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Induction of c-*fos* expression through JNK-mediated TCF/Elk-1 phosphorylation

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Growth factors induce c-*fos* transcription by stimulating phosphorylation of transcription factor TCF/Elk-1, which binds to the serum response element (SRE). Under such conditions Elk-1 could be phosphorylated by the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2. However, c-*fos* transcription and SRE activity are also induced by stimuli, such as UV irradiation and activation of the protein kinase MEKK1, that cause only an insignificant increase in ERK1/2 activity. However, both of these stimuli strongly activate two other MAPKs, JNK1 and JNK2, and stimulate Elk-1 transcriptional activity and phosphorylation. We find that the JNKs are the predominant Elk-1 activation domain kinases in extracts of UV-irradiated cells and that immunopurified JNK1/2 phosphorylate Elk-1 on the same major sites recognized by ERK1/2, that potentiate its transcriptional activity. Finally, we show that UV irradiation, but not serum or phorbol esters, stimulate translocation of JNK1 to the nucleus. As Elk-1 is most likely phosphorylated while bound to the c-*fos* promoter, these results suggest that UV irradiation and MEKK1 activation stimulate TCF/Elk-1 activity through JNK activation, while growth factors induce c-*fos* through ERK activation.

Keywords: c-*fos*/growth factor/transcription regulation

Introduction

As a prototypical immediate early gene c-*fos* transcription is rapidly induced in response to a wide array of extracellular stimuli, including growth factors, cytokines, neurotransmitters, ion fluxes, phorbol esters and UV irradiation. The ability of c-*fos* to respond to a large number of diverse stimuli is explained by the presence of at least three distinct *cis*-acting response elements within its promoter that bind transcription factors whose activity is regulated by different signal transduction pathways (reviewed by Treisman, 1992). Two of these elements, the cAMP response element (CRE) and the serum response element (SRE) are constitutively occupied in non-stimulated cells, while the third element, the Sis-inducible enhancer (SIE) is occupied only after cell stimulation with certain growth factors and cytokines (Hayes *et al.*, 1987; Herrera *et al.*, 1989). The CRE is most likely recognized by transcription factor CREB or other related proteins, such as CREM or ATF1 (reviewed by Mayer and Habener, 1993), whereas the SRE is recognized by a protein complex which includes the serum response factor (SRF) and ternary complex factors (TCFs) (reviewed by Treisman, 1992, 1994). The SIE is recognized by members of the STAT family of transcription factors (reviewed by Darnell *et al.*, 1994). The activities of all of these proteins are stimulated in response to their phosphorylation by a variety of signal-activated protein kinases (reviewed by Hill and Treisman, 1995; Karin and Hunter, 1995).

Although the SRF is rapidly phosphorylated in response to mitogenic stimulation and this phosphorylation event affects its DNA binding properties (Rivera *et al.*, 1993), the major regulatory input received by the SRE can be attributed to TCF phosphorylation (Hill *et al.*, 1993). Among the TCFs, most attention has been given to Elk-1, the first TCF to be identified (Hipskind *et al.*, 1991). Elk-1 is rapidly phosphorylated at several sites located in its C-terminal activation domain (Gille *et al.*, 1992; Marais *et al.*, 1993; Zinck *et al.*, 1993). Phosphorylation at these sites increases the ability of Elk-1 to form a ternary complex with the SRE and SRF (Gille *et al.*, 1992, 1995) and, most importantly, increases its ability to activate transcription (Hill *et al.*, 1993; Marais *et al.*, 1993). Several lines of evidence indicate that the most likely protein kinases to phosphorylate Elk-1 in response to mitogens are the ERK1 and ERK2 mitogen-activated protein kinases (MAPKs). Purified ERK1/2 can phosphorylate Elk-1 *in vitro* at the same sites whose phosphorylation increases after cell stimulation with growth factors and the kinetics of ERK1/2 activation correlate with the kinetics of Elk-1 phosphorylation (Gille *et al.*, 1992, 1995; Marais *et al.*, 1993; Zinck *et al.*, 1993).

Another group of MAPKs are the JNKs (also known as SAPKs), whose activity is stimulated in response to growth factors, cytokines, UV irradiation, heat shock, certain inhibitors of protein synthesis and T cell activators (Dérijard *et al.*, 1994; Kallunki *et al.*, 1994; Kyriakis *et al.*, 1994; Su *et al.*, 1994). Although, like the ERKs, the JNKs are activated by dual phosphorylation at conserved threonine and tyrosine residues (Dérijard *et al.*, 1994), they are activated by different upstream kinases (Minden *et al.*, 1994a; Sanchez *et al.*, 1994; Dérijard *et al.*, 1995; Lin *et al.*, 1995). While some stimuli, such as growth factors, activate the ERKs more efficiently than they activate the JNKs, other stimuli, such as UV irradiation, preferentially activate the JNKs (Minden *et al.*, 1994b). The JNKs were first identified by their ability to phosphorylate c-Jun and stimulate its transcriptional activity (Hibi *et al.*, 1993). More recently they were shown to phosphorylate and stimulate ATF2 activity (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). c-Jun and ATF2 form a heterodimer that is believed to mediate c-*jun* induction (van Dam *et al.*, 1993). Here we show that two preferential activators of JNK activity, UV irradiation and expression of MEKK, induce c-*fos*

