Neither ERK nor JNK/SAPK MAP kinase subtypes are essential for histone H3/HMG-14 phosphorylation or c-fos and c-jun induction

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SUMMARY

The effects of EGF, TPA, UV radiation, okadaic acid and anisomycin on ERK and JNK/SAPK MAP kinase cascades have been compared with their ability to elicit histone H3/HMG-14 phosphorylation and induce c-fos and c-jun in C3H 10T1/2 cells. EGF and UV radiation activate both ERKs and JNK/SAPKs but to markedly different extents; EGF activates ERKs more strongly than JNK/SAPKs, whereas UV radiation activates JNK/SAPKs much more strongly than ERKs. Anisomycin and okadaic acid activate JNK/SAPKs but not ERKs, and conversely, TPA activates ERKs but not JNK/SAPKs. Nevertheless, all these agents elicit phosphorylation of ribosomal and pre-ribosomal S6, histone H3 and HMG-14, and the induction of c-fos and c-jun, showing that neither cascade is absolutely essential for these responses. We then analysed the relationship between ERKs, JNK/SAPKs and the transcription factors Elk-1 and c-Jun, implicated in controlling c-fos and c-jun, respectively. JNK/SAPKs bind to GST-cJun1-79, and ERKs, particularly ERK-2, to GST-Elk1307-428; there is no cross-specificity of binding. Further, GST-Elk1307-428 binds preferentially to active rather than inactive ERK-2. In vitro, JNK/SAPKs phosphorylate both GST-cJun1-79 and GST-Elk1307-428, whereas ERKs phosphorylate GST-Elk1307-428 but not GST-cJun1-79. Thus, neither ERKs nor JNK/SAPKs are absolutely essential for nuclear signalling and c-fos and c-jun induction. The data suggest either that activation of a single MAP kinase subtype is sufficient to elicit a complete nuclear response, or that other uncharacterised routes exist.

Key words: growth factor, UV irradiation, MAP kinase, histone H3/HMG-14 phosphorylation, c-fos/c-jun induction

INTRODUCTION

Protein kinase cascades implicated in mediating mitogen-stimulated nuclear responses such as histone H3 or HMG-14 phosphorylation and the induction of immediate-early (IE) genes have been the subject of intense recent study (Pulverer et al., 1991; Smeal et al., 1991; Marais et al., 1993; Zinck et al., 1993; Kyriakis et al., 1994; Hibi et al., 1993; Derijard et al., 1994; Deng and Karin, 1994; Kardalinou et al., 1994; Cano et al., 1994), with some contentious conclusions (discussed by Cano et al., 1994; Cooper, 1994; Cano and Mahadevan, 1995). The activation of MAP (mitogen-activated protein) kinases, involving Ras-GTP mediated translocation of an upstream kinase to the plasma membrane as the initial event, has been reviewed at length (Nishida and Gotoh, 1993; Blumer and Johnson, 1994; Marshall, 1994; Avruch et al., 1994). MEK, a dual-specificity kinase, phosphorylates the MAP kinases ERK-1 and -2 (extracellular-regulated kinase-1 and -2) on threonine and tyrosine residues (reviewed by Blumer and Johnson, 1994; Marshall, 1994; Avruch et al., 1994). ERKs can phosphorylate the ternary complex factor (TCF) Elk-1 (Gille et al., 1992; Marais et al., 1993; Zinck et al., 1993; Rao and Reddy, 1994), which together with serum response factor (SRF) is constitutively resident on the serum response element (SRE) controlling c-fos expression (reviewed by Treisman, 1994). Thus, the chain from plasma membrane to the c-fos gene is proposed to close.

First indications of additional complexity came from studies of a second IE gene, c-jun, controlled primarily by constitutively occupied AP-1 sites (Rozeck and Pfeifer, 1993), although other occupied upstream sites are also present (see Discussion). c-Jun protein on these AP-1 sites can be phosphorylated on serines 63 and 73 by a second subtype of MAP kinases, called JNK/SAPKs (Jun NH-terminal kinase/stress-activated protein kinase). More recent work indicates that ATF-2, a potential partner for c-Jun on these AP-1 sites, can also be phosphoryl-
ated by JNK/SAPKs (Livingstone et al., 1995; van Dam et al., 1995). Originally described as p54 MAP kinase, JNK/SAPKs are 50-60% homologous to ERKs, with at least six mRNAs reported in rat (Kyriakis et al., 1994; Derijard et al., 1994; reviewed by Cano and Mahadevan, 1995). Where ERKs are phosphorylated on the motif T-E-Y, JNK/SAPKs have T-P-Y and the MEK that phosphorylates ERKs is incapable of activating JNK/SAPKs (Kyriakis et al., 1994; Han et al., 1994). Minden et al. (1994a,b) provide evidence that MEKK, originally proposed as an upstream kinase for MEK in the ERK cascade, may in fact exclusively activate the JNK/SAPK cascade. Further, a MEK equivalent for the JNK/SAPK cascade, called SEK (SAPK/ERK kinase) or JNKK (JNK kinase) has been cloned, and shown to be phosphorylated and activated by MEKK (Sanchez et al., 1994; Yan et al., 1994). This leads to the postulation of parallel MAP kinase cascades that are simultaneously activated and targeted to distinct transcription factors, and thereby, to distinct IE genes (Cooper, 1994; reviewed by Cano and Mahadevan, 1995).

