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The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-*fos* transcription in response to UV light

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We investigated the activation of c-*fos* transcription following UV irradiation, a 'stress' stimulus. In both HeLa TK⁻ and NIH 3T3 cells the Serum Response Element is required for efficient UV-induced c-*fos* transcription, and in HeLa TK⁻ cells the Ternary Complex Factor (TCF) binding site contributes substantially to activation. Consistent with this, UV irradiation activates LexA–TCF fusion proteins more strongly in HeLa TK⁻ than in NIH 3T3 cells. The TCF C-termini of the TCFs are substrates for UV-induced MAP kinases: both the Elk-1 and SAP-1a C-termini are efficiently phosphorylated by the p38 MAPK, but only the Elk-1 C-terminus is a good substrate for the SAPK/JNKs. The specificity and activation kinetics of TCF C-terminal kinases, and the susceptibility of transcriptional activation by LexA–TCF fusion proteins to specific inhibitors of different MAPK pathways, show that both the ERK and p38 MAPK pathways contribute to TCF activation in response to UV irradiation. Activity of both these pathways is also required for the response of the c-*fos* gene itself to UV stimulation.

Keywords: c-*fos*/ERK/MAP kinase/p38/SAPK/JNK/ Ternary Complex Factor/UV light

Introduction

The Mitogen-Activated Protein Kinases (MAPKs) comprise a family of kinases that phosphorylate a common core consensus motif, S/T-P, and which are regulated via evolutionarily conserved kinase cascades. In mammalian cells three MAPK cascades are characterized at present, which regulate the activity of the ERK MAPKs, the SAPK/JNK MAPKs and p38/RK/MPK2/CSBP (hereafter referred to as p38 MAPK) in response to different environmental or developmental signals (Waskiewicz and Cooper, 1995). Studies over the last several years have indicated that these pathways are an important mechanism by which extracellular signals can lead to alterations in gene expression, since many MAPK substrates are transcription factors (for review see Treisman, 1996).

The c-*fos* gene is activated by diverse stimuli known to activate one or more MAPK cascades. Many of these stimuli act through the Serum Response Element (SRE), which also controls many genes co-regulated with c-*fos* (for review see Treisman, 1990). The c-*fos* SRE binds a transcription factor ternary complex comprising Serum Response Factor (SRF; Norman *et al.*, 1988) and a member of the Ternary Complex Factor (TCF) family of ETS-domain proteins (Herrera *et al.*, 1989; Shaw *et al.*, 1989; Graham and Gilman, 1991). Each of the three known TCFs, Elk-1, SAP-1 and NET/ERP/SAP-2, contains a conserved C-terminal transcriptional activation domain with multiple S/T-P motifs (Dalton and Treisman, 1992). This domain is a good substrate for the ERKs *in vitro*; moreover, activation of the ERK MAPK pathway by growth factor stimulation *in vivo* causes its phosphorylation at the S/T-P motifs and thereby potentiates transcriptional activation (Gille *et al.*, 1992, 1995a; Janknecht *et al.*, 1993, 1995; Marais *et al.*, 1993; Kortenjann *et al.*, 1994; Price *et al.*, 1995; for review see Treisman, 1994). The TCFs act to connect the c-*fos* promoter to the ERK MAPK pathway: SRE mutations that prevent ternary complex formation block c-*fos* activation via the pathway, and they can be complemented by expression of appropriate altered-specificity mutants of the Elk-1 TCF (Hill *et al.*, 1994; Kortenjann *et al.*, 1994).

The SAPK/JNKs and p38 MAPK are strongly activated in response to chemical and environmental stress stimuli (Derijard *et al.*, 1994; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Kyriakis *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). The SAPK/JNKs potentiate transcriptional activation by c-Jun and ATF-2 transcription factors (Adler *et al.*, 1992; Hibi *et al.*, 1993; Derijard *et al.*, 1994; Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). Given the similarities between the core recognition sequences for these MAPKs and the ERKs, and the involvement of the SRE in c-*fos* induction in response to stress stimuli such as UV irradiation (Buscher *et al.*, 1988) or protein synthesis inhibitors (Subramaniam *et al.*, 1989), it is likely that stress-induced MAPKs might also regulate TCF activity. Indeed, the Elk-1 C-terminus can be phosphorylated *in vitro* by the SAPK/JNKs, and its ability to activate transcription *in vivo* can be potentiated by MEKK, a kinase that can activate both ERK and SAPK/JNK pathways (Cavigelli *et al.*, 1995; Gille *et al.*, 1995b; Price *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). However, although many stress stimuli that activate the SAPK/JNK pathway also activate the p38 MAPK pathway, and may also activate the ERK pathway to some extent (see Radler-Pohl *et al.*, 1993), the relative contributions of the different pathways to c-*fos* transcriptional activation have not been evaluated.

In this paper we have investigated the role of different MAPK pathways in TCF and c-*fos* transcription in response to a stress stimulus, UV irradiation. We show that all the TCFs are substrates for the p38 MAPK *in vitro*. Moreover, in UV-irradiated HeLa TK⁻ cells activity of the

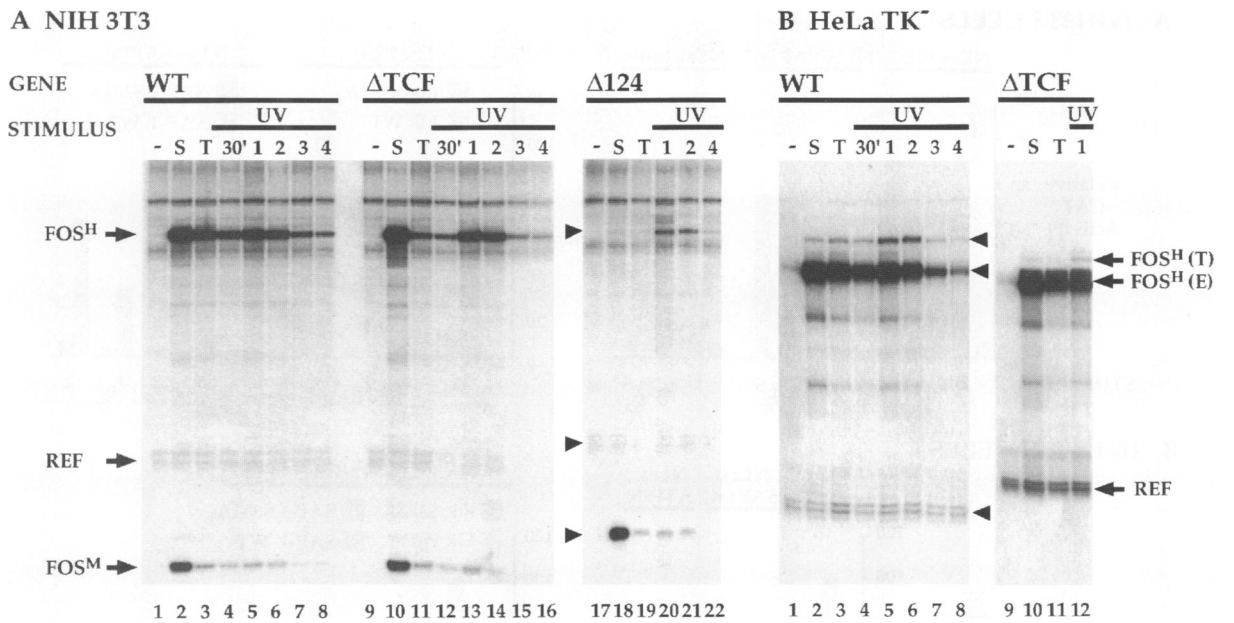

Fig. 1. Sequence requirements for c-*fos* transcriptional activation by UV light. Transcripts were analysed by RNase protection; the positions of protected fragments generated by the different transcripts are indicated. (**A**) NIH 3T3 cells. Cells were transfected with the wild-type human c-*fos* gene containing 711 bp 5′ flanking sequence (WT; lanes 1–8); ΔTCF, its derivative containing a mutated TCF binding site (lanes 9–16) or a c-*fos* gene containing only 124 bp 5′ flanking sequence (Treisman, 1985), together with an α-globin reference plasmid. Following maintainance in medium containing 0.5% serum for 40 h, and RNA prepared either directly (lanes 1, 9 and 17), 30 min following stimulation by 15% serum (lanes 2, 10 and 18) or 50 ng/ml TPA (lanes 3, 11 and 19), or at the indicated times following UV irradiation (lanes 4–8, 12–16 and 20–22). In NIH 3T3 cells, transcripts of the transfected human gene (c-fos^H) are easily distinguished from those of the endogenous mouse gene (c-fos^M). In this experiment, the serum-, TPA- and UV-induced RNA levels from the ΔTCF mutant were 118%, 26% and 75% of the levels from the wild-type gene. Similar results were obtained in a second experiment. (**B**) HeLa TK⁻ cells. Cells were transfected with a wild-type human c-*fos* gene containing 711 bp 5′ flanking sequence (WT; lanes 1–8), or a derivative in which the TCF binding site is mutated to a LexA half-operator (ΔTCF; lanes 9–16), together with an α-globin reference plasmid. These experiments used derivatives of the human c-*fos* gene pFM711 (Wilson and Treisman, 1988), which carries a small deletion–insertion in the 5′ untranslated region to enable transcripts of the transfected gene [c-$fos^H(T)$] to be distinguished from those of the endogenous human gene [c-$fos^H(E)$]. Cells were maintained in medium containing 0.5% serum (lanes 1 and 9) or stimulated as indicated with 15% serum (lanes 2, 10), 50 ng/ml TPA (lanes 3 and 11) or by UV irradiation for the indicated times (lanes 4–8 and 12) before RNA preparation. In this experiment, the serum-, TPA- and UV-induced RNA levels from the ΔTCF mutant were 15%, 7% and 15% of the levels from the wild-type gene.

p38 MAPK and ERK MAPK pathways is necessary both for maximal transcriptional activation by the Elk-1 and SAP-1a TCFs, and for transcription of the c-*fos* gene itself.