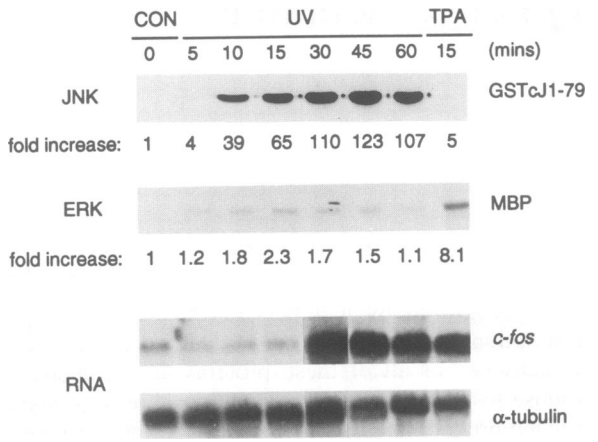

Fig. 1. Activation of JNK, but not ERK, correlates with c-*fos* mRNA induction after UV irradiation. Serum-starved HeLa cells were exposed to UV light (40 J/m^2 UV-C) or TPA (100 ng/ml). The cells were harvested at the indicated times (min) after stimulation. Activation of JNK or ERK was determined by an immune complex kinase assay with GST–cJun(1–79) or MBP as substrate respectively. The fold activation was calculated after quantitation using a phosphorimager. From the same lysates total cytoplasmic RNA was isolated and subjected to Northern analysis with cDNA probes specific for c-*fos* or α-tubulin.

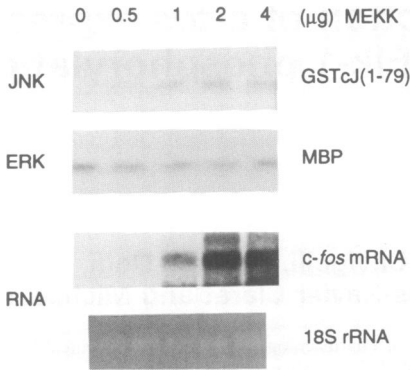

Fig. 2. Activation of JNK, but not of ERK, correlates with c-*fos* mRNA induction by transiently transfected MEKK1. HeLa cells cultured on 6 cm dishes were transfected with different amounts of MEKK1 expression vector by the lipofectamine method. After 48 h cells were harvested and subjected to the same analyses as described in Figure 1. The integrity of the RNA is shown by the ethidium bromide staining of 18S rRNA. The lower levels of JNK activation and c-*fos* induction compared with Figure 1 are due to transfection of no more than 10–20% of the cells.

transcription and Elk-1 transcriptional activity in the absence of considerable ERK activation. We also show that JNK can phosphorylate Elk-1 on sites that are also phosphorylated by ERK1/2 and are known to stimulate its transcriptional activity.

Results

Efficient c-fos induction without ERK activation

Irradiation of cultured mammalian cells with UV results in efficient induction of c-*jun* (Devary *et al.*, 1991) and c-*fos* (Büscher *et al.*, 1988) mRNAs. Deletion analysis indicated that the effect of UV on the c-*fos* promoter is mediated through its SRE (Büscher *et al.*, 1988). As UV irradiation of HeLa cells leads to only weak ERK activation, while resulting in strong JNK activation (Minden *et al.*, 1994b), we compared the time course of c-*fos* induction and ERK2 and JNK activation in response to UV in the same cells (Figure 1). As expected, UV irradiation led to a large increase in JNK activity, as measured by an immune complex kinase assay with a GST–cJun(1–79) fusion protein as substrate. A very small increase in JNK activity was observed in cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). At the same time, UV irradiation had a marginal effect on ERK2 activity, which was measured by an immune complex kinase assay with myelin basic protein (MBP) as substrate. MBP is not phosphorylated by the JNKs (Hibi *et al.*, 1993). In contrast to UV, ERK2 activity was stimulated by TPA. A transient increase in ERK2 phosphorylation, which is much smaller in magnitude than the effect of TPA, was also reported by Radler-Pohl *et al.* (1993). Northern blot analysis indicated that UV irradiation induced c-*fos* expression at least as efficiently as TPA (Figure 1). However, for unknown reasons the kinetics of c-*fos* induction by UV were somewhat slower than the kinetics of its induction by TPA.

Another way to preferentially activate the JNKs with little or no effect on ERK activity is expression of MEKK1, a protein kinase that phosphorylates and activates the JNK kinase (JNKK or SEK1; Minden *et al.*, 1994a; Yan *et al.*, 1994; Lin *et al.*, 1995). We have transiently transfected HeLa cells with a MEKK1 expression vector using the lipofectamine method and 48 h later prepared cell extracts to measure JNK and ERK activities and c-*fos* expression. Transfection with 1–4 μg MEKK1 expression vector/6 cm plate resulted in JNK activation and c-*fos* induction, without a measureable increase in ERK2 activity (Figure 2). The weaker response seen in this case, compared with the UV effect, is due to transfection of no more than 10% of the cells, as determined by indirect immunofluorescence (unpublished results).

UV and MEKK stimulate SRE activity

Previous analysis of the c-*fos* promoter identified the SRE as the *cis* element which mediates the response to UV (Büscher *et al.*, 1988). To determine whether the SRE can also mediate the response to MEKK we co-transfected an SRE-dependent reporter (Büscher *et al.*, 1988) with a MEKK1 expression vector into HeLa cells. As shown in Figure 3A, co-transfection with the MEKK1 vector stimulated expression of the SRE–CAT reporter 5-fold above the level seen in cells co-transfected with non-specific DNA. MEKK1 was as efficient in stimulating SRE activity as TPA or UV irradiation. When co-transfected with different amounts of the MEKK1 expression vector, the SRE–CAT reporter exhibited a dose-dependent increase in its expression (Figure 3B). Transfection of even the highest doses of the MEKK1 vector used in these experiments resulted only in strong JNK1 activation and had hardly any effect on ERK2 activity, as measured by co-transfection of expression vectors encoding epitope tagged versions of both kinases and immune complex kinase assays (Figure 3C).

