The EMBO Journal Vol.16 No.5 pp.1009–1022, 1997

CREB is activated by UVC through a p38/HOG-1-dependent protein kinase

M.Iordanov[1,2], K.Bender[1], T.Ade[3], W. Schmid[4], C.Sachsenmaier[1,5], K.Engel[6], M.Gaestel[6], H.J.Rahmsdorf[1] and P.Herrlich[1,7,8]

[1]Forschungszentrum Karlsruhe, Institute für Genetik and [3]für Toxikologie, and [7]Universität Karlsruhe, Institut für Genetik, Postfach 3640, D-76021 Karlsruhe, [4]Deutsches Krebsforschungszentrum, Abteilung für Molekularbiologie der Zelle I, Im Neuenheimer Feld 280, D-69120 Heidelberg, [6]Max-Delbrück-Centrum für Molekulare Medizin, AG Stressproteine, Robert-Rössle-Strasse 10, D-13122 Berlin, Germany and [2]Oregon Health Sciences University, Department of Cell and Developmental Biology, Portland, OR 97201, USA

[5]Present address: Fred Hutchinson Cancer Research Center, A2-025, 1124 Columbia Street, Seattle, WA 98104, USA

[8]Corresponding author

Changes in environmental conditions such as the addition of growth factors or irradiation of cells in culture first affect immediate response genes. We have shown previously that short wavelength UV irradiation (UVC) elicits massive activation of several growth factor receptor-dependent pathways. At the level of the immediate response gene c-*fos*, these pathways activate the transcription factor complex serum response factor (SRF)–p62^{TCF} which mediates part of the UV-induced transcriptional response. These studies have, however, suggested that more that one pathway is required for full UV responsiveness of c-*fos*. Using appropriate promoter mutations and dominant-negative cAMP response element (CRE)-binding protein (CREB), we now find that UVC-induced transcriptional activation depends also on the CRE at position –60 of the c-*fos* promoter and on the functionality of a CREB. Upon UV irradiation, CREB and ATF-1 are phosphorylated at serines 133 and 63, respectively, preceded by and dependent on activation of p38/RK/HOG-1 and of a p38/RK/HOG-1-dependent p108 CREB kinase. Although p90^{RSK1} and MAPKAP kinase 2 are also activated by UV, p90^{RSK1} does not, at least not decisively, participate in this signalling pathway to CREB and ATF-1 as it is not p38/RK/HOG-1 dependent, and CREB is a poor substrate for MAPKAP kinase 2 *in vitro*. On the basis of resistance to the growth factor receptor inhibitor suramin and of several types of cross-refractoriness experiments, the UVC-induced CREB/ATF-1 phosphorylation represents an as yet unrecognized route of UVC-induced signal transduction, independent of suramin-inhibitable growth factor receptors and different from the Erk 1,2–p62^{TCF} pathway.

Keywords: c-*fos*/CREB/MAPKAP kinase 2/p108 CREB kinase/UV

Introduction

Many of the cellular reactions to short wavelength radiation (UVC) such as skin erythema and carcinogenesis, UVC-induced programmed cell death (apoptosis) and cellular survival seem to require UVC-induced gene expression (Dévary *et al.*, 1992; Friedberg *et al.*, 1995; Schreiber *et al.*, 1995). Two series of experiments support this interpretation. The changes of gene expression after UVC irradiation depend on the activity of protein tyrosine kinases (Dévary *et al.*, 1992; Sachsenmaier *et al.*, 1994). Accordingly, tyrosine kinase inhibitors prevent gene induction and also increase the sensitivity of cells to UVC killing (Dévary *et al.*, 1992). The second type of evidence is based on elimination of a critical transcription factor. Both mutagenic reactions and survival are impaired in the absence of the transcription factor subunit Fos. Antisense c-*fos* oligonucleotides suppress UVC-induced chromosomal aberrations (van den Berg *et al.*, 1991) and embryonic fibroblasts from c-*fos* –/– mice show increased sensitivity to UVC (Schreiber *et al.*, 1995; Haas and Kaina, 1995). In addition, UVC influences not only the programme of genes expressed in the irradiated cells, but also the fate of inner organismic cells that are not reached directly by UVC, by inducing the synthesis and secretion of growth factors (Schorpp *et al.*, 1984; Rotem *et al.*, 1987; Krämer *et al.*, 1993).

The fact that adverse agents such as UVC can trigger complex macromolecule synthesis requires the existence of primary sensors which react with the adverse agent or absorb the radiation energy and then translate into cellular language, i.e. activate molecules normally involved in signalling and gene regulation. There has been only limited progress in unravelling the primary target(s) of UVC absorption relevant for signal transduction to the transcription factors. Comparison of dose–response curves for induced gene expression in DNA repair-proficient and -deficient cells has yielded support for DNA damage as an intermediate of induction (Miskin and Ben-Ishai, 1981; Schorpp *et al.*, 1984; Stein *et al.*, 1989). Thus DNA seems to be one of the relevant absorbing molecules. A second class of 'sensors' has been found recently in the plasma membrane by systematically following the signal pathways in the 'upstream' direction starting from transcription factors mediating the UV response (Mai *et al.*, 1989; Dévary *et al.*, 1992, 1993; Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994). On the basis of the inhibition of the UV response by either suramin (a drug that poisons plasma membrane receptors) or by pre-treatment of cells with growth factors (a procedure which induces, after transient activation, the inactivation and internalization of growth factor receptors), and from the finding that several growth factor receptors are phosphorylated at tyrosine residues within a fraction of a minute after UV irradiation,

it has been concluded that growth factor receptors are part of the relevant pathway(s) used by UV to reach, in particular, the immediate response genes (Sachsenmaier *et al.*, 1994; Knebel *et al.*, 1996). According to these data, UVC-induced transcription of c-*fos* in HeLa cells is mediated to a large extent by epidermal growth factor receptor (EGFR), interleukin-1 receptor (IL-1R) and basic fibroblast growth factor receptor (bFGFR).

The c-*fos* promoter elements required for UVC-induced transcription have been delimited by deletion mutation (Büscher *et al.*, 1988). The serum response element (SRE) is required and its deletion reduces induction to ~50%. Indeed, the UV-induced growth factor-dependent signal transduction pathways lead to activation of the ternary complex factor $p62^{TCF}$ (Elk-1; Sap-1; Sachsenmaier *et al.*, 1994; Price *et al.*, 1996) which, in conjunction with the serum response factor (SRF), binds to the SRE (reviewed by Treisman, 1994). Using new promoter mutants, we now find that the response of the c-*fos* promoter depends also on the presence of the cAMP response element (CRE)-binding protein (CREB) binding site at position –60 of the c-*fos* promoter. This not only indicates that promoter elements are complex cooperating assemblies but also shows the existence of still another UVC signalling pathway to c-*fos* and possibly to other CRE-regulated promoters. CREB and ATF-1 are indeed phosphorylated in response to UVC, at serines 133 and 63 respectively. A p38/RK/HOG-1-dependent p108 serine/threonine protein kinase is identified as a UVC-induced CREB kinase in HeLa cells. The UVC-induced CREB and ATF-1 phosphorylation, on the basis of suramin resistance and of various types of cross-refractoriness experiments, represents a novel UVC signalling pathway independent of activation of the growth factor receptors described so far.

Results

Several promoter elements contribute to the UVC response of the c-fos promoter: the proximal CRE as a new UV response element

Dissection of the promoter has yielded evidence that the $p62^{TCF}$/Elk-1–$(SRF)_2$ complex formed over the SRE (at positions –320/–300), is important for the UVC response of c-*fos* (Büscher *et al.*, 1988; Sachsenmaier *et al.*, 1994; Price *et al.*, 1996). When cloned in front of a heterologous UVC-non-responsive promoter, the SRE suffices to confer UVC inducibility, and inducibility of the c-*fos* promoter in 3T3 cells is inhibited by 50% upon deletion of the SRE. To screen for other UVC targets in the c-*fos* promoter, we examined deletion mutants of the proximal CRE (–60 CRE). In HeLa cells transiently transfected with c-*fos* promoter–CAT reporter constructs, deletion of the CRE reduced both the UVC and the cAMP inducibility to <50% (Figure 1, compare –711/+45 *fos* CAT, lanes 1–3, with –711/ΔCRE/+45 *fos* CAT, lanes 7–9), implicating the CRE in mediating part of the response to both stimuli, and suggesting that factors so far known to be specifically activated by cAMP (Montminy *et al.*, 1986) could also be addressed by UVC.

The importance of the CRE is documented further by experiments overexpressing a dominant-negative mutant of CREB (CREBM1 which carries a serine→alanine substitution at position 133, Gonzales and Montminy, 1989). This mutant reduced by >50% both the UVC and the cAMP response of the full-length promoter (Figure 1, compare lanes 1–3 with lanes 4–6). This suggests that a factor with binding ability similar to CREBM1 was involved in the UVC response and that CREBM1 could not be activated by UVC (nor by cAMP) treatment of cells. The specificity of the inhibition by CREBM1 is demonstrated by the fact that it does not interfere with the UVC induction of a NF-κB-dependent promoter construct (–105/–79 HIV TATA CAT; Figure 1, compare lanes 14 and 17). Similar roles for the –60 CRE and for a CRE-binding factor in the UVC response of c-*fos* were observed in NIH3T3 mouse fibroblasts and in JEG-3 human chorionic carcinoma cells (not shown).

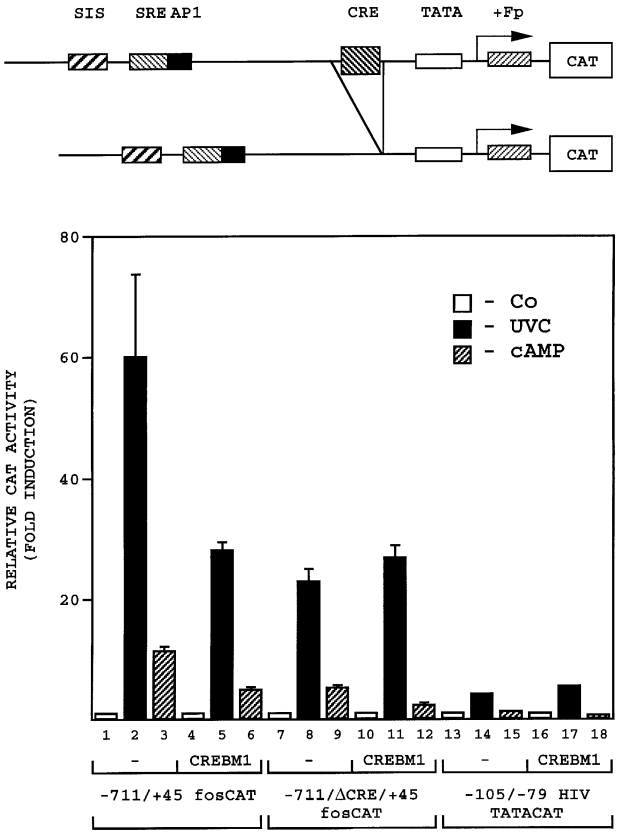

Fig. 1. The proximal CRE in the c-*fos* promoter and CREB mediate part of the UVC response of the gene. HeLa tk^- cells were transfected with 5 μg of –711/+45 *fos* CAT or –711/Δ–65/–52/+45 (–711/ΔCRE/+45) *fos* CAT promoter constructs. Five μg of RSV0 (–) or a RSV-driven expression vector coding for a dominant-negative CREB mutant (CREBM1) were co-transfected where indicated. The cells were serum-starved for 24 h post-transfection and then stimulated with 30 J/m^2 UVC or treated with forskolin and IMX (10 μM and 0.5 mM cAMP) for an additional 24 h. Average relative CAT activities (induction factors) of three independent experiments (±SEM) are presented. SIS, responsive element participating in the c-*fos* transcriptional induction by conditioned medium from oncogene v-*sis*-transformed cells; SRE, serum responsive element which mediates the transcriptional induction of c-*fos* in response to a plethora of stimuli such as serum, growth factors, pro-inflammatory cytokines, hormones (e.g. insulin), Ca^{2+}, phorbol esters, oxidative stress, protein synthesis inhibitors and UVC; AP-1, sequence with as yet undefined function located immediately downstream of the SRE and bearing homology to AP-1 and CREB binding sites; CRE, '–60 CRE' (the proximal cAMP responsive element); +Fp, intragenic enhancer element (+18/+38) participating in the UVC and cAMP inducibility of the promoter. The arrow indicates to the start of transcription.