Results

c-fos sequences required for transcriptional activation by UV light

We used transient transfection of intact or mutated human c-*fos* genes to evaluate the contribution of the SRE to c-*fos* induction in mouse NIH 3T3 cells and HeLa TK⁻ cells, analysing c-*fos* mRNA levels directly by RNase protection mapping. In both cell types, transcription of both the endogenous and transfected c-*fos* genes was induced by UV irradiation; maximal c-*fos* mRNA levels, which were comparable with those following TPA stimulation, were reached 60 min following stimulation (Figure 1A and B, lanes 1–8). To evaluate the contribution of TCF to UV-induced transcriptional activation we examined a promoter mutant, ΔTCF, that blocks TCF binding *in vitro* (Hill *et al.*, 1994). In NIH 3T3 cells, this mutation had only a small effect on the transcription following UV irradiation, significantly impaired the response to TPA, but left serum-induced transcription largely unaffected (Figure 1A, lanes 9–16; see Hill and Treisman, 1995). Deletion of sequences including the SRE effectively blocked serum- and TPA-induced transcription and substantially inhibited UV inducibility (Figure 1A, lanes 17–22 and data not shown; see Discussion). In contrast, in HeLa TK⁻ cells the ΔTCF mutation substantially inhibited c-*fos* transcription following stimulation by serum, TPA or UV irradiation (Figure 1B, lanes 9–12). These results show c-*fos* upstream sequences including the SRE are required for the response to UV in both NIH 3T3 and HeLa TK⁻ cells, and that at least in HeLa TK⁻ cells, TCF binding contributes substantially to UV-induced transcription.

UV light activates the C-termini of both SAP-1 and Elk-1 TCFs

We next examined the ability of UV irradiation to potentiate transcriptional activation by LexA–TCF fusion proteins, using a LexA operator-controlled reporter gene (Marais *et al.*, 1993; Price *et al.*, 1995). We reasoned that the greater dependence of transcriptional activation upon the SRE TCF site in HeLa TK⁻ cells might reflect more efficient activation of the TCF C-termini in these cells, and therefore again performed experiments in both cell lines. As controls, we examined activation by fusion proteins containing the N-termini of c-Jun or ATF-2, known substrates for the UV-induced SAPK/JNK MAP kinases (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995).

In NIH 3T3 cells, transcriptional activation by both

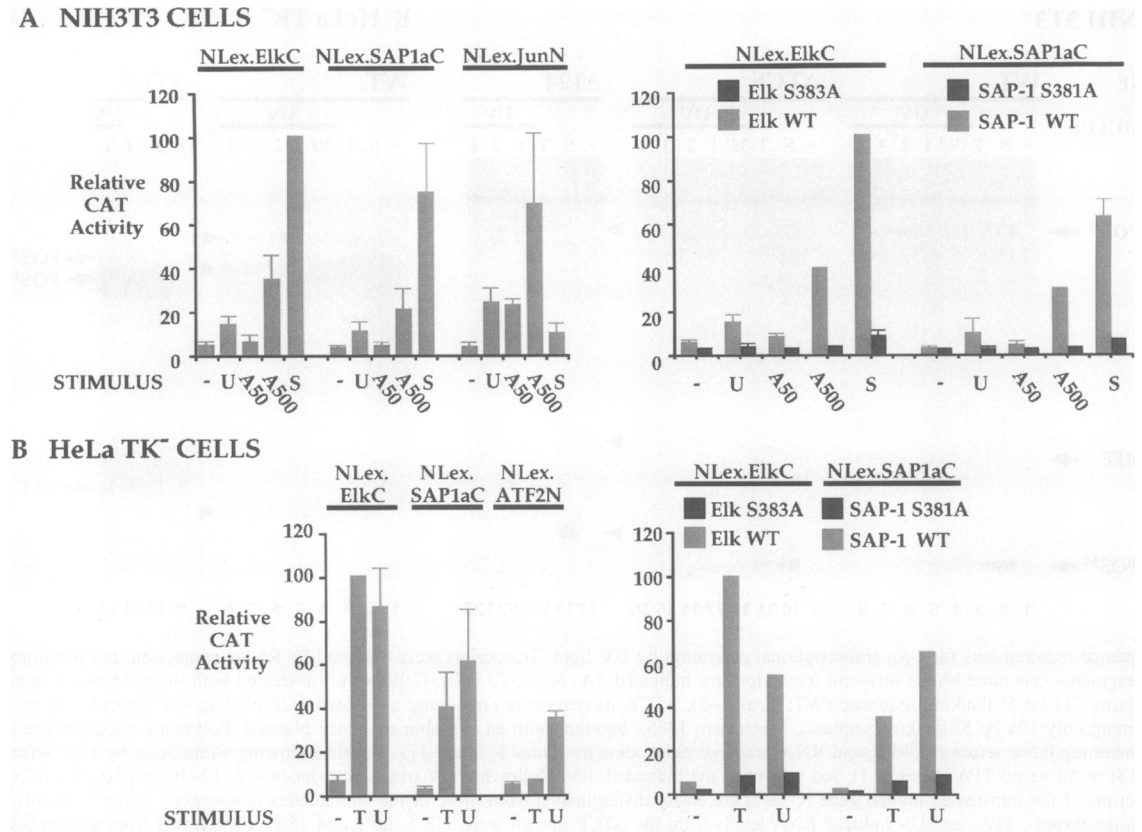

Fig. 2. Transcriptional activation by LexA fusion proteins. Cells were transfected with plasmids encoding various LexA fusion proteins, together with a *TK.CAT* gene controlled by two copies of the LexA operator. Left-hand panels: following maintainance in medium containing 0.5% serum for 40 h, transfected cells were harvested for CAT analysis either directly (bars –) or after an additional 8 h following stimulation by 50 J/m^2 UV light (bars U), 50 ng/ml anisomycin (bars A50), 500 ng/ml anisomycin (bars A500), 15% serum (bars S), or 50 ng/ml TPA (bars T). Right-hand panels: activities of the wild-type LexA–TCF fusion proteins (grey bars) were compared with those fusions containing Ser-Ala mutations at Elk-1 residue 383 or SAP-1a residue 381 (black bars). (**A**) NIH 3T3 cells. Activities of NLex.ElkC, NLex.SAP1C and N.Lex.JunN were compared. Activities are taken relative to TPA-induced NLex.ElkC. Error bars show SEM from 6–7 (left panels) or 2 (right panel) independent experiments. (**B**) HeLa TK$^-$ cells. Activities of NLex.ElkC, NLex.SAP1C and NLex.ATF2N were compared. Activities are taken relative to TPA-induced NLex.ElkC; error bars show SEM from 6–7 (left panels) independent experiments. In the right-hand panel, 3 μg of each LexA–TCF plasmid were transfected; similar results to these were obtained in a second experiment in which 1 μg each plasmid was used.

Elk-1 and SAP-1a fusion proteins following UV irradiation was much less efficient than following serum stimulation; induction by anisomycin, another stress stimulus, was inefficient at concentrations insufficient to inhibit protein synthesis, but occurred efficiently at a higher, inhibitory concentration (Figure 2A, left panel). In these cells activity of a LexA–Jun fusion protein was potentiated both by UV irradiation and either dose of anisomycin, although the extent of activation at high concentrations was somewhat variable (Figure 2A, left panel). For each stimulus, maximal activity of the TCF fusion proteins was dependent on the integrity of multiple TCF C-terminal phosphorylation sites, including Elk-1 Ser383/SAP-1a Ser381 (Figure 2A, right panel and data not shown).