A third mammalian MAP kinase subtype represented by p38 (Han et al., 1994), RK (Rouse et al., 1994) or p40 (Freshapey et al., 1994) with the motif T-G-Y instead of T-E-Y in the activation domain was later discovered. In addition, two drug-binding proteins, CSBP1 and CSBP2, were cloned and found to be MAP kinases of this subtype, following which it was shown that the drug potently and specifically inhibits this subtype of MAP kinases (Lee et al., 1994; Cuenda et al., 1995). Although the yeast homologue of this subtype, HOG-1 is singularly implicated in the response to hyperosmolarity, the consensus is that in mammalian cells all three kinase cascades are activable to varying extents by such stress (Han et al., 1994; Gargova et al., 1994). The conclusion from all these studies is that as opposed to yeast cells, in which different MAP kinase subtypes appear to control separate events, they are simultaneously activable in mammalian cells and may run in parallel to distinct ends, raising formidable issues of specificity and targeting within these cascades (reviewed by Cooper, 1994; Cano and Mahadevan, 1995).

While the above is largely accepted, the issue of how distinct MAP kinase subtypes are deployed towards specific IE genes is more contentious. Although ERKs, by phosphorylating TCFs, can be involved in c-fos induction (Gille et al., 1992; Marais et al., 1993; Zinck et al., 1993), there are clear circumstances when c-fos is induced without ERK activation (Cano et al., 1994), and conversely where ERK activation does not result in c-fos induction (Campos-Gonzalez and Glenn, 1992; Selva et al., 1993). Similarly, claims that JNK/SAPK-mediated phosphorylation of c-Jun is required for c-jun induction run contrary to observations of c-jun induction in the absence of JNK/SAPK activation (reviewed by Cano and Mahadevan, 1995). The two major sources of contention are first, that much of these studies have been performed in different cell lines, and second, that the many agents used to activate IE genes are generally regarded as equivalent, with little rigorous regard to the strength and time-course of activation by each. There has not been strict comparison of the relative abilities of different agents to activate IE genes with their capacity to activate different MAP kinase subtypes in the same cells. Here, we present such an analysis, comparing the relative abilities of EGF, TPA, okadaic acid, anisomycin and UV irradiation to elicit nuclear responses such as histone H3/HMG-14 phosphorylation and c-fos/c-jun induction with their ability to activate ERKs and JNK/SAPKs in C3H 10T1/2 cells. Because we found that neither MAP kinase subtype was essential for activation of either IE gene, we tested for cross-talk, the ability of ERKs to bind to and phosphorylate c-Jun and conversely, JNK/SAPKs to bind and phosphorylate Elk-1. These results show that neither ERKs nor JNK/SAPKs are essential for nuclear signalling and IE gene induction, and suggest either that activation of one MAP kinase subtype is sufficient to elicit a complete nuclear response or that there are other as yet uncharacterised pathways activated by all the stimuli tested here.

MATERIALS AND METHODS

Cell culture, mitogen stimulation and extraction of C3H 10T1/2 cells
C3H 10T1/2 mouse fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) foetal calf serum (FCS). Confluent cultures were rendered quiescent by incubation for 12-18 hours in DMEM containing 0.5% (v/v) FCS. Cells were stimulated as indicated with EGF (50 ng/ml; kindly provided by G. Panayotou, Ludwig Institute for Cancer Research, London), TPA (100 nM; Sigma), anisomycin (10 μg/ml; Sigma) or okadaic acid (500 nM; Sigma). For UV irradiation, 100 mm diameter dishes of quiescent cells in 3 ml of DMEM were exposed to the doses indicated, using a Spectrolinker (XL-1000; Spectronics Corp.). At indicated times after stimulation, medium was aspirated and cells harvested as described below.

Sequential extraction and separation of labelled proteins
Quiescent cells were labelled for 3 hours in phosphate-free DMEM containing 170 μCi/ml [32P]phosphate (carrier free; NEN). After stimulation as indicated, cells were lysed in cold Triton-deoxycholate (DOC) buffer (20 mM Hepes, pH 7.2, 1% Triton X-100 (TX-100), 1% sodium DOC, 100 mM sodium chloride, 50 mM sodium fluoride (NaF), 5 mM EDTA, 100 μM sodium molybdate) with protease inhibitors (Mahadevan and Bell, 1990) and subjected to sequential extraction as described by Kardalainou et al. (1994). Aliquots of protein were solubilized in Laemmli sample buffer containing 4.5 M urea for SDS-PAGE gels or 8 M urea-5% acetic acid for analysis on acid-urea gels (Barratt et al., 1994a,b).