Phosphorylation of Elk-1 by JNK

Stimulation of SRE activity by UV or MEKK1 in the absence of considerable ERK activation suggested that

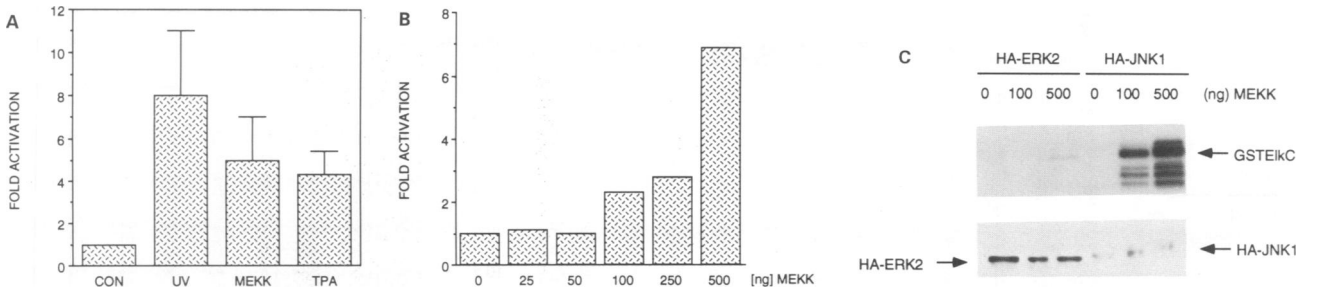

Fig. 3. Activation of the SRE by UV and MEKK1 correlates with JNK activation. (**A**) Transcriptional activation of SRE–CAT after TPA and UV treatments and by transient MEKK1 expression. Sub-confluent monolayers of HeLa cells were transiently transfected with an SRE–CAT reporter (1 μg) and MEKK1 expression vector (0.5 μg) as indicated. The total amount of DNA was kept constant with herring testes DNA. The transfected cells were serum starved for 16 h and left untreated (CON) or treated with 100 ng/ml TPA or exposed to 40 J/m^2 UV-C. After 12 h the cells were harvested and CAT activity was determined. In the case of the MEKK1 co-transfection and its corresponding control the cells were harvested after 48 h. The data represent the means ± SE of three independent experiments done in triplicate. Fold stimulation was calculated relative to that of cells transfected with the reporter and non-specific DNA and subjected to no further treatment. (**B**) Dose-dependent stimulation of the SRE–CAT reporter in MEKK-transfected HeLa cells. Cells were transiently co-transfected with SRE–CAT (1 μg) and increasing amounts of MEKK1 expression vector (ng). After 48 h the cells were harvested and CAT activity was determined. Fold stimulation was calculated as described above. (**C**) MEKK1 activates JNK, but not ERK. HeLa cells were transiently transfected with the HA–ERK2 or HA–JNK1 expression vectors (1 μg each) and 100 or 500 ng MEKK1 expression vector. After 48 h the cells were harvested, HA–ERK2 and HA–JNK1 were immunoprecipitated with an anti-HA antibody and their activities determined by immune complex kinase assays using GST–ElkC as substrate (upper panel). A sample of each lysate was analyzed for expression of HA–JNK1 or HA–ERK2 by immunoblotting with anti-HA antibody (lower panel).

the JNKs, whose activity was strongly elevated in response to UV irradiation or MEKK1 expression, may phosphorylate and activate one of the transcription factors that interact with the SRE. Previous studies have pointed out Elk-1 as the major mediator of stimulation of SRE activity seen after treatment with growth factors (Gille *et al.*, 1992; Hill *et al.*, 1993; Marais *et al.*, 1993; Zinck *et al.*, 1993). We used an 'in-gel' kinase assay to identify the major renaturable protein kinases in extracts of UV-irradiated cells that can phosphorylate the C-terminal activation domain of Elk-1. The use of a GST–ElkC fusion protein containing residues 307–428 of Elk-1 (Marais *et al.*, 1993) as substrate revealed strong phosphorylation by 46 and 55 kDa UV-stimulated protein kinase activities (Figure 4). These activities were identical in their electrophoretic mobilities to JNK1 and JNK2 revealed by the use of GST–cJun(1–79) as an 'in-gel' substrate.

To examine whether purified JNKs can phosphorylate Elk-1 and determine the sites of phosphorylation we incubated GST–ElkC with activated JNK1/2 immunopurified from UV-irradiated HeLa cells in the presence of [γ-^{32}P]ATP. For comparison we phosphorylated GST–ElkC with ERK2 immunopurified from TPA-stimulated HeLa cells. We found that both protein kinases phosphorylated GST–ElkC with similar efficiencies (Figure 5A). Two-dimensional phosphopeptide mapping indicated that the major sites of ElkC phosphorylation by JNK1/2 are identical to the sites phosphorylated by ERK2 (Figure 5B). However, the relative efficiency with which these sites were phosphorylated appears to differ between JNK and ERK. To examine whether the JNK phosphorylation sites include Ser383 and Ser389, the major residues whose phosphorylation is responsible for enhancement of Elk-1 transcriptional activity (Marais *et al.*, 1993; Gille *et al.*, 1995), we examined phosphorylation of a mutant ElkC protein in which these serines were replaced with alanine residues. One of the major phosphopeptides seen in tryptic phosphopeptide maps of wild-type ElkC phosphorylated by either JNK or ERK was no longer found in tryptic digests of ElkC(Ala383/389) phosphorylated

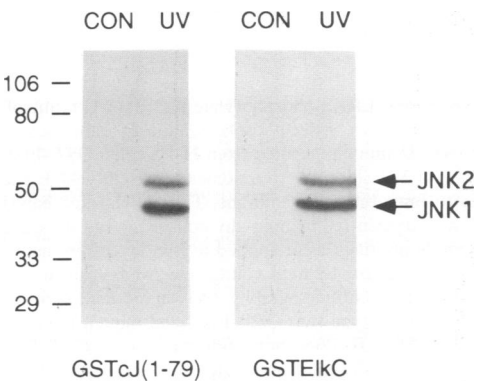

Fig. 4. JNK1 and JNK2 are the major UV-activated ElkC kinases. Lysates from untreated or UV-irradiated HeLa cells were fractionated on SDS gels containing either GST–cJun(1–79) or GST–ElkC within the gel matrix. After renaturation the gels were assayed for kinase activity *in situ*. The positions of molecular mass markers are indicated.

by either JNK or ERK (Figure 5B). Thus, the JNK phosphorylation sites include Ser383 and Ser389.