The residual cAMP response of the CRE deletion mutant was reduced further by CREBM1 (Figure 1, compare lanes 9 and 12), probably because of the existence of two upstream CRE-like sequences located at positions –343/–336 and –295/–288 which presumably bind members of the CREB/ATF family (Berkowitz *et al.*, 1989). Whether the upstream CREB/ATF-binding elements do participate in the UVC response cannot be decided unequivocally, since the contribution of the transcription factor complex binding to the SRE dominates (Figure 1, compare lanes 5, 8 and 11), but they probably do. The remaining cAMP response seen in lane 12 may be due to a non-homologous intragenic CRE which does not bind CREB (+18/+38, Härtig *et al.*, 1991).

UVC induces the transactivating property of CREB

To show directly that the transactivating property of one of the CRE-binding factors, CREB (Sassone-Corsi *et al.*, 1988), can be induced by UVC, we tested whether CREB fused to a different DNA-binding domain, that of the yeast transcription factor GAL4, could be activated by UVC to initiate transcription of a GAL4-dependent reporter. HeLa cells were transiently co-transfected with expression vectors encoding either the DNA-binding domain (DBD) of GAL4 or a chimeric protein consisting of the GAL4 DBD fused to the full-length CREB protein (Figure 2A) or to a CREB protein lacking the bZip region (Figure 2B), together with a GAL4 element-driven CAT reporter gene construct (Chrivia *et al.*, 1993). Both cAMP and UVC promoted GAL4–CREB-dependent transcription but not that by the GAL4 DBD alone (Figure 2), indicating efficient activation of CREB by both UVC and cAMP. With constructs containing the complete CREB including the bZip region, UVC induction was less efficient than cAMP induction while the reverse was found for the truncated CREB (compare Figure 2A and B). The reason for this difference is unknown.

Activation of CREB by cAMP depends on the phosphorylation of Ser133 by the cAMP-dependent protein kinase A (PKA; Gonzales and Montminy, 1989; Taylor *et al.*, 1990). Accordingly, a GAL4–CREB gene construct in which Ser133 was mutated to alanine responded less well to cAMP treatment (Figure 2B, lane 11). UVC induction of this hybrid transcription factor was reduced to the same extent (lane 10), indicating that Ser133 is also instrumental in UV-induced transactivation of CREB. It is not clear how the residual activity in the Ala133 mutant is generated. Perhaps some CBP (see below) can interact with the mutant protein. Residual activation could then be caused by CBP activation (see below). Interestingly, a second phosphorylation site, Ser142, usually found to be inhibitory and the target of Ca^{2+}/calmodulin-dependent protein kinase II (Sun *et al.*, 1994), does not seem to be addressed by UVC. Mutation of this serine to alanine did not significantly enhance forskolin- or UV-induced transactivation of GAL4–CREB (Figure 2B, compare lanes 7 and 15 and lanes 6 and 14).

The activated CREB communicates with the basal transcription factor TFIIB through the bridging protein CBP (Kwok *et al.*, 1994). A GAL4–CBP fusion protein should be able to activate transcription directly. When fused to the DNA-binding domain of GAL4, the C-terminal portion of CBP (amino acids 1678–2441)

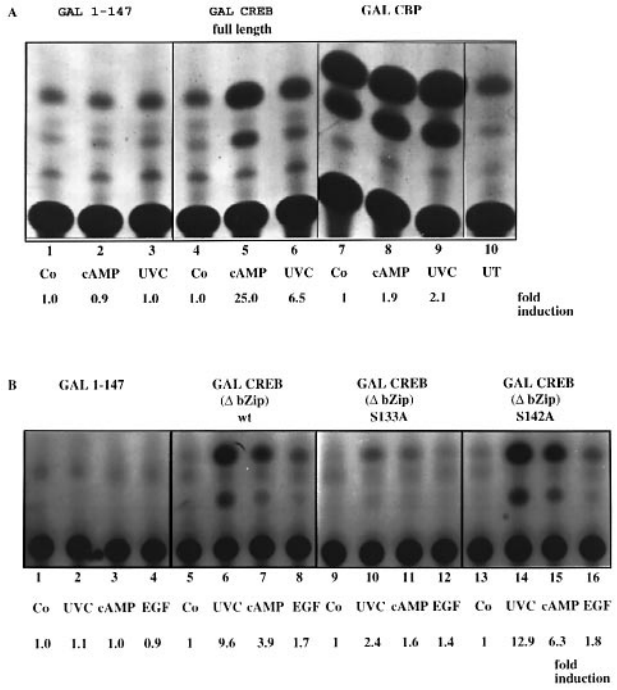

Fig. 2. (**A**) UVC induces the transactivation potential of CREB and of CBP. HeLa cells were transfected with 2 μg of GAL4–CAT reporter construct together with 3 μg of either GAL4 DBD (GAL 1–147), GAL4–CREB (full-length) or GAL4–CBP expression vectors as indicated. The cells were serum-starved for 24 h post-transfection and then stimulated with 30 J/m^2 UVC or forskolin and IMX (20 μM and 1 mM, cAMP). After 24 h, CAT determinations were performed. (**B**) UV-induced activation of CREB depends on the presence of Ser133. HeLa cells were transfected with 2 μg of GAL4–CAT reporter construct together with 3 μg of either GAL4 DBD (GAL 1–147), GAL4–CREBΔbZip wild-type, GAL4–CREBΔbZip S133A or GAL4–CREBΔbZip S142A expression vectors as indicated. The cells were serum-starved for 24 h post-transfection and then stimulated with either 30 J/m^2 UVC, forskolin and IMX (20 μM and 1 mM, cAMP) or EGF (20 ng/ml). After 24 h, CAT determinations were performed. Induction factors are indicated. The experiment presented is one of several independently performed determinations.

could indeed activate transcription, strongly suggesting the presence in this region of an autonomous spontaneously active transactivation domain. Interestingly, both cAMP and UVC enhanced transcription ~2-fold, suggesting that the cAMP- and UVC-induced signalling pathways also caused an activating modification of CBP (Figure 2A).

In conclusion, our experiments demonstrate that UVC activates a signalling pathway at the end of which CREB (and possibly other CRE-binding proteins) are located, and that CREB activation contributes to UVC-induced c-*fos* transcription.

UVC is a potent inducer of CREB Ser133 and ATF-1 Ser63 phosphorylation

At the c-*fos* promoter, the transcription factors are DNA bound at most or all times (Herrera *et al.*, 1989). Activation must therefore involve post-translational modification of the factors in the DNA-bound state. The experiments with GAL4–CREB wild-type or with the Ser133 mutation (Figure 2) and with the dominant-negative CREBM1 (Figure 1) have already pointed to Ser133 as the site of modification after UVC as well as cAMP treatment. With cAMP as the inducer, Ser133 phosphorylation by the catalytic subunit of PKA (Gonzales and Montminy, 1989)

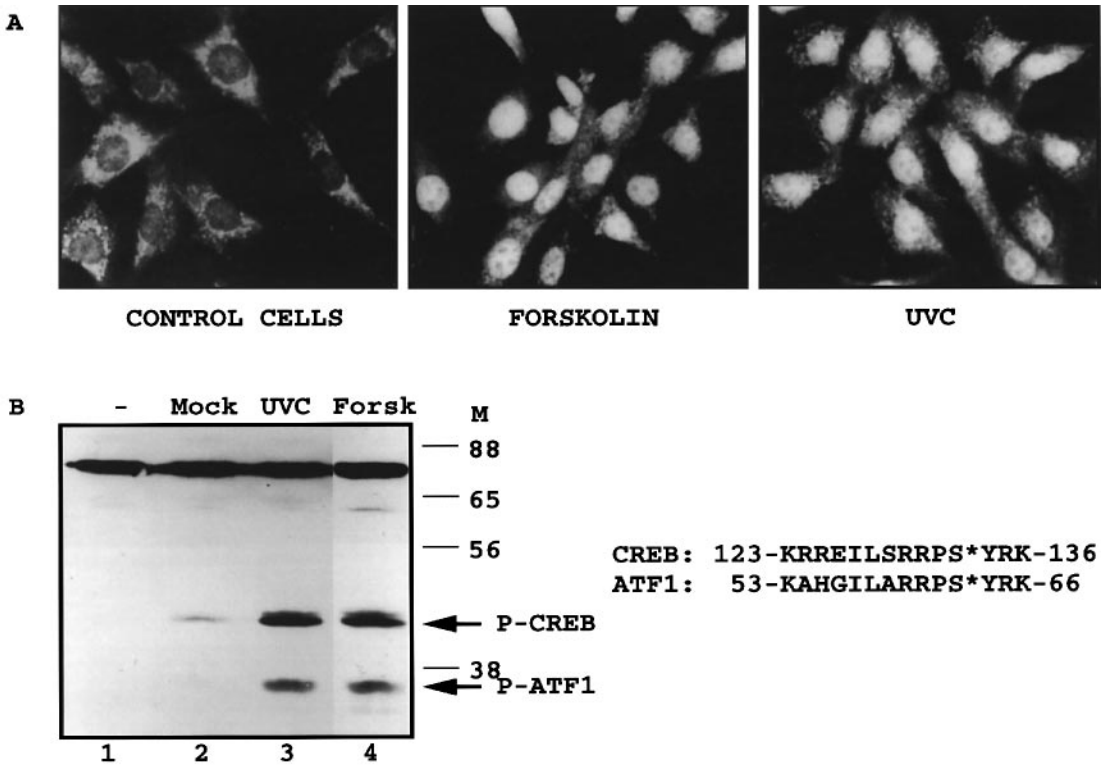

Fig. 3. Forskolin and UVC irradiation induce Ser133 phosphorylation of CREB and Ser63 phosphorylation of ATF-1 in NIH3T3 mouse fibroblasts. (**A**) NIH3T3 cells were serum-starved for 24 h (DMEM, 0.5% FCS) and then stimulated with forskolin (10 μM) or UVC (30 J/m^2). 15 min later the cells were fixed in 4% *p*-formaldehyde and processed for immunofluorescence analysis. The antibody used recognizes only CREB and ATF-1 phosphorylated at serines 133 and 63, respectively. The dark-stained nuclei are indicative of the non-phosphorylated state of CREB and ATF-1. The forskolin- and UVC-induced phosphorylations are manifested by positive nuclear staining. (**B**) Western blot using the same antibody. The cells were treated as in (A) (forskolin = Forsk), lysed in boiling Laemmli buffer to avoid dephosphorylation and degradation of the proteins and processed for Western blot analysis. The amino acid sequences of phosphorylated CREB and ATF-1 recognized by the antibody are indicated. Mock: treated as for UV irradiation, but without irradiation.

has been shown to cause binding of CBP (Chrivia *et al.*, 1993; Kwok *et al.*, 1994). To examine directly the Ser133 phosphorylation state of CREB after UVC stimulation of cells, we used an antibody recognizing only the phosphorylated form of CREB: the amino acid sequence 123–136 (KRREILSRRPS^{133}YRK, Herdegen *et al.*, 1994) with Ser133 carrying the phosphate group, and investigated by immunofluorescence the presence of this epitope in NIH3T3 mouse fibroblasts. Since the phosphorylated epitope is conserved to 100% between CREB and its family member ATF-1, the antibody used also recognizes ATF-1 phosphorylated at Ser63 [this residue corresponds functionally to Ser133 of CREB (see sequence in Figure 3) and is phosphorylated by PKA; Lee and Masson, 1993, and references therein]. In untreated cells, the nuclei remained dark after staining with antibody (Figure 3A), consistent with the assumption that CREB and ATF-1 were in the non-phosphorylated state. Treatment of the cells with either forskolin or UVC (30 J/m^2, a dose which leads to efficient c-*fos* transcription in these cells) resulted in strongly positive nuclear staining consistent with the efficient phosphorylation of nuclear CREB and/or ATF-1 at serines 133 and 63, respectively. A Western blot analysis with this antibody, using whole cell extracts, indeed revealed both cAMP- and UVC-induced phosphorylation of the same proteins (Figure 3B), with the characteristic molecular weights of CREB (43 kDa, Montminy and Bilezikjian, 1987) and ATF-1 (35 kDa, Hai *et al.*, 1989).