Northern blot analysis
C3H 10T1/2 cells were prepared as described above and stimulated as indicated. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (1987). Aliquots containing 3 μg RNA were resolved on formaldehyde/agarose gels (Sambrook et al., 1989) except 0.41 M formaldehyde was used, as described by Chomczynski (1992) and transferred onto nylon membranes (Hybond-N+, Amersham); hybridisation was performed essentially as described by Church and Gilbert (1984) using a 32P-labelled BglII/SalI fragment of v-fos (Curran et al., 1982) at pAT153. Mouse c-jun pAH119 (Ryseck et al., 1988) was generously provided by Rodrigo Bravo (Roche). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (Edwards and Mahadevan, 1992) was a 1 kbp fragment of murine cDNA cloned in pBluescript KS- (Stratagene).

Plasmids
Plasmids encoding GST and GST-c-Jun,79 fusion proteins were provided by M. Karin (Hibi et al., 1993) and GST fused to Elk-1807-428 was provided by R. Treisman (Marais et al., 1993).

Protein purification
GST-fusion proteins were purified by affinity chromatography on
were resuspended in 30 mM beta-glycerophosphate, 0.1 mM Na vanadate, 2 mM DTT), the beads were diluted so that the final concentration of buffer was 20 mM Hepes, pH 8.0, 75 mM NaCl, and 3-5 mM MgCl₂, 0.1 mM EDTA, 0.05% TX-100, 0.5 mM DTT, 20 mM beta-glycerophosphate, 0.1 mM Na vanadate, with protease inhibitors (Mahadevan and Bell, 1990). The cell suspension was washed in kinase buffer (20 mM Hepes, pH 8.0, 20 mM MgCl₂, 20 mM beta-glycerophosphate, 0.1 mM Na vanadate, 2 mM DTT), the beads were resuspended in 30 μl of kinase buffer containing 20 μM ATP and 3-5 μCi [γ-³²P]ATP. After 30-40 minutes at 25°C the reaction was terminated by washing with HBIB buffer.

For experiments in which unbound kinase was required, beads were resuspended in 30 μl of kinase buffer containing either 200 mM NaCl, 60 μM cold ATP, 1% TX-100 or 0.01% SDS, as indicated, and left on ice with frequent mixing for 1 hour. The beads were then diluted with 3 volumes of kinase buffer to a final concentration of 20 μM cold ATP. The kinase assay was performed as described above and terminated by acetonitrile precipitation at -20°C. Phosphorylated proteins were boiled in 30 μl 2× Laemmli buffer and resolved on 10% SDS-polyacrylamide gels, followed by autoradiography.

Western blotting analysis
Proteins were electrophoresed on 14% minigels (8x12×0.075cm) (Anderson et al., 1973) and transferred overnight to polyvinylidene difluoride membranes (Immobilon-P, Millipore), in transfer buffer (12.5 mM Tris, 192 mM glycine, 0.05% SDS, 20% methanol). Membranes were blocked with 5% Marvel in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBST) for 1 hour, then probed with mouse monoclonal antibody recognizing human, mouse and rat ERK-1 and -2 (Zymed), diluted 1:10,000 in TBST for 1 hour. The membranes were rinsed twice then washed 3 times for 5 minutes each time in TBST. After washing, blots were incubated with sheep anti-mouse antibody coupled to horseradish peroxidase (1:10,000 in TBST) for 1 hour, washed as before and visualised using the Renaissance detection system (NEN).

In-gel kinase assay
Quiescent C3H 10T₁/₂ cells were treated as indicated and lysed as described by Cano et al. (1994). Bacterially expressed GST-SAP-1 (Treisman, 1994) were obtained and purified as described by Cano et al. (1994), and copolymerized at 100 μg/ml in SDS-polyacrylamide gels for in-gel kinase assays. Kinase assays were carried out as described previously (Cano et al., 1994).

Immunoprecipitation with anti-JNK rabbit antiserum
Control or stimulated cells (100 mm dishes) were lysed in 500 μl of 20 mM Tris-HCl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 0.5% NP-40 and 0.1 mM Na vanadate, 50 mM NaF plus protease inhibitors (Mahadevan and Bell, 1990), for 30 minutes on ice. The cells were pelleted and the supernatants incubated with 5 μl of anti-JNK rabbit antiserum (247), kindly provided by M. Karin, for 2 hours at 4°C with rotation. The immunocomplexes were recovered using 10 μl Protein A Sepharose, rotated for 2 hours at 4°C. The beads were washed 6 times with lysis buffer and once with kinase buffer, then resuspended in 50 μl of kinase buffer containing 20 μM cold ATP and 5 μCi [γ-³²P]ATP. The reaction was terminated by acetonitrile precipitation.

RESULTS

Confluent, quiescent C3H 10T₁/₂ cells were used for these experiments because many characteristics of intracellular and chromatin-associated signalling biochemistry and IE gene induction in these cells have been previously established (Mahadevan and Edwards, 1991; Edwards and Mahadevan, 1992; Kardalinou et al., 1994; Barratt et al., 1994a,b; Cano et al., 1994).