To determine whether UV and MEKK1 can stimulate Elk-1 transcriptional activity through phosphorylation of the same sites recognized by the ERKs, we co-transfected expression vectors encoding two different GAL4–Elk-1 fusion proteins, one containing the wild-type version of the Elk-1 activation domain (GAL4–ElkC) and the other the mutant version in which Ser383 and Ser389 were replaced by alanines (Marais *et al.*, 1993), with a GAL4-dependent reporter plasmid. Both UV irradiation and co-expression of MEKK1 stimulated activation by the wild-type GAL4–ElkC fusion protein, but not by the mutant GAL4–ElkC(Ala383/389) protein (Figure 6). Both MEKK1 and UV irradiation led to a larger increase in GAL4–ElkC activity than TPA. Similar results were obtained in COS-7 cells (data not shown). Further evidence that MEKK1 expression and UV irradiation stimulate phosphorylation of GAL4–ElkC was obtained by examining their effects on its electrophoretic mobility. Previous

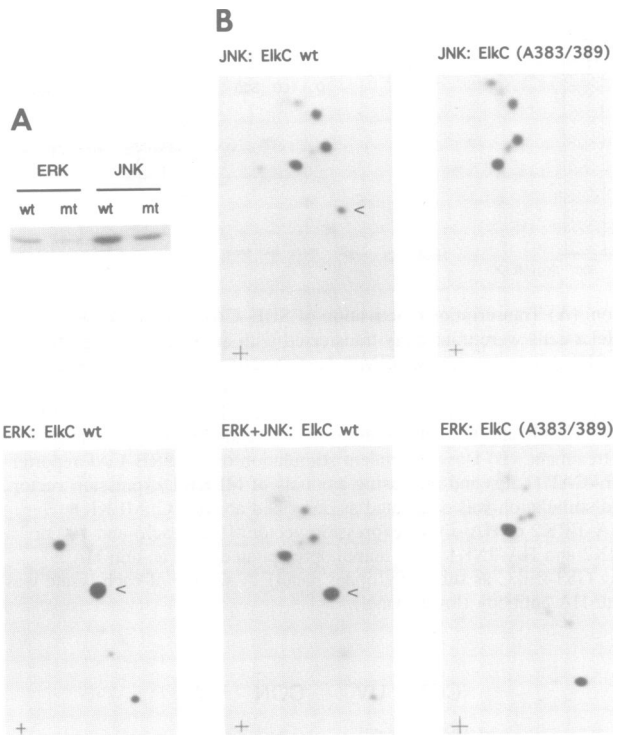

Fig. 5. JNK1/2 and ERK2 phosphorylate the Elk-1 C-terminal domain at the same major sites. After phosphorylation by either activated ERK2 or JNK1/2 immunopurified from HeLa cells, GST–ElkC and GST–ElkC(Ala 383/389) were fractionated by SDS–PAGE and transferred to Immobilon-P membrane. The radiolabeled bands were incubated with trypsin (containing chymotrypsin) and the eluted phosphopeptide mixtures were applied to thin layer chromatography plates and resolved first by high voltage electrophoresis and then by ascending thin layer chromatography. (**A**) An autoradiogram of phosphorylated wild-type (wt) GST–ElkC and mutant (mt) GST–ElkC(Ala 383/389). (**B**) Phosphopeptide maps of wt GST–ElkC and GST–ElkC(Ala 383/389) phosphorylated by either JNK1/2 or ERK2. The crosses show the origins and the arrowhead indicates the phosphopeptide that is missing in the mutant substrate.

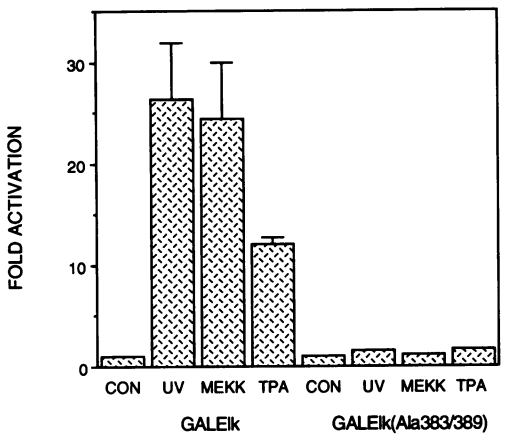

Fig. 6. Transcriptional activity of the Elk-1 C-terminal domain is induced by UV irradiation, transient expression of MEKK1 and TPA treatment. HeLa cells were transfected with wild-type GAL4–ElkC or mutant GAL4–ElkC(Ala 383/389) expression vectors (0.5 μg each) and a GAL–CAT reporter (1 μg). The cells were exposed to either UV (40 J/m^2) or TPA (100 ng/ml) and 12 h later CAT activity was determined. Stimulation of CAT activity by MEKK1 was determined 48 h after co-transfection with either the MEKK1 vector or non-specific carrier DNA. Results reflect the average ± SD of two to three different experiments done in triplicate.