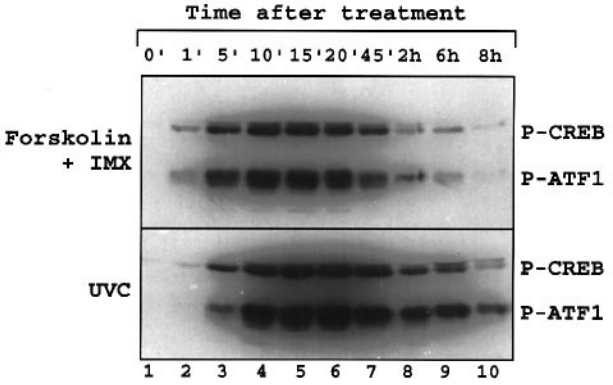

Fig. 4. Kinetics of UV- and cAMP-induced CREB and ATF-1 phosphorylation. HeLa cells were treated with forskolin and IMX (20 μM and 1 mM) or irradiated with UVC (30 J/m^2) as indicated. At the time points indicated, cells were harvested and assayed for the phosphorylation of CREB and ATF-1 as described in Figure 3.

The β-splice form of CREB (Blendy *et al.*, 1996) and the CRE modulator protein CREM (Foulkes *et al.*, 1991) were not detected. A protein of 80 kDa and of unknown nature reacted strongly with the antibody irrespective of the source of the extract. In both untreated and treated cells, this crossreactivity is equally distributed in nucleus and cytoplasm and there is no induced translocation (not shown).

We conclude that CREB and ATF-1 are indeed

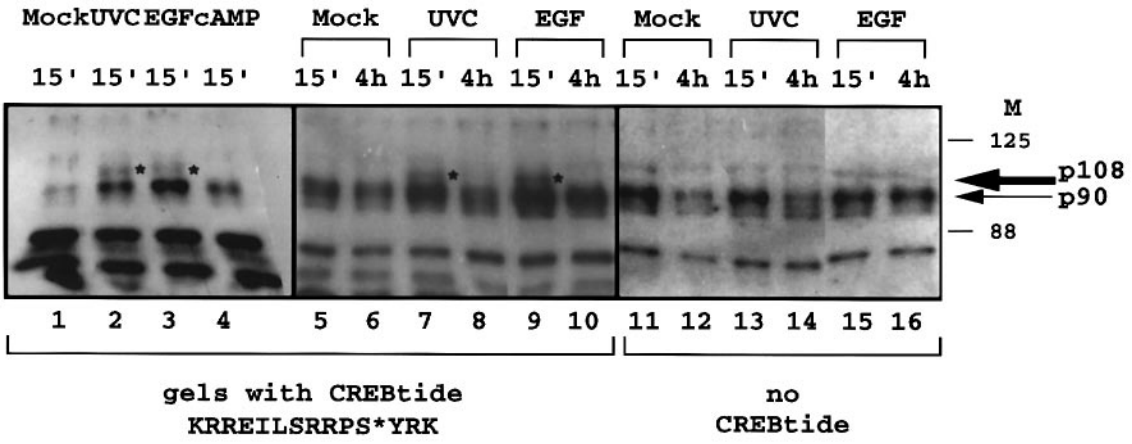

Fig. 5. UVC induces a 108 kDa CREB kinase in HeLa cells. Serum-starved HeLa cells were treated with either UVC (60 J/m^2), EGF (20 ng/ml) or forskolin plus IMX (10 μM and 0.5 mM, cAMP) for 15 min or, where indicated, for 4 h. Nuclear extracts were prepared and 'in gel kinase assays' were performed as described in Materials and methods. In lanes 11–16, no CREBtide has been added to the gel. Mock = mock-treated cells. The p90 kDa CREB kinase is indicated by a thin arrow, the thick arrow points to the p108 kDa CREB kinase. Where detectable, the p108 CREB kinase is also indicated in the gel by an asterisk. The peptide used for the in-gel kinase assay ('CREBtide') is shown below the figure.

phosphorylated at the appropriate serines after UVC irradiation, which can explain the mechanism of UVC-induced activation of CRE-binding proteins and their transcriptional role in the UV response of c-*fos*.

UV-induced CREB and ATF-1 phosphorylation is an immediate early event. It occurs with a lag period of <5 min after irradiation of 3T3 (not shown) or HeLa cells, with initial phosphorylations already seen at 1 min (Figure 4). The kinetics and magnitude of CREB/ATF-1 phosphorylation and of subsequent dephosphorylation after cAMP and UV resemble each other.

UV-induced p108 CREB kinase

Which protein kinase(s) could be responsible for UVC-induced CREB activation? A major criterion for its (their) identification could be that UVC enhanced its (their) activity. We therefore examined CREB-accepting protein kinases by 'in-gel' kinase assay (see Materials and methods). Nuclear extracts from untreated or from UVC-irradiated HeLa cells were resolved by SDS–PAGE with specific substrate added prior to acrylamide polymerization: the non-phosphorylated CREB peptide KRREILSRRPS^{133}YRK (Figure 5, left two panels). In control gels, the peptide was omitted (Figure 5, right panel) or an irrelevant peptide was added to the gel (not shown). As one might expect from the resolution of total nuclear extracts, several protein kinase activities are detectable. Bands of ~90 and 75 kDa autophosphorylate in the absence of CREB peptide (Figure 5, right panel). The important finding is that only two protein kinase activities are enhanced by UVC irradiation of the cells and that this enhancement depended entirely on the presence of the CREB peptide: a 90 kDa band (thin arrow) was increased after UVC treatment, a band of ~108 kDa (thick arrow) is only visible in the lanes with extracts from UVC-irradiated cells (Figure 5, lanes 2 and 7, marked with an asterisk). We did not detect renaturable UVC-induced kinase activities at lower molecular weights (not shown). The UVC-induced enhancements of protein kinase activities were transient, raised at 15 min (lane 7) and gone at 4 h (lane 8). Both protein kinases were not activated in cells treated with forskolin plus iso-butylmethylxanthine (IMX) (Figure 5, lane 4, cAMP).

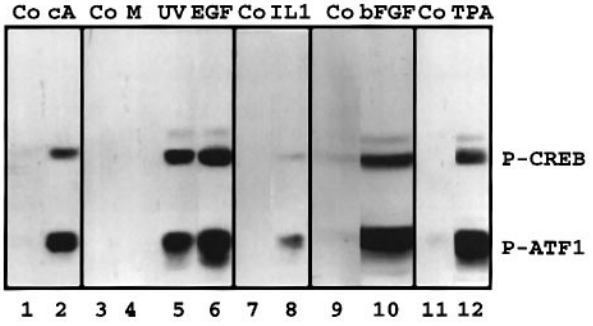

Fig. 6. EGF, IL-1α, bFGF and the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induce phosphorylation of CREB and ATF-1 in HeLa cells. Serum-starved HeLa cells were stimulated with either forskolin and IMX (10 μM and 0.5 mM, cA), UVC (30 J/m^2), EGF (20 ng/ml), IL-1α (2 ng/ml), bFGF (10 ng/ml) or TPA (200 ng/ml). After 15 min, cell lysates were prepared for Western blot analyses. M = mock-treated cells.

The protein kinases known to phosphorylate CREB, the activity of the catalytic subunit of PKA and the Ca^{2+}/calmodulin-dependent kinases, were not enhanced in UVC-treated cells (not shown, below the molecular weight range of the gel in Figure 5). Experiments addressing the upstream pathway to be described below also speak against an involvement of either PKA or Ca^{2+}/calmodulin-dependent protein kinases.

CREB phosphorylation upon growth factor stimulation

Interestingly, the 90 and 108 kDa protein kinases were also transiently activated upon EGF treatment of cells (Figure 5, lanes 3 and 9). This finding prompted an examination of the CREB and ATF-1 phosphorylation states in cells treated with one of several growth stimuli. CREB Ser133 and ATF-1 Ser63 were indeed phosphorylated in response to phorbol ester, bFGF, IL-1α and EGF, as well as UVC (Figure 6) and tumour necrosis factor (TNF)-α (not shown). EGF also causes Ser133-dependent

activation of GAL4–CREB (Figure 2B, lanes 8, 12 and 16), although the enhancement was only slight. Thus CREB and ATF-1 phosphorylation is not at all exclusive for elevated cAMP and it appears that several signalling pathways address CREB and ATF-1, and probably involve the same protein kinases. These data match those of other laboratories demonstrating CREB Ser133 phosphorylation in PC12 cells in response to nerve growth factor (NGF) and in melanocytes in response to several growth factors (Ginty *et al.*, 1994; Böhm *et al.*, 1995). A protein kinase induced after NGF migrated with 105 kDa (Ginty *et al.*, 1994) and could be identical with p108 CREB kinase.

Dissection of upstream signalling

Could one of these putative growth factor-triggered pathways be relevant for the induction by UVC? We have shown previously that several UVC-initiated signal transduction cascades inducing c-*fos* and c-*jun* gene transcription depend on the presence on the cell surface of activatable growth factor receptors (Sachsenmaier *et al.*, 1994). UVC strongly stimulates ligand-independent tyrosine phosphorylation of several receptor tyrosine protein kinases (Sachsenmaier *et al.*, 1994; Coffer *et al.*, 1995; Knebel *et al.*, 1996). To try and identify putative signalling components shared by the UVC response of CREB, specific down-modulation experiments were performed (Sachsenmaier *et al.*, 1994) and inhibitors were applied.

Specific down-modulation experiments are based on the following principle: a stimulus, e.g. EGF, activates components of a signal transduction pathway in a transient manner. In the case of EGF, the EGFR undergoes autophosphorylation followed by receptor internalization. Restimulation by the same stimulus, e.g. EGF, is not possible for some period of time. Such down-modulation has been found for many stimuli. Pre-treatment of cells with one stimulus makes cells refractory to a second stimulation by the same agent. A second different stimulus can, however, be successful if it utilizes a component other than the down-modulated one. Refractoriness to UVC would result if UVC required one of the down-modulated components of the previous stimulus.

