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Sequential DNA damage-independent and -dependent activation of NF-κB by UV

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NF-κB activation in response to UV irradiation of HeLa cells or of primary human skin fibroblasts occurs with two overlapping kinetics but totally different mechanisms. Although both mechanisms involve induced dissociation of NF-κB from IκBα and degradation of IκBα, targeting for degradation and signaling are different. Early IκBα degradation at 30 min to ~6 h is not initiated by UV-induced DNA damage. It does not require IκB kinase (IKK), as shown by introduction of a dominant-negative kinase subunit, and does not depend on the presence of the phosphorylatable substrate, IκBα, carrying serines at positions 32 and 36. Induced IκBα degradation requires, however, intact N- (positions 1–36) and C-terminal (positions 277–287) sequences. IκB degradation and NF-κB activation at late time points, 15–20 h after UV irradiation, is mediated through DNA damage-induced cleavage of IL-1α precursor, release of IL-1α and autocrine/paracrine action of IL-1α. Late-induced IκBα requires the presence of Ser32 and Ser36. The late mechanism indicates the existence of signal transfer from photoproducts in the nucleus to the cytoplasm. The release of the 'alarmone' IL-1α may account for some of the systemic effects of sunlight exposure.

Keywords: alarmone/CHUK/IκB/IKK/interleukin-1α

Introduction

In most types of cells, the dimeric transcription factor NF-κB, whose subunits belong to the Rel family, is kept in the cytoplasm through interaction with IκB proteins (reviewed by Bäuerle and Henkel, 1994; Bäuerle and Baltimore, 1996; Barnes and Karin, 1997). In response to one of numerous stimuli, e.g. cytokines, intracellular parasitic infections or phorbol ester, the inhibitory proteins (shown for IκBα and β) are rapidly degraded, and NF-κB is released from its inhibitory association and taken up by the nucleus where it activates the transcription of, for example, genes important for the inflammatory and immune responses (reviewed by Verma *et al*., 1995; Bäuerle and Baltimore, 1996; Gilmore *et al*., 1996; Wulczyn *et al*., 1996; Barnes and Karin, 1997). Upon treatment with tumor necrosis factor α (TNFα), interleukin-1α (IL-1α), or phorbol ester, IκBα is targeted for degradation by phosphorylation of the N-terminal serines at positions 32 and 36 (Brown *et al*., 1995; Traencker *et al*., 1995; DiDonato *et al*., 1996). Recently an IκB kinase (IKK, CHUK) has been characterized and shown, by antisense deprivation and dominant-negative mutation, to be required for the activation of NF-κB by TNFα, IL-1α, okadaic acid or phorbol ester (DiDonato *et al*., 1997; Régnier *et al*., 1997; Zandi *et al*., 1997). The pathway to NF-κB may comprise still other protein kinases, e.g. NIK (Malinin *et al*., 1997; Régnier *et al*., 1997), presumably acting upstream of IKK. An alternative mechanism of NF-κB activation seems to occur in oxygen-deprived cells upon reoxygenation; IκBα appears to dissociate from NF-κB without degradation as a result of tyrosine phosphorylation (Imbert *et al*., 1996).

Ultraviolet (UV) irradiation, UV-B or UV-C, of mammalian cells causes the activation of several transcription factors including NF-κB and the subsequent transcription of many genes, collectively called the UV response (reviewed by Bender *et al*., 1997; Herrlich *et al*., 1997). The original evidence for NF-κB regulation by UV stems from the observation that UV exposure of cells in culture or of mice induces transcription from the long terminal repeat (LTR) promoter of human immunodeficiency virus 1 (HIV-1; Valerie *et al*., 1988; Stein *et al*., 1989a; Morrey *et al*., 1991). The transcriptional activation of HIV-1 depends on the two NF-κB-binding sites located between positions –105 and –79 of the LTR, since their destruction abolishes the UV response. Also, NF-κB-binding sites cloned in front of a heterologous UV-non-responsive reporter render the construct UV-inducible (Stein *et al*., 1989a) and nuclear extracts isolated from UV-irradiated HeLa cells contain elevated NF-κB DNA-binding activity with maximal levels at 4 h after irradiation (Stein *et al*., 1989a). These experiments indicate that UV, like TNFα or IL-1α, targets NF-κB to the nucleus.

Our laboratory has been concerned with the signal transduction pathways elicited by UV, ultimately activating transcription factors (Schorpp *et al*., 1984; Herrlich and Karin, 1988; Karin and Herrlich, 1989; Mai *et al*., 1989; Sachsenmaier *et al*., 1994; Knebel *et al*., 1996). The analysis led from UV-responsive elements and transcription factors to signal transduction components and to the 'origins' of signaling. UV acts on more than one primary target molecule linked to signal transduction. Major primary target molecules generating UV-induced signaling are located at the plasma membrane (reviewed in Herrlich *et al*., 1997) leading to the ligand-independent activation of several receptor tyrosine kinases within fractions of a minute after UV irradiation, including receptors which may be relevant for the stimulation of NF-κB: the receptors for IL-1α and TNFα (Sachsenmaier *et al*., 1994; Rosette and Karin, 1996; Tobin *et al*., 1998). In agreement with UV induction through components in the plasma

membrane, UV irradiation causes NF-κB activation in cytoplasts (Dévary *et al*., 1993) and activates receptor tyrosine kinases, possibly also those relevant for NF-κB activation, in solubilized plasma membranes (Knebel *et al*., 1996) or in cytosolic extracts supplied with cellular membranes (Simon *et al*., 1994). UV-induced activation of receptor tyrosine kinases can be explained by transient or permanent inhibition of tyrosine phosphatases (Knebel *et al*., 1996; S.Groß, A.Knebel, T.Tenev, A.Neininger, A.Deck, P.Herrlich, M.Gaestel and F.D.Böhmer, unpublished) which shifts the balance between negative and positive control elements towards increased signal flow.

Inconsistent with the notion of a signaling pathway originating in the plasma membrane is a series of experiments which can only be interpreted as involving the nucleus in signaling to NF-κB. For instance, far lower doses of UV-C (2 J/m^2) were required for NF-κB-dependent HIV-LTR activation in cells from patients with Xeroderma pigmentosum group A (XPA) than in cells from healthy human individuals (20 J/m^2; Stein *et al*., 1989a). Since these two types of cells differ only in their ability to repair UV-induced DNA photoproducts, the density of such lesions in the DNA must be a determinant of HIV transcription and presumably of NF-κB activation. Also in support of a role for DNA damage in NF-κB activation, lipofection of UV-irradiated pSV2-gpt DNA, but not of non-irradiated plasmid, into XPA cells stably transfected with a HIV-LTR-CAT plasmid induced CAT expression, which in turn was inhibited upon introduction by liposomes of the pyrimidine dimer excision enzyme, T4 endonuclease V (Yarosh *et al*., 1993).

Our present study aims at defining the mechanism of UV-induced activation of NF-κB and of IκB degradation in primary human skin fibroblasts and in HeLa cells. We show that UV causes IκB degradation by two totally different sequentially occurring mechanisms: an early phase does not involve the phosphorylation of IκB by IκB kinase while a late mechanism established in cultured cells beyond 15 h, requires the phosphorylatable IκBα Ser32 and Ser36, the DNA damage-induced processing of presynthesized IL-1α precursor protein and release of IL-1α. The action of secreted IL-1α on other cells including inflammatory cells could explain some of the systemic effects of sun exposure.

Results

Time-course of UV-induced NF-κB activity

Despite the fact that UV seemingly activates the IL-1α and TNFα receptors in HeLa cells (Sachsenmaier *et al*., 1994; Rosette and Karin, 1996), the time-course of NF-κB activation by UV differed from that induced by IL-1α or TNFα. While IL-1α, TNFα or phorbol ester caused rapid (15 min) and transient (60 min) IκBα degradation (not shown) and NF-κB activation (Figure 1A), UV radiation induced a delayed (30–60 min; Figure 1A) and long-lasting (still elevated at 42 h; Figure 1B) NF-κB response and reduction of IκBα levels (measured by Western blot, not shown; see below) in HeLa cells. Also in primary human skin fibroblasts, the response to TNFα was rapid while it took 60 min for the first appearance of active NF-κB after UV (not shown).

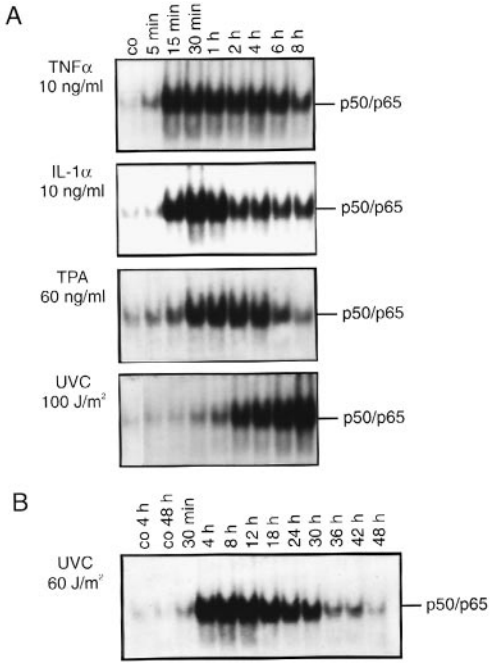

Fig. 1. Time-course of NF-κB activation. (**A**) HeLa cells were not treated or treated with TNFα (10 ng/ml), IL-1α (10 ng/ml), TPA (60 ng/ml) or UV-C (100 J/m^2). Nuclear extracts were prepared at the indicated time points and equal amounts of proteins (5 μg) were analysed for NF-κB activity by electrophoretic mobility shift assay (EMSA). (**B**) HeLa cells were irradiated with 60 J/m^2 UV-C and analysed at the indicated time points for NF-κB activity as described in (A). The position of the shift produced by the p50/p65 heterodimer is indicated.