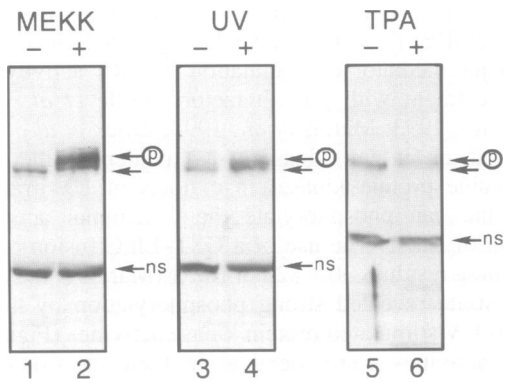

Fig. 7. MEKK1, UV irradiation and TPA stimulate ElkC phosphorylation. COS-7 cells were lipofected with 5 μg GAL4–ElkC (lanes 3–6) and either 0.5 μg empty vector (lanes 1 and 3–6) or 0.5 μg MEKK1 expression vector (lane 2). After 36 h some of the transfected cells were exposed to either UV-C (40 J/m^2) or TPA (100 ng/ml) as indicated. After 30 min whole cell extracts were prepared, separated by SDS–PAGE, blotted and probed with an anti-GAL4 antibody. ns, non-specific cross-reactive band. The position of the slower migrating GAL4–Elk is shown by P.

studies have shown that phosphorylation of GAL4–ElkC on the stimulatory C-terminal phosphoacceptors retards its electrophoretic mobility (Price *et al.*, 1995). As shown in Figure 7, both MEKK1 expression and UV irradiation, like TPA, induced a retardation in the electrophoretic mobility of GAL4–ElkC.

Selective nuclear translocation of ERK and JNK

At very early times after UV irradiation weak and highly transient ERK activation can be detected (Radler-Pohl *et al.*, 1993; Minden *et al.*, 1994b; Figure 1). As Elk-1 is most likely phosphorylated while bound to the c-*fos* promoter and other DNA targets (Hill and Treisman, 1995; Karin and Hunter, 1995), we examined the effect of UV irradiation on the subcellular localization of ERK2 and JNK1. While nuclear translocation of the ERKs has been described (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993), the effect of activation on the subcellular distribution of the JNKs was hitherto unknown. First we used cell fractionation and immunoblotting to examine the distribution of JNK1 and ERK2 in HeLa cells stimulated with either UV or serum. As shown in Figure 8A, UV irradiation resulted in the rapid appearance of JNK1 in the nuclear fraction. Cell stimulation with serum did not result in any increase in JNK1 immunoreactive material in the nuclear fraction above its low basal level. In contrast, the nuclear appearance of ERK2 immunoreactivity was rapidly stimulated by serum, but not by UV irradiation. Next we examined the distribution of the two MAPKs by indirect immunofluorescence. Due to their rounded morphology and the presence of non-specific cross-reacting antigens, HeLa cells were found to be unsuitable for this analysis. We therefore used MRC5 human fibroblasts for these experiments. In these cells both the JNK1 and ERK2 antibodies reacted only with their respective antigens (Figure 8B). While ERK2 was essentially cytoplasmic in unstimulated cells, with very little or no protein detected in the nucleus, JNK1 immunoreactivity was both cytoplasmic and nuclear in unstimulated cells (Figure 8C). While UV