Induction of c-fos and c-jun in response to EGF, TPA, anisomycin, okadaic acid and UV radiation
Although EGF, TPA, anisomycin, okadaic acid and UV irradiation are all capable of inducing c-fos and c-jun, direct comparison reveals differences in the characteristics of each response (Fig. 1). EGF, the only physiological agent tested here (Fig. 1A), induces both genes to approximately equivalent levels within 15 minutes, peaking at 30 minutes. EGF-induced c-fos mRNA is substantially lost by 45 minutes, whereas c-jun mRNA is more persistent, being present at 45 minutes (Fig. 1A) and lost at 60 minutes (C.A.H and L.C.M, not shown). TPA induction of c-fos is similar to EGF, but mRNA is still present at 45 minutes, whereas c-jun induction is slightly delayed, peaking at 45-60 minutes (Fig. 1A and B), and remaining up to 90 minutes (Fig. 1B). With both agents, the general strength of induction is approximately equivalent. However, given that c-Fos and c-Jun dimerise to form AP-1 complexes, contributing both to transcriptional activation (Hirai et al., 1990) and repression (Schonthal et al., 1989), it is relevant that the ratio of c-fos to c-jun mRNA is distinctive at each time-point tested, a finding which extends to other members of the fos (fosB) and jun (junB, junD) families (C.A.H and L.C.M, not shown). If these mRNA ratios result in differing proportions of Fos- to Jun-family proteins, it is conceivable that distinct AP-1 complexes would result. Anisomycin (Fig. 1A), by a combination of inducing intracellular signals, stabilising transcripts and delaying transcriptional shut-off, induces c-fos and c-jun sustainedly, but, as reported previously, has a much stronger effect on c-jun (Mahadevan and Edwards, 1991; Edwards and Mahadevan, 1992). The combination of anisomycin and EGF superinduces c-fos and c-jun (Fig. 1A), this data being included here for comparison only as this phenomenon and its causes have been described in detail elsewhere (Mahadevan and Edwards, 1991; Edwards and Mahadevan, 1992).

In contrast to EGF, TPA or anisomycin which rapidly induce c-fos and c-jun, induction by okadaic acid (reviewed by Cohen, 1990) is very much slower (Fig. 1B), despite its very strong activation of kinases in these cells (Mahadevan et al., 1991; Cano et al., 1994). Levels of mRNA comparable to that seen at 15-30 minutes of EGF treatment are only seen at 60-90 minutes after okadaic acid treatment. To eliminate the possibility that okadaic acid also produced secondary toxic effects
that delayed or impeded $c$-$fos$ and $c$-$jun$ induction, okadaic acid-treated cells were stimulated with TPA, under which conditions there was no delay in $c$-$fos$/c-$jun$ induction (Fig. 1B). In fact, the response, particularly for $c$-$fos$, is stronger and prolonged upon co-treatment; we find no mRNA-stabilising effect that can account for this (C.A.H and L.C.M, not shown). Like okadaic acid, UV irradiation (Fig. 1C) also elicited more gradual and weaker activation of $c$-$fos$ and $c$-$jun$ (reviewed by Karin, 1994). Both IE gene mRNAs begin to be detectable at 30 minutes after UV treatment, that for $c$-$fos$ being transient and lost by 90 minutes, whereas $c$-$jun$ mRNA peaks at 45-60 minutes and persists to 2 hours. Even at their maximum, induction by UV irradiation is much poorer than with other agents. A possible complication here is that UV treatment can potentially interfere with mRNA, perhaps crosslinking it in the cell, as evidenced by the loss of control GAPDH mRNA at higher doses of irradiation (not shown). However, at the dose used here, GAPDH mRNA is unaffected, suggesting that the $c$-$fos$ and $c$-$jun$ mRNA signals observed are valid measures of induction.

**Analysis of intracellular and chromatin-associated phosphorylation events elicited by UV radiation**

Apart from UV radiation, we have shown that all the other agents used here elicit phosphorylation of ribosomal and pre-ribosomal S6, and in chromatin, phosphorylation of the nucleosomal

![Fig. 1](image_url). Northern blot analysis of $c$-$fos$ and $c$-$jun$ expression in response to different agents. C3H 10T1/2 cells were treated as indicated: (A) EGF (50 ng/ml); TPA (100 nM); anisomycin (An; 10 μg/ml); or EGF plus anisomycin (EGF + An), for 15 to 60 minutes. (B) okadaic acid (OA; 500 nM); TPA (100 nM); or okadaic acid plus TPA (OA + TPA), for 15 to 90 minutes. (C) UV irradiation (200 J/m²) for 15 minutes to 2 hours, and anisomycin (An; 10 μg/ml), 45 minutes. C, control (unstimulated). GAPDH was used as a loading control.
activate these kinases (Fig. 3B). Activation by EGF was transient, peaking at 15 minutes and substantially terminated within 15 minutes (Fig. 3A). Anisomycin and okadaic acid did not activate ERKs (Fig. 3A; see also Cano et al., 1994). UV irradiation elicited weak activation of JNK/SAPKs, to c-jun induction, whereas TPA elicits no JNK/SAPK activation but strongly induces c-jun; these results dissociate the activation of the JNK/SAPK cascade from c-jun induction, and suggest that alternative routes to c-jun must exist.