In HeLa TK$^-$ cells, activity of the NLex.ElkC fusion protein was efficiently stimulated by both UV light and TPA (14- to 16-fold induction; Figure 2B). The activity of the NLex.SAP-1a fusion protein was comparable with that of NLex.ElkC, although in this case induction by UV was more efficient than by TPA, albeit somewhat more variable (23-fold induction for UV, 16-fold for TPA). UV-induced transcriptional activation by both SAP-1 and Elk-1 C-termini was dependent on all the conserved C-terminal S/T-P motifs tested, including Elk-1 Ser383 (SAP-1a Ser381; Figure 2B, right panel), Elk-1 Thr363 and/or Thr368, Elk-1 Thr417 and/or Ser422, and SAP-1a Ser387 (data not shown). We found that in HeLa TK$^-$ cells the LexA–Jun fusion protein exhibited very high constitutive transcriptional activity, and therefore used as a control a LexA–ATF2 fusion gene (Livingstone *et al.*, 1995). The LexA–ATF2 fusion protein was efficiently activated by UV irradiation, but not by TPA (Figure 2B, left panel). Thus, transcriptional activation by the TCFs correlates with the greater contribution of TCF binding sites to UV-induced, c-*fos* transcriptional activation in HeLa TK$^-$ cells.

TCF C-termini are substrates for multiple MAPKs

The ability of both SAP-1a and Elk-1 fusion proteins to respond to UV stimulation suggests that both TCF C-termini are substrates for UV-activated kinases. We tested the ability of the p38, SAPK/JNK and ERK2 MAPKs to phosphorylate different GST fusion proteins containing the C-termini of either Elk-1, SAP-1a or SAP-2, the third TCF closely related to SAP-1a (Price *et al.*, 1995). Both p38 MAPK immune complexes and MEK-activated recombinant ERK2 phosphorylated each of the TCF C-termini but not the Jun N-terminus (Figure 3A,

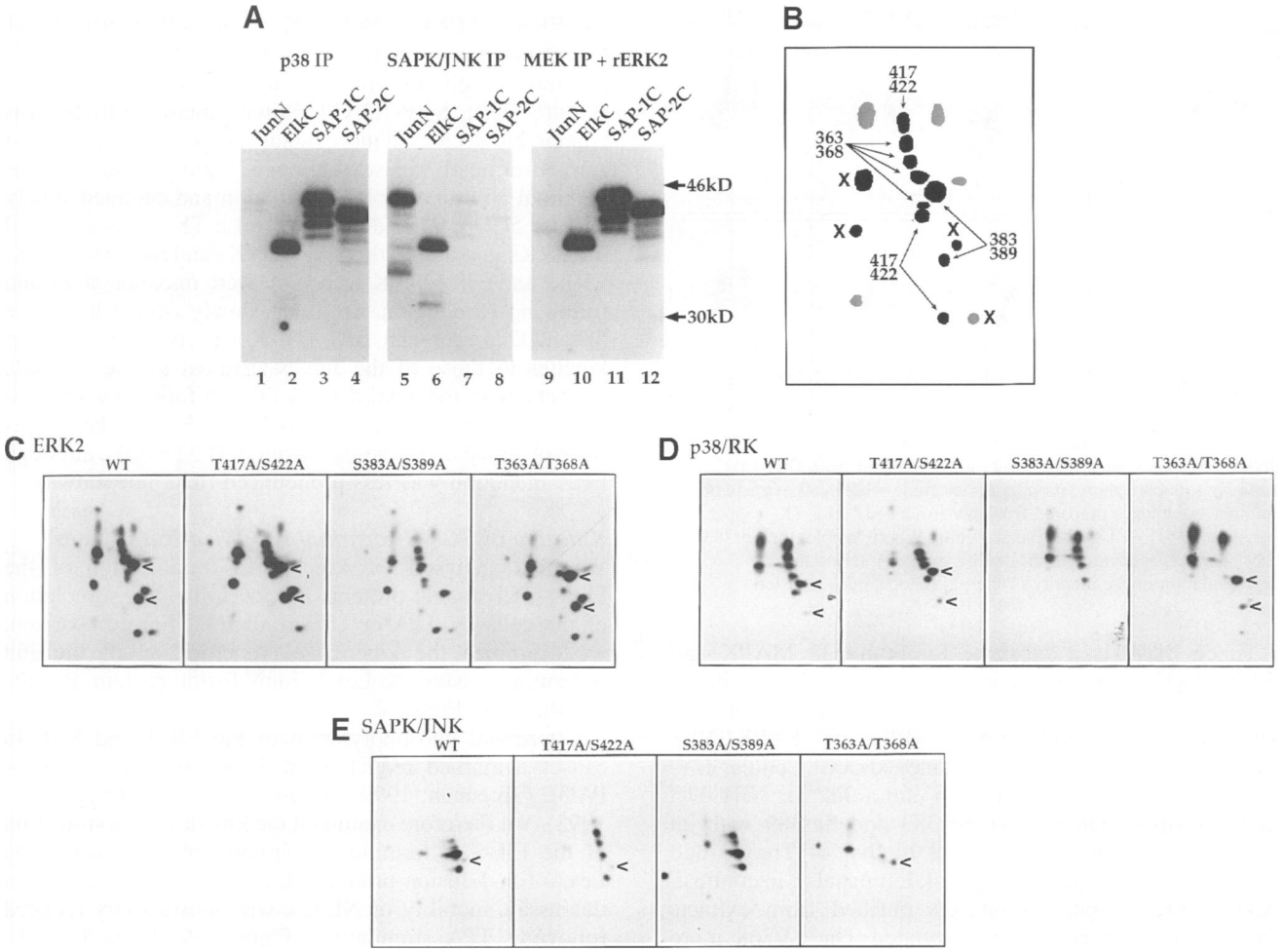

Fig. 3. (**A**) TCFs are substrates for multiple MAPKs. GST fusion proteins containing the N-terminal region of c-Jun (lanes 1, 5 and 9) or the C-terminal regions of Elk-1 (residues 307–428; lanes 2, 6 and 10), SAP-1a (residues 268–431; lanes 3, 7 and 11) or SAP-2 (residues 222–407; lanes 4, 8 and, 12) were phosphorylated in immune complex kinase assays using SAPK/JNK or p38 MAPK immunoprecipitated from extracts of NIH 3T3 cells prepared 60 min following UV irradiation. For comparison, the same substrates were phosphorylated using recombinant ERK2, activated by a MEK immune complex prepared from NIH 3T3 cells 15 min following serum stimulation. (**B**) Identities of Elk-1 C-terminal phosphopeptides. The identities of Elk-1 chymotryptic/tryptic phosphopeptides deduced from analysis of Ser→Ala point mutant derivatives of the Elk-1 C-terminus are indicated. Phosphopeptides unaffected my mutation of S/T-P motifs at 363 and/or 368, 383 and/or 389, or 417 and/or 422 are indicated by 'X'. (**C**) Phosphopeptide fingerprints of GST-ElkC derivatives phosphorylated by ERK2. Either wild-type or the indicated Ser/Thr→Ala point mutant derivatives of GST-ElkC were phosphorylated using 9E10-tagged ERK2 precipitated from serum-stimulated cells, then subjected to fingerprinting using a mixture of chymotrypsin and trypsin. For reference the phosphopeptides characteristic of phosphorylation at Ser383 and/or Ser389 are identified by carets, where visible. (**D**) Phosphopeptide fingerprints of GST-ElkC derivatives phosphorylated by p38 MAPK. (**E**) Phosphopeptide fingerprints of GST-ElkC derivatives phosphorylated by SAPK/JNK.

lanes 1–4 and 9–12); similar results were obtained with immunoprecipitated ERK2 (data not shown). In contrast, SAPK/JNK immune complexes phosphorylated the Jun N-terminus and the Elk-1 C-terminus, but not SAP-1a or SAP-2 (Figure 3A, lanes 5–8). Similar substrate preferences were observed with MAPK immune complexes from both HeLa TK$^-$ and NIH 3T3 cells, and when immune complexes were purified from UV-stimulated cells expressing epitope-tagged p54SAPKβ or p38 kinases (data not shown).

The different substrate preferences of the p38 MAPK and the SAPK/JNKs suggested that these kinases might differentially phosphorylate the different Elk-1 S/T-P motifs. To investigate this, we performed peptide fingerprinting studies of GST-ElkC, using point mutants to identify phosphopeptides associated with phosphorylation at particular sites. Results are shown in Figure 3B–E. ERK2-phosphorylated Elk-1 gave rise to a complex pattern of phosphopeptides, including products characteristic of labelling at Ser383 and/or Ser389, Thr363 and/or Thr368, and Thr417 and/or Ser422, with relative efficiencies 383/9 > 363/8 > 417/422 (Figure 3C). Other products, which presumably reflect phosphorylation at other S/T-P motifs within the Elk-1 C-terminal region, were also generated (Figure 3B, spots 'X'). The p38 MAPK-phosphorylated Elk-1 generated products characteristic of phosphorylation at Ser383 and/or Ser389, Thr363 and/or Thr368 and Thr417 and/or Ser422 (Figure 3D). In this case residues 417/422 were more efficiently labelled than by ERK2, while some other unidentified ERK2 products were not generated (compare Figure 3C and D). In contrast, SAPK/JNK-phosphorylated Elk-1 efficiently generated only a limited number of phosphopeptide products. Phosphorylation at Thr363 and/or Thr368 was much more efficient than at Ser383/389, while the other products were only weakly discernible (Figure 3E, see also Figure 4).