One problem in such down-modulation experiments is the choice of downstream endpoints, because restimulation can only be measured using components with transient induction kinetics. Suitable transiently active components are the transcription factors, e.g. CREB and ATF-1, and protein kinases, e.g. Erk 1,2. In the experiment shown as Figure 7, we show first the induction by EGF of Erk 1,2 activation (at 15 min, Figure 7A, lane 6), then the decay of activity (the EGF-induced Erk 1,2 activation has disappeared at 4 h; Figure 7A, lane 7) followed by demonstration of refractoriness to the same stimulus (Figure 7A, lane 8). As described, stimulation with UVC as the second stimulus should then clarify whether the two stimuli share signalling components (Figure 7A, lane 9). This type of analysis has demonstrated that EGF alone (Figure 7A, lane 9) or a cocktail of growth factors (EGF, IL-1α and bFGF; Figure 7B, lane 9) cannot inhibit UVC-induced CREB and ATF-1 phosphorylation, while blocking the UVC-dependent reactivation of Erk 1,2 in the same cells (Sachsenmaier *et al.*, 1994; and Figure 7A, lane 9). This indicates that the UVC-induced pathway to CREB and ATF-1 differs from those used by the growth factor receptors, and from that used by UVC in the activation of Erk 1,2. Accordingly, a specific inhibitor of the EGFR, AG1478 (Levitzki and Gazit, 1995), inhibited UVC- and EGF-induced EGFR phosphorylation (not shown, see Knebel *et al.*, 1996) and Erk 1,2 activation (Figure 7D, lower panel), but not the UVC-induced phosphorylation of CREB and ATF-1 (Figure 7D, upper panel). Down-modulation experiments similar to the one above indicate that the UVC-dependent pathway to CREB and ATF-1 does not share components with those of TNF-α, of IMX plus forskolin (both not shown) and of phorbol ester (Figure 7C).

Suramin, a drug that poisons plasma membrane receptors (Betsholtz *et al.*, 1986; Coffey *et al.*, 1987; Fantini *et al.*, 1989), has been used to inhibit UVC-induced reactions which require the function of growth factor receptors (Sachsenmaier *et al.*, 1994). The UVC-induced CREB/ATF-1 phosphorylation turned out to be suramin-resistant (Figure 8A), while UVC-induced Erk 1,2 activation was sensitive (Figure 8B, see also the control with growth factor-rich conditioned medium, EPIF, from UV-irradiated cells whose effect is suramin-sensitive, Figure 8 and Krämer *et al.*, 1993). That suramin still inhibits most of the UV-induced c-*fos* transcription (Sachsenmaier *et al.*, 1994) can be explained by assuming that the suramin-resistant pathway to the CRE does not on its own suffice to induce c-*fos* transcription. This is indeed the case for UVC induction (not shown; and Büscher *et al.*, 1988; and see Discussion) as well as for induction by NGF (Ginty *et al.*, 1994).

The possibility remained that UVC triggered the release of calcium from endoplasmic reticulum or the entry from extracellular sources in a growth factor receptor-independent manner, increasing intracellular calcium and thereby activating Ca^{2+}/calmodulin-dependent protein kinases which accept CREB and ATF-1 as substrates. Elevation of the intracellular Ca^{2+} concentration indeed leads, in numerous cell types, to transcriptional activation of c-*fos* both through the SRE (Bading *et al.*, 1993) and through the CRE (Sheng *et al.*, 1990; Bading *et al.*, 1993). Consistently, increased cytosolic calcium was found to cause both Erk 1,2 activation (Bading and Greenberg, 1991; Chao *et al.*, 1992; Fiore *et al.*, 1993; Rosen *et al.*, 1994) and CREB Ser133 phosphorylation (Sheng *et al.*, 1990, 1991). Indeed, disturbing intracellular Ca^{2+} levels by treatment of cells with the Ca^{2+} ionophore A23187 (Pressman, 1976) inhibited UV-induced c-*fos* transcription (Büscher *et al.*, 1988) and UV-induced CREB phosphorylation (not shown). However, this result may be due to a basic need for Ca^{2+} rather than to UV-induced activation of Ca^{2+}-dependent signalling components, since UVC irradiation, in contrast to the Ca^{2+} ionophore A23187, did not induce a detectable rise in intracellular Ca^{2+} levels in HeLa cells at doses between 30 and 2000 J/m^2. Ca^{2+} was measured by spectroscopic analysis with the fluorescent-free Ca^{2+}-binding dye fluor-3 and by video-imaging with the same dye (not shown; Iordanov, 1995). It is unlikely, therefore, that UVC-induced CREB and ATF-1 phosphorylations are mediated through a UVC-induced rise in Ca^{2+} concentration and through the activation of known Ca^{2+}/calmodulin-dependent protein kinases.

All these data suggest that the UVC-induced signalling pathway to CREB and ATF-1 does not overlap with those

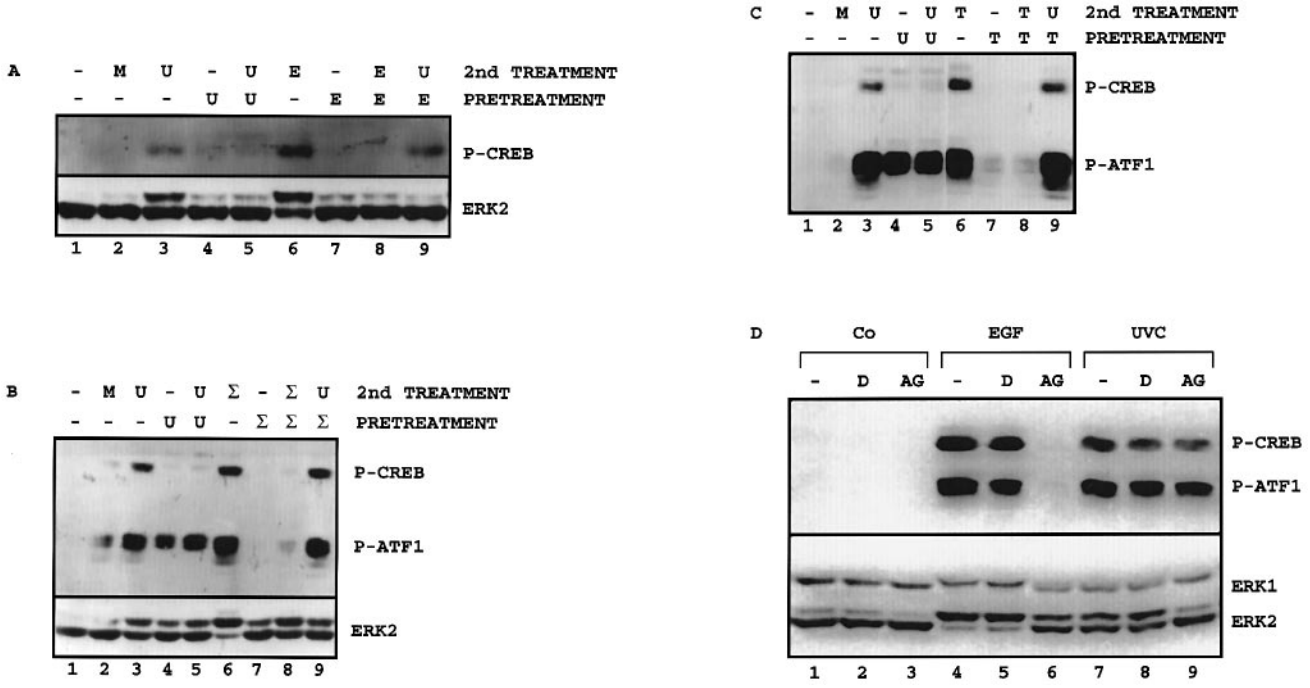

Fig. 7. Down-modulation of growth factor receptor-induced signal transduction pathways hampers the UVC responsiveness of Erk 1,2 but not of CREB. (**A**) HeLa cells were not pre-treated (–) or pre-treated with UVC (30 J/m^2) or EGF (20 ng/ml) followed 4 h later by a second treatment with either UVC (30 J/m^2) or EGF (20 ng/ml) for 15 min. The cell lysates were analysed by Western blot using the antibody directed against phosphorylated CREB (upper panel) or using the Erk 2-specific antibody αIcp^{42} (lower panel). M; treated as for UV-irradiation but without irradiation. (**B**) The same design as in (A), except that a combination of three growth factors (Σ = EGF, 20 ng/ml; HIL-1α, 2 ng/ml; bFGF, 10 ng/ml) was used instead of EGF. (**C**) The same design as in (A), except that TPA (200 ng/ml) was used instead of EGF. (**D**) The EGFR inhibitor AG1478 does not inhibit UVC-induced CREB phosphorylation. HeLa cells, which were starved in 0.5% FCS for 24 h, were either not treated, or treated with DMSO or with the EGFR-specific inhibitor AG1478 (100 nM). Two minutes later, the cells were treated with EGF (20 ng/ml), irradiated with UVC (30 J/m^2) or left untreated. After 15 min, cell extracts were prepared and the activation state of Erk 1,2 (with antibody K-23 recognizing Erk 1 and Erk 2) and CREB phosphorylation were determined by Western blot analysis.

examined above. In particular, the receptor tyrosine kinase/Erk 1,2 pathway is clearly different from the pathway to CREB and ATF-1. Suramin resistance implies that either a receptor at the cell surface was not involved, or that this specific receptor could not be blocked by suramin.

p38/HOG-1 dependence of UVC-induced p108 CREB kinase

To define the new UV pathway to CREB, we attempted to link the CREB kinases to known components of signal transduction. p90 CREB kinase is active in control cells prior to UVC or EGF treatment (in contrast to its *in vitro* substrates CREB and ATF-1 which are not phosphorylated prior to such treatment, see Figure 5, lanes 1, 5 and 6) and therefore perhaps not the prime candidate for the *in vivo* response to UVC. Nevertheless, we considered its contribution to CREB and ATF-1 phosphorylation *in vivo*. The molecular weight obviously corresponds to that of p90^{RSK1}. p90 CREB kinase migrates in PAGE to a position identical with p90^{RSK1} as revealed in a Western blot type experiment with antibodies directed against p90^{RSK1} (compare Figure 9C with D, see legend for experimental details). The activation of p90 CREB kinase by UVC or EGF goes along with the disappearance of the faster migrating species of p90^{RSK1} (Figure 9D). Thus this modification of p90^{RSK1} could be the physical basis of p90 CREB kinase activation. p90^{RSK1} has been shown to be activated by the Ras–Raf–MEK1,2–Erk1,2 pathway (for review, see Treisman, 1996) which is activated by UVC with kinetics preceding the rapid induction of c-*fos* transcription. Could UV-induced CREB and ATF-1 phosphorylation be mediated through the UV-induced Ras–Raf–MEK1,2–Erk1,2, p90^{RSK} pathway? This is not likely since under conditions of inhibition of this pathway (pre-treatment of cells with growth factors, phorbol esters or suramin, specific receptor inhibition) when Erk1,2 is not activatable by UVC, UVC-induced CREB phosphorylation is not affected.

Thus p90 CREB kinase is either not relevant for CREB and ATF-1 phosphorylation *in vivo* or it is part of still another pathway. In addition to the Raf–MEK1,2–Erk 1,2 pathway, UV irradiation strongly activates the MEKK1–MKK4–JNK/SAPK pathway (Hibi *et al.*, 1993; Dérijard *et al.*, 1994). The kinetics are slower than those of CREB phosphorylation (D.Wilhelm and P.Angel, personal communication; Price *et al.*, 1996). Further, UV also activates p38/RK/HOG-1 (Raingeaud *et al.*, 1995; and Figure 9A; for reviews on proline-directed kinases, see Cano and Mahadevan, 1995; Treisman, 1996). In order to distinguish between the JNK/SAPK pathway and reactions depending on p38/RK/HOG-1, we used an inhibitor (SB203580) which specifically inhibits p38/RK/HOG-1 without affecting Erk1,2 or JNKs/SAPKs (Lee *et al.*, 1994; Cuenda *et al.*, 1995). SB203580 reduced significantly the UVC- and EGF-induced CREB and ATF-1 phosphorylations (Figure 9B), without interfering with several other UV-induced reactions such as c-Jun phosphorylation (Radler-Pohl *et al.*, 1993), NF-κB activation (Stein *et al.*,

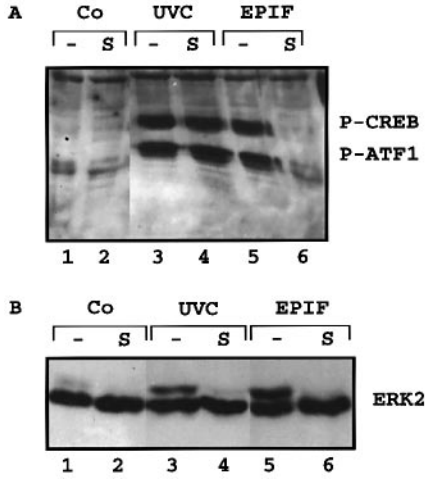

Fig. 8. The UVC-induced CREB and ATF-1 phosphorylations are suramin resistant. (**A**) HeLa cells were pre-incubated with suramin (0.3 mM) for 45 min where indicated (S) and then treated, with either UVC (30 J/m^2) or conditioned medium from UVC-irradiated HeLa cells (30 J/m^2) that had been allowed to synthesize and secrete EPIF for 48 h (EPIF). Then, 15 min after treatment, cell lysates were prepared for Western blot analysis using the antibody directed against phosphorylated CREB and ATF-1. (**B**) The same cell lysates were used to monitor the effect of suramin on Erk 2 activation, using the Erk 2-specific antibody αIcp[42].