**Binding of kinases to c-Jun and Elk-1 and the substrate-specificity of bound kinases**

To explain the ability of anisomycin, which does not activate ERKs, to induce c-fos, or that of TPA, which does not activate JNK/SAPKs, to induce c-jun, we asked if ERKs and JNK/SAPKs could each bind and/or phosphorylate transcription factors controlling both genes. In the same way that
Methods. After solid phase kinase assay, GST-cJun were then isolated as described in Materials and indicated and GST-cJun UV and TPA, for 5 minutes to 1 hour as treated with EGF, anisomycin, okadaic acid, cell extracts (WCEs) were prepared from cells treated with EGF, anisomycin, okadaic acid, UV and TPA, for 5 minutes to 1 hour as indicated and GST-cJun binding kinases were then isolated as described in Materials and Methods. After solid phase kinase assay, GST-cJun proteins phosphorylated by bound kinase were analyzed by SDS-PAGE and autoradiography. The position of GST-cJun1-79 on these gels, as determined by Coomassie Blue staining, is indicated.

JNK/SAPks will bind to and phosphorylate c-Jun (Fig. 3B), the ability of kinases to bind and phosphorylate the ternary complex factor Elk-1 was assessed (Fig. 4A). For these experiments, a GST-fusion protein encompassing the box-C region of Elk-1 (residues 307-428) which contains several MAP kinase sites implicated in c-fos activation (Marais et al., 1993) was used (kindly provided by R. Treisman, ICRF, London). Lysates from cells stimulated with either anisomycin (which does not activate ERKs) or EGF (which strongly activates ERKs) were incubated with GST-Elk1,307-428 binding kinases. Whole cell extracts (WCEs) were prepared from cells treated with EGF, anisomycin, okadaic acid, UV and TPA, for 5 minutes to 1 hour as indicated and GST-cJun1-79 binding kinases were then isolated as described in Materials and Methods. We next analysed the ability of the bound kinases to cross-phosphorylate additional substrates provided during the in vitro kinase assays (Fig. 4B). Lysates from cells treated with EGF (Fig. 4I, lane 2), anisomycin (lane 3), okadaic acid (lane 4) and UV irradiation (lane 5) were incubated with either GST-Elk1,307-428 (upper panel), GST-cJun1-79 (centre panel) or GST (lower panel), and bound kinases pelletted and washed as described. Before bound kinases were assayed, aliquots of GST-cJun1-79 or GST-Elk1,307-428 were added so that all tubes had equivalent amounts of both substrates present. This showed that GST-cJun1-79-bound JNK/SAPks were clearly capable of phosphorylating GST-Elk1,307-428, whereas the kinase(s) that bound to GST-Elk1,307-428 did not phosphorylate GST-cJun1-79 (Fig. 4BI, upper panel), further indication that two different kinases are involved. To confirm this, we also showed that immunoprecipitated JNK/SAPks (antibody kindly provided by Dr M. Karin, UCSF, San Diego) could clearly phosphorylate both GST-cJun1-79 and GST-Elk1,307-428 in these assays (Fig. 4BII, lower panel). Note that we observe some non-specific binding (discussed further below) of the EGF-activated kinases but not of the anisomycin-, okadaic acid- or UV-activated kinases to the affinity matrices used both for GST-fusion proteins (Fig. 4BI, lower panel) and for immunoprecipitation (Fig. 4BII, upper panel).

A potential complication here is that the bound kinases may have restricted access to subsequently added substrates. Attempts to circumvent this by washing bound kinases off these beads prior to assay failed because the kinases bound to GST-Elk1,307-428 and GST-cJun1-79 were very poorly released by a variety of washes (not shown). In particular for the GST-Elk1,307-428-bound kinases, the fraction released was negligible (not shown, see also Fig. 5C). Kinase buffer supplemented with ATP, which would allow phosphorylation of the substrate, did release some JNK/SAPk from its substrate (Hibi et al., 1993) but this represented a small fraction of bound kinase. Kinase buffer containing 1% TX-100 released approximately 20% of the bound JNK/SAPks, which when assayed against GST-Elk1,307-428 and GST-cJun1-79 in solution showed identical phosphorylation characteristics to that obtained on the beads. Due to the very low levels of kinase released from the GST-Elk1,307-428 beads by these procedures, a similar attempt to assay Elk-binding kinases in solution was not possible.

Identification of kinases bound to GST-Elk1,307-428

The above shows that kinase(s) bound to GST-Elk1,307-428 are strongly active under conditions where ERKs are known to be

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active, but not when ERKs are inactive, such as upon anisomycin or okadaic acid treatment (see Fig. 3). To identify these kinases, GST-Elk1\textsubscript{307-428}-bound proteins from EGF-stimulated cells were first analysed by in-gel kinase assays (Fig. 5A, lane 3). This revealed the presence of renaturable kinase activity only at a position on the gel corresponding to ERKs-1 and -2 (Cano et al., 1994). Note that the GST-cJun\textsubscript{1-79}-binding JNK/SAPKs, which are very slightly active at this timepoint (see Fig. 3), produced no signal (Fig. 5A, lane 4).