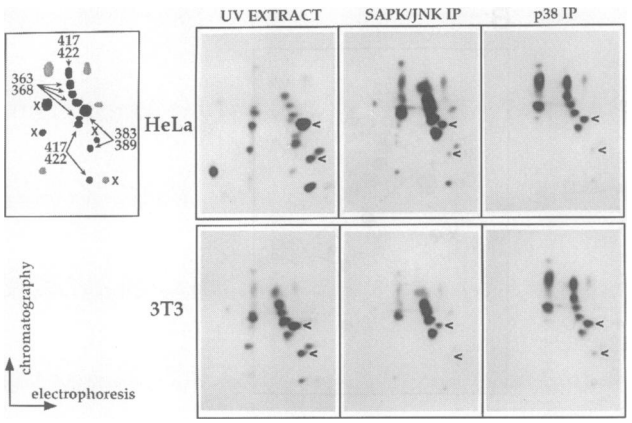

Fig. 4. UV-induced cellular Elk-1 kinases are distinct from SAPK/JNKs. Phosphopeptide fingerprints were prepared from GST-ElkC following its phosphorylation by either crude whole cell extracts or immune complexes prepared from UV-irradiated HeLa TK$^-$ (upper panels) or NIH 3T3 (lower panels) cells. Where visible, the Ser383/389 phosphopeptides are indicated by carets. A schematic representation of the identity of the phosphopeptides is at left.

Since Elk-1 is a substrate for both p38 MAPK and SAPK/JNKs we were interested to compare the specificity of the Elk-1 kinases present in crude extracts of UV-stimulated cells with p38 MAPK and SAPK/JNKs immunoprecipitated from the same extracts. Cellular UV-induced kinases present in both HeLa TK$^-$ or NIH 3T3 cells phosphorylated Elk-1 Ser383 and Ser389 with an efficiency greater than or equal to that of Thr363 and Thr368 phosphorylation (Figure 4, left panels). In contrast, SAPK/JNK immune complexes purified from extracts from either cell type phosphorylated Thr363/368 more efficiently than Ser383/389 (Figure 4, centre panels). The pattern of products generated following phosphorylation of GST-ElkC by p38 immune complexes was more similar to that obtained following phosphorylation by crude UV-induced extracts, although phosphorylation at Ser383 and Ser389 was somewhat less efficient relative to other sites (Figure 4, right panels). Taken together, these data show that the SAPK/JNKs are unlikely to be the sole Elk-1 C-terminal kinase in these extracts, and are suggestive of a significant contribution by ERKs to UV-induced Elk-1 C-terminal phosphorylation (compare Figure 4 left panels with Figure 3C).

Taken together, these results show that p38 MAPK can efficiently phosphorylate the C-terminus of each TCF, and can efficiently phosphorylate many but not all the Elk-1 C-terminal residues whose phosphorylation is required for transcriptional activation. In contrast, the SAPK/JNKs show a marked specificity for Elk-1 TCF, and preferentially phosphorylate a subset of those residues whose phosphorylation is required for transcriptional activation, particularly Thr363 and/or Thr368. The restricted specificity of Elk-1 phosphorylation by SAPK/JNK, and the ability of p38 MAPK to phosphorylate each TCF C-terminus, strongly suggests that the p38 MAPK contributes to the UV-induced phosphorylation of the TCF C-termini.

Kinetics of UV-inducible TCF C-terminal kinase activation

Next we compared the kinetics of activation of cellular TCF C-terminal kinases with those of the p38, SAPK/JNK and ERK MAPKs. In HeLa TK$^-$ whole cell extracts, Elk-1 C-terminal kinase activity was greatest 15 min after UV treatment, declining gradually to basal levels within 2 h (Figure 5A); SAP-1a C-terminal kinase activity was similar, but decreased more rapidly (Figure 5B). In contrast Jun N-terminal kinase, although rapidly induced, was maximal 30 min after UV irradiation and declined slowly (Figure 5C). UV irradiation of HeLa TK$^-$ cells activated the ERKs in addition the SAPK/JNKs and the p38 MAPK. ERK2 and p38 MAPK activation were maximal at 15 min following stimulation, declining slowly over 2 h (Figure 5D and E); in contrast, SAPK/JNK activity showed similar kinetics to those of the Jun N-terminal kinase in crude extracts, with maximal activity 30 min following stimulation (Figure 5; compare panels C and F). Similar results were obtained using extracts from NIH 3T3 cells, although ERK induction was less pronounced (data not shown).

Kinetics of TCF C-terminal modification in vivo

We next analysed the kinetics of modification of the LexA–TCF fusion proteins *in vivo* following stimulation of the cells by TPA or UV irradiation. For comparison, we examined the kinetics of modification of the Jun N-terminus, using the LexA–JunN fusion protein. Results are shown in Figure 6.

C-terminal phosphorylation of the Elk-1 and SAP-1a causes a marked reduction in their mobilities in SDS–PAGE (Treisman, 1990; Marais *et al.*, 1993; Price *et al.*, 1995). We therefore examined the kinetics of modification of the Elk-1 C terminus by immunoblotting, using the LexA–Elk-1 fusion protein NL.Elk (Hill *et al.*, 1993). In this assay, mobility of NL.Elk was substantially reduced following TPA stimulation (Figure 6A, lanes T). UV-induced phosphorylation of NL.Elk was less efficient than that induced by TPA, and only a fraction of the protein was retarded to the same extent; it appeared maximal 15 min following stimulation (Figure 6A, UV lanes). Next, we used an antibody which specifically recognizes only the phosphorylated form of Ser383 (F.H.Cruzalegui and R.Treisman, unpublished data). Ser383 phosphorylation was maximal at 15 min following UV irradiation, and subsequently declined (Figure 6B). We also assessed modification of NL.Elk using a ternary complex gel mobility shift assay. Phosphorylation of NL.Elk, even at substoichiometric levels, reduces the mobility, in non-denaturing gels, of the ternary complexes that it forms with SRF (Hill *et al.*, 1993). In this assay, some modification of the protein was detectable even in unstimulated cells; however, unmodified protein was no longer detectable 15 min after UV irradiation, but began to reappear during the subsequent 2 h (Figure 6E, compare lanes 1 and 3).

We used an analogous fusion protein, NL.SAP1a, to analyse the kinetics of modification of the SAP-1a C-terminus. In unstimulated cells three predominant forms of NL.SAP1a were produced, migrating with different mobilities on SDS–PAGE; which represent different phosphorylated forms of the protein (M.A.Price, unpublished observations; Price *et al.*, 1995). Following TPA stimulation, the majority of the protein migrated as low mobility forms (Figure 6C, lane T). UV-induced phosphorylation of the SAP-1a C-terminal sequences was clearly much less extensive than that induced by TPA, as judged by the proportion of protein migrating most slowly (Figure 6C,

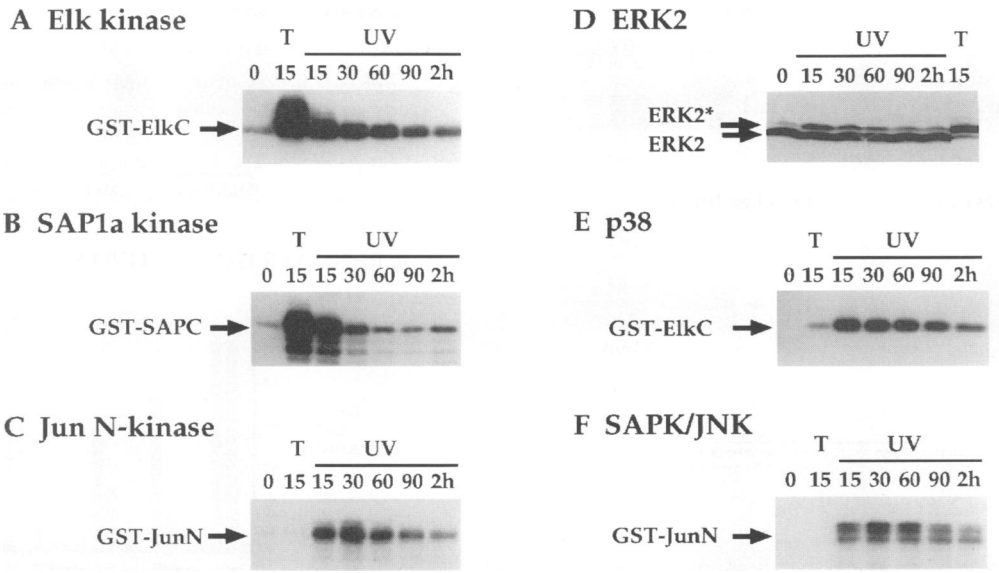

Fig. 5. UV-induced kinases in HeLa TK⁻ cells. Whole cell extracts or SDS-lysates were prepared at the indicated times (minutes unless specified) following UV irradiation or TPA stimulation. The extracts were used directly to phosphorylate GST-ElkC (**A**), GST-SAP1C (**B**) or GST-JunN (**C**). The SDS lysates were analysed for ERK activation by immunoblot (**D**); the upper band is phosphorylated, activated ERK2; the lower band is unphosphorylated, inactive ERK2; a less intense band above the activated ERK2 is ERK1. The extracts were analysed for p38 activity by immune complex kinase assay with GST-ElkC as substrate (**E**) and SAPK/JNK activity by immune complex kinase assay with GST-JunN as substrate (**F**).

lanes UV); some modification was discernible as judged by the shift in the relative intensities of the various bands, but the assay did not permit the kinetics of SAP-1a modification to be assessed. We therefore again turned to the gel mobility-shift ternary complex assay. Phosphorylation of NL.SAP1a increases its ability to form ternary complexes with SRF (M.A.Price, unpublished observations; Figure 6E, compare lanes 8 and 9). UV irradiation increased NL.SAP1a ternary complex activity with maximal activity at 15 min following stimulation, followed by a decline to basal level (Figure 6E, lanes 10–14). Thus, at least those kinases giving rise to the increased NL.SAP-1a ternary complex activity must be maximally active at early times following UV stimulation.