1989) and IκBα degradation (K.Bender, unpublished; all not shown). Most importantly, SB203580 did not interfere with the UVC-induced activation of the p90 CREB kinase and the modification of p90^{RSK1} (Figure 9C and D). As one might have expected, SB203580 did not affect forskolin-induced CREB phosphorylation either (not shown). These data indicate that the UVC action on CREB and ATF-1 depends on p38/HOG-1, and that p90^{RSK1} (and the p90 CREB kinase) is not part of the pathway.

Recently, a new protein kinase has been shown to be activated in conjunction with p38/RK/HOG-1: the 50 kDa MAPKAP kinase 2 (Stokoe *et al.*, 1992; see Cuenda *et al.*, 1995 for references). MAPKAP kinase 2 is the major enzyme responsible for the phosphorylation of the small heat shock proteins in cells treated with IL-1, arsenite or sorbitol. Although the 'in gel' kinase assay revealed no band of this size, we examined the putative involvement of MAPKAP kinase 2 in UV-induced CREB and ATF-1 phosphorylation, a possibility made plausible by its activation in stressed cells. A constitutively active form of MAPKAP kinase 2, GST–MAPKAP kinase 2 Δ3BPC (Engel *et al.*, 1995), renatures only poorly in an 'in gel' kinase assay with Hsp25 as substrate (M.Cahill, A.Nordheim and M.Gaestel, unpublished), suggesting that this may also be the case for the UV-regulated enzyme. In order to assay for the activation of MAPKAP kinase 2 in UV-irradiated cells and for its activity towards CREB, we immunoprecipitated the kinase from HeLa cell extracts at different time points after UV irradiation and offered His-tagged CREB or Hsp25 as target proteins. Indeed, UV irradiation activated MAPKAP kinase 2 strongly when tested with its substrate Hsp25, detectable at 2 min after UV irradiation and inhibited by the p38/RK/HOG-1 inhibitor SB203580 (Figure 10A, lanes 1–6). Also, treatment of cells with EGF activated the kinase through the p38/RK/HOG-1 pathway (Figure 10A, lanes 7 and 8). In contrast, phosphorylation of His-tagged CREB protein by the immunoprecipitated MAPKAP kinase 2 was not detectable (not shown). We investigated, therefore, whether constitutively active MAPKAP kinase 2 produced in and purified from *Escherichia coli* accepted the CREB protein *in vitro*. While the recombinant enzyme phosphorylated the Hsp25 protein strongly [Figure 10A, lane 9; as shown earlier (Gaestel *et al.*, 1991) Hsp25 is also phosphorylated, to a lower extent, by PKA, lane 10], it phosphorylated the CREB protein only poorly (Figure 10B, lane 1; which was, however, a good substrate for PKA, Figure 10B, lane 2). Table I summarizes the enzymatic parameters for MAPKAP kinase 2 towards the relevant substrate peptides derived from either Hsp25 or CREB, in comparison with PKA. The data show that MAPKAP kinase 2 phosphorylates the CREB peptide much less efficiently than the peptide derived from the Hsp25 protein. We conclude that MAPKAP kinase 2 is strongly activated in UV-irradiated HeLa cells, but that CREB (and possibly ATF-1) is only a poor substrate for this enzyme *in vitro*. Thus MAPKAP kinase 2 may participate but is unlikely to account for the massive phosphorylation of CREB and ATF-1 after UV irradiation.

Could p108 be the decisive kinase? Several arguments speak for the *in vivo* significance of p108 in the UVC response of CREB. UVC activation of p108 CREB kinase is also strongly inhibited by SB203580 (Figure 9C), as is UVC-induced CREB and ATF-1 phosphorylation (Figure 9B). Also, activation of p108 CREB kinase in response to EGF is inhibited by SB203580 (Figure 9C). Thus p108 must be downstream of p38/RK/HOG-1. Another argument concerns the strict correlation of induced p108 activity and CREB (and ATF-1 phosphorylation) after stimuli (EGF, UVC, but not cAMP), and the absence of activity prior to treatment. Thus p108 is likely to contribute the largest portion of the relevant UVC response to CREB.

Discussion

We show here that the transcription factors CREB and ATF-1 are phosphorylated at serines 133 and 63, respectively, when cells are treated with one of various growth factors (EGF, bFGF, IL-1α, TNF-α, and conditioned medium from UVC-treated cells, EPIF, Schorpp *et al.*, 1984) or with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). It has been shown previously that CREB is also activated by NGF in PC12 cells (Ginty *et al.*, 1994) and by several growth factors in melanocytes (Böhm *et al.*, 1995). Our central point is the identification in several cell lines of CREB and ATF-1 as functional targets of a novel signal transduction chain elicited by short wavelength UV irradiation (UVC). CREB is thus not only a target for a limited set of signals (that work through cAMP and Ca^{2+} as 'second messengers') but is addressed by numerous stimuli.

The UVC response pathway to CREB/ATF-1 involves p38-HOG-1

Within 15 min after treatment of HeLa cells with cAMP- or Ca^{2+}-elevating agents, with phorbol esters, with one of several growth factors or with UVC, the transcription factors CREB and ATF-1 are phosphorylated at serines 133 and 63, respectively. The kinases responsible differ

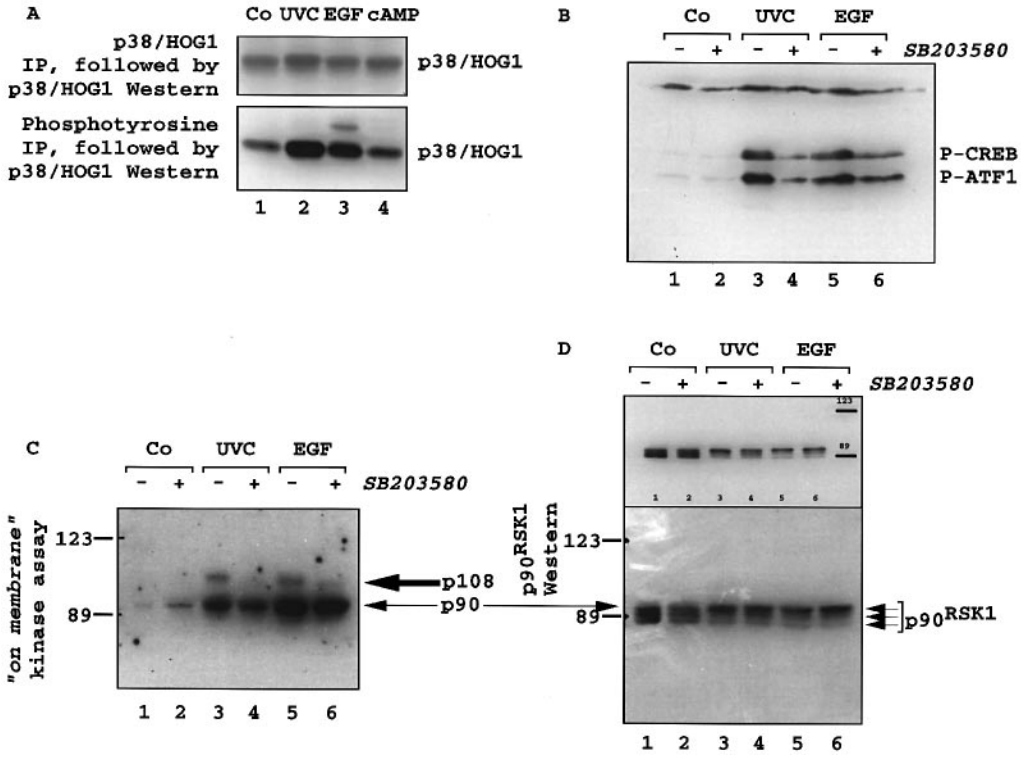

Fig. 9. The p108 CREB kinase, but not p90^{RSK1}, is activated by UVC and EGF through the p38/HOG1 pathway. (**A**) Activation of p38/HOG1 by UVC and EGF in HeLa cells. Cells were treated with UVC (30 J/m^2), EGF (20 ng/ml) or forskolin plus IMX (20 μM and 1 mM, cAMP) as indicated. Then, 15 min after treatment, cells were lysed in 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 1 mM Na vanadate, 1 mM PMSF. The lysates were divided and precipitated with an antibody directed against p38/RK/HOG1 (upper panel) or with an anti-phosphotyrosine-specific antibody (gift of Dr B.Druker). The immunoprecipitates were separated in a 10% SDS–PAGE, followed by Western blotting using an anti-p38/RK/HOG1 antibody (Santa Cruz, # sc-535). (**B**) UVC- and EGF-induced CREB phosphorylation is inhibited by pre-treatment of the cells with the p38/HOG1-specific inhibitor SB203580. Cells were pre-treated with SB203580 (25 μM) or with DMSO for 15 min as indicated. They were then treated with UVC (30 J/m^2) or EGF (20 ng/ml) and lysed after 15 min. Cell lysates were prepared for Western blot analysis using the antibody directed against phosphorylated CREB and ATF-1. (C) and (D) UVC- and EGF-induced activation of p108 but not of p90 is inhibited by SB203580. The p90 kinase co-migrates with the UVC- and EGF-modified low mobility form of p90^{RSK1}. Cells were treated with the indicated agonists (as before) with and without pre-treatment with SB203580. After lysis, cell lysates were subjected to an 'on membrane' kinase assay with membrane-bound CREBtide as described in Materials and methods (**C**), followed by a p90^{RSK1} Western blot (Santa Cruz, # sc-231-G) performed on the same membrane (**D**). The insert in the upper part of (D) shows a separate p90^{RSK1} Western blot using less protein to ensure better separation of the different p90^{RSK1} species.

between different inducers: in the case of cAMP-elevating agents it is PKA (Gonzales and Montminy, 1989); Ca^{2+}-elevating agents induce the Ca^{2+}/calmodulin-dependent kinases II and IV (Dash *et al.*, 1991; Sheng *et al.*, 1991; Matthews *et al.*, 1994; Sun *et al.*, 1994). Mitogens and UVC are known to activate the proline-directed protein kinases Erk 1,2, JNKs/SAPKs and p38/RK/HOG-1 which accept the substrate sequence (P)-X-S*/T*-P-X (reviewed by Karin, 1994; Treisman, 1996). The sequence around CREB Ser133 and ATF-1 Ser63 reads RRPS*YRK and is not likely to be accepted by proline-directed protein kinases. As shown here, growth factors and UVC activate CREB (and ATF-1)-accepting protein kinases in the 90–110 kDa range as well as MAPKAP kinase 2. The p90^{RSK1} has been implicated previously in Ser133 phosphorylation in response to certain growth factors in human melanocytes (Böhm *et al.*, 1995); and NGF activates, in PC12 phaeochromocytoma cells, a p105 CREB kinase (Ginty *et al.*, 1994). Here, we have identified a UVC- and EGF-induced p108 CREB-specific protein kinase from HeLa cells and we show that a UV- and EGF-activated protein kinase with the molecular weight of p90^{RSK1} also seems to accept CREB *in vitro*. In contrast, MAPKAP kinase 2, which is strongly activated in UV-irradiated cells, phosphorylates CREB *in vitro* only poorly, as compared with its preferred substrate, the small heat shock proteins.