DNA damage-independent and -dependent induction of NF-κB activity

As the induction of HIV-1 promoter activity depended on DNA photoproducts (Stein *et al*., 1989a), we first attempted to show similar dependence for UV-induced NF-κB activity. We exploited the specific difference in the repair kinetics (but not generation) of photoproducts between primary cells from a healthy human individual and those from a patient with XPA. As for the HIV-1-CAT reporter plasmid, NF-κB activity should be inducible in XPA cells at a lower dose than in wild-type cells, provided that DNA photoproducts were indeed intermediates and the repair time until harvest for NF-κB determination was sufficient to establish, by repair in the healthy cells, a damage-density difference. In wild-type human fibroblasts, 6–4-photoproducts are repaired with a half-life of 60–90 min, pyrimidine dimers with a half-life of 4–6 h (Friedberg *et al*., 1995). To be certain that sufficient DNA lesion density was reached, we determined in parallel the stabilization of p53 which is known to depend on DNA damage (Yamaizumi and Sugano, 1994). p53 accumulation, clearly visible at 6 h, was maximally induced by 5–10 J/m^2 in XPA cells and by 30 J/m^2 in wild-type fibroblasts (Figure 2, upper two panels, lanes 1–7) reflecting the photoproduct density difference between XPA and wild-type cells reached by repair in wild-type cells over the 6 h time period. This dose difference becomes of course even more pronounced with repair time (Figure 2, upper two panels, lanes 8–14).

However, NF-κB activation at 6 h after UV irradiation in the same extracts occurred with identical UV dose in

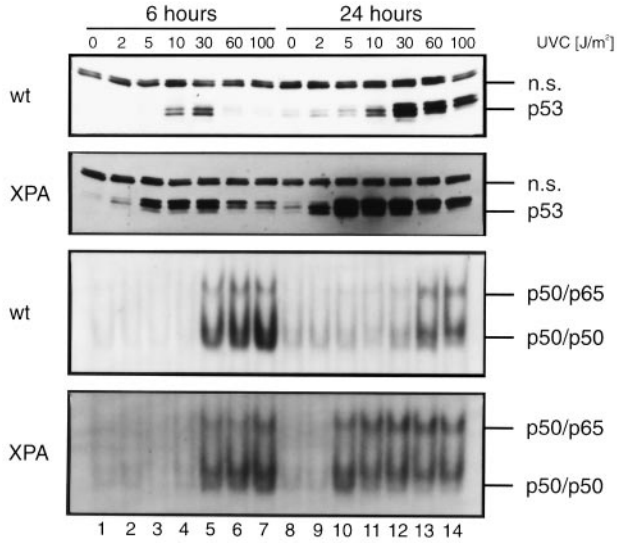

Fig. 2. Two mechanisms of UV-C-induced NF-κB activation. Wild-type fibroblasts and XPA fibroblasts were irradiated with the indicated doses of UV-C. Nuclear extracts were prepared 6 and 24 h after irradiation. For detection of p53 equal amounts of nuclear extracts (35 μg) were analyzed by Western blotting with the human p53-specific monoclonal antibody Ab-2 (upper two panels). The position of the p53 protein is indicated. n.s., non-specific signal. For detection of NF-κB activity equal amounts of protein (5 μg) were analyzed by EMSA (lower two panels). The heterodimeric p50/p65 and the homodimeric p50/p50 complexes are indicated.

XPA and wild-type cells, reaching maximal activity at ~30 J/m^2 (Figure 2, lower two panels, lanes 1–7; note that in primary fibroblasts p50 is in excess, but not in HeLa cells, see Figure 1). In contrast, NF-κB activity measured at later time points, e.g. at 24 h, was induced in XPA cells by 5 J/m^2 while the dose required in normal cells remained high, 60 J/m^2 (Figure 2, lower two panels, lanes 8–14). These results imply that NF-κB activity in fibroblasts was induced by UV through two different mechanisms, one responsible for early activation, the other for delayed induction. Only the latter mechanism involved DNA photoproducts as necessary intermediates. The time-course of NF-κB activation (Figure 1) may thus result from the fusion of at least two induction kinetics. Subsequently we explored which signal transduction pathways to NF-κB were exploited by the two types of UV induction. The early mechanism was studied in HeLa cells and in primary human fibroblasts in parallel while the delayed mechanism was resolved from the early induction in primary fibroblasts from a patient with XPA.

The early mechanism of UV-induced NF-κB activation

Suramin resistance. The possibility that UV-induced ligand-independent receptor tyrosine kinase activation (Sachsenmaier *et al*., 1994) was involved, was tested by the non-specific surface receptor poison, suramin, which abolishes UV-induced receptor tyrosine kinase activation (Sachsenmaier *et al*., 1994; Iordanov *et al*., 1997a). The early activation of NF-κB by UV was resistant to suramin, in contrast to that by growth factor containing conditioned medium (not shown). Thus a suramin-sensitive receptor tyrosine kinase was not involved in early UV induction of NF-κB in HeLa cells or in fibroblasts.

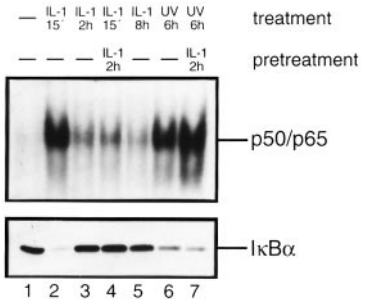

Fig. 3. Pretreatment with IL-1α does not interfere with UV-C-induced NF-κB activation. HeLa cells were not treated (–) or treated with IL-1α (10 ng/ml) or UV-C (100 J/m^2) for the indicated times. These pretreatments were followed by a second treatment with either IL-1α (10 ng/ml) or UV-C (100 J/m^2) where indicated. NF-κB activity of equal amounts of nuclear extract (5 μg) was analyzed by EMSA (upper panel). The amount of IκBα proteins was determined in cytoplasmic extracts by Western blotting with the anti-IκBα antibody sc-371 (lower panel).

No cross-refractoriness with IL-1α or TNFα. The suramin resistance suggested that the receptors for IL-1α or TNFα, both of which are activated by UV in HeLa cells and keratinocytes (Sachsenmaier *et al*., 1994; Rosette and Karin, 1996; Tobin *et al*., 1998), were not involved in the UV-activation of NF-κB. Nevertheless, we explored their putative involvement in a second type of approach. As shown previously (see also Sachsenmaier *et al*., 1994), most induced pathways become transiently refractory to a second stimulation by the same agent. Cross-refractoriness occurs if two stimuli address the same limiting signaling component. IL-1α induced transient NF-κB activity (15 min; Figure 3, lane 2). After 2 or 8 h, NF-κB activity has disappeared almost completely (Figure 3, lanes 3 and 5) which begs the question as to whether re-treatment with a second stimulus re-induces NF-κB. Restimulation with IL-1α did not activate NF-κB (Figure 3, lane 4). Pretreatment with either IL-1α or TNFα, however, could not prevent UV-dependent rapid degradation of IκBα and NF-κB activation [Figure 3, compare lane 7 (UV-induced after IL-1α pretreatment) with lane 6 (UV only); data for TNFα not shown]. Thus components of signaling downregulated upon cytokine treatment, are not required for UV-induced signaling but are required for reinduction by cytokine. Restimulation with TNFα after pretreatment with IL-1α or the reverse did, however, induce a second response (not shown), indicating that the two growth factor pathways involve different limiting components, both of which are not involved in the UV response.

We conclude that degradation of IκBα and activation of NF-κB at early time points after UV irradiation did not occur, at least not predominantly, through DNA damage nor through activation of components of the IL-1α or TNFα receptor-driven pathways.

The early activation of NF-κB does not require the phosphorylation of IκBα Ser32 and Ser36 and cannot be inhibited by dominant-negative IκB kinase. The data presented so far suggest that the mechanism by which UV causes IκBα degradation, differs from that utilized by IL-1α or TNFα. IL-1α and TNFα are known to cause phosphorylation of IκBα at Ser32 and Ser36 (Figure 5A) which then leads to degradation through a ubiquitin-dependent pathway (Brown *et al*., 1995; Chen *et al*.,

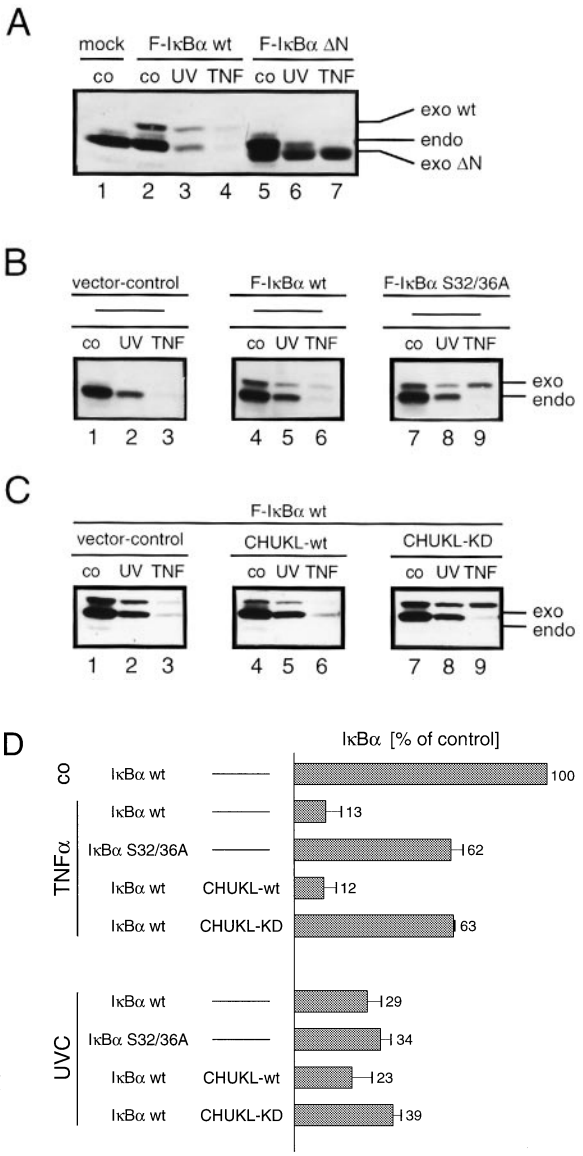

Fig. 4. IκBα Ser32 and Ser36 and CHUKL are required for TNFα- but not for UV-induced degradation. (**A**) HeLa cells were non-transfected (mock) or transiently transfected with epitope-tagged derivatives of the indicated IκBα cDNA expression vectors (2 μg). The cells were subsequently stimulated with UV-C (100 J/m^2) or with human TNFα (10 ng/ml) and harvested after 6 h or 30 min, respectively. The amount of IκBα proteins was determined in cytoplasmic extracts by Western blotting with the anti-IκBα antibody sc-371. The positions of the exogenous and endogenous IκBα-proteins are indicated. (**B**) HeLa cells were transiently transfected with cDNA expression vector encoding epitope-tagged IκBα (2μg) or with the parental vector (pCMV4; 2 μg). The cells were stimulated and analyzed as described in (A). (**C**) HeLa cells were transiently transfected with a cDNA expression vector (1 μg) for epitope-tagged wild-type (wt) IκBα together with a cDNA expression vector (5 μg) for IKK-β/CHUKwt or for IKK-β/CHUK with a mutated kinase domain (CHUKL-KD). The cells were stimulated and analyzed as described in (A). (**D**) Quantitative evaluation of IκBα levels by densitometry.