These results demonstrate that the kinetics of TCF phosphorylation closely follow those of the TCF-C terminal kinases detected in crude extracts as described in the preceding section. Similarly, when an immunoblotting assay was used to examine the kinetics of c-Jun N-terminal modification, which also retards mobility of the protein in SDS–PAGE (Radler-Pohl *et al.*, 1993), we found that c-Jun N-terminal modifications closely match those of the Jun N-terminal kinases detectable in crude extracts of UV-irradiated cells (Figure 6D). Taken together, these data are most consistent with a role for ERK and/or p38, rather than the SAPK/JNKs in TCF activation following UV irradiation.

Activation of TCFs by MEKK

The data presented above suggest other kinases in addition to the SAPK/JNKs must contribute to activation of the TCF C-termini in response to UV irradiation. We next wished to evaluate the ability of the SAPK/JNKs alone to activate the different TCFs. MEKK overexpression potentiates both SAPK/JNK and ERK, but not the p38, MAPK pathways; moreover the SAPK/JNK pathway is more sensitive to MEKK expression than the ERK pathway (Lange-Carter *et al.*, 1993; Minden *et al.*, 1994; Yan *et al.*, 1994; Lin *et al.*, 1995). We therefore titrated MEKK expression plasmid into the reporter gene assays with different LexA–TCF fusion proteins, using LexA.ATF2N as a control. In parallel, we assessed the ability of MEKK to activate epitope-tagged MAPKs. In HeLa TK⁻ cells, activation of 9E10-ERK2 by MEKK was detectable only at the highest plasmid input used, and was weak (<2-fold). In contrast, activation of HA-p54SAPKβ was much more efficient (up to 20-fold) and also was detectable at lower MEKK plasmid inputs, at which ERK activation did not occur. Activation of FLAG-p38 by MEKK was not detectable above its very low basal level (Figure 7A).

Transcriptional activation by LexA.ATF2N, a target for the SAPK/JNK pathway (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995) was clearly detectable even at the lowest inputs of MEKK plasmid, and activation level at the highest MEKK input surpassed that by UV light (Figure 7B). In contrast, NLex.ElkC was activated less efficiently: activation was detected only at higher MEKK plasmid inputs and was comparable with the UV-induced level only at the highest MEKK input (Figure 7B). Activation of NLex.SAP1C was very weak, occurring only at the highest MEKK plasmid input. These results suggest that intracellular activation of the SAPK/JNKs can indeed be sufficient for activation of the Elk-1, but not the SAP-1a C-terminus, consistent with the *in vitro* phosphorylation studies. However, the activation of NLex.ElkC by MEKK appears relatively inefficient when compared with NLex.ATF2N (see Discussion).

Maximal UV-induced TCF activation requires MEK and p38

Next we evaluated the contribution of different MAPK pathways to transcriptional activation by the TCF C-termini *in vivo*. To do this, we exploited two recently described pathway-specific inhibitors: PD98059, which

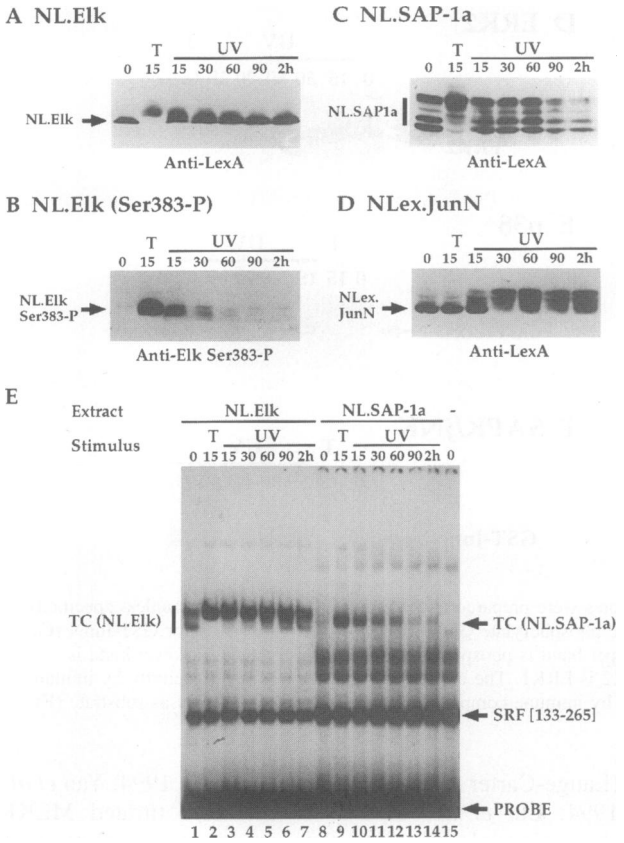

Fig. 6. Kinetics of UV-induced TCF C-terminal modification *in vivo*. Whole cell extracts were prepared from cells transfected with 8 μg expression plasmids expressing NL.Elk, NLex.SAP1A and NLex.JunN at the indicated times (min unless otherwise stated) following stimulation. (**A**) Immunoblotting analysis of NL.Elk with anti-LexA. (**B**) Immunoblotting analysis of NL.Elk with anti-Elk-1 Phosphoserine 383. (**C**) Immunoblotting analysis of NL.SAP1a with anti-LexA. (**D**) Immunoblotting analysis of NLex.JunN with anti-LexA. (**E**) Gel mobility-shift analysis of ternary complex formation by NL.Elk (lanes 1–7) and NL.SAP1a (lanes 8–14). Ternary complexes were formed between the SRF DNA binding domain fragment SRF[133–265] and the NL.Elk or NL.SAP1 fusion proteins (1.6 μg and 16 μg cell extracts respectively) on the SRE derivative LSRE (Hill *et al.*, 1993) and resolved by native gel electrophoresis. The positions of the probe, SRF[133–265] binary complex and the ternary complexes formed with the fusion proteins are indicated.

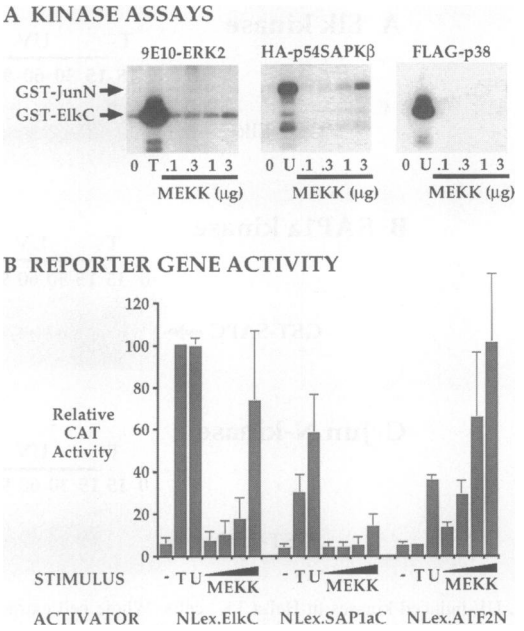

Fig. 7. Activation of MAPKs and TCFs by MEKK overexpression. (**A**) Activation of MAPKs. HeLa TK$^-$ cells were transfected with plasmids expressing 9E10-tagged ERK, HA-p54SAPKβ or FLAG-p38 MAPK, together with the indicated amounts of a MEKK expression plasmid. Kinase activity was analysed by immune complex kinase assays with GST-ElkC as substrates for ERK2 and p38 MAPK and GST-JunN as substrate for p54SAPKβ. For comparison, cells were stimulated by either serum (ERK) or UV irradiation (SAPK/JNK and p38). In two experiments, the range of MEKK concentrations used on average increased ERK activity 0.9-, 0.8-, 1.0- and 1.8-fold; p54SAPKβ activity at least 2.4-, 3.1-, 8.6- and 19-fold; p38 MAPK activation was not detectable. TPA stimulation increased ERK activity 35-fold, while UV stimulation increased activity of p54SAPKβ and p38 42-fold and at least 65-fold respectively. (**B**) Activity of LexA fusion proteins. HeLa TK$^-$ cells were transfected with expression plasmids producing NLexElkC, NLex.SAP1C and NLex.ATF2N together with a LexA controlled reporter gene and the indicated amounts of MEKK expression plasmid. Extracts were prepared for CAT analysis 40 h following transfection. As control, cells were stimulated with TPA or UV irradiation. Activities are relative to TPA-stimulated NLex.ElkC; error bars indicate SEM for three independent experiments.

prevents activation of MEK1 by Raf, (Alessi *et al.*, 1995; Dudley *et al.*, 1995) and SB203580, which specifically inhibits the p38 MAPK (Lee *et al.*, 1994; Cuenda *et al.*, 1995). We tested their effect on the UV- or TPA-induced activation of the LexA–TCF fusion proteins in HeLa TK$^-$ cells. Both PD98059 and SB203580 partially inhibited UV-induced transcriptional activation, and a combination of both compounds reduced activation to basal levels (Figure 8A, left panel). In contrast, TPA-induced transcriptional activation by both LexA–TCF fusion proteins was strongly inhibited in cells treated with PD98059, but not by SB203580; indeed, the latter compound consistently increased the activity of both proteins (Figure 8A, right panel). Control experiments showed that PD98059 and SB203580, singly or in combination, did not inhibit activation of the SAPK/JNKs in immune complex kinase assays (Figure 8B); SB203580, but not PD98059, inhibited the p38 MAPK, as assessed by activation of MAPKAPK2 (data not shown).