Although proline-directed protein kinases will not phosphorylate CREB, CREB kinases may well be downstream of proline-directed kinases. We therefore considered whether the p42 and p44 mitogen-activated protein kinases (Erk 1,2; reviewed by Marshall, 1994), the p46 and p54 Jun N-terminal kinases (JNKs, Hibi *et al.*, 1993; Dérijard *et al.*, 1994; also referred to as stress-activated protein kinases, SAPKs, Kyriakis *et al.*, 1994) and the p38/RK/HOG-1-kinase (also named CSBP kinase, Lee *et al.*, 1994; for review, see Cano and Mahadevan, 1995) addressed one of the CREB kinases detected in UV-irradiated cells. All three proline-directed protein kinases are activated in UV-irradiated cells (Hibi *et al.*, 1993; Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994; Raingeaud *et al.*, 1995; and Figure 9A). While Erk 1,2 and p38 are activated rapidly after UVC (Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994; M.Iordanov, unpublished; Price *et al.*, 1996), JNK activation may be too slow (D.Wilhelm and P.Angel, personal communication; Price *et al.*, 1996) to account for UVC-induced CREB phosphorylation which is detectable in minutes after treatment (Figure 4). In order to distinguish between the

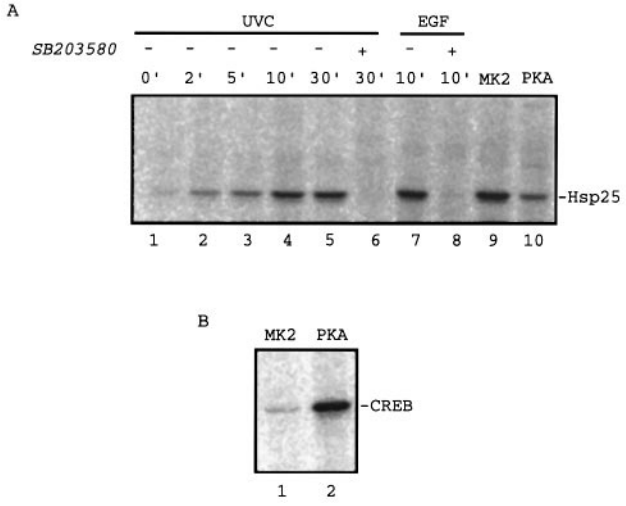

Fig. 10. MAPKAP kinase 2 is activated by UVC but phosphorylates CREB *in vitro* only poorly. (**A**) The kinetics of UV induction (30 J/m^2) as well as the EGF induction (20 ng/ml) of MAPKAP kinase 2 were analysed by immunoprecipitation of the enzyme from lysates of HeLa cells prepared at different time points after UVC treatment and a subsequent kinase assay using recombinant Hsp25 as substrate (lanes 1–5 and 7). Stimulation of MAPKAP kinase 2 is suppressed in the presence of the inhibitor SB203580 (lanes 6 and 8). As controls, constitutively active recombinant MAPKAP kinase 2 (MK2) and PKA were used to phosphorylate Hsp25 (lanes 9 and 10). (**B**) His-tagged CREB was phosphorylated *in vitro* by constitutively active MAPKAP kinase 2 (MK2) and protein kinase A (PKA). The amount of enzyme used and the assay conditions are identical to those in (A), lanes 9 and 10. The relative degree of phosphorylation was analysed by SDS–PAGE and Phospho-Imaging.

Erk 1,2 and the p38/HOG-1 pathway, we used various cellular conditions in which the Erk 1,2 pathway was not activatable and asked whether CREB still responds to UVC. Such conditions included treatment with the growth factor receptor poison suramin, pre-treatment of cells with growth factors and treatment with an inhibitor of the EGF receptor tyrosine kinase, AG1478. In all these cellular conditions, in which Erk 1,2 was not activatable by UVC, UVC was fully effective in inducing CREB/ATF-1 phosphorylation. These findings strongly suggest that the Erk 1,2 pathway was not or not to a significant extent involved in UVC-induced CREB/ATF-1 phosphorylation. Interestingly, $p90^{RSK}$ is activated by Erk 1,2 (Sturgill *et al.*, 1988; for review, see Blenis, 1993; Treisman, 1996), and, therefore, perhaps not part of the UVC-induced pathway to CREB.

Further support that neither the Erk 1,2 pathway nor the JNK pathway are involved, but that UVC reaches CREB through p38/RK/HOG-1 came from the use of the inhibitor of p38/RK/HOG-1, SB203580 (Lee *et al.*, 1994; Cuenda *et al.*, 1995). The inhibitor blocked p108 CREB kinase and MAPKAP kinase 2 activation, as well as CREB and ATF-1 phosphorylation, but did not affect the UVC-induced activation of the p90 CREB kinase nor the UVC-induced modification of $p90^{RSK1}$. Irrespective of the specificity of the inhibitor, SB203580 dissociates the UV-induced activation of p90 CREB kinase/$p90^{RSK1}$ from UV-induced CREB and ATF-1 phosphorylation. The inhibitor did not interfere with UV-induced c-Jun modification, phorbol ester-induced c-Fos modification, UV-induced degradation of IκBα and forskolin-induced CREB/ATF-1 phosphorylation. We conclude that p108 CREB kinase,

Table I. *In vitro* phosphorylation of peptides—enzymatic parameters

Substrate peptide kinase	CREBtide KRREILSRRPS*YRK	Hsp25 peptide KKKALNRQLS*VAA
PKA	180/1700	1500/152
MAPKAP kinase 2	97.2/4	31.9/60

The values represent the parameters K_m (μM)/V_{max} (nmol/min×mg).

MAPKAP kinase 2 and CREB/ATF-1 are downstream of UV-induced p38/RK/HOG-1 activation. A dominant-negative mutant of MAPKAP kinase 2 (M.Müller and M.Gaestel, unpublished) interfered with UV-induced transactivation of a transiently transfected GAL4–CREB protein (not shown), which would be compatible with a role for MAPKAP kinase 2 in the pathway. However, the mutant seems also to affect other p38-dependent activities (e.g. $p62^{TCF}$ activation) and the PKA-dependent phosphorylation of CREB. The experiment therefore does not allow for a definitive conclusion on the order of components and on the role of MAPKAP kinase 2.

We conclude that both enzymes, p108 and MAPKAP kinase 2, may account for UV-induced CREB/ATF-1 phosphorylation, with MAPKAP kinase 2 probably less significant because of its low activity on CREB *in vitro*. Irrespective of which protein kinase carries out UVC-induced, p38-dependent CREB phosphorylation (p108 CREB kinase or MAPKAP kinase-2), our findings allow the important conclusion that mitogen-activated protein kinases do not always phosphorylate target transcription factors directly. They can act through downstream protein kinases.

What is upstream of p38?

Our data indicate that a new UVC response pathway leads to the activation of p38 and hence to the phosphorylation of CREB/ATF-1. It is not clear what is upstream of p38. Two UVC pathways have been delimited in the past: DNA damage-dependent processes (e.g. p53 stabilization; Lu and Lane, 1993; Nelson and Kastan, 1994; Yamaizumi and Sugano, 1994; late responding genes; Miskin and Ben-Ishai, 1981; Schorpp *et al.*, 1984; Stein *et al.*, 1989) and the activation of growth factor receptor pathways (Dévary *et al.*, 1992; Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994; Knebel *et al.*, 1996). As detailed above, in various conditions in which growth factor receptors were down-modulated, UVC could still induce CREB/ATF-1 phosphorylation. This suggests that either suramin-resistant growth factor receptors do exist, or that UV addresses new, possibly intracellular targets. We have found previously that UVC induces elevated phosphorylation of growth factor receptors at tyrosine residues by inhibiting the dephosphorylation of these receptors, presumably by inactivating the SH group in the catalytic centre of protein tyrosine phosphatases (Knebel *et al.*, 1996). As a result, UVC may prolong not only the lifetime phosphorylated (activated) receptor tyrosine kinases but also that of intracellular protein tyrosine kinases and other tyrosine phosphate-bearing proteins. Such a general mechanism could explain how so many adverse agents can trigger Erk 1,2, JNK and p38 pathways. For instance, efficient activation of the EGFR–Erk 1,2 pathway can be achieved by oxidants and an SH-alkylating agent (Knebel

et al., 1996), JNK activation has been observed after treatment of cells with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) or methyl methanesulfonate (MMS) (van Dam *et al.*, 1995) and CREB activation has been postulated to be responsible for the transcription of the β-DNA polymerase gene after MNNG, MMS, *N*-acetoxyacetylaminofluorene and H_2O_2. (Fornace *et al.*, 1989; Kedar *et al.*, 1991). The β-DNA polymerase gene could, however, according to these reports, not be activated by UVC.

A difference between single CRE promoters and complex enhancers

Another observation is not yet understood. UVC (as shown here) and NGF (Ginty *et al.*, 1994) cause similarly effective CREB phosphorylation at Ser133 and GAL4–CREB activation as does cAMP. Moreover, CREB and possibly other CRE-binding proteins contribute to the transcriptional activation of the c-*fos* gene by UVC (as shown here) and by NGF (Ginty *et al.*, 1994; Bonni *et al.*, 1995). Strangely, however, both UVC (not shown) and NGF (Ginty *et al.*, 1994; Bonni *et al.*, 1995) induce a CRE-driven reporter only poorly, in contrast to cAMP. The UVC-induced contribution of CREB to c-*fos* transcription also cannot be measured in promoter constructs lacking the SRE and the +18/+38 element (Büscher *et al.*, 1988). We have not yet been able to find a difference in phosphorylation between CREB activated by cAMP and that activated by UVC. In particular, there is no inhibitory Ser142 phosphorylation (P.Sassone-Corsi, personal communication) as has been observed as a result of activation of calmodulin-dependent protein kinase type II (Sun *et al.*, 1994). Accordingly, mutation of Ser142 to alanine does not enhance UV-induced GAL4–CREB activation (Figure 2B). Also we have no evidence that the activation of CBP differs between UVC and cAMP (Figure 2A). We need to postulate, however, that an as yet undetected difference exists. This difference may distinguish single CRE promoters from complex enhancers where many transcription factors interact. Obligatory collaboration between transcription factors has been reported previously, e.g. Ets with AP-1 in the polyoma promoter (Wasylyk *et al.*, 1990), and $p62^{TCF}$ with SRF at the c-*fos* SRE (Treisman, 1994). UVC- or NGF-activated CREB may represent an example of conditional dependence on collaborating factors. The UVC- or NGF-modified form of CREB must collaborate, the 'cAMP form' does not need to. The prominent collaborating partner is the $p62^{TCF}$–$(SRF)_2$ complex. The c-*fos* promoter may represent an exquisite example of such collaborative assembly in that transgenic experiments in mice and derived cell lines have documented that most promoter elements are required for any c-*fos* promoter function (Robertson *et al.*, 1995). The complex assembly of the c-*fos* enhancer–promoter (as well as of other complex promoters) may be built to contract multiple signalling pathways and to permit nevertheless fine-tuning in that the relative contributions of individual stimuli depend strongly on the context of the other activated transcriptional elements.