To confirm this, kinases bound to GST-cJun\textsubscript{1-79} and GST-Elk1\textsubscript{307-428} under various conditions of stimulation were western-blotted using anti-ERK antibodies (Fig. 5B, lanes 1-5). This showed unexpectedly that ERK-binding to GST-Elk1\textsubscript{307-428} was enhanced in cells in which these kinases were activated by EGF (Fig. 5B, lane 2); less binding is seen in control cells (lane 1), or in cells where these kinases are inactive (anisomycin, lane 3 and okadaic acid, lane 4; see Fig. 3A) or weakly active (UV radiation, lane 5). During these procedures, partial de-activation of ERKs in EGF-stimulated lysates is seen, giving rise to four bands in total lysates corresponding to active and inactive ERK-1 and -2 (compare Fig. 5B, lane 7 with Fig. 3A). The band of GST-Elk1\textsubscript{307-428}-bound immunoreactivity corresponded in position only to active ERK-2 (Fig. 5B, lane 2). Thus, GST-Elk1\textsubscript{307-428} shows clear preferential binding to active rather than inactive ERKs, and there also seems to be some selection for ERK-2 over ERK-1.

Note that there is a small amount of inactive ERK-2 bound to the fusion protein in these experiments (Fig. 5B, lanes 1-5). Because there is some non-specific binding of ERKs to our immuno- and GST-matrices (see Fig. 4B), we tested various washing protocols, and found that with additional washing, GST-Elk1\textsubscript{307-428}-bound ERKs are observed only from lysates of stimulated cells and not from controls (Fig. 5C, lanes 1-12). Again, the position of the GST-Elk1\textsubscript{307-428}-bound kinase cor-

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**Fig. 4.** Analysis of kinase activities that bind to GST, GST-Elk1\textsubscript{307-428} and GST-cJun\textsubscript{1-79}. (A) WCEs from unstimulated (control) cells or from cells stimulated with anisomycin (An; 10 \( \mu \)g/ml) for 1 hour, or EGF (50 ng/ml) for 5 minutes, were mixed with GSH-agarose containing either GST (lanes 1-3 and 7-8), GST-Elk1\textsubscript{307-428} (lanes 4-6) or GST-cJun\textsubscript{1-79} (lanes 9-10). Kinases binding specifically to these fusion proteins were then incubated with additional GSH-agarose bound substrates as follows: GST-Elk1\textsubscript{307-428} (lanes 1-3); GST- (lanes 4-6 and 9-10); GST-cJun1\textsubscript{1-79} (lanes 7-8). Kinase assays were then performed as described in Materials and Methods. Positions of fusion proteins, as indicated by Coomassie Blue staining, are arrowed. (B) Cells were treated as follows: unstimulated, control (lane 1) or stimulated with EGF (50 ng/ml), 5 minutes (lane 2); anisomycin (An; 10 \( \mu \)g/ml), 1 hour (lane 3); okadaic acid (OA; 500 nM), 1 hour (lane 4); and UV (400 J/m\(^2\)), 1 hour (lane 5). I. WCEs were mixed with different GST-fusion protein-GSH agarose beads as indicated in each panel, after washing (see Materials and Methods), bound kinases were supplemented with a mixture of GST-Elk1\textsubscript{307-428}, GST-cJun\textsubscript{1-79} and GST-GSH agarose as substrates for subsequent kinase assays (see Materials and Methods). The positions of these proteins are indicated. II. Phosphorylation of GST-fusion proteins by JNK activity. Cell lysates were immunoprecipitated with normal rabbit antiserum (NRS) or with an antiserum raised against JNK (kindly provided by M. Karin). The immunoprecipitates were supplemented with a mixture of GST-Elk1\textsubscript{307-428}, GST-cJun1\textsubscript{1-79} and GST-GSH-agarose beads as substrates and resuspended in 50 \( \mu \)l of kinase assay buffer containing 20 \( \mu \)M cold ATP plus 5 \( \mu \)Ci [\( \gamma \)-\( \text{32} \)P]ATP. Positions of fusion proteins, as indicated by Coomassie Blue staining, are arrowed.
responds only to active ERK-2 (Fig. 5C, single band in lanes 2, 4, 6, 8, and 10), although, due to the more protracted procedures post-lysis, there is both active and inactive kinase (four bands, Fig. 5C, lane 14) in the lysates. Longer exposures of these gels show trace amounts of active ERK-1 also bound, but a complete absence of inactive enzyme (not shown). Thus, GST-Elk1<sub>307-428</sub> binds preferentially to active rather than inactive ERKs and also shows preferential binding to ERK-2.

Incubation of these complexes with 60 μM ATP, which would allow phosphorylation of GST-Elk1<sub>307-428</sub>, does not release the kinase (Fig. 5C, lanes 9-12). Dephosphorylation and inactivation of ERK-2 may be essential for release, a possibility testable using the CL100/3CH134 phosphatase in vitro.

Although ERK-2/GST-Elk1<sub>307-428</sub> complexes described here are stable at 4°C (at which temperature these experiments were done) they become unstable at room temperature (M. Price and R. Treisman, ICRF, Lincoln’s Inn Fields, London; personal communication; E.C and L.C.M, not shown). This raises the question of whether selective stable binding of active ERK-2 to Elk-1 occurs in intact cells, a problem exceedingly difficult to address, both in the context of the highly constrained intranuclear chromatin milieu and in view of the speed with which these events occur. It is also unclear if it is the on/off rate of ERK kinases on Elk-1 that is of significance, rather than any stable binding. However, the in vitro conditions used here permit the clear observation of this unique specificity, indicating that dual-phosphorylated active ERKs, more especially active ERK-2, exists in a conformation that favours its interaction with Elk-1.