A further control experiment was performed with the muscarinic m1 receptor, which in NIH 3T3 cells activates SAPK/JNKs and ERKs, and both LexA–TCF and LexA–Jun fusion proteins, upon stimulation by carbachol (Coso, 1995; R.Treisman, unpublished observations). We found that carbachol-stimulated activity of the NLex.ElkC fusion protein was completely inhibited by PD98059 but unaffected by SB203580, while in contrast, activity of NLex.JunN was unaffected by either inhibitor (Figure 8C). Taken together, these results show that both MEK and p38 MAPK pathways contribute to the activation of the Elk-1 and SAP-1 TCF C-termini, and that UV- or carbachol-induced SAPK/JNK activation is insufficient for the activation of Elk-1.

Maximal UV-induced c-fos activation requires MEK and p38

Finally we used the inhibitors to examine the roles of MEK1 and p38 in activation of the c-*fos* gene itself. Cells were transfected with the intact human c-*fos* gene, pretreated with inhibitors, stimulated and then transcription of both the transfected and endogenous c-*fos* genes ana-

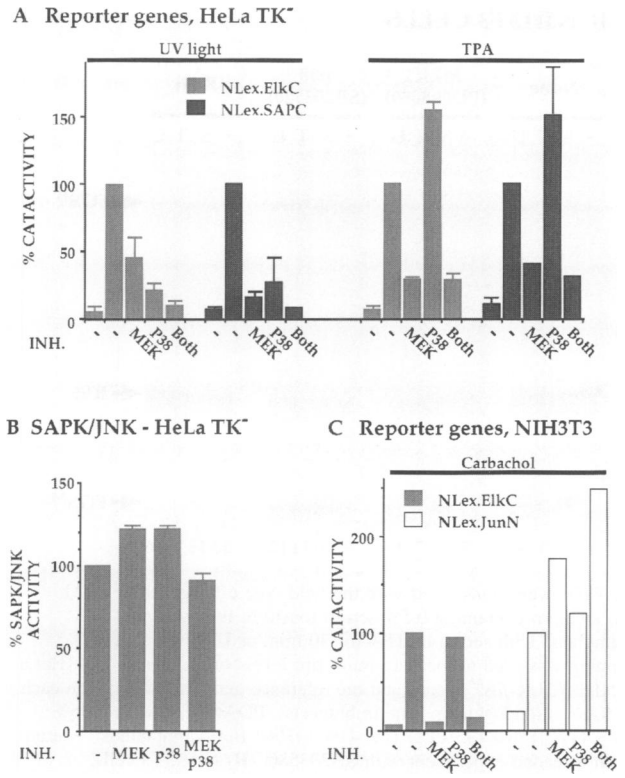

Fig. 8. MEK1 and p38 are required for maximal UV-induced activation of LexA–TCF fusion proteins. (A) Effect of inhibitors on transcriptional activation by LexA fusion proteins. HeLa TK⁻ cells were transfected with expression plasmids producing either LexA–TCF or LexA–ATF2 fusion proteins and maintained in 0.5% serum for 40 h. Cells were then either left unstimulated (first bar of each group) or, following a 1 h pretreatment with either PD 98059 (MEK inhibitor), SB203580 (p38 inhibitor) or both as indicated, were stimulated by UV irradiation or TPA. Extracts were prepared for CAT analysis 8 h later. Activities are taken relative to the response in the absence of inhibitors. Error bars indicate SEM for two or three independent experiments. (**B**) Effect of the inhibitors on SAPK/JNK and p38 activation. HeLa TK⁻ cells were maintained in medium containing 0.5% FCS for 24 h, then pretreated for 1 h with the indicated inhibitors before UV irradiation. Extracts were prepared 30 min later and SAPK/JNK activity assessed by immune complex kinase assay with GST-JunN as substrate. Results are the average of two independent experiments. Inclusion of the inhibitors in the kinase reactions did not impair SAPK/JNK activity. (**C**) Inhibitors do not affect m1 receptor-mediated NLex.JunN activation. NIH 3T3 cells were transfected with an expression plasmid encoding the m1 muscarinic acetylcholine receptor, together with the Lex operator-controlled reporter and expression plasmids producing either NLex.ElkC or NLex.JunN fusion proteins, and maintained in 0.5% serum for 40 h. Cells were then either left unstimulated (first bar of each group) or, following a 1 h pretreatment with inhibitors as indicated, were stimulated with 100 μM carbachol. Extracts were prepared for CAT analysis 8 h later.

lysed by RNase protection. In HeLa TK⁻ cells pretreatment with SB203580 reduced induction by UV light by 2- to 3-fold but slightly increased induction by serum or TPA (Figure 9A, lanes 9–12). In contrast, PD98059 slightly reduced induction of both transfected and endogenous genes by each stimulus (Figure 9A, lanes 5–8). Substantial inhibition of each response was only observed in the presence of both PD98059 and SB203580 (Figure 9A, lanes 13–16). We note that even though TPA-induced TCF activation was sensitive only to PD98059, both inhibitors were required to block activation of c-*fos* transcription by TPA in these cells (see Discussion). We also examined the effects of these inhibitors on c-*fos* induction in NIH 3T3 cells. In these cells, efficient UV-induced c-*fos* transcriptional activation occurs under conditions where the LexA–TCF fusion proteins do not efficiently activate transcription. Treatment with PD98059 reduced serum-induced transcription of both endogenous and transfected c-*fos* genes by 3- to 5-fold, and TPA-induced transcription by >10-fold, but caused only a 2- to 3-fold reduction in UV-induced transcription (Figure 9B, lanes 5–8). In contrast, treatment with SB203580 enhanced the serum and TPA response by up to 2-fold, but reduced the response to UV by some 30% (Figure 9B, lanes 5–9). In combination both compounds blocked the response to UV irradiation and significantly inhibited the response to serum (Figure 9B, lanes 9–16). These results support the notion that the p38 and ERK pathways both contribute to c-*fos* activation in response to UV irradiation.

Discussion

In this work we investigated the regulation of the c-*fos* gene by a stress stimulus, UV light. Upstream regulatory sequences including the SRE are required for activation of the gene in response to UV irradiation, in agreement with previous results (Buscher *et al.*, 1988). However, binding of the SRF accessory factor TCF makes a substantial contribution to UV-induced c-*fos* transcriptional activation in HeLa TK⁻ cells, but not in NIH 3T3 cells. UV irradiation activates both Elk-1 and SAP-1a C-terminal kinases in both cell types, and specificity, kinetic and inhibitor studies show that these include both the ERKs and p38 MAPKs. Our studies show that both the ERK and p38 MAPK pathways contribute to c-*fos* activation in response to UV irradiation, and demonstrate that the SAPK/JNK activation induced by this stimulus is sufficient neither for TCF activation nor c-*fos* transcription in these cells.

Elk-1 and SAP-1 are substrates for multiple MAPKs

We found that the C-termini of all three known TCFs are substrates for the p38 and ERK2 MAPKs, while by contrast, only the Elk-1 C-terminus is a good substrate for SAPK/JNKs. Several previous studies, which used in-gel rather than immune complex kinase assays to investigate Elk-1 phosphorylation by stress-induced kinases, did not detect Elk-1 phosphorylation by the p38 MAPK (Cano *et al.*, 1995; Cavigelli *et al.*, 1995; Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). This may reflect the sensitivity of p38 to the denaturation–renaturation process used in the in-gel kinase assay, and cautions against the exclusive use of this technique to evaluate MAPK activities. Phosphopeptide fingerprinting studies showed that phosphorylation of the Elk-1 C-terminus by p38 MAPK is efficient at many, but not all, of the sites phosphorylated by ERK2; conversely, certain sites appear better substrates for p38 MAPK.