Materials and methods

Plasmid constructs

The –711/+45 *fos* CAT and –711/Δ–65/–52/+45 *fos* CAT constructs (Härtig *et al.*, 1991) and the –105/–79 HIV TATA CAT construct (Stein *et al.*, 1989) have been described. The CREBM1 expression vector has been described by Gonzales and Montminy (1989). Rous sarcoma virus (RSV) GAL4 1–147 and RSV GAL4–CBP (1678–2441), as well as the reporter GAL4–CAT were as reported (Chrivia *et al.*, 1993; Kwok *et al.*, 1994). RSV GAL4–CREB is described in Sun *et al.* (1994), and GAL4–CREBΔbZip and the respective mutants in Ser133 and 142 in Sun and Maurer (1995). Constitutively active MAPKAP kinase 2 was expressed in *E.coli* using the vector pGEX-5X-3-MK2 Δ3BPC (Engel *et al.*, 1995).

Cell culture and transient transfections

HeLa tk^- and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Transfection of HeLa tk^- cells, using the DEAE–dextran method, followed by chloramphenicol acetyltransferase (CAT) assays were performed as described (Angel *et al.*, 1987) except that the chloroquine step was omitted. CAT activity was quantified using a Fuji Bio-Imaging Analyzer.

Chemicals, growth factors and treatment of cells

TPA (Sigma) was stored as a 200 μg/ml stock solution in dimethylsulfoxide (DMSO, Fluka) at –20°C and was applied at a final concentration (f.c.) of 200 ng/ml. EGF (human, recombinant, from *Saccharomyces cerevisiae*; Sigma), bFGF (human, recombinant, from *E.coli*; British Biotechnology), IL-1α (human, recombinant, from *E.coli*; British Biotechnology) and TNF-α (Saxon Biochemicals) were stored as 10 μg/ml stock solutions in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) at –20°C. Forskolin and IMX (Sigma) were stored as 10 mM and 0.5 M stock solutions in DMSO, respectively, at –20°C. In order to stimulate cAMP-dependent signal transduction, the cells were treated with a combination of forskolin and IMX (10 μM and 0.5 mM f.c.). Suramin (Germanin, Bayer) (0.3 mM f.c. in DMEM) was freshly prepared and used only once. The EGFR inhibitor AG1478 was stored in DMSO at 4°C at a concentration of 100 μM. EPIF medium was prepared as described previously (Krämer *et al.*, 1993). UVC irradiation of cells was performed as described previously (Radler-Pohl *et al.*, 1993), except that the PBS washing step was omitted. The p38/RK/HOG-1 inhibitor SB203580 was a generous gift of Dr J.C.Lee, King of Prussia, PA.

Antibodies and Western blotting procedure

The antibody used to detect phosphorylated CREB is an affinity-purified rabbit polyclonal serum raised against a CREB peptide phosphorylated at Ser133 (peptide 123-KRREILSRRS^{133}YRK-136; Herdegen *et al.*, 1994). It recognizes only CREB phosphorylated at Ser133 and ATF-1 phosphorylated at Ser63 but does not cross-react with the non-phosphorylated forms of these proteins. The anti-p42 MAP kinase-specific antibody αIcp^{42} was a generous gift of Dr C.Marshall, Institute of Cancer Research, London, UK. The anti-Erk 1,2 (K-23)-, the anti-p38/RK/HOG-1 (#sc-535)- and the anti-$p90^{RSK1}$ (# sc-231-G)-specific antibodies were from Santa Cruz Biotechnology. The anti-phosphotyrosine-specific antibody was given by Dr P.Druker, Portland. The anti-MAPKAP kinase 2 antibody was raised in rabbits against recombinant GST–MAPKAP kinase 2 Δ3B (Plath *et al.*, 1994). Anti-rabbit immunoglobulins from goat and anti-mouse immunoglobulins from rabbits, both peroxidase-coupled, were from Dako (Glostrup, Denmark) and detected using the enhanced chemiluminescence method (Amersham). Whole cell lysates from treated or untreated cells for Western blot analyses were prepared by lysing the cells directly in 400 μl of boiling Laemmli buffer per 10 cm Petri dish. Fifty μl of the lysate were resolved by 10% SDS–PAGE. Equal loading was ascertained by staining with Coomassie blue. Electrophoretic transfer and detection with specific antibodies were performed as described previously (Radler-Pohl *et al.*, 1993; Sachsenmaier *et al.*, 1994).

Immune complex kinase assays of MAPKAP kinase

A total of 1×10^6 HeLa tk^- cells were harvested, lysed in 100 μl of lysis buffer [20 mM Tris acetate, pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM pyrophosphate, 1% Triton X-100, 1 mM benzamidine, 0.27 M sucrose, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] on ice for 15 min and centrifuged at 15 000 *g* for 15 min. Then 25 μl of the supernatant was diluted in 500 μl of immunoprecipitation buffer [IP buffer plus BSA; 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1% (w/v) BSA)] and incubated with 5 μl of a rabbit antiserum against MAPKAP kinase 2 at 4°C overnight. Immunocomplexes were precipitated by incubation with 25 μl of 50% slurry of protein A–Sepharose in PBS (Pharmacia, Uppsala,

Sweden) for 1 h. The IP pellet was washed four times with 500 µl of IP buffer minus BSA and redissolved in 25 µl of assay mixture (50 mM β-glycerophosphate, 0.1 mM EDTA, 0.1 mM ATP, 10 mM magnesium acetate, 1.5 µCi of [γ-^{33}P]ATP) containing 2 µg of recombinant Hsp25 (Gaestel *et al*., 1989). The kinase reaction was incubated for 15 min at 30°C. The reaction was terminated by addition of 8 µl of 4× SDS loading buffer. ^{33}P labelling of the substrate was detected and quantified after SDS–PAGE using the Bio Imaging Analyser BAS 2000 (Fuji, Tokyo, Japan).

Peptide assay and in vitro phosphorylation of proteins

Assays using the peptides KKKALNRQLSVAAamide and KRRE-ILSRRPSYRKamide and recombinant MAPKAP kinase 2 Δ3BPC (Engel *et al*., 1995) or the catalytic subunit of PKA (Sigma) were performed as phosphocellulose filter binding assays (Stokoe *et al.*, 1992). Enzymatic parameters were calculated according to Lineweaver–Burk analysis.

Two µg of recombinant Hsp25 (Gaestel *et al*., 1989) or His-CREB were incubated with either 0.5 µg of MAPKAP kinase 2 or 0.1 µg of PKA catalytic subunit (Sigma) in the phosphorylation assay mixture for 15 min at 30°C. The reaction was stopped by addition of 8 µl of 4× SDS loading buffer. Phosphorylation products were analysed by SDS–PAGE and subsequent Phospho-Imaging.

Preparation of nuclear extracts, 'in gel' kinase assays and 'on membrane' kinase assays

Nuclear extracts were prepared according to Dignam *et al.* (1983) with modifications. HeLa tk$^-$ cells were grown to confluency in 10 cm Petri dishes and then serum starved (0.5% FCS) for 24 h. After treatment, the medium was removed, the cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 0.7 mM $CaCl_2$, 6.5 mM NaH_2PO_4, 1.5 mM KH_2PO_4, pH 7.6) and were harvested in 5 ml of ice-cold PBS. All subsequent steps were carried out at 4°C. The cells were centrifuged at 250 *g*. The volume of the cell pellet was determined (packed cell volume = PCV). The cells were resuspended in 3× PCV buffer A [10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 10 µg/ml of each aprotinin, leupeptin and pepstatin] and incubated on ice for 15 min. The cells were lysed by pushing them five times through a 26 gauge needle and the nuclei were pelleted by centrifugation at 5600 *g* for 45 s. The nuclei were resuspended in 1.5× PCV buffer C (20 mM HEPES pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, 200 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 µg/ml of each aprotinin, leupeptin and pepstatin) and rotated for 30 min. The nuclear membranes were pelleted by centrifugation at 5600 *g* for 5 min. The supernatant (nuclear extract) was diluted 1:1 in 2× Laemmli buffer. Five to 10 µl of nuclear extract were used for 'in gel' kinase assays which were performed as described previously (van Dam *et al.*, 1995) except that the proteins were resolved by 7.5% SDS–PAGE with or without co-polymerized substrate (400 µg/ml separating gel solution). For 'on membrane' kinase assays, CREBtide was co-polymerized in a 5 mm thick 1% agarose gel (prepared in 1× electrotransfer buffer for SDS–PAGE gels) at a concentration of 400 µg/ml, and then electrotransferred onto Immobilon™ membrane. Whole cell lysates (prepared as for Western analysis) from untreated and treated HeLa tk$^-$ cells were separated in a 7.5% SDS–PAGE and then electrotransferred onto the membrane with immobilized CREBtide. The membrane was then subjected to the same experimental procedure as for the 'in gel' kinase assay.

Immunofluorescence

NIH3T3 cells were plated out at low density onto round cover slips (diameter 10 mm) in 6 cm Petri dishes, grown for 24 h and then serum starved for an additional 24 h. At 15 min post-treatment with forskolin or UVC, the cells were washed with PBS and were fixed to the cover slips with 4% *p*-formaldehyde at room temperature for 8 min. After washing three times with PBS, the cells were permeabilized with 0.2% Triton X-100 for 10 min, and washed again four times with PBS. In order to prevent unspecific binding of the antibody, the cells were incubated with 10% FCS in PBS for 10 min. The cover slips were washed three times with PBS and transferred into 24-well tissue culture plates (one slip per well). The cells were then incubated with 30 µl of 1:1000 dilution of the first antibody in PBS/10% FCS at room temperature for 30 min. After washing three times with PBS, the cells were incubated with a 1:50 dilution of the rhodamine-coupled secondary antibody (anti-rabbit IgG, from goat, Dianova) at room temperature for 30 min. The cover slips were washed three times with PBS and once with H_2O, and then mounted onto microscopic slides using pre-warmed (50°C) glycergel (Dako). The microscopic analysis of the slides was carried out using a Diaplan fluorescent microscope (Zeiss) and photographs were taken using Kodak Ektachrome P800/1600 film.

Acknowledgements

We thank Dr R.Goodman, Portland, for the gene constructs pRSVGAL4 1–147, pRSVGAL4 CBP and pGAL4CAT; Dr R.Maurer, Portland, for the GAL4–CREB gene constructs and for the recombinant His-CREB protein; Dr B.Druker, Portland, for the anti-phosphotyrosine specific antibody; Dr C.Marshall, London, for the anti-MAP kinase antibody; Dr A.Ullrich, München, for the inhibitor of EGFR tyrosine kinase AG1478; Dr J.C.Lee, King of Prussia, PA, for giving us the p38/RK/HOG-1-specific inhibitor SB203580; and Dr R.Benndorf, Berlin, for the Hsp25 peptide. We thank the members of our laboratories, in particular Axel Knebel, for discussions and Dr P.Sassone-Corsi, Strasbourg, for communicating results prior to publication. M.Litfin and G.Schwederski contributed excellent technical assistance. We also thank Ingrid Kammerer and Diane Nichol for help with the text. H.J.R. was supported by the DFG (Ra 247/6-1) and the Dr Mildred Scheel-Stiftung (W86/94 Ra I), M.G. by the DFG (Ga 453/2-4 and SFB273/YE4) and P.H. by Fonds der Chemischen Industrie.

References

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.

Bading,H. and Greenberg,M.E. (1991) Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science*, **253**, 912–914.

Bading,H., Ginty,D.D. and Greenberg,M.E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science*, **260**, 181–186.

Berkowitz,L.A., Riabowol,K.T. and Gilman,M.Z. (1989) Multiple sequence elements of a single functional class are required for cyclic AMP responsiveness of the mouse c-fos promoter. *Mol. Cell. Biol.*, **9**, 4272–4281.