**DISCUSSION**

By comparing responses to EGF, TPA, anisomycin, okadaic acid and UV irradiation in C3H 10T<sub>1/2</sub> cells, we show here that there is no strict correlation between ERK activation and c-fos induction nor between JNK/SAPK activation and c-jun induction. Despite differentially activating ERKs and JNK/SAPKs, all these agents induce c-fos and c-jun, and elicit a common subset of responses including S6 phosphorylation, and the phosphorylation of histone H3 and HMG-14 on nucleosomes (Barratt et al., 1994a,b). The observation of these phosphorylation events in response to UV irradiation is described here for the first time. We show that ERKs bind to GST-Elk1<sub>307-428</sub> and JNK/SAPKs to GST-cJun<sub>1-79</sub> with no cross-specificity, but the formal possibility, at least, of cross-talk is raised by the demonstration that JNK/SAPKs can phosphorylate GST-Elk1<sub>307-428</sub> in vitro. Finally, and unexpectedly, we find that binding of ERKs, particularly ERK-2, to GST-Elk1<sub>307-428</sub> is favoured when the kinase is in the active conformation, representing the first demon-

![Fig. 5. Characterization of GST-Elk1<sub>307-428</sub>-binding kinases.](image-url)
Stratification of this type of affinity between an active kinase and its substrate.

**Signalling to c-fos via the ERK and JNK/SAPK cascades**

The c-fos gene contains multiple upstream regulatory elements that are, with the exception of the c-fos SRE (Sadowski et al., 1993; Fu and Zhang, 1993), constitutively occupied by various transcription factors (Herrera et al., 1989). For the c-fos SRE, these include TCF and SRF, both of which can be phosphorylated by mitogen-activated kinases, the former by ERKs and the latter by pp90rsk/MAPKAP kinase-1, which lies downstream of the ERKs (reviewed by Treisman, 1994). The SRE and its associated proteins have been shown to be the target of multiple signalling pathways directed to c-fos (Graham and Gilman, 1991). Anisomycin does not activate ERKs or pp90rsk/MAPKAP kinase-1, and therefore cannot utilise either of these mechanisms to activate the c-fos gene (Blenis et al., 1991; Cano et al., 1994). Two possibilities arise. The first is that other anisomycin-activated MAP kinases, such as the JNK/SAPKs, and possibly other uncharacterised MAPKAP kinases downstream of JNK/SAPKs, will function in place of ERKs or pp90rsk/MAPKAP kinase-1 to phosphorylate TCF or SRF, in which regard we demonstrate that JNK/SAPKs phosphorylate GST-Elk1307-428 in vitro may be relevant. Whitmarsh et al. (1995) have recently provided evidence that supports this conclusion, but direct proof that JNK/SAPKs phosphorylate Elk1 in intact cells remains lacking. Although JNK/SAPKs do not bind to the GST-Elk1307-428 fusion protein used here, it is possible that they will bind to full-length Elk1. Proof that this is relevant will only come from detailed analysis of TCF phosphorylation and site utilisation in intact anisomycin-treated cells. The second possibility is that anisomycin may utilise a pathway entirely distinct from the ERK kinase–TCF route (Gille et al., 1992; Marais et al., 1993; Zinck et al., 1993; reviewed by Treisman, 1994). A point that weighs against other genetic elements being involved is that Subramaniam et al. (1989) have shown that cycloheximide, which shows similar but weaker signalling responses to anisomycin (Mahadevan and Edwards, 1991; Edwards and Mahadevan, 1992) will activate a reporter gene driven only by a c-fos SRE, indicating that these compounds can induce c-fos via the TCF/ERK route. We have also tested the role of SIE and SIF in controlling c-fos (Sadowski et al., 1993; Fu and Zhang, 1993) and found that neither EGF nor anisomycin utilises this route in these cells (not shown). A final consideration here is that anisomycin may also activate other kinases, such as the p38/RK subtype of MAP kinase (Han et al., 1994; Rouse et al., 1994) and MAPKAP-kinase 2, all of which represent potential alternative routes to SRF or TCF phosphorylation.

**Activation-dependent binding of ERKs to TCFs**

In the course of these studies, we found that GST-Elk1307-428 binds selectively to active rather than inactive ERKs, and further that ERK-2 is preferred over ERK-1. The observation of preferential binding between individual members of a MAP kinase subfamily and their transcription factor substrate has also been reported for the JNK/SAPK subfamily, where JNK-2 binds preferentially to c-Jun compared to JNK-1 (Kallunki et al., 1994). Binding is extremely stable at 4°C; the kinases are resistant to being washed out with a variety of buffers. In fact, the enhanced activation-dependent binding of ERKs is predicted from the recently-reported atomic structure of ERK-2. This showed that Thr-183 and Tyr-185 form part of the linker LI2 region comprising a ‘phosphorylation lip’ that overhangs the ATP-binding and catalytic cleft of the kinase, such that Thr-183 faces outwards whereas Tyr-185 faces the catalytic site (Zhang et al., 1994). These authors suggest that the lip occludes substrate binding, and predicted that phosphorylation of these sites, particularly Tyr-185, might cause a conformational change that allowed enhanced substrate–interaction and consequent phosphorylation (Zhang et al., 1994). The observation of kinases, and more specifically ERKs, bound to TCFs has been previously reported (Zinck et al., 1993; Rao and Reddy, 1994), but this represents the first demonstration of enhanced binding of active ERK-2 to Elk1, providing biochemical evidence supporting predictions made on the basis of the structural studies of ERK-2 (Zhang et al., 1994).