The different TCF C-terminal regions are extensively conserved, so the observation that only Elk-1 is phosphorylated efficiently by SAPK/JNKs suggested that these MAPKs might show a preference for Elk-1 MAPK sites poorly conserved with the other TCFs. Phosphopeptide mapping studies showed that immunopurified SAPK/JNKs and p54SAPKβ preferentially phosphorylate Elk-1 Thr363

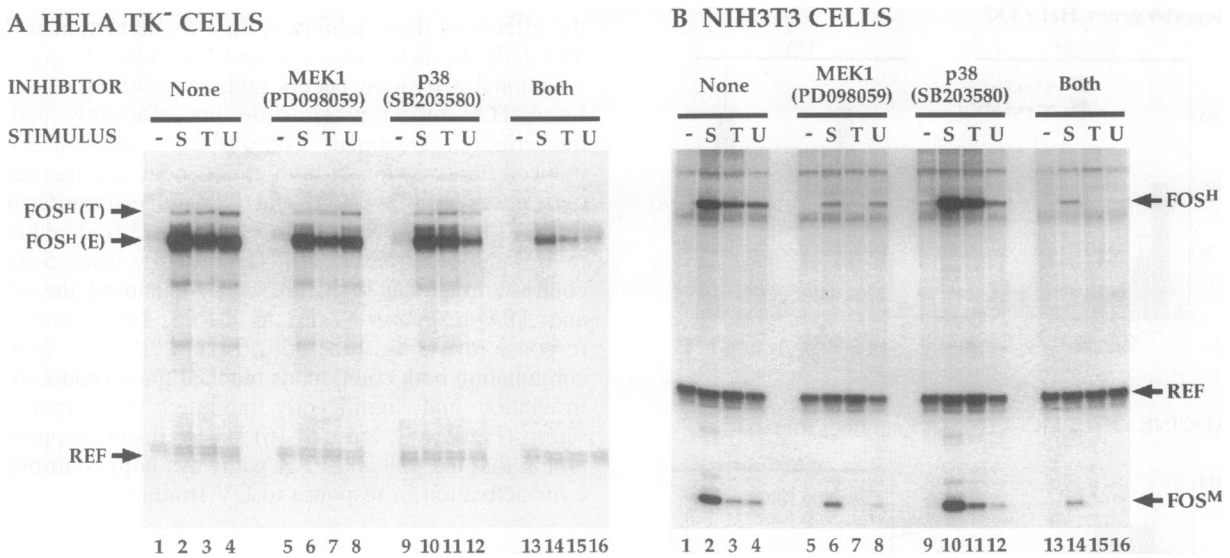

Fig. 9. MEK1 and p38 are required for maximal UV-induced c-*fos* transcription. Cells were transfected with the wild type c-*fos* genes pFM711 (**A**) or pF711 (**B**) together with an α-globin reference plasmid and maintained in medium containing 0.5% serum for 40 h. Following a 1 h pretreatment with the indicated inhibitors, the cells were either left untreated, stimulated with serum or TPA for 30 min, or UV-irradiated and incubated for a further 60 min before RNA was prepared. RNase protection mapping was used to measure transcript levels of the transfected [HeLa TK⁻, c-$fos^H(T)$; NIH 3T3, c-fos^H] and endogenous c-*fos* [HeLa TK-, c-$fos^H(E)$; NIH 3T3, c-fos^M] genes and the reference gene as indicated on each panel. (**A**) HeLa TK⁻ cells. Transcript levels, relative to untreated cells were as follows (endogenous gene in brackets). PD98059, +serum, 55% (80%); +TPA, 63% (70%); +UV 78% (82%). SB203580, +serum 87% (58%), +TPA, 156% (95%); +UV, 41% (33%); Both compounds: +serum, 19% (17%), +TPA, 27% (24%); +UV, 9% (9%). Similar results were obtained in a second experiment with SB203580. (**B**) NIH 3T3 cells. Transcript levels, relative to untreated cells were as follows (endogenous gene in brackets). PD98059, +serum, 17.6% (34.7%); +TPA, <4% (12%); +UV 24% (39%). SB203580, +serum 192% (115%), +TPA, 264% (172%); +UV, 71% (79%); Both compounds: +serum, 16% (15%), +TPA, <4% (6%); +UV, <3% (5%). Similar results were obtained in a second experiment.

and/or Thr368, consistent with the finding that SAPK/JNK preferentially phosphorylates Elk-1 on threonines (Whitmarsh *et al.*, 1995). Interestingly, Thr363 is poorly conserved between Elk-1 and the other TCFs (Dalton and Treisman, 1992). In contrast, other Elk-1 C terminal S/T-P motifs, including Ser383 and/or Ser389, were labelled inefficiently, as observed previously (Cavigelli *et al.*, 1995). Our findings contradict a previous suggestion that Ser383 is the predominant SAPK/JNK target (Gille *et al.*, 1995b); however, the methods used in that study do not resolve peptides phosphorylated at Thr363 and Thr368 (Gille *et al.*, 1995a).

The nature of UV-induced TCF C-terminal kinases

Previous studies of TCF activation by stress stimuli concluded that in the case of Elk-1 the SAPK/JNKs constitute the predominant cellular stress-induced Elk-1 C-terminal kinases; however, they did not address the potential contributions of the ERK and/or p38 pathways to regulation (Cavigelli *et al.*, 1995; Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995). We found that UV irradiation activates the ERKs and the p38 MAPK in addition to the SAPK/JNKs.

Several observations suggest that it is the p38 MAPK and the ERKs, rather than the SAPK/JNKs, that constitute the predominant UV-induced TCF C-terminal kinase activities in HeLa TK⁻ cells. First, kinase activities capable of phosphorylating both the SAP-1a and Elk-1 C-termini are present in these extracts, although only Elk-1 is a good substrate for the SAPK/JNKs. Second, phosphopeptide mapping shows that, in the case of Elk-1, the specificity of the UV-induced kinase(s) present in crude cell extracts is distinct from that of SAPK/JNKs immunoprecipitated from those extracts. Third, the activity of both SAP-1a and Elk-1 C-terminal kinases detectable in crude extracts from UV-irradiated cells parallels the modification of the two TCF C-termini, and is maximal before the peak activity of the SAPK/JNKs. Fourth, UV-induced transcriptional activation by both Elk-1 and SAP-1 fusion proteins is partially inhibited both by PD98059, which inhibits MEK activation by Raf, and by SB203580, which inhibits activity of p38; a combination of both inhibitors almost completely blocks activity. In contrast, TPA-induced transcriptional activation by either TCF C-terminus is sensitive only to inhibition of MEK activation. Obviously, the inhibitor studies are subject to the caveat that other cellular kinases distinct from the target molecules are affected, and a recent study has demonstrated that p38β, which is 75% indentical to p38 MAPK and co-regulated with it, is also a target for bicyclic imidazole inhibitors of the SB203580 family (Jiang *et al.*, 1996). However, our control experiments showed that the inhibitors affected neither activation nor the activity of SAPK/JNKs in our cells (see also Alessi *et al.*, 1995; Cuenda *et al.*, 1995).

Activation of TCFs by multiple MAPKs in vivo

Our results suggest that both the p38 MAPK and ERK pathways cooperate to promote TCF phosphorylation in response to stress stimuli, while the ERK pathway alone is responsible for activation in response to TPA. The mechanism of cooperation remains to be elucidated. One possibility is suggested by slightly different sequence preferences of the different MAPKs, which appear to be overlapping but not identical. Perhaps the strength of the UV-induced stimulus is such that neither p38 MAPK nor ERKs are activated to the extent sufficient for TCF

activation. According to this view, both p38 MAPK and the ERKs would be required for activation in response to UV light, but the strong activation of the ERKs induced by TPA stimulation would by itself be sufficient to potentiate TCF activity. Stimulation of the ERKs alone by co-expression of activated MEK is sufficient to activate each TCF (Price *et al.*, 1995), and while this manuscript was in preparation it was reported that co-expression of both an activated MKK3 mutant and p38 MAPK is also sufficient to activate the Elk-1 C terminus (Raingeaud *et al.*, 1996).

Our data show that UV-induced activation of the SAPK/JNKs is insufficient for activation of LexA–TCF fusion proteins. Is activation of the SAPK/JNKs ever sufficient to potentiate TCF activity? One such situation may be the activation of Elk-1 by MEKK. MEKK can activate both the ERK and SAPK/JNK pathways, but the SAPK/JNK pathway appears more sensitive than the ERK pathway to MEKK stimulation (Lange-Carter *et al.*, 1993; Minden *et al.*, 1994) while the p38 pathway appears insensitive (Lin *et al.*, 1995). MEKK titration experiments have suggested that selective SAPK/JNK activation can be sufficient for Elk-1 activation (Cavigelli *et al.*, 1995; Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995). In agreement with this we found that the LexA–Elk-1 fusion protein was much more efficiently activated by MEKK overexpression than was the corresponding LexA–SAP-1a fusion protein, in line with their relative abilities to act as SAPK/JNK substrates. However, significant activation of another SAPK/JNK substrate, ATF-2, occurred at inputs of MEKK plasmid insufficient to cause detectable Elk-1 activation. Thus, even under conditions where the SAPK/JNK pathway is constitutively and strongly activated, activation of Elk-1 appears relatively inefficient compared with other SAPK/JNK substrates such as the ATF2 N-terminus.