1995; Traencker *et al*., 1995; DiDonato *et al*., 1996). To investigate whether Ser32 and Ser36 were required for the UV response, we transiently expressed in HeLa cells (Figure 4) or XPA fibroblasts (see Figure 7) FLAG-tagged human wild-type or mutant IκBα driven by the cytomegalovirus (CMV) promoter. The exogenous wild-type protein (larger than endogenous IκBα) was degraded with similar efficiency and kinetics as the endogenous protein upon treatment of cells with either IL-1α (not shown), TNFα or UV (Figure 4A and B, middle panel). Deleting 36 N-terminal amino acids (IκBαΔN) stabilized IκBα and made it non-responsive to any treatment including UV (Figure 4A). The N-terminus is apparently required for recognition by the proteasome pathway (e.g. comprising the lysines at positions 21 and 22 which serve as ubiquitination substrates; Alkalay *et al*., 1995; Chen *et al*., 1995; Scherer *et al*., 1995; Baldi *et al*., 1996). A double point mutant of IκBα with Ser32 and Ser36 replaced by alanines (S32/36A), was resistant to TNFα- or IL-1α-induced degradation (shown for TNFα in Figure 4B, right panel; see also Figure 7), in agreement with published results (Brockman *et al*., 1995; Brown *et al*., 1995; Traencker *et al*., 1995). IκBαS32/36A was, however, still degraded in response to UV (Figure 4B, right panel; see quantitation in Figure 4D; see also Figure 7). Consistent with this result, transient co-transfection of HIV-CAT reporter with an expression vector encoding FLAG-tagged IκBαS32/36A reduced the response to TNFα but not to UV while co-expression of IκBα wild-type affected both inductions (not shown). Thus, UV-induced IκBα degradation does not appear to require phosphorylation of Ser32 and Ser36, in contrast to that induced by the cytokines.

A consequence of this result is that the UV-dependent degradation of IκBα may not involve IκB kinase-driven phosphorylation of the serines at positions 32 and 36 (DiDonato *et al*., 1997; Zandi *et al*., 1997). IKK does, however, phosphorylate a C-terminal serine (DiDonato *et al*., 1997). To test whether IKK was needed for the UV response, we aimed at inactivating the enzyme. The active form of IκB kinase consists of at least two subunits, IKKα and IKKβ, coupled by a leucine zipper (Zandi *et al*., 1997). Both share ATP-binding motifs and contribute to catalytic activity. If UV could target IκB for degradation by a mechanism distinct from that requiring phosphorylation, a dominant-negative mutant of IκB kinase should not interfere with the early UV response of NF-κB. In our transfection conditions, a D→N mutant of IKKβ (CHUKL-KD) which is unable to bind ATP, severely inhibited IκB degradation after treatment of cells with TNFα (Figures 4C and D; note that these are transient transfections. The determination of endogenous IκB protein in this case reflects the behavior in the bulk of cells. IκB levels are therefore not visibly affected by dominant-negative IKKβ). The dominant-negative mutant caused the same degree of inhibition of TNFα-induced IκBα degradation as the S32/36A mutation of IκB. The UV response was, however, not inhibited (Figures 4C and D) indicating that IKK was not involved in UV-induced signal transduction in the cells examined here, despite the fact that the response to TNFα involved IKK. This result also eliminates the involvement of IKK-dependent C-terminal phosphorylation.

C-terminal sequence requirement. Because of the reported IκBα tyrosine (Tyr42) phosphorylation after either reoxygenation of Jurkat cells or treatment with the tyrosine phosphatase inhibitor, pervanadate (Imbert *et al*., 1996), we explored the possibility that IκBα could be directly phosphorylated at tyrosines rather than at the serines, in

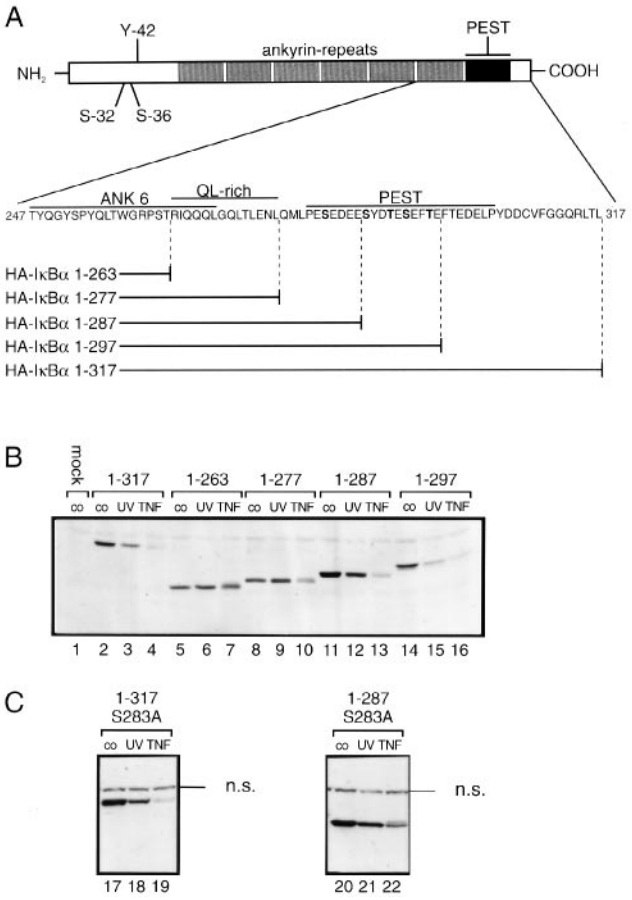

Fig. 5. Amino acids between IκBα positions 277 and 287 are required for UV-induced degradation. (**A**) IκBα mutants. The upper graph shows a scheme of the IκBα protein with S32 and Ser36, and Tyr42 which is phosphorylated upon reoxygenation of cells. The ankyrin repeats are indicated in gray, the PEST sequence is indicated in black. HA is tagged to the N-terminal end of the protein. The lower graph shows the amino acid sequence between positions 247 and 317 and the deletion mutants which were used in this study. (**B**) HeLa cells were transiently transfected with 2 μg of the indicated gene constructs encoding the HA-IκBα fusion proteins (lanes 2–16) or with 2 μg of the parental vector pCMV4 (mock; lane 1). The cells were subsequently stimulated with UV-C (100 J/m^2, 6 h) or with human TNFα (10 ng/ml, 15 min). The amount of IκBα proteins encoded by the exogenous gene constructs was determined in cytoplasmic extracts by Western blotting with a monoclonal antibody specific for the HA epitope (clone 12 CA 5). (**C**) Ser283 is not required for UV- or TNFα-induced IκBα degradation. Ser283 was mutated to alanine, either in the complete IκBα protein (lanes 17–19) or in the deletion mutant HA-IκBα 1–287 (lane 20–22). The mutated gene constructs were analyzed for IκBα expression as in (B). n.s., non-specific signal.

response to UV. In both HeLa and Jurkat cells treated with pervanadate, IκBα phosphorylated at tyrosines was detected with either an IκBα-specific or a tyrosine phosphate-specific antibody. After UV irradiation, however, IκBα was not phosphorylated at tyrosines either in Jurkat or in HeLa cells (data not shown).

To screen for other sequences in IκBα that might be relevant for UV-induced turnover, we focused on the C-terminal region which is, in addition to the N-terminus, required for the TNFα-induced degradation (Aoki *et al.*, 1996; Sun *et al.*, 1996). The C-terminus harbors a QL-rich region (aa 264–277) and a PEST sequence (aa 281–304) including several putative casein kinase II phosphorylation sites (Figure 5A) which seem not to exert a regulatory role (Barroga *et al.*, 1995; Brown *et al.*, 1995; Rodriguez *et al.*, 1995; Whiteside *et al.*, 1995; Aoki *et al.*, 1996; Krappmann *et al.*, 1996; Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996; Sun *et al.*, 1996). In accordance with published observations, TNFα could not trigger degradation of a long C-terminal truncation (IκBα aa 1–263, Figure 5B). Also UV did not induce degradation of this mutant protein. To dissect the area of the C-terminus absent in the 1–263 truncation, progressive deletions were tested. These deletion mutants revealed loss of UV-induced destabilization of IκBα prior to that by TNFα (Figure 5B) or IL-1α (not shown). Clearly, deletion to position 277 had no effect on TNFα-induced turnover (Figure 5B) while all deletions reaching into the stretch between positions 277 and 287 (e.g. IκBα 1–284; not shown) were stable in response to UV. IκBα 1–287 was, however, destabilized by UV. Since the stretch between positions 277 and 287 contains one serine at position 283, a putative target of casein kinase II (Barroga *et al.*, 1995; Lin *et al.*, 1996; McElhinny *et al.*, 1996; Schwarz *et al.*, 1996) and of a lipopolysaccharide-inducible protein kinase (Kuno *et al.*, 1995), and since one might expect UV-induced phosphorylation to be the initiating event, we point-mutated this serine (S283A). In the context of the complete or truncated (to 287) IκBα protein, UV destabilized IκBαS283A (Figure 5C), suggesting, at least, that Ser283 does not play a prominent role in UV-induced turnover.

