NotI sites of pGEX-4T-1 to generate pGEX-IκBα(FL;WT), pGEX-IκBα(1-277), and pGEX-IκBα(241-317), which were used for producing the respective GST-IκBα fusion proteins. The different constructs were confirmed by sequencing. Bacterial expression vector for GST-IκBα(1-54) was described (DiDonato et al., 1997). To generate pGEX-IκBα(6m) the codons specifying Ser283, Ser288, Thr291, Ser293, Thr296, and Thr299 of human IκBα were converted to alanine codons by site-directed mutagenesis (Zandi et al., 1997). Murine cDNAs for WT IκBα and its five alanine substitution mutant were described elsewhere (Pando and Verma, 2000). These DNAs were subcloned into the pLPC-X retroviral expression vector (CLONETECH), which confers puromycin resistance.

Anti-CK2β antibody was from BD Transduction Laboratories (San Diego, CA). Anti-CK2α antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-p38 MAPK antibody was from Cell Signaling Technology (Beverly, MA). Anti-p38α (C-20), anti-IκBα (C-21), and anti-histone 2B (FL-126) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-actin and anti-α-tubulin antibodies were from Sigma (St Louis, MO).

GST-fusion proteins were produced in *E. coli* BL21(DE3) cells. Glutathione (GSH)-bound GST-fusion proteins were purified as described (Hibi et al., 1993).

Immnoprecipitation, Immunoblotting, Kinase Assays, and Phospho-Peptide Mapping

Cells were lysed in ice-cold lysis buffer (50 mM HEPES-OH, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, 0.5% deoxycholate, 20 mM NaF, 1 mM EGTA, 20 mM β-glycerophosphate, 0.5 mM DTT, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 U/ml aprotinin, 10 μg/ml leupeptin, 4.17 μg/ml bestatin, and 0.83 μg/ml pepstatin) to prepare whole-cell lysates. Immunecomplex kinase assays with anti-CK2β antibody were performed as described (Delhase et al., 1999). Solid-phase kinase assays were performed as described (Hibi et al., 1993), except that GST-IκBα proteins were used as the ligands and substrates. In vivo labeling and phosphopeptide mapping of ^{32}P-labeled polypeptides were performed as described (DiDonato et al., 1996). Immunoblotting was performed by resolving whole-cell lysates or immunecomplex kinase reactions by SDS-PAGE and blotting onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). The membranes were probed with the appropriate antibodies. Antibody-antigen complexes were detected by SuperSignal Western Pico Luminoll/Enhancer Solution (Pierce, Rockford, IL).

Pull-down experiments to identify the CK2 docking site on IκBα were performed by incubating cell lysates with GSH-agarose bound GST-IκBα proteins as described above for the solid-phase kinase assay except that we used five times more affinity matrix to ensure complete immunodepletion. After the incubation period, the beads were precipitated and the supernatant was collected and subjected to immunecomplex kinase assay.

RNA Interference Experiments

To "knock down" CK2β expression, siRNA oligonucleotides against CK2β mRNA were designed using "siRNA Target Finder and Design Tool" (Ambion's WEB site: http://www.ambion.com/RNAi/index.

html). The sequences of oligonucleotides were; 5′-UGAAUUCUU CUGUGAAGUGdTdT-3′ and 5′-CACUUCACAGAAGAAUUCAdTdT-3′, and they were chemically synthesized by Proligo (Boulder, CO). 2.5–3.0 × 10^5 of HeLa cells were inoculated in 35 mm dishes for 24 hr prior to transfection of siRNA oligos, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 24 hr after transfection of siRNA, cells were mock treated or exposed to UVC (20 J/m^2) and further incubated before harvesting. Whole-cell lysates were prepared and subjected to immunoblotting with the indicated antibodies.

Retroviral Transduction

Recombinant retroviruses used to reconstitute *IκBα*$^{-/-}$ 3T3 cells were produced using Phoenix packaging cells cotransfected with retroviral IκBα expression vectors and pCL-Eco (Naviaux et al., 1996) using Lipofectamine PLUS Reagent (Invitrogen, Carlsbad, CA). After 48 hr, supernatants were collected, centrifuged to remove cell debris, and filtered to serve as viral stocks. Polybrene (Sigma) was added to a final concentration of 8 μg/ml and cells were infected overnight (~18 hr). Medium was replaced and cells were further incubated for 24 hr before addition of puromycin (Calbiochem-Novabiochem Corporation, San Diego, CA) to a final concentration of 2 μg/ml to select the transfectants.

Electrophoretic Mobility Shift Assay

Nuclear cell extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL). To monitor the level of cytoplasmic contamination, nuclear extracts were immunoblotted with anti-IKKγ antibody (data not shown). Mobility shift assays were performed as described (Li and Karin, 1998), except that 5 μg of nuclear cell extract was used.

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