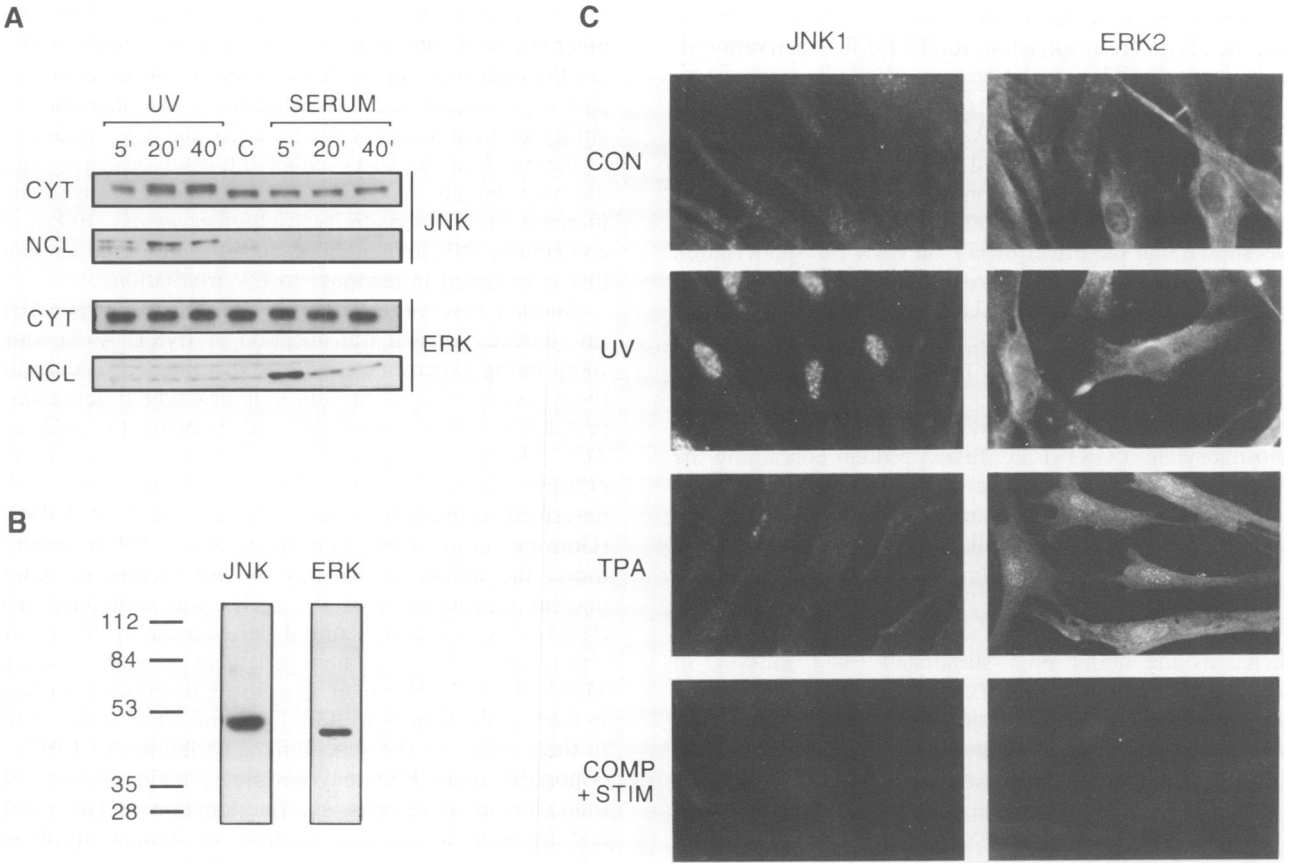

Fig. 8. Subcellular distribution of JNK1 and ERK2 before and after cell stimulation. (**A**) HeLa cells, grown as described in Materials and methods, were starved for 36–48 h and stimulated with either UV (40 J/m^2) or 20% fetal bovine serum for the indicated times or left unstimulated (C). Nuclei were isolated and nuclear (NCL) and cytoplasmic (CYT) extracts were prepared. The purity of the nuclear fractions was assayed using LDH as a cytosolic marker. Extracts were separated by SDS–PAGE, transferred to Immobilon-P membranes and immunoblotted with either anti-JNK1 333.8 or anti-ERK2 C-14 (Santa Cruz) antibodies. (**B**) Whole cell extracts of MRC5 cells were separated by SDS–PAGE, transferred to Immobilon-P membranes and immunoblotted with either anti-JNK1 333.8 or anti-ERK2 C-14 antibodies as indicated. A single major band is seen in both cases. (**C**) Subcellular localization of JNK1 and ERK2 in MRC5 fibroblasts. MRC5 human fibroblasts were serum starved for 36–48 h. After that cells were either not stimulated (CON) or exposed to either UV light (40 J/m^2) or TPA (200 ng/ml) as indicated. After 20 min the cells were fixed, permeabilized and immunostained with anti-JNK1 or anti-ERK2 antibodies, as described in Materials and methods. Nuclear translocation of JNK1 protein is evident after UV light irradiation, while no significant differences in its subcellular distribution are detected in TPA-stimulated cells. In either non-stimulated or UV-irradiated cells very little ERK2 is detected in the nucleus, but following TPA treatment its nuclear entry is detected. Blocking experiments (COMP + STIM) were carried out by immunostaining the cells after pre-incubating the anti-JNK1 and anti-ERK2 antibodies with GST–JNK1 and GST–ERK2 respectively.

irradiation did not affect the subcellular localization of ERK2, it clearly stimulated the translocation of JNK1 immunoreactivity from the cytoplasm to the nucleus. On the other hand, treatment with TPA or serum (data not shown) resulted in nuclear entry of ERK2, but had only a minor effect on the subcellular location of JNK1. Competition experiments indicated that the staining by both antibodies was specific. Thus, like the ERKs, JNK1 translocates to the nucleus once activated. It should be noted, however, that a considerable amount of ERK2 remained in the cytoplasmic compartment, even in TPA-treated cells.

Discussion

The SRE is the major focal point through which growth factors and other mitogens (e.g. TPA) induce transcription of c-*fos* and presumably other immediate early genes whose promoters contain SREs (Treisman, 1992). The SRE is recognized by a protein complex containing the SRF and TCF (Treisman, 1992, 1994). Work done in several laboratories has shown that phosphorylation of TCFs, in particular Elk-1, is the primary mechanism by which mitogenic signaling pathways stimulate SRE activity (Gille *et al.*, 1992; Hill *et al.*, 1993; Marais *et al.*, 1993; Zinck *et al.*, 1993; Price *et al.*, 1995). The kinases responsible for phosphorylation and stimulation of Elk-1 transcriptional activity in response to mitogenic signals are believed to be the ERK1/2 MAPKs (Gille *et al.*, 1992, 1995; Marais *et al.*, 1993; Zinck *et al.*, 1993). The results described above indicate that ERK1/2 are unlikely to be the only protein kinases responsible for stimulation of TCF/Elk-1 activity. We show that stimuli, such as UV irradiation or expression of MEKK1, which do not lead to a significant increase in ERK activity or its nuclear translocation can nevertheless induce c-*fos* transcription and stimulate SRE activity. Both UV irradiation and MEKK1 expression are very potent activators of two other MAPKs, JNK1 and JNK2 (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Minden *et al.*, 1994a,b; Yan *et al.*, 1994).

Several lines of evidence, listed below, strongly suggest that the JNKs are responsible for TCF/Elk-1 activation in response to UV irradiation or MEKK1 expression. Although other investigators (Sachsenmaier *et al.*, 1994) have previously attributed UV induction of c-*fos* to ERK activation, it should be noted that these authors did not measure the effect of UV on ERK activity. In another report, however, the same group (Radler-Pohl *et al.*, 1993) has shown that the effect of UV on ERK phosphorylation is rather miniscule in comparison with the effect of TPA. When compared with the activation of JNK, which is clearly the predominant UV-activated MAPK, the increase in ERK activity appears to be rather insignificant (Minden *et al.*, 1994b; Figure 1).