Thus we can conclude that the early onset of NF-κB activation after UV irradiation of primary human skin fibroblasts and HeLa cells does involve prior degradation of IκB, but by a mechanism different from that used by TNFα or IL-1α. The degradation mechanism involves the N-terminal region, presumably the N-terminal lysines, and the stretch between amino acid positions 277 and 287. The C-terminal requirements also differ from those needed in TNFα-dependent degradation. Ser32, Ser36 and Ser283 as well as tyrosine phosphorylation are irrelevant for the early UV-induced turnover.

The late mechanism of UV-induced NF-κB activation

DNA damage-mediated degradation of IκBα and activation of NF-κB are mediated through the release of IL-1α. The late induction of NF-κB activity can be dissociated from the early induction in XPA fibroblasts. In contrast to HeLa cells, human fibroblasts appear to carry an excess of p50 such that p65/p50 heterodimers and p50 homodimers are generated. Antibody supershifts are shown in Figure 6A; compare the constituents of NF-κB gelshifts of HeLa and XPA cells. Specificity of the gelshift and of the antibodies was ascertained by control gelshifts using an Oct-1 oligonucleotide and by adding an excess of specific or non-related oligonucleotides (Figure 6A). Induced NF-κB activity in XPA fibroblasts was resolved into two bands which, according to supershift experiments, represent p65/p50 and p50/p50 (Figure 6A). Also, the activity induced in HeLa cells was resolved into two complexes, which according to the supershifts can be identified as a major p65/p50 heterodimer and a minor p50/p50 homodimer. It is not clear why the HeLa homodimer migrated slower than that of XPA cells. UV enhanced only the heterodimer in HeLa cells (Figures 1 and 6A),

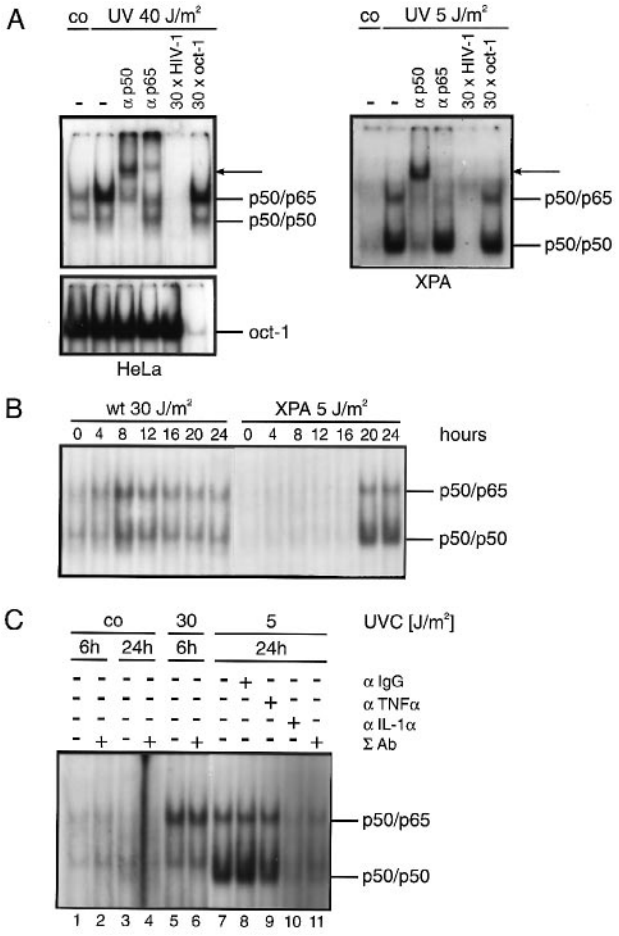

Fig. 6. Composition of NF-κB, kinetics and IL-1α-dependence of the late UV response of NF-κB. (**A**) In order to investigate the composition of the complexes obtained, extracts of HeLa cells irradiated with 40 J/m^2 and harvested at 6 h, and extracts from XPA cells irradiated with 5 J/m^2 and harvested at 24 h, were preincubated with the p50-specific antibody sc-1190X or with the p65-specific antibody sc-372. To determine specificity of binding, we used a 30-fold excess of the non-labeled NF-κB-binding oligonucleotide or of the Oct-1-binding oligonucleotide as competitors. (**B**) Late activation of NF-κB in XPA cells with low UV doses. Wild-type and XPA fibroblasts were irradiated with 30 and 5 J/m^2, respectively. At the indicated time points nuclear extracts were prepared and equal amounts of protein (5 μg) were analyzed for NF-κB activity by EMSA. The heterodimeric p50/p65 and the homodimeric p50/p50 complexes are indicated. (**C**) IL-1α mediates the late UV signal to NF-κB. XPA fibroblasts were not irradiated or irradiated with UV-C as indicated. Immediately after irradiation the cells were treated with 10 μg/ml of neutralizing antibodies directed against TNFα (αTNFα), IL-1α (αIL-1α) or both (ΣAb). As controls the cells were treated with an unrelated immunoglobulin (αIgG) or not treated with antibodies. Six h (30 J/m^2) and 24 h (5 J/m^2) after irradiation nuclear extracts were prepared and equal amounts of protein (5 μg) were analyzed for NF-κB activity by EMSA.

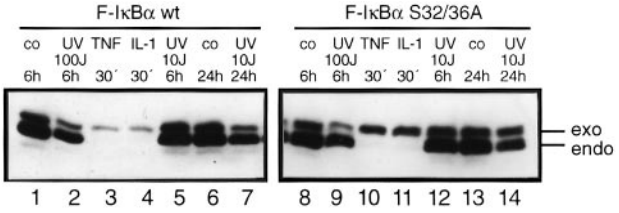

Fig. 7. IκBα Ser32 and Ser36 are required for the late UV-induced IκBα degradation. XPA fibroblasts were transiently transfected with a IκBα cDNA expression vector (2 μg) encoding the FLAG-tagged wild-type IκBα protein or the Ser32/36Ala mutant of IκBα. The cells were mock treated (lanes 1, 6, 8 and 13) or treated with TNFα (10 ng/ml), IL-1α (10 ng/ml), or UV-C (100 or 10 J/m^2). Six or 24 h after irradiation and 30 min after treatment with TNFα or IL-1α cytoplasmic extracts were prepared and equal amounts of proteins (80 μg) were analyzed for the amounts of exogenous and endogenous IκBα proteins by Western blotting with the anti-IκBα antibody sc-371. The positions of the exogenous and endogenous IκBα-proteins are indicated.

but hetero- and homodimers in fibroblasts (Figures 2 and 6).

With 5 J/m^2 an early induction in XPA cells was below detection level, while the DNA damage-dependent activation occurred with a lag period of 20 h (Figure 6B). With 30 J/m^2 in wild-type fibroblasts, one may expect both mechanisms to occur. However, the early DNA damage-independent response obviously concealed, by merging, the second wave of induction (Figure 6B). What causes the delay in XPA cells irradiated with 5 J/m^2 of UV? After all, DNA damage by UV is inflicted instantaneously. An interesting possibility has been suggested by previous experiments showing UV-induced and apparently DNA damage-dependent synthesis and release of cytokines (termed EPIF, Schorpp *et al*., 1984; or UVIS, Rotem *et al*., 1987; damage dependence, Yarosh *et al*., 1993; M.Litfin and H.J.Rahmsdorf, unpublished). UV-irradiated HeLa cells for instance release IL-1α and bFGF (Krämer *et al*., 1993). UV-induced release, accumulation in the medium and autocrine or paracrine action of cytokines could well account for the slow time-course of NF-κB activation. To test this possibility, we examined whether we could interfere with the induction of NF-κB in XPA cells by neutralizing anti-cytokine antibodies. Of several antibodies tested, only those directed against IL-1α could obliterate the delayed response to 5 J/m^2 UV in XPA fibroblasts (of both hetero- and homodimers, Figure 6C, lanes 10 and 11; controls shown with antibodies to TNFα, lane 9, and IgG, lane 8). The early induction by 30 J/m^2 was not affected (Figure 6C, compare lanes 5 and 6). Thus, these data indicate that IL-1α release is an intermediate in the delayed DNA damage-dependent UV response and that IL-1α is the predominant cytokine secreted from UV-irradiated primary fibroblasts, mediating NF-κB activation.

Requirement for IκBα Ser32 and Ser36. If IL-1α mediated the late DNA damage-dependent UV signal to NF-κB, late IκBα degradation should depend on the induced phosphorylation at Ser32 and Ser36. Introduced transiently into XPA fibroblasts, FLAG-tagged IκBαS32/36A was resistant to TNFα, IL-1α and to UV determined at 24 h, compatible with its being mediated by IL-1α, while at an early time point (6 h) it was degraded in response to UV (Figure 7; compare mutant lanes with wild-type lanes: e.g. 14 with 7, 9 with 2 etc.; compare also with the behavior of the endogenous IκBα which migrates faster than FLAG-tagged IκBα; the data correspond to those obtained in HeLa cells in Figure 4).

IL-1α precursor cleavage in the cytoplasm. The question remains as to how DNA damage can induce IL-1α release. We have reported earlier that IL-1α transcription is stimulated in HeLa cells upon UV irradiation (Krämer *et al*., 1993). In XPA cells an effect on transcription was

barely detectable (not shown). Nevertheless, IL-1α protein was released as revealed by immunoprecipitation with IL-1α-specific antibodies (not shown). Since IL-1α is synthesized as a precursor protein, we asked whether UV-induced DNA damage enhanced cleavage of the precursor. To improve the detection of IL-1α precursor, we transiently transfected into XPA fibroblasts an expression clone encoding the FLAG-tagged IL-1α precursor protein. UV irradiation caused significant precursor cleavage as determined by Western blot experiments (Figure 8A). UV irradiation caused the decrease of the exogenous precursor protein coinciding with an increase in tagged IL-1α followed by cytokine release into the medium. The cleavage occurred with an amazingly long lag period of 18 h, suggesting that the delay in NF-κB activation was caused by steps preceding IL-1α processing rather than IL-1α accumulation in the medium.