**Signalling to c-jun by JNK/SAPK-dependent and -independent routes**

In contrast to c-fos, for which the upstream transcription factors are the target of multiple signalling systems, signalling to c-jun has mostly been studied with respect to upstream AP-1/TRE sites constitutively occupied by c-Jun-containing complexes (Angel et al., 1988; Han et al., 1992; Rozek and Pfeifer, 1993), possibly with ATF-2 as its partner (van Dam et al., 1995; Livinstonstone et al., 1995). JNK/SAPKs are proposed to bind to c-Jun via the ß-domain, phosphorylating it on serines 63 and 73 (Kyrkakis et al., 1994; Hibi et al., 1993; Derijard et al., 1994). It is a particular irony that JNK/SAPKs are widely proposed to be targeted to c-Jun (Kyrkakis et al., 1994; Hibi et al., 1993; Derijard et al., 1994) and/or ATF-2 (van Dam et al., 1995; Livinstonstone et al., 1995) on this TPA-responsive element, whereas TPA is the only agent tested here that does not activate JNK/SAPKs. There is some generality here, as TPA does not activate JNK/SAPKs in several other cell lines including NIH 3T3, although it does in JURKAT cells (Su et al., 1994). TPA also does not activate the p38/RK subtype of MAP kinases in some cell types (Han et al., 1994). Thus, the ERKs are the only MAP kinase subtype clearly activated by TPA, but its role in TPA-stimulated c-jun induction remains unknown.

**The relevance of MAP kinase cascades to IE gene induction and histone H3/HMG-14 phosphorylation**

It is important to stress that no evidence is presented, nor the claim made here, that ERKs and JNK/SAPKs are not involved in c-fos and c-jun induction (Pulverer et al., 1991; Smeal et al., 1991; Gille et al., 1992; Marais et al., 1993; Zinck et al., 1993; Kyrkakis et al., 1994; Hibi et al., 1993; Derijard et al., 1994; Deng and Karin, 1994). On the contrary, the fact that JNK/SAPKs specifically bind and phosphorylate c-Jun, whereas ERKs, particularly ERK-2, bind and phosphorylate TCFs, strongly implicates these kinases in signalling to c-fos and c-jun. What is shown here is that neither subtype is indispensable for signalling to these genes, which does raise the possibility that there may exist other unknown kinases invariably activated by every one of these agents which are essential for IE gene induction. Alternatively, it may be that although all these agents do activate some responses in common, they
utilise distinct uncharacterised kinase cascades, transcription factors and/or regulatory elements to activate c-fos and c-jun. We have presented data for at least three kinases, p70/85 S6k, histone H3 kinase and HMG-14 kinase, that are activated in common by every one of the agents described here, irrespective of which MAP kinase cascade is active. We have also described two EGF- and anisomycin-activated kinases p45 and p55 whose detection by in-gel kinase assays is enhanced in the presence of poly-glu/tyr or poly-glu/phe; although similar to JNK/SAPKs in some respects (discussed by Cano et al., 1994), present evidence indicates that p45 and p55 are distinct from JNK/SAPKs (to be published elsewhere). The list of kinases invariably associated with c-fos/c-jun induction may be restricted still further, as the use of rapamycin to ablate p70/85 S6k has no effect on c-fos/c-jun induction (Kardalinou et al., 1994; Cano et al., 1994). Taking all the data into account, this leaves the nucleosomal kinases, phosphorylating histone H3 and HMG-14, as the only ones at present invariably associated with IE gene induction. Further, the nucleosomal response is not a consequence of gene activation, as it occurs even when transcription is ablated with α-amanitin or actinomycin D (Mahadevan et al., 1991; Edwards and Mahadevan, 1992; Barratt et al., 1994b). One model would be that irrespective of which MAP kinase cascade is involved in getting the signal to regulatory elements upstream of c-fos and c-jun, the IE gene activation that follows requires H3 and HMG-14 phosphorylation as an essential prerequisite.

We are greatly indebted to Dr M. Price and Dr R. Treisman for kindly providing the GST-Elk1[378-428] fusion construct, and for many helpful discussions, particularly for information on temperature-dependent ERK/Elk-1 binding stabilities prior to publication. We thank Dr Michael Karin and members of his laboratory for the kind gift of anti-JNK antibodies and GST-c-Jun1-79 plasmid used in this work, others in our laboratory for helpful discussions and Dr Michael J Barratt for criticisms of this paper. This work is funded by the Cancer Research Campaign, Wellcome Trust, and BBSRC.

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(Received 25 July 1995 - Accepted 6 September 1995)