Betsholtz,C., Johnsson,A., Heldin,C.-H. and Westermark,B. (1986) Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. *Proc. Natl Acad. Sci. USA*, **83**, 6440–6444.

Blendy,J.A., Kaestner,K.H., Schmid,W., Gass,P. and Schütz,G. (1996) Targeting of the CREB gene leads to up-regulation of a novel CREB mRNA isoform. *EMBO J.*, **15**, 1098–1106.

Blenis,J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl Acad. Sci. USA*, **90**, 5889–5892.

Böhm,M., Moellmann,G., Cheng,E., Alvarez-Franco,M., Wagner,S., Sassone-Corsi,P. and Halaban,R. (1995) Identification of p90^{RSK} as the probable CREB-ser^{133} kinase in human melanocytes. *Cell Growth Differ.*, **6**, 291–302.

Bonni,A., Ginty,D.D., Dudek,H. and Greenberg,M.E. (1995) Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol. Cell. Neurosci.*, **6**, 168–183.

Büscher,M., Rahmsdorf,H.J., Litfin,M., Karin,M. and Herrlich,P. (1988) Activation of the c-*fos* gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. *Oncogene*, **3**, 301–311.

Cano,E. and Mahadevan,L.C. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem. Sci.*, **20**, 117–122.

Chao,T.-S., Byron,K., Lee,K.-M., Villereal,M. and Rosner,M. (1992) Activation of MAP kinases by calcium-dependent and calcium-independent pathways. *J. Biol. Chem.*, **267**, 19876–19883.

Chrivia,J.C., Kwok,R.P.S., Lamb,N., Hagiwara,M., Montminy,M. and Goodman,R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*, **365**, 855–859.

Coffer,P.J., Burgering,B.M.T., Peppelenbosch,M.P., Bos,J.L. and Kruijer,W. (1995) UV activation of receptor tyrosine kinase activity. *Oncogene*, **11**, 561–569.

Coffey,R.J.,Jr, Leof,E.B., Shipley,G.D. and Moses,H.L. (1987) Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. *J. Cell. Physiol.*, **132**, 143–148.

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.

Dash,P., Karl,K., Colicos,M., Prywes,R. and Kandel,E. (1991) cAMP response element-binding protein is activated by Ca^{2+}/calmodulin- as well as cAMP-dependent protein kinase. *Proc. Natl Acad. Sci. USA*, **88**, 5061–5065.

Dérijard,B., Hibi,M., Wu,I.-H., Barrett,T., Su,B., Deng,T., Karin,M. and Davis,R. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.

Dévary,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.

Dévary,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.

Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.

Engel,K., Schultz,H., Martin,F., Kotlyarov,A., Plath,K., Hahn,M., Heinemann,U. and Gaestel,M. (1995) Constitutive activation of mitogen-activated protein kinase-activated protein kinase 2 by mutation of phosphorylation sites and an A-helix motif. *J. Biol. Chem.*, **270**, 27213–27221.

Fantini,J., Rognoni,J.B., Roccabianca,M., Pommier,G. and Marvaldi,J. (1989) Suramin inhibits cell growth and glycolytic activity and triggers differentiation of human colic adenocarcinoma cell clone HT29-D4. *J. Biol. Chem.*, **264**, 10282–10286.

Fiore,R., Murphhy,T., Sanghera,J., Pelech,S. and Baraban,J. (1993) Activation of p42 mitogen-activated protein kinase by glutamate receptor stimulation in rat primary cortical cultures. *J. Neurochem.*, **61**, 1626–1633.

Fornace,A.J.,Jr, Zmudzka,B., Hollander,M.C. and Wilson,S.H. (1989) Induction of β-polymerase mRNA by DNA-damaging agents in Chinese hamster ovary cells. *Mol. Cell. Biol*., **9**, 851–853.

Foulkes,N.S., Borrelli,E. and Sassone-Corsi,P. (1991) CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell*, **64**, 739–749.

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

Gaestel,M. *et al.* (1989) Molecular cloning, sequencing and expression in *E.coli* of the 25 kDa growth-related protein of Ehrlich ascites tumor and its homology to mammalian stress proteins. *Eur. J. Biochem.*, **179**, 209–213.

Gaestel,M., Schröder,W., Benndorf,R., Lippmann,C., Buchner,K., Hucho,F., Erdmann,V.A. and Bielka,H. (1991) Identification of the phosphorylation sites of the murine small heat shock protein hsp 25. *J. Biol. Chem*., **266**, 14721–14724.

Ginty,D., Bonni,A. and Greenberg,M. (1994) Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-*fos* transcription via phosphorylation of CREB. *Cell*, **77**, 713–725.

Gonzalez,G.A. and Montminy,M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*, **59**, 675–680.

Haas,S. and Kaina,B. (1995) c-Fos is involved in the cellular defence against the genotoxic effect of UV radiation. *Carcinogenesis*, **16**, 985–991.

Hai,T., Liu,F., Coukos,W.J. and Green,M.R. (1989) Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.*, **3**, 2083–2090.

Härtig,E., Loncarevic,I.F., Büscher,M., Herrlich,P. and Rahmsdorf,H.J. (1991) A new cAMP response element in the transcribed region of the human c-*fos* gene. *Nucleic Acids Res.*, **19**, 4153–4159.

Herdegen,T., Gass,P., Brecht,S., Neiss,W.F. and Schmid,W. (1994) The transcription factor CREB is not phosphorylated at serine 133 in axotomized neurons: implications for the expression of AP-1 proteins. *Mol. Brain Res.*, **26**, 259–270.

Herrera,R.E., Shaw,P.E. and Nordheim,A. (1989) Occupation of the c-fos serum response element *in vivo* by a multi-protein complex is unaltered by growth factor induction. *Nature*, **340**, 68–70.

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.

Iordanov,M. (1995) The cAMP responsive element binding protein (CREB) as a participant in the mammalian UVC response and as a target for repression by the activated glucocorticoid receptor. Ph.D. thesis, Universität Karlsruhe.

Karin,M. (1994) Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell Biol.*, **6**, 415–424.

Kedar,P.S., Widen,S.G., Englander,E.W., Fornace,A.J.,Jr and Wilson,S.H. (1991) The ATF/CREB transcription factor-binding site in the polymerase β promoter mediates the positive effect of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine on transcription. *Proc. Natl Acad. Sci. USA*, **88**, 3729–3733.

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.

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.

Kwok,R.P.S., Lundblad,J.R., Chrivia,J.C., Richards,J.P., Bächinger,H.P., Brennan,R.G., Roberts,S.G.E., Green,M.R. and Goodman,R.H. (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature*, **370**, 223–226.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of cJun kinases. *Nature*, **369**, 156–160.

Lee,J.C. *et al*. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, **372**, 739–746.

Lee,K.A. and Masson,N. (1993) Transcription regulation by CREB and its relatives. *Biochim. Biophys. Acta*, **1174**, 221–233.

Levitzki,A. and Gazit,A. (1995) Tyrosine kinase inhibition: an approach to drug development. *Science*, **267**, 1782–1788.

Lu,X. and Lane,D.P. (1993) Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell*, **75**, 765–778.

Mai,S. *et al*. (1989) Mechanisms of the ultraviolet light response in mammalian cells. *J. Cell Sci.*, **94**, 609–615.

Marshall,C. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.

Matthews,R.P., Guthrie,C.R., Wailes,L.M., Zhao,X., Means,A.R. and McKnight,G.S. (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol.*, **14**, 6107–6116.

Miskin,R. and Ben-Ishai,R. (1981) Induction of plasminogen activator by UV light in normal and xeroderma pigmentosum fibroblasts. *Proc. Natl Acad. Sci. USA*, **78**, 6236–6240.

Montminy,M.R. and Bilezikjian,L.M. (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature*, **328**, 175–178.

Montminy,M.R., Sevarino,K.A., Wagner,J.A., Mandel,G. and Goodman,R.H. (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl Acad. Sci. USA*, **83**, 6682–6686.

Nelson,W.G. and Kastan,M.B. (1994) DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Mol. Cell. Biol.*, **14**, 1815–1823.

Plath,K., Engel,K., Schwedersky,G. and Gaestel,M. (1994) Characterization of the proline-rich region of mouse MAPKAP kinase 2: influence on catalytic properties and binding to the c-abl SH3 domain *in vitro*. *Biochem. Biophys. Res. Commun.*, **203**, 1188–1194.

Pressman,B.C. (1976) Biological applications of ionophores. *Annu. Rev. Biochem.*, **45**, 501–530.

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.

Robertson,L., Kerppola,T., Vendrell,M., Luk,D., Smeyne,R., Bocchiaro,C., Morgan,J. and Curran,T. (1995) Regulation of c-*fos* expression in transgenic mice requires multiple interdependent transcription control elements. *Neuron*, **14**, 241–252.

Rosen,L., Ginty,D.D., Weber,M.J. and Greenberg,M.E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron*, **12**, 1207–1221.

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.

Sassone-Corsi,P., Visvader,J., Ferland,L., Mellon,P.L. and Verma,I.M. (1988) Induction of proto-oncogene *fos* transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes Dev.*, **2**, 1529–1538.

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.

Schreiber,M., Baumann,B., Cotten,M., Angel,P. and Wagner,E.F. (1995) Fos is an essential component of the mammalian UV response. *EMBO J.*, **14**, 5338–5349.

Sheng,M., McFadden,G. and Greenberg,M. (1990) Membrane depolarization and calcium induce c-*fos* transcription via phosphorylation of transcription factor CREB. *Neuron*, **4**, 571–582.

Sheng,M., Thompson,M.A. and Greenberg,M.E. (1991) CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*, **252**, 1427–1430.

Stein,B., Rahmsdorf,H.J., Steffen,A., Litfin,M. and Herrlich,P. (1989) 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.

Stokoe,D., Campbell,D.G., Nakielny,S., Hidaka,H., Leevers,S.J., Marshall,C. and Cohen,P. (1992) MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. *EMBO J.*, **11**, 3985–3994.

Sturgill,T.W., Ray,L.B., Erikson,E. and Maller,J.L. (1988) Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, **334**, 715–718.

Sun,P. and Maurer,R.A. (1995) An inactivating point mutation demonstrates that interaction of cAMP response element binding protein (CREB) with the CREB binding protein is not sufficient for transcriptional activation. *J. Biol. Chem.*, **270**, 7041–7044.

Sun,P., Enslen,H., Myung,P.S. and Maurer,R.A. (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.*, **8**, 2527–2539.

Taylor,S.S., Buechler,J.A. and Yonemoto,W. (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.*, **59**, 971–1005.

Treisman,R. (1994) Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.*, **4**, 96–101.

Treisman,R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.*, **8**, 205–215.

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.

van den Berg,S., Kaina,B., Rahmsdorf,H.J., Ponta,H. and Herrlich,P. (1991) Involvement of Fos in spontaneous and ultraviolet light induced genetic changes. *Mol. Carcinogen.*, **4**, 460–466.

Wasylyk,B., Wasylyk,C., Flores,P., Begue,A., Leprince,D. and Stehelin,D. (1990) The c-*ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. *Nature*, **346**, 191–193.

Yamaizumi,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.

Received on August 9, 1996; revised on November 1, 1996

Note added

After submission of this manuscript, two reports characterizing CREB-phosphorylating protein kinases appeared. Xing *et al.* (*Science*, **273**, 959) identified $p90^{RSK}$ family member RSK2 as a functional CREB kinase in phorbol ester-treated K562 cells and in NGF-treated PC12 cells. Tan *et al.* (*EMBO J.*, **15**, 4629–4642) demonstrated that in FGF- or arsenite-treated human neuroblastoma SK-N-MC-cells, MAPKAP kinase 2 is the only detectable SB203580-sensitive CREB kinase.