In an attempt to define the preceding steps we used a number of inhibitors. As one might have expected from the long lag period, UV-induced IL-1α processing was sensitive to cycloheximide (not shown), indicating the need for protein synthesis. The final step in the processing of IL-1α precursor involves proteolytic cleavage. The cysteine protease calpain has been proposed to be the processing enzyme (Kobayashi *et al*., 1990; Carruth *et al*., 1991; Kavita and Mizel, 1995). Inhibitors of this class of proteases, E64-d, Z-D.dcbmk and Z-VAD.fmk (Mehdi, 1991; Zhu *et al*., 1995; Nicholson and Thornberry, 1997), inhibited the cleavage and release of IL-1α (shown for Z-VAD.fmk in Figure 8B). In keeping with the essential role of IL-1α in the delayed UV response, the inhibitors also blocked the delayed UV-induced activation of NF-κB (Figure 8C, compare lanes 10–12 with lane 9). These inhibitors are likely to interfere with IL-1α processing alone rather than with p105 cleavage, as E64-d has been shown not to affect p105 (Palombella *et al*., 1994).

Is the release of the alarmone IL-1α by enhanced processing the consequence of apoptosis or an independent process? The time-course suggests a late event. IL-1α processing preceded phosphatidylserine presentation on the cell surface (annexin V staining, not shown) which is an early sign of apoptosis. Only 5% of the cells showed annexin V staining at 24 h. This number of cells could not account for the degree of cleavage of IL-1α precursor in the transfectants. Also the magnitude of the NF-κB response in XPA cells at 24 h after UV suggests that a considerable part of the response occurs in cells by autocrine stimulation either prior to apoptosis or in surviving cells.

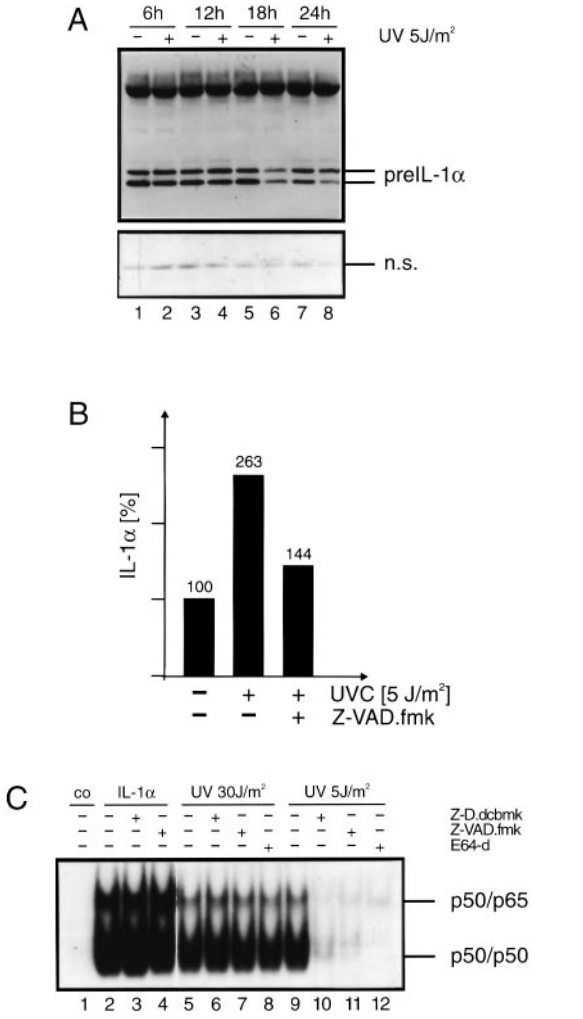

Fig. 8. The UV-induced release of IL-1α depends on the cleavage of IL-1α precursors. (**A**) XPA fibroblasts were transiently transfected with a cDNA expression vector (5 μg) encoding the FLAG-IL-1α precursor fusion protein. Twenty-four hours after transfection the cells were not irradiated or irradiated with 5 J/m^2. At the indicated time points cell lysates were prepared and immunoprecipitated with anti-IL-1α antiserum (#1190) followed by Western blot with an anti-IL-1α polyclonal antibody (AB-200NA). The two IL-1α precursor proteins are indicated. The slow-migrating band represents the Ig heavy chain, the n.s. (non-specific signal) band served as loading control. (**B**) XPA fibroblasts were transiently transfected with an IL-1α-precursor expression vector as in (A). Immediately after irradiation the cells were not treated or treated with the caspase inhibitor Z-VAD.fmk (50 μM). Twenty-four hours later mature IL-1α was immunoprecipitated from the culture medium using a FLAG epitope-specific antibody (sc-807; Santa Cruz). The level of IL-1α detected in the culture medium of non-treated cells was set at 100%. (**C**) Several caspase inhibitors prevent late NF-κB activation. XPA fibroblasts were not treated, treated with IL-1α (10 ng/ml) or irradiated with 5 J/m^2 or 30 J/m^2 UV. Caspase inhibitors Z-VAD.fmk (50 μM) or Z-D.dcbmk (100 μM) and the calpain inhibitor E 64-d (50 μM) were added to the cells immediately after treatment as indicated. Nuclear extracts were prepared 30 min after IL-1α addition, 6 h after irradiation with 30 J/m^2 and 24 h after irradiation with 5 J/m^2 and analyzed for NF-κB activity by EMSA. The positions of the shifts produced by p50/p65 heterodimers and p50/p50 homodimers are indicated.

Discussion

Here we have shown that UV irradiation of primary human skin fibroblasts and of HeLa cells in culture activates the transcription factor NF-κB through two different pathways, both of which lead to IκB degradation. Early activation within the first 6 h after UV does not depend, at least mainly, on UV-induced DNA photoproducts and does not utilize components known to participate in cytokine-dependent NF-κB activation such as IKK and its IκB substrate Ser32 and Ser36. Sequences in the N- and C-termini of IκB, however, need to be intact. Relevant UV absorption by a yet unknown suramin-resistant membranal or cytoplasmic chromophore may link to a signal transduction component that reaches IκB (see also below). In contrast, the delayed UV response of NF-κB requires DNA damage as an intermediate which sets off a signaling chain out of the nucleus leading to the processing and

release of the alarmone IL-1α, followed by autocrine/paracrine induction of the IL-1α signaling pathway to NF-κB. This late mechanism of induction is measured predominantly in the UV-induced expression of NF-κB reporter with long-term accumulation of CAT (Stein *et al*., 1989a; Yarosh *et al*., 1993). Similarly, late activation of other transcription factors mediating UV-induced expression of urokinase, metallothionein or collagenase (Miskin and Reich, 1980; Schorpp *et al*., 1984; Stein *et al*., 1989a,b) may be explained by IL-1α release. The dual mechanism of NF-κB activation thus solves the apparent discrepancy between previous studies proposing nuclear and non-nuclear pathways (Stein *et al*., 1989a; Devary *et al*., 1993).

The early pathway to NF-κB

Following irradiation of HeLa cells (Figure 1) or human primary fibroblasts (Figure 6B) with UV, NF-κB activity is detectable with a lag period of 30–60 min, followed by massive increases at 2–6 h. This time-course is slow in comparison with the activation of other early UV targets (Devary *et al*., 1992; Hibi *et al*., 1993; Radler-Pohl *et al*., 1993; Sachsenmaier *et al*., 1994; Gupta *et al*., 1995; Livingstone *et al*., 1995; Raingeaud *et al*., 1995; van Dam *et al*., 1995; Price *et al*., 1996; Iordanov *et al*., 1997a). It is also slow in comparison with NF-κB activation after TNFα or IL-1α. The delay of NF-κB activation (30–60 min) resembles the induction of signaling through collagen receptors (Vogel *et al*., 1997). It is not known which rate-limiting step causes the delay.

The time-course difference (between TNFα and UV induction of NF-κB) matches a difference in the mechanism. IκBα is phosphorylated at Ser32 and Ser36 upon TNFα treatment, but not after UV. Nevertheless it is probably ubiquitinated since the N-terminus (with the lysine residues) is required for degradation. We do not yet know how ubiquitination is triggered by UV. Different sequence requirements in the IκBα C-terminus between TNFα and UV suggest recognition by a different ubiquitin-conjugating enzyme. Interestingly, the appearance of p50 homodimers is also enhanced by UV irradiation, demanding that the UV-induced early mechanism encompasses p105 processing.

The level of IκB is the net result of synthesis and degradation. Since IκBα, whether complexed with NF-κB (Krappmann *et al*., 1996; van Antwerp and Verma, 1996) or in excess of NF-κB, is an intrinsically unstable protein (IκBα mRNA half-life was 30–45 min, protein half-life was 120–160 min for endogenous, complexed IκB, and 45 min for overexpressed, free IκB; not shown), a block of resynthesis would cause disappearance of IκBα and a delayed release and activation of NF-κB. UV treatment of cells indeed affects both transcription and translation (Sauerbier and Hercules, 1978; Iordanov *et al*., 1998). One could hypothesize that this inhibition was the cause of a net loss of IκB. A simply passive induction of NF-κB by loss of IκBα due to UV-induced translational inhibition is however highly unlikely: (i) even 100 J/m^2 of UV inhibited overall translation by only 50% (not shown, and Iordanov *et al*., 1998); (ii) inhibition of IκB translation to a similar degree by emetine, which is not supposed to cause ribosome-borne signal transduction (Iordanov *et al*., 1997b), could not mimic NF-κB induction by UV (not shown); (iii) a non-specific reduction of IκB by blocking synthesis should not be vulnerable to the introduction of IκB mutations. However, several mutant proteins are stable after UV irradiation, e.g. IκBαΔN and IκBα1-277, whereas slightly different mutants, e.g. IκBα1-287, expressed at similar levels, are readily degraded upon UV irradiation (Figure 5B). Moreover, all mutant proteins were degraded with similar kinetics in non-irradiated cells treated with the protein synthesis inhibitors, cycloheximide or emetine (not shown).