Cooperation between MAPK pathways in c-fos transcription

The SRE is required for efficient UV-induced transcriptional activation in both NIH 3T3 and HeLa TK$^-$ cells, in agreement with previous studies (Buscher *et al.*, 1988). However, the contribution of TCF to transcriptional activation appeared small in NIH 3T3 cells, since disruption of the SRF–TCF ternary complex severely inhibited TPA- but not UV-induced activation. In HeLa TK$^-$ cells, by contrast, we found that TCF binding was required for the efficient transcriptional induction of genes in response to stimulation by UV irradiation. These results correlate with the greater efficiency with which UV irradiation activates LexA–TCF fusion proteins in the HeLa TK$^-$ cells.

We found that UV-induced c-*fos* transcriptional activation is reduced following inhibition of MEK or p38 MAPK in both HeLa TK$^-$ and NIH 3T3 cells, even though TCF contributes little to activation in the latter case. These results suggest that other c-*fos* promoter elements must also receive UV-induced MAPK signals. It is likely that the TATA-box proximal CRE may be one such target: proteins binding this element are targets both for a novel ras-dependent NGF-responsive signal pathway (Ginty *et al.*, 1994) and for MAPKAPK2, an effector of the p38 MAPK (Tan *et al.*, 1996). Indeed, in NIH 3T3 cells, a truncated c-*fos* promoter containing only 124 bp of 5′ flanking sequence exhibits a residual response to UV-, but not serum- and TPA-induced signals, consistent with a strong signal input via this region. These results are consistent with the proposal that proteins binding to both SRE and CRE regions appear to be required for full activity of the promoter (Ginty *et al.*, 1994; Bonni *et al.*, 1995; R.Treisman, unpublished observations). Our results show that in HeLa TK$^-$ cells, but not NIH 3T3 cells, only inhibition of both the ERK and p38 MAPK pathways is sufficient to block transcriptional activation by TPA stimulation, while TPA-induced activation of the TCF C-termini is sensitive only to blockade of the ERK pathway. This may also reflect activation of the CRE via the p38 MAPK pathway, which is weakly activated by TPA in the HeLa TK$^-$ cells.

Why do the promoter-proximal sequences make a greater contribution to activation in NIH 3T3 cells than in HeLa TK$^-$ cells? Perhaps this reflects a weaker UV response by these cells. In this view, transcription factors such as the TCFs, which are activated by kinases such as p38 MAPK or ERKs, might be activated weakly compared with those such as CREB, which are activated by kinases further down the kinase cascade such as p90Rsk or MAPKAPK2. It will be interesting to evaluate the relative contributions of the CRE and SRE to transcriptional activation in response to signals of different strengths.

Materials and methods

Plasmids

Expression plasmids encoding the following fusion proteins: NLex.ElkC, Elk-1 residues 307–428 (Marais *et al.*, 1993); NLex.SAP1C, SAP-1a residues 268–431, NLex.JunN, Jun codons 1–194 (both Price *et al.*, 1995); NLex.ATF2N, ATF2 codons 19–96 (mutant C2; Livingstone *et al.*, 1995), were derived from MLVplink (Dalton and Treisman, 1992) or EFplink (R.Marais, unpublished). In NL.ELK and NL.SAP1a, the Ets domain is replaced by LexA residues 1–87 (Hill *et al.*, 1993). GST–TCF fusion proteins contained the same sequences as NLex- series of genes; GST-SAP2C contains SAP-2 residues 222–407 (Marais *et al.*, 1993; Price *et al.*, 1995). Reporter plasmid LexOP2.tkCAT contains a dimeric LexA operator (Marais *et al.*, 1993). Kinase expression plasmids were as follows: EFMEKK (Price *et al.*, 1995); EXVERK2tag (9E10; Howe *et al.*, 1992); pMT2HA-SAPKp54β (Sanchez *et al.*, 1994); pCMV5-FLAGp38 (Derijard *et al.*, 1995). The EF-M1 expression plasmid comprises the entire coding region of the human m1 receptor inserted into EFplink.

pF711 and pF124 contains the intact human c-*fos* transcription unit with 711 or 124 bp of 5′ flanking sequence respectively; pF711ΔTCF contains point mutations that prevent TCF binding (Treisman, 1985; Hill and Treisman, 1995). For HeLa cell studies, we used analogous derivatives of pFM711, which contains a small deletion–insertion in its 5′ untranslated region that allows its transcripts to be distinguished from those of the human c-*fos* gene; RNase protection probes were as described (Treisman, 1985; Wilson and Treisman, 1988).

Transfections, extracts and other methods

Transient transfections and CAT assays were done as described (Hill *et al.*, 1993; Marais *et al.*, 1993) but with 0.1 mg/ml and 0.5 mg/ml final DEAE-Dextran concentrations for NIH 3T3 and HeLa TK$^-$ cells respectively and 20% DMSO/DME shock for the HeLa TK$^-$ cells. Unless otherwise stated 1 μg each LexA fusion gene expression plasmid was used for transcriptional activation studies. Cells were stimulated with 15% serum, 50 ng/ml TPA, 50 ng/ml or 500 ng/ml anisomycin, 100 μM Carbachol or 40–50 J/m^2 UV light. Prior to high anisomycin or UV stimulation, half or all the medium was removed and replaced either after 60 min or immediately following stimulation. Inhibitor pretreatments were at 50 μM PD98059 and 10 μM SB203580 for 1 h before stimulation. For SDS lysates, cells were harvested in boiling SDS–PAGE buffer containing 0.4 mM Na_3VO_4 and 5 mM NaF. Phosphopeptide fingerprinting was as described (Marais *et al.*, 1993), but used 10 μg each TLCK-treated chymotrypsin and TPCK-treated trypsin (Sigma

C3142, 22H7110). RNA preparation and analysis, and gel mobility shift assays were as described (Hill *et al.*, 1993; Marais *et al.*, 1993; Hill and Treisman, 1995).

Kinase assays

Immune complex kinase assays for JNK/SAPK, p38 MAPK and 9E10-tagged ERK2 were performed by methods derived from those of J.Kyriakis and J.Woodgett (personal communication). For JNK/SAPK and p38 MAPK cells were washed with PBS and lysed in 20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na_3VO_4, 10% glycerol, 1% Triton X-100, 2 μM leupeptin, 400 μM PMSF, 5 μg/ml aprotinin, 0.1 μg/ml okadaic acid (200 μl per 9 cm plate). After 5 min on ice, the lysate was clarified and 500 μg extract protein used for immunoprecipitation. The immunoprecipitates were washed thrice each in lysis buffer, lysis buffer with 150 mM NaCl (p38 MAPK), and finally in assay buffer (SAPK/JNK: 20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100; p38 MAPK: 20 mM MOPS pH 7, 1 mM EDTA, 5% glycerol, 0.1% β-mercaptoethanol, 0.01% Brij 35, 0.1 μg/ml okadaic acid). Kinase reactions contained 20 μl kinase buffer with 1 μg GST fusion protein and Mg-ATP mixes as follows: SAPK/JNK, 7.5 μl 50 mM $MgCl_2$, 100 μM ATP, 2 μCi [γ-^{32}P]ATP; p38 MAPK, 6 μl 60 mM Mg acetate, 300 μM ATP and 2 μCi [γ-^{32}P]ATP. After 20 min at 30°C, the reactions were stopped by addition of 4× SDS–PAGE sample buffer. MAPKAPK2 was immunoprecipitated from 500 μg cell lysates, prepared as above, using anti-MAPKAPK2 serum (a generous gift from P.Cohen) and its activity measured exactly as described previously (Stokoe *et al.*, 1992). For analysis of whole cell extract kinase activity, 13.2 μg HeLa TK$^-$ or 6.6 μg NIH 3T3 cell extract protein was used under SAPK/JNK reaction conditions; products were recovered from the reaction by incubation with glutathione–Sepharose beads for 15–60 min. The beads were washed with 200 μl lysis buffer once and twice with 100 μl lysis buffer with 150 mM NaCl and analysed by SDS–PAGE. Gels were stained to confirm equal sample recovery from each reaction.

Epitope-tagged proteins were detected using anti-HA (12CA5, Boehringer; 2 μg), FLAG (IBI; 1.5 μg) or 9E10 (0.5 μg) with either protein G– (9E10, FLAG) or protein A– (HA) Sepharose. Antibodies were as follows: for SAPK/JNKs, a polyclonal antiserum raised against p54SAPKβ which reacts with SAPKs α, β and γ (JNK1,2; Kyriakis *et al.*, 1994); similar results were obtained using the C17 antibody (Santa Cruz) which predominantly recognizes p54SAPKγ; for p38 MAPK, AN3, a polyclonal C-terminal peptide antiserum (Rouse *et al.*, 1994); for LexA, anti-LexA polyclonal (Neosystem IC516100); for Elk-1 phosphoserine 383, RT25 (F.Cruzalegui and R.Treisman, unpublished); for ERKs, pan-ERK (Transduction laboratories, E17120).

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