As shown, the only renaturable protein kinases that can phosphorylate a GST–ElkC fusion protein containing the C-terminal activation domain of Elk-1, found in extracts of UV-irradiated HeLa cells are JNK1 and JNK2 (Figure 4). The same fragment of Elk-1 was previously used by Marais *et al.* (1995) to identify a 44 kDa serum-stimulated protein kinase, most likely ERK1, and by Gille *et al.* (1995) to assay and follow the purification of ERK1 and ERK2 as the major NGF-stimulated Elk-1 kinases. *In vitro*, we find that immunopurified JNK1/2 phosphorylated GST–ElkC on the same major sites phosphorylated by immunopurified ERK2, including Ser383 and Ser389. It should be noted, however, that the two MAPKs show different site preferences, as the relative intensities of the JNK- and ERK-generated phosphopeptides are not identical. Substitution of Ser383 and Ser389 by non-phosphorylatable alanine residues abolishes or severely reduces stimulation of Elk-1 transcriptional activity by growth factors (Marais *et al.*, 1993; Gille *et al.*, 1995). As shown above, in addition to eliminating one of the major JNK-generated phosphopeptides, these mutations also abolish stimulation of Elk-1 transcriptional activity by UV irradiation or MEKK1 expression. In addition to stimulating the transcriptional activity of GAL4–ElkC, both UV irradiation and MEKK1 expression induce a retardation in the electrophoretic mobility of this fusion protein, a modification previously shown to reflect phosphorylation of the Elk-1 activation domain (Price *et al.*, 1995).

Although an absolute identification of which kinase phosphorylates Elk-1 under any given physiological or pathological condition will require the development of novel approaches, the present studies indicate that Elk-1 phosphorylation and transcriptional activity are likely to be stimulated by more than a single protein kinase. In addition, these studies add Elk-1 and possibly other members of the TCF family whose phosphorylation sites and mode of regulation are very similar to those of Elk-1 (Price *et al.*, 1995), to the list of potential JNK targets. While c-Jun was the first transcription factor shown to be regulated by these protein kinases (Hibi *et al.*, 1993; Su *et al.*, 1994), ATF2 was recently shown to also be a potential JNK target (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; van Dam *et al.*, 1995). Interestingly, phosphorylation of all three factors, c-Jun, ATF2 and TCF/Elk-1, contributes to the stimulation of AP-1 activity, albeit by somewhat different mechanisms (Karin and Hunter, 1995). Through phosphorylation of all three proteins, the JNKs appear to play a key role in induction of AP-1 activity in response to UV irradiation and probably other stressful conditions. Recent work by Schreiber *et al.* (1995) indicates that both c-*fos* and c-*jun* induction are part of a natural defense mechanism that increases the ability of mammalian cells to withstand UV irradiation. Mutations that inactivate either the c-*fos* or c-*jun* genes, the two targets of JNK action, cause a considerable increase in UV sensitivity (Schreiber *et al.*, 1995). As conjecture, JNK activation is part of a prototype response that is mounted in response to UV irradiation.

Another new finding described in this report is activation-induced nuclear translocation of JNK1. As genomic footprinting experiments indicate that the SRE is constitutively occupied by a protein complex which generates a protection pattern consistent with it being the SRF and TCF (Herrera *et al.*, 1989), the most likely site of TCF phosphorylation is the nucleus. Previous studies have described activation-induced nuclear entry of ERK1/2 (Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993). Furthermore, the ability of various growth factors to induce nuclear translocation of the ERKs was correlated with their ability to induce neural differentiation of PC12 cells, a program that requires new gene activation (reviewed by Marshall, 1995). However, as c-*fos* transcription is induced in these cells equally well by EGF and NGF, which differ in their effect on the subcellular distribution of ERKs, it is possible that ERKs may not play a major role in c-*fos* induction in these cells. By fractionation of HeLa cells and immunoblotting and staining of human fibroblasts with a monoclonal antibody to JNK1 we show that JNK1 is concentrated in the nucleus upon its activation, suggesting that activation-dependent nuclear entry is a common property of all MAPKs. Interestingly, neither the ERKs nor the JNKs contain obvious nuclear localization signals. As nuclear entry of both ERK2 and JNK1 appears to be related to the efficiency of their activation, it represents another important point of control that provides biological specificity to the action of these protein kinases. While many extracellular stimuli can cause small and transient increases in JNK and ERK activities, the dependence of their nuclear entry on efficient activation ensures that only a small subset of these stimuli will eventually lead to phosphorylation of nuclear transcription factors and other important nuclear targets in a manner that is specific to each MAPK type.

Material and methods

Immunoprecipitation, kinase assays and phosphopeptide mapping

HeLa S3 cells were lysed in 50 mM HEPES, pH 7.6, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 100 μM Na_3V0_4, 0.5% NP-40 (lysis buffer), supplemented with proteinase inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin]. After 20 min, lysates were centrifugated (13 000 r.p.m. for 2 min) and 1 μl antiserum raised against JNK1 (but recognizing also JNK2) or 5 μl polyclonal antibody against ERK2 (C-14; Santa Cruz Biotechnology) were added to the supernatant, together with 30 μl of a 50% suspension of protein A–Sepharose beads. After 3 h incubation at 4°C, the beads were washed three times with lysis buffer and once with 50 mM HEPES, pH 7.6, 10 mM $MgCl_2$ and immune complex kinase assays were performed in 20 μl kinase buffer consisting of 50 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 10 μM ATP and 1 μCi [γ-^{32}P]ATP containing either 10 μg GST–ElkC, 10 μg GST–cJun(1–79) or 5 μg MBP as substrates. After incubation at 30°C for 20 min, SDS sample buffer was added and the mixture was boiled for 5 min and the samples separated by SDS–