Translational inhibition by UV-C is also not sufficient to explain the late induction of NF-κB-dependent promoters which were measured by CAT expression (Stein *et al*., 1989a). Further, translational inhibition should not depend on DNA damage-repair; the dose of UV required should be identical between wild-type and XPA cells. However, 2 J/m^2 UV-C induce a maximum of HIV-1 transcription in XPA cells while 30 J/m^2 are required in wild-type cells.

Similarly, loss of IκB by transcriptional inhibition cannot explain UV-induced NF-κB activation; there is no major drop of IκBα mRNA in HeLa cells after irradiation even with doses as high as 100 J/m^2 (not shown), at which dose IκBα protein is degraded and NF-κB is activated. In summary, these data suggest the existence of a specific early signaling pathway which destabilizes IκBα and which is distinct from that originating from known surface receptors stimulated by their ligands.

It is puzzling that UV irradiation induces activation of the receptors for TNFα and IL-1α in HeLa cells and in mouse keratinocytes (Sachsenmeier *et al*., 1994; Rosette and Karin, 1996; Tobin *et al*., 1998). Nevertheless the pathways to UV-induced early induction of NF-κB differ from those elicited by the ligands. This conclusion is based on the finding that IKK and the substrate serines are not required and that there is no cross-refractoriness of pathways. Differences in signal transduction between different types of cells, as have been found for UV-induced AP-1 activation (Sachsenmaier *et al*., 1994; Huang *et al*., 1997), are possible but do not seem to apply here, as keratinocytes, where TNFα and IL-1α receptor activations have been measured, and HeLa cells should share pathways. The pathways triggered by the TNFα receptor are not totally clear. While TNFα induces JNK activity through receptor-TRADD/TRAF-2, NF-κB seems to be activated mainly by a different, as yet unknown, pathway (Lee *et al*., 1997; Yeh *et al*., 1997). The report that UV-B induction of NF-κB was inhibitable by dominant-negative TNFα-receptor and TRAF-2 (Tobin *et al*., 1998) could indicate that UV-B activates the receptor and that this activation is required for UV induction along with a second signal, not involving IKK. A role for the TNFα receptor even in the UV-induced JNK activation has also been suggested recently (Iordanov *et al*., 1998), since inhibitors of the generation of ribosome-borne signaling blocked JNK activation. Thus, a more complete understanding of TNFα receptor activation by UV compared with ligand activation is required to dissect the pathways to NF-κB.

Interestingly, suramin-resistant activation of cellular signal transduction has also been observed in the UV-induced activation of p38/HOG1 (Iordanov *et al*., 1997a), in contrast to the suramin-sensitive activation of Erk in the same cells. NF-κB is, however, not downstream of

p38 since a p38 inhibitor, SB203580 (Cuenda *et al*., 1995), could not block UV-induced IκBα degradation (not shown).

The delayed pathway to NF-κB

In contrast to the transient activation of NF-κB by most inducers, activation by UV irradiation is prolonged over 24–48 h. As shown by dose differences between wild-type and XPA cells, the expression of an NF-κB-dependent reporter determined at 42 h after UV irradiation (Stein *et al*., 1989a) and the delayed IκBα degradation and NF-κB activation described here, depend on UV-induced DNA damage.

Work on UV-induced transcription of collagenase and metallothionein, as well as on UV-induced stabilization of p53, has identified DNA damage in the transcribed strand of active genes as relevant for the signaling process (Yamazumi and Sugano, 1994; Blattner *et al*., 1998) and thus suggested that stalled transcription complexes may generate the signal. Subsequently a chain of events is induced that ultimately, after a considerable lag period, activate proteases, one of which processes the IL-1α precursor. Mature IL-1α then activates, through its membrane receptor, IKK to phosphorylate IκBα and to initiate degradation.

The nature of the signal generated by stalled transcription complexes is unfortunately not yet known. We do not wish to speculate as to its nature as this paper does not contribute to the question of signal generation except for indicating the existence of a signal flow out of the nucleus into the cytoplasm. Whereas DNA damage by radiation is inflicted instantaneously, IL-1α processing occurs after a long lag period. The susceptibility to protease inhibitors could mean that the processing itself was inhibited, or that IL-1α was downstream of caspase action induced as part of the apoptotic program. Interestingly CD95-induced JNK activation was also sensitive to inhibition of cysteine proteases (Cahill *et al*., 1996).

The delayed release of an alarmone, IL-1α, may account for several delayed responses after UV irradiation: transcription of collagenase, metallothionein and, in part, mutagenesis (Maher *et al*., 1988; Boesen *et al*., 1992; Krämer *et al*., 1993). Conditioned medium from UV-irradiated cells induces the transcription of plasminogen activator, collagenase I, HIV-1 and metallothionein IIA (Schorpp *et al*., 1984; Rotem *et al*., 1987; Stein *et al*., 1989b; Krämer *et al*., 1993; Yarosh *et al*., 1993). IL-1α release is certainly a good candidate for some of the systemic effects of sun exposure. Both precursor cleavage and free IL-1α could serve as targets for strategies that would prevent unwanted side-effects of radiation and of treatments with other DNA damaging agents, for example in the course of cancer therapy.

Materials and methods

Plasmids

The IκBα expression plasmids pCMV-FLAG-IκBα wt and pCMV-FLAG-IκBα Ser32/36Ala, in which Ser32 and Ser36 were mutated to alanines (Scherer *et al*., 1995), were obtained from Dr Dean Ballard (Vanderbilt University, Nashville, TN). The IκBα C-terminal truncation mutants (Sun *et al*., 1996) pCMV-HA-IκBα (1–317), pCMV-HA-IκBα (1–263), pCMV-HA-IκBα (1–277), pCMV-HA-IκBα (1–287) and pCMV-HA-IκBα (1–297) were generously given by Dr Warner Greene (University of California, San Francisco, CA). Site-specific mutations of pCMV-HA-IκBα (1–317/Ser283Ala) and pCMV-HA-IκBα (1–287/Ser283Ala) and a D145N mutant of IKKβ (CHUKL) unable to bind ATP were created by PCR cloning and site-directed mutagenesis (Stratagene). The pRc/RSV-pIL1αFLAG encoding the IL-1α precursor (Siders *et al*., 1993) was a gift of Dr S.Mizel (Wake Forest University, NC).

Cell culture and transfections

Cells were grown at 37°C and 6% CO_2. HeLa tk$^-$ cells (Angel *et al*., 1987) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS). Primary human fibroblasts from a healthy donor (a gift from Dr Eife, München) and fibroblasts from a patient with XPA (a gift of Dr J.Cleaver, CA) were grown in DMEM supplemented with 10 and 20% FCS, respectively. The human leukemic T cell line, Jurkat, was maintained in RPMI 1640 containing 10% heat-inactivated FCS, 1 mM sodium pyruvate and 2 mM L-glutamine (Gibco-BRL). All culture media were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (from Gibco-BRL).

HeLa cells were plated 20–24 h prior to transfection at a density of 1×10^6 cells per 100 mm dish. Transfection was performed using DEAE-Dextran (Kawai and Nishizawa, 1984). Fibroblasts were plated 20–24 h prior to transfection at a density of 8×10^5 cells per 100 mm dish. Transfection was performed using Lipofectamine^{TM} Reagents (Gibco-BRL).

Antibodies, reagents and UV irradiation

The following antibodies were used: anti-IκBα rabbit polyclonal (sc-371, Santa Cruz); anti-phosphotyrosine rabbit monoclonal (PY-20, Transduction Labs); anti-FLAG rabbit polyclonal (sc-807, Santa Cruz); anti-HA mouse monoclonal (clone 12CA5, Boehringer Mannheim); anti-IL-1α and anti-TNFα rabbit polyclonal (BioTech Trade, St Leon-Rot); anti-IL-1α rabbit monoclonal (clone #1190, a gift from Dainippon Pharmaceutical Co., Ltd, Japan); anti-IL-1α goat polyclonal (R&D systems); anti-IgG rabbit polyclonal (Dianova); anti-p53 mouse monoclonal (clone Ab-2, Calbiochem); anti-p50 antibody (sc-1190X, Santa Cruz); and the anti-p65 antibody (sc-372, Santa Cruz). Cells were incubated with 10 ng/ml human recombinant TNFα (Calbiochem), 10 ng/ml human recombinant IL-1α (Sigma) or 60 ng/ml TPA (Sigma). Pervanadate was prepared as previously described (Imbert *et al*., 1996) with freshly dissolved Na_3VO_4 pH10 (Sigma). For UV irradiation, cells were washed with phosphate-buffered saline (PBS) and irradiated without PBS with a monochromatic UV lamp (15W Hg lamp) obtained from Vetter (Wiesloch, Germany) emitting a wavelength of 254 nm (half-maximal width, 2.3 nm).

Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared and used in EMSAs as previously described (Stein *et al*., 1989a). Briefly, cells were washed twice with ice-cold PBS, scraped from the plate with a rubber policeman and suspended in 100 μl lysis buffer (10mM HEPES pH 7.9, 1 mM EDTA, 60 mM KCl, 0.5% Nonidet P-40, 1 mM DTT, 1 mM PMSF). After 5 min on ice, nuclei were sedimented at 1200 *g* for 5 min. The supernatant was diluted with 50 μl of nuclear buffer (250 mM Tris–HCl pH 7.8, 60 mM KCl, 1 mM DTT, 1 mM PMSF), cleared by centrifugation at 13 000 *g* for 15 min and used as cytoplasmic extracts. The nuclei were washed with lysis buffer without NP-40 and suspended in 100 μl nuclear buffer. Nuclei were lysed by three cycles of freezing and thawing in liquid nitrogen and ice. The nuclear extracts were cleared by centrifugation at 13 000 *g* for 15 min.

EMSAs were done as described previously (Stein *et al*., 1989a), with slight modifications: binding was performed in a volume of 20 μl with 5–6 μg nuclear protein in a buffer containing 12 mM HEPES pH 7.8, 62.5 mM Tris–HCl pH 7.8, 60 mM KCl, 0.6 mM EDTA, 12% glycerol, 5 mM DTT, 2 μg BSA and 1 μg poly(dI–dC). Approximately 10 fmol (100 000 c.p.m.) of ^{32}P-radiolabeled double-stranded oligonucleotide (5′-AGCTTGGGGACTTTCCAGCCG-3′) derived from the HIV-LTR, or (5′-TCGACTCGAGATGCAAATAAG-3′) for Oct-1, were used per reaction. Resultant DNA–protein complexes were resolved on 5% polyacrylamide gels and detected by autoradiography.

Immunoprecipitation and Western blot analysis

Cells were lysed in a buffer containing 137 mM NaCl, 20 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 50 mM NaF, 10 mM NaPPi, 10 μM Na_2MoO_4, 50 μM $ZnCl_2$, 20 mM β-glycerolphosphate, 1 mM PMSF, 1 mM Na_3VO_4, 1 μg/ml aprotinin and 10 μg/ml leupeptin. To separate nuclear and cytoplasmic extracts the

nuclei were pelleted by centrifugation at 13 000 *g* for 20 min at 4°C. The protein concentration of each sample was measured.

Immunoprecipitations were performed by using 20 µl protein A beads (Pharmacia) and 5 µl of antibody in a volume of 800 µl lysis buffer for 2 h at 4°C. The beads were pelleted and washed three times with lysis buffer and once with PBS before mixing with 45 µl 2× SDS sample buffer. The samples were boiled for 5 min and resolved by SDS–polyacrylamide gel electrophoresis (PAGE), followed by Western blot analysis.

For Western blot analyses equal amounts of protein extracts were fractionated by SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked (1 h at room temperature) with PBS containing 0.3% Tween 20 and 10% powdered milk and then incubated with a specific antiserum. Specific proteins were visualized by enhanced chemiluminescence (Amersham Life Science Inc., Cleveland, OH).

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References

Alkalay,I., Yaron,A., Hatzubai,A., Orian,A., Ciechanover,A. and Ben-Neriah,Y. (1995) Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc. Natl Acad. Sci. USA*, **92**, 10599–10603.

Angel,P., Baumann,I., Stein,B., Delius,H., Rahmsdorf,H.J. and Herrlich,P. (1987) 12-*O*-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5′-flanking region. *Mol. Cell. Biol.*, **7**, 2256–2266.

Aoki,T., Sano,Y., Yamamoto,T. and Inoue,J.I. (1996) The ankyrin repeats but not the PEST-like sequences are required for signal-dependent degradation of IκBα. *Oncogene*, **12**, 1159–1164.

Bäuerle,P.A. and Baltimore,D. (1996) NF-κB: ten years after. *Cell*, **87**, 13–20.

Bäuerle,P.A. and Henkel,T. (1994) Function and activation of NF-κB in the immune system. *Annu. Rev. Immunol.*, **12**, 141–179.

Baldi,L., Brown,K., Franzoso,G. and Siebenlist,U. (1996) Critical role for lysines 21 and 22 in signal-induced, ubiquitin-mediated proteolysis of IκBα. *J. Biol. Chem.*, **271**, 376–379.

Barnes,P.J. and Karin,M. (1997) Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.*, **336**, 1066–1071.

Barroga,C.F., Stevenson,J.K., Schwarz,E.M. and Verma,I.M. (1995) Constitutive phosphorylation of IκBα by casein kinase II. *Proc. Natl Acad. Sci. USA*, **92**, 7637–7641.

Bender,K., Blattner,C., Knebel,A., Iordanov,M., Herrlich,P. and Rahmsdorf,H.J. (1997) UV-induced signal transduction. *J. Photochem. Photobiol. B*, **37**, 1–17.

Blattner,C., Bender,K., Herrlich,P. and Rahmsdorf,H.J. (1998) Photoproducts in transcriptionally active DNA induce signal transduction to the delayed UV-induced genes for collagenase and metallothionein. *Oncogene*, **16**, 2827–2834.

Boesen,J.J., Dieteren,N., Bal,E., Lohman,P.H. and Simons,J.W. (1992) A possible factor in genetic instability of cancer cells: stress-induced secreted proteins lead to decrease in replication fidelity. *Carcinogenesis*, **13**, 2407–2413.

Brockman,J.A., Scherer,D.C., McKinsey,T.A., Hall,S.M., Qi,X., Lee,W.Y. and Ballard,D.W. (1995) Coupling of a signal response domain in IκBα to multiple pathways for NF-κB activation. *Mol. Cell. Biol.*, **15**, 2809–2818.

Brown,K., Gerstberger,S., Carlson,L., Franzoso,G. and Siebenlist,U. (1995) Control of IκBα proteolysis by site-specific, signal-induced phosphorylation. *Science*, **267**, 1485–1488.

Cahill,M.A., Peter,M.E., Kischkel,F.C., Chinnaiyan,A.M., Dixit,V.M., Krammer,P.H. and Nordheim,A. (1996) CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. *Oncogene*, **13**, 2087–2096.

Carruth,L.M., Demczuk,S. and Mizel,S.B. (1991) Involvement of a calpain-like protease in the processing of the murine interleukin 1-α precursor. *J. Biol. Chem.*, **266**, 12162–12167.

Chen,Z., Hagler,J., Palombella,V.J., Melandri,F., Scherer,D., Ballard,D. and Maniatis,T. (1995) Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin-proteasome pathway. *Genes Dev.*, **9**, 1586–1597.

Cuenda,A., Rouse,J., Doza,Y.N., Meier,R., Cohen,P., Gallagher,T.F., Young,P.R. and Lee,J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229–233.

Devary,Y., Gottlieb,R.A., Smeal,T. and Karin,M. (1992) The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell*, **71**, 1081–1091.

Devary,Y., Rosette,C., DiDonato,J.A. and Karin,M. (1993) NF-κB activation by ultraviolet light not dependent on a nuclear signal. *Science*, **261**, 1442–1445.

DiDonato,J., Mercurio,F., Rosette,C., Wu-Li,J., Suyang,H., Ghosh,S. and Karin,M. (1996) Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell. Biol.*, **16**, 1295–1304.

DiDonato,J.A., Hayakawa,M., Rothwarf,D.M., Zandi,E. and Karin,M. (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature*, **388**, 548–554.

Friedberg,E., Walker,G. and Siede,W. (1995) *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Gilmore,T.D., Koedood,M., Piffat,K.A. and White,D.W. (1996) Rel/NF-κB/IκB proteins and cancer. *Oncogene*, **13**, 1367–1378.

Gupta,S., Campbell,D., Dérijard,B. and Davis,R.J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, **267**, 389–393.

Herrlich,P. and Karin,M. (1988) Regulation of gene expression by posttranslational modification of transcription factors. In Kleinkauf,H., von Döhren,H. and Jasenicke,L. (eds), *The Roots of Modern Biochemistry.* Walter de Gruyter, Berlin, Germany, pp. 431–439.

Herrlich,P., Blattner,C., Knebel,A., Bender,K. and Rahmsdorf,H.J. (1997) Nuclear and non-nuclear targets of genotoxic agents in the induction of gene expression. Shared principles in yeast, rodents, man and plants. *Biol. Chem.*, **378**, 1217–1229.

Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135–2148.

Huang,C., Ma,W. and Dong,Z. (1997) Signal transduction through atypical PKCs, but not the EGF receptor, is necessary for UVC-induced AP-1 activation in immortal murine cells. *Oncogene*, **14**, 1945–1954.

Imbert,V. *et al.* (1996) Tyrosine phosphorylation of IκBα activates NF-κB without proteolytic degradation of IκBα. *Cell*, **86**, 787–798.

Iordanov,M., Bender,K., Ade,T., Schmid,W., Sachsenmaier,C., Engel,K., Gaestel,M., Rahmsdorf,H.J. and Herrlich,P. (1997a) CREB is activated by UVC through a p38/HOG-1-dependent protein kinase. *EMBO J.*, **16**, 1009–1022.

Iordanov,M.S., Pribnow,D., Magun,J.L., Dinh,T.H., Pearson,J.A., Chen,S.L. and Magun,B.E. (1997b) Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the α-sarcin/ricin loop in the 28S rRNA. *Mol. Cell. Biol.*, **17**, 3373–3381.

Iordanov,M.S., Pribnow,D., Magun,J.L., Dinh,T.H., Pearson,J.A. and Magun,B.E. (1998) Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. *J. Biol. Chem.*, **273**, 15794–15803.

Karin,M. and Herrlich,P. (1989) *Cis*- and *trans*-acting genetic elements responsible for induction of specific genes by tumor promotors, serum factors and stress. In Colburn,N.H. (ed.), *Genes and Signal Transduction in Multistage Carcinogenesis*. Marcel Dekker Inc., New York-Basel, NY, pp. 415–440.

Kavita,U. and Mizel,S.B. (1995) Differential sensitivity of interleukin-1α and -β precursor proteins to cleavage by calpain, a calcium-dependent protease. *J. Biol. Chem.*, **270**, 27758–27765.

Kawai,S. and Nishizawa,M. (1984) New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Mol. Cell. Biol.*, **4**, 1172–1174.

Knebel,A., Rahmsdorf,H.J., Ullrich,A. and Herrlich,P. (1996) Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *EMBO J.*, **15**, 5314–5325.

Kobayashi,Y., Yamamoto,K., Saido,T., Kawasaki,H., Oppenheim,J.J. and Matsushima,K. (1990) Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1α. *Proc. Natl Acad. Sci. USA*, **87**, 5548–5552.

Krämer,M., Sachsenmaier,C., Herrlich,P. and Rahmsdorf,H.J. (1993) UV-irradiation-induced interleukin-1 and basic fibroblast growth factor synthesis and release mediate part of the UV response. *J. Biol. Chem.*, **268**, 6734–6741.

Krappmann,D., Wulczyn,F.G. and Scheidereit,C. (1996) Different mechanisms control signal-induced degradation and basal turnover of the NF-κB inhibitor IκBα *in vivo*. *EMBO J.*, **15**, 6716–6726.

Kuno,K., Ishikawa,Y., Ernst,M.K., Ogata,M., Rice,N.R., Mukaida,N. and Matsushima,K. (1995) Identification of an IκBα-associated protein kinase in a human monocytic cell line and determination of its phosphorylation sites on IκBα. *J. Biol. Chem.*, **270**, 27914–27919.