PAGE and visualized by autoradiography. For 'in-gel' kinase assays HeLa cells were serum starved for 16 h and than irradiated with 40 J/m^2 UV-C. After 40 min the cells was harvested, lysed as described above and 30 μg protein/lane were separated by SDS–PAGE on gels containing either GST–ElkC or GST–cJun(1–79). 'In-gel' kinase assays were performed as described (Hibi *et al.*, 1993). For phosphopeptide analysis GST–ElkC and GST–ElkC(Ala383/389) were phosphorylated, as described above, by activated ERK2 or JNK1/2 immunoprecipitated from TPA- or UV-stimulated HeLa cells and separated by SDS–PAGE. After transferring to an Immobilon-P membrane the radiolabeled bands were excised, the peptides eluted using trypsin (Sigma T8918) and subjected to tryptic peptide mapping, as described (Boyle *et al.*, 1991; Marais *et al.*, 1993). A polyclonal antibody raised against bacterially expressed JNK1 was used to immunoprecipitate JNK1 and JNK2. The polyclonal anti-ERK2 antibody C-14 was purchased from Santa Cruz Biotechnology. For immunoprecipitation of JNK1 and ERK2 tagged with the HA epitope the monoclonal antibody 12CA5 was used, as described (Minden *et al.*, 1994a).

RNA extraction and Northern blot analysis

HeLa cells were subjected to various treatments as indicated. Total cytoplasmic RNA was extracted as described (Devary *et al.*, 1991). RNA samples (10 μg) were fractionated on a 1% agarose gel, transferred to Zetabind Nylon membrane (CUNO Labs) and hybridized to ^{32}P-labeled cDNA probes specific for c-*fos* and α-tubulin.

Transfections and cell culture

HeLa and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfections with the MEKK1 expression vector were carried out by the lipofectamine method as recommended by the manufacturer (Gibco-BRL). Other transfections were carried out by the calcium phosphate co-precipitation method. For determination of reporter activity 6-well plates were used; for other analyses we used 6 cm dishes. To determine transcription activity, 1 μg SRE–CAT or 1 μg GAL–CAT reporters and 500 ng GAL4–ElkC (wild-type and mutant) expression vectors were used. The amounts of MEKK1 expression vector used are indicated in the legends to the figures. The reporter plasmids and expression vectors, GAL–CAT, SRE–CAT [(–320/–299)-pBLCAT4 in Büscher *et al.*, 1988], GAL4–ElkC, GAL4–ElkC(Ala383/389), GST–ElkC, GST–ElkC(Ala383/389) and MEKK1 have been described (Büscher *et al.*, 1988; Lillie *et al.*, 1989; Marais *et al.*, 1993; Minden *et al.*, 1994a,b). The MRC5 human fibroblasts were incubated in MEM Earl's medium supplemented with 10% fetal bovine serum. Quiescent cells were obtained by incubating cultures in serum-free medium for 36–48 h. Cells were then stimulated as described.

Western blot analysis

Whole cell extracts were prepared in a lysis buffer supplemented with a cocktail of protease and phosphatase inhibitors, as described (DiDonato *et al.*, 1995). Equal amounts of proteins were suspended in SDS sample buffer and resolved on 10% SDS–PAGE. The gel was electrotransferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA). After blocking in 5% dried milk the GAL4 fusion proteins were detected with a chemoluminescent detection kit (Amersham) using an anti-GAL4 antibody (a gift from Dr Y.Shaul, Weizman Institute of Science). Confluent MRC5 and HeLa cells, either quiescent or stimulated, as described in Figure 8, were lysed in 25 mM HEPES, pH 7.7, 0.1% Triton X-100, 0.3 M NaCl, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 1 mM Na_3V0_4, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 5 μg/μl leupeptin, 1 mM PMSF, centrifugated for 10 min at 4°C and supernatant proteins fractionated by SDS–PAGE. The proteins were electrotransferred to Immobilon-P membrane and immunoprobed with mouse monoclonal IgG 333.8 antibody to detect JNK1 or rabbit polyclonal antibody C-14 (Santa Cruz Biotechnology) to detect ERK2.

Cell fractionation

HeLa cells, serum starved for 36–48 h, were stimulated as described in Figure 8 and lysed in nuclear precipitation buffer (NPB; 10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, 140 mM NaCl, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/μl leupeptin) with 0.5% Nonidet P-40 by freezing in liquid nitrogen and thawing at 37°C. Lysate was layered onto 50% sucrose/NPB and centrifuged at 13 000 r.p.m. for 10 min. Pellets were washed with NPB and nuclear proteins were extracted with Dignam C buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 5 μg/μl leupeptin). Both the post-nuclear fraction and nuclear proteins were collected and examined by Western blot, as described above.

Purity of the nuclear fractions was tested by lactate dehydrogenase (LDH) assay as a cytosolic marker (Storrie and Madden, 1990). Results showed <5% LDH activity in the nuclear fractions.

Immunocytochemistry

MRC5 fibroblasts were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde/PBS at room temperature for 30 min. Cells were then rehydrated in PBS containing bovine serum albumin (BSA) or goat serum (Gibco-BRL) and permeabilized with 0.25% Triton X-100. Cells were washed twice with PBS and incubated with the primary antibody (either mouse monoclonal anti-JNK1 333.8 or rabbit polyclonal anti-ERK2 C-14; Santa Cruz Biotechnology) in PBS containing BSA or goat serum at room temperature for 2 h. Cells were washed with PBS and incubated with the TRITC-conjugated secondary antibody (anti-mouse or anti-rabbit). After three washes with PBS the cells were mounted on Mowiol (containing DABCO) and examined with a Biorad MRC-1024 Confocal System with an excitation–emission filter for rhodamine. The same results were obtained by fixing cells with ethanol/acetic acid 95:5 (v/v) at room temperature for 10 min followed by rehydration and incubation with the antibodies, as described above. Specificity of JNK1 and ERK2 detection was determined by the use of different secondary antibodies giving the same results and by antigen competition experiments. Anti-JNK1 333.8 and anti-ERK2 C14 antibodies were pre-incubated with GST–JNK1 and GST–ERK2 fusion proteins respectively for 30 min at 37°C.

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