Lee,S.Y., Reichlin,A., Santana,A., Sokol,K.A., Nussenzweig,M.C. and Choi,Y. (1997) TRAF2 is essential for JNK but not NF-κB activation and regulates lymphocyte proliferation and survival. *Immunity*, **7**, 703–713.

Lin,R., Beauparlant,P., Makris,C., Meloche,S. and Hiscott,J. (1996) Phosphorylation of IκBα in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol. Cell. Biol.*, **16**, 1401–1409.

Livingstone,C., Patel,G. and Jones,N. (1995) ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.*, **14**, 1785–1797.

Maher,V.M., Sato,K., Kateley-Kohler,S., Thomas,H., Michaud,S., McCormick,J.J., Kraemer,M., Rahmsdorf,H.J. and Herrlich,P. (1988) Evidence of inducible error-prone mechanisms in diploid human fibroblasts. In Moses,R.E. and Summer,W.C. (eds), *DNA Replication and Mutagenesis*. American Society of Microbiology, Washington, DC, pp. 465–471.

Mai,S., Stein,B., van den Berg,S., Kaina,B., Lücke-Huhle,C., Ponta,H., Rahmsdorf,H.J., Kraemer,M., Gebel,S. and Herrlich,P. (1989) Mechanisms of the ultraviolet light response in mammalian cells. *J. Cell Sci.*, **94**, 609–615.

Malinin,N.L., Boldin,M.P., Kovalenko,A.V. and Wallach,D. (1997) MAP3K-related kinase involved in NF-κB induction by TNF, CD95 and IL-1. *Nature*, **385**, 540–544.

McElhinny,J.A., Trushin,S.A., Bren,G.D., Chester,N. and Paya,C.V. (1996) Casein kinase II phosphorylates IκBα at S-283, S-289, S-293 and T-291 and is required for its degradation. *Mol. Cell. Biol.*, **16**, 899–906.

Mehdi,S. (1991) Cell-penetrating inhibitors of calpain. *Trends Biochem. Sci.*, **16**, 150–153.

Miskin,R. and Reich,E. (1980) Plasminogen activator: induction of synthesis by DNA damage. *Cell*, **19**, 217–224.

Morrey,J.D., Bourn,S.M., Bunch,T.D., Jackson,M.K., Sidwell,R.W., Barrows,L.R., Daynes,R.A. and Rosen,C.A. (1991) *In vivo* activation of human immunodeficiency virus type 1 long terminal repeat by UV type A (UV-A) light plus psoralen and UV-B light in the skin of transgenic mice. *J. Virol.*, **65**, 5045–5051.

Nicholson,D.W. and Thornberry,N.A. (1997) Caspases: killer proteases. *Trends Biochem. Sci.*, **22**, 299–306.

Palombella,V.J., Rando,O.J., Goldberg,A.L. and Maniatis,T. (1994) The ubiquitin-proteasome pathway is required for processing the NF-κ B1 precursor protein and the activation of NF-κB. *Cell*, **78**, 773–785.

Price,M.A., Cruzalegui,F.H. and Treisman,R. (1996) The p38 and Erk Map kinase pathways cooperate to activate ternary complex factors and c-fos transcription in response to UV-light. *EMBO J.*, **15**, 6552–6563.

Radler-Pohl,A., Sachsenmaier,C., Gebel,S., Auer,H.-P., Bruder,J.T., Rapp,U., Angel,P., Rahmsdorf,H.J. and Herrlich,P. (1993) UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. *EMBO J.*, **12**, 1005–1012.

Raingeaud,J., Gupta,S., Rogers,J.S., Dickens,M., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.*, **270**, 7420–7426.

Régnier,C.H., Song,H.Y., Gao,X., Goeddel,D.V., Cao,Z. and Rothe,M. (1997) Identification and characterization of an IκB kinase. *Cell*, **90**, 373–383.

Rodriguez,M.S., Michalopoulos,I., Arenzana-Seisdedos,F. and Hay,R.T. (1995) Inducible degradation of IκBα *in vitro* and *in vivo* requires the acidic C-terminal domain of the protein. *Mol. Cell. Biol.*, **15**, 2413–2419.

Rosette,C. and Karin,M. (1996) Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science*, **274**, 1194–1197.

Rotem,N., Axelrod,J.H. and Miskin,R. (1987) Induction of urokinase-type plasminogen activator by UV light in human fetal fibroblasts is mediated through a UV-induced secreted protein. *Mol. Cell. Biol.*, **7**, 622–631.

Sachsenmaier,C., Radler-Pohl,A., Zinck,R., Nordheim,A., Herrlich,P. and Rahmsdorf,H.J. (1994) Involvement of growth factor receptors in the mammalian UVC response. *Cell*, **78**, 963–972.

Sauerbier,W. and Hercules,K. (1978) Gene and transcription unit mapping by radiation effects. *Annu. Rev. Genet.*, **12**, 329–363.

Scherer,D.C., Brockman,J.A., Chen,Z., Maniatis,T. and Ballard,D.W. (1995) Signal-induced degradation of IκBα requires site-specific ubiquitination. *Proc. Natl Acad. Sci. USA*, **92**, 11259–11263.

Schorpp,M., Mallick,U., Rahmsdorf,H.J. and Herrlich,P. (1984) UV-induced extracellular factor from human fibroblasts communicates the UV response to nonirradiated cells. *Cell*, **37**, 861–868.

Schwarz,E.M., Van Antwerp,D. and Verma,I.M. (1996) Constitutive phosphorylation of IκBα by casein kinase II occurs preferentially at serine 293: requirement for degradation of free IκBα. *Mol. Cell. Biol.*, **16**, 3554–3559.

Siders,W.M., Klimovitz,J.C. and Mizel,S.B. (1993) Characterization of the structural requirements and cell type specificity of IL-1 α and IL-1 β secretion. *J. Biol. Chem.*, **268**, 22170–22174.

Simon,M.M., Aragane,Y., Schwarz,A., Luger,T.A. and Schwarz,T. (1994) UVB light induces nuclear factor κB (NF κB) activity independently from chromosomal DNA damage in cell-free cytosolic extracts. *J. Invest. Dermatol.*, **102**, 422–427.

Stein,B., Rahmsdorf,H.J., Steffen,A., Litfin,M. and Herrlich,P. (1989a) UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos and metallothionein. *Mol. Cell. Biol.*, **9**, 5169–5181.

Stein,B., Krämer,M., Rahmsdorf,H.J., Ponta,H. and Herrlich,P. (1989b) UV-induced transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat and UV-induced secretion of an extracellular factor that induces HIV-1 transcription in nonirradiated cells. *J. Virol.*, **63**, 4540–4544.

Sun,S., Elwood,J. and Greene,W.C. (1996) Both amino- and carboxyl-terminal sequences within IκBα regulate its inducible degradation. *Mol. Cell. Biol.*, **16**, 1058–1065.

Tobin,D., van Hogerlinden,M. and Toftgard,R. (1998) UVB-induced association of tumor necrosis factor (TNF) receptor 1/TNF receptor-associated factor-2 mediates activation of Rel proteins. *Proc. Natl Acad. Sci. USA*, **95**, 565–569.

Traenckner,E.B., Pahl,H.L., Henkel,T., Schmidt,K.N., Wilk,S. and Bäuerle,P.A. (1995) Phosphorylation of human IκBα on serines 32 and 36 controls IκBα proteolysis and NF-κB activation in response to diverse stimuli. *EMBO J.*, **14**, 2876–2883.

Valerie,K., Delers,A., Bruck,C., Thiriart,C., Rosenberg,H., Debouck,C. and Rosenberg,M. (1988) Activation of human immunodeficiency virus type 1 by DNA damage in human cells. *Nature*, **333**, 78–81.

van Antwerp,D.J. and Verma,I.M. (1996) Signal-induced degradation of IκBα: association with NF-κB and the PEST sequence in IκBα are not required. *Mol. Cell. Biol.*, **16**, 6037–6045.

van Dam,H., Wilhelm,D., Herr,I., Steffen,A., Herrlich,P. and Angel,P. (1995) ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.*, **14**, 1798–1811.

Verma,I.M., Stevenson,J.K., Schwarz,E.M., Van Antwerp,D. and Miyamoto,S. (1995) Rel/NF-κB/IκB family: intimate tales of association and dissociation. *Genes Dev.*, **9**, 2723–2735.

Vogel,W., Gish,G.D., Alves,F. and Pawson,T. (1997) The discoidin receptor tyrosine kinases are activated by collagen. *Mol. Cell*, **1**, 13–23.

Whiteside,S.T., Ernst,M.K., LeBail,O., Laurent-Winter,C., Rice,N. and Israel,A. (1995) N- and C-terminal sequences control degradation of MAD3/IκBα in response to inducers of NF-κB activity. *Mol. Cell. Biol.*, **15**, 5339–5345.

Wulczyn,F.G., Krappmann,D. and Scheidereit,C. (1996) The NF-κB/Rel and IκB gene families: mediators of immune response and inflammation. *J. Mol. Med.*, **74**, 749–769.

Yamazumi,M. and Sugano,T. (1994) UV-induced nuclear accumulation of p53 is evoked through DNA damage of actively transcribed genes independent of the cell cycle. *Oncogene*, **9**, 2775–2784.

Yarosh,D.B., Alas,L., Kibitel,J., O'Connor,A., Carrier,F. and Fornace,A.J. (1993) Cyclobutane pyrimidine dimers in UV-DNA induce release of soluble mediators that activate the human immunodeficiency virus promoter. *J. Invest. Dermatol.*, **100**, 790–794.

Yeh,W.C. *et al.* (1997) Early lethality, functional NF-κB activation and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity*, **7**, 715–725.

Zandi,E., Rothwarf,D.M., Delhase,M., Hayakawa,M. and Karin,M. (1997) The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. *Cell*, **91**, 243–252.

Zhu,H., Fearnhead,H.O. and Cohen,G.M. (1995) An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett.*, **374**, 303–308.

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