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UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase

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Irradiation of cells with ultraviolet light (UV) leads to modifications of c-Jun resembling those elicited by phorbol esters or oncogenes, and to enhanced transcription of AP-1-dependent genes. The UV-induced signal also triggers activation of Raf-1 and MAP-2 kinases. A dominant-negative Raf-1 kinase mutant strongly interferes with both phorbol ester and UV-induced AP-1 activation, indicating obligatory involvement of identical components in cytoplasmic signal transduction. Thus, from a presumably nuclear site of energy absorption, a signal needs to be transmitted to the cytoplasm in order to achieve activation of a nuclear transcription factor. Further, signals elicited from different primary sites merge prior to or at the level of activation of Raf-1 kinase.

Key words: AP-1/MAP-2 kinase/Raf-1 kinase/transcription factor/UV light

Introduction

The transcription factor AP-1 (Jun/Fos) represents the prototype of a regulatory protein that converts extracellular signals into changes in the program of gene expression (reviewed by Angel and Karin, 1991). AP-1-dependent promoters are rapidly activated within a few minutes in response to serum growth factors or phorbol esters. This immediate induction also occurs in the presence of an inhibitor of protein synthesis, demonstrating that the immediate activation of AP-1 is due to post-translational modification. Further, the synthesis of AP-1 subunits, e.g. of c-Fos and c-Jun (Curran and Franza, 1988), is induced ensuring prolonged expression of target genes. Prior to an activating modification, both subunits of the prototype AP-1, Fos and Jun, carry already modifications: phosphate (Curran and Teich, 1982; Curran *et al.*, 1987; Müller *et al.*, 1987; Boyle *et al.*, 1991) and glycosyl groups (Jackson and Tjian, 1988). Upon treatment with phorbol ester or overexpression of oncogenes, the phosphorylation pattern of c-Jun is changed: phosphorylation of the DNA binding domain on Thr231, Ser243 and Ser249 is decreased resulting in an enhanced DNA binding activity (Boyle *et al.*, 1991; Lin *et al.*, 1992). In contrast, phosphorylation on Ser63 and Ser73 located in the transactivation domain, is enhanced (Binétruy *et al.*, 1991; Pulverer *et al.*, 1991). Phosphates at both positions, 63 and 73, appear necessary for full transactivation.

Treatment of cells with DNA damaging agents, e.g. UV irradiation, alkylating agents or bulky adduct formers, causes massive regulatory changes that by and large mimic the proliferative response induced by phorbol esters or growth factors (Herrlich *et al.*, 1992). For UV and O^6-guanine-DNA alkylation it has been shown that the lesions in DNA are required in order to induce transcription of a number of target genes: in cells deficient for the repair of pyrimidine dimers or O^6-alkylguanine in DNA, gene expression is induced by lower doses of the agents than in proficient cells (Miskin and Ben-Ishai, 1981; Schorpp *et al.*, 1984; Mai *et al.*, 1989; Stein *et al.*, 1989a) indicating that the difference in lesion density reached after several hours of repair translates into a difference in enhancer/promoter activity. Despite the fact that the slow repair kinetics do not prove DNA damage as an intermediate in very early events, we consider it likely that the initial steps also depend on DNA damage.

By examining target promoters it has been shown that members of the AP-1 family mediate part of the DNA damage (or UV) response (Stein *et al.*, 1989a; Holbrook and Fornace, 1991). The UV response is, however, not restricted to the nucleus: NFκB, whose activity is also greatly enhanced in response to UV, is activated from its cytoplasmic location (Stein *et al.*, 1989a). This prompted us to examine the signal transduction pathway(s) elicited by UV and received by the nuclear transcription factor AP-1. We show here that phorbol esters and UV cause similar post-translational modifications of AP-1 within minutes. We also demonstrate that UV- and phorbol ester-induced signal transduction depends on the presence of Raf-1 kinase, thus indicating common pathways at the level of and downstream of Raf-1 activation. Furthermore, UV leads to immediate activation of MAP-2 kinase, similarly to phorbol ester. Hence we provide evidence that UV causes AP-1 activation via cytoplasmic components.

Results

Rapid post-translational activation of pre-existing AP-1

UV induces the transcription of a number of cellular genes as well as promoter constructs carrying single or multiple sites for a specific transcription factor. For some of these genes transcription occurs in the absence of protein synthesis, indicating that transcription factors are direct targets of an UV-induced signal transduction cascade and that even long-term induction of these promoters is achieved by immediate changes of pre-existing transcription factors by post-translational modification. The transcription factor AP-1 is such a direct target. The activity state of the heterodimer Fos-Jun or of the homodimer Jun-Jun, the main components of AP-1, is reflected by transcription from

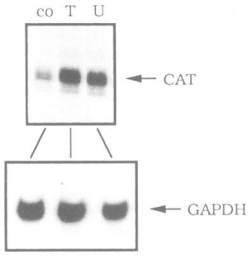

Fig. 1. Rapid induction of AP-1 dependent transcription. SP6 RNase protection analysis of specific RNA from HeLa *tk*$^-$ cells harvested at 30 min after treatment with either 60 ng/ml TPA (T) or 30 J/m^2 UV (256 nm) (U). The specific RNA tested for is transcribed from the transiently transfected 5 × TRE-TATA−CAT construct. 40 μg of CsCl-purified total RNA were hybridized to 1 × 10^6 c.p.m. of coll−CAT−SP6 riboprobe. The amounts of RNA were controlled by Northern RNA transfer hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as probe (Fort *et al.*, 1985). Lanes were scanned and induction factors were calculated after correction for the amount of GAPDH mRNA (UV, 5.2-fold; TPA, 3-fold).

promoters containing the AP-1 binding site [TPA (12-*O*-tetradecanoylphorbol-13-acetate) response element, TRE; Angel *et al.*, 1987a, 1988a; Bohmann *et al.*, 1987]. We have measured the characteristics of UV- and TPA-induced activation of AP-1 by RNase protection analysis of transcripts from the 5 × TRE−CAT construct (Figure 1). AP-1 activity is low in serum-starved cells, as shown by the relatively poor transcription of the transiently transfected gene construct. Rapid activation of transcription (and of AP-1 activity) is induced by either serum (not shown), phorbol ester or ultraviolet irradiation within minutes, as shown by the accumulation of CAT reporter mRNA in the first 30 min after treatment (Figure 1). Previous run-on analyses had shown full transcriptional activity 15 min after stimulation (König *et al.*, 1989; data not shown). The time-course of induction resembles that of the dyad symmetry element (DSE) factor-dependent activation of the c-*fos* promoter (Büscher *et al.*, 1988). Results qualitatively similar to the 5 × TRE−CAT construct have been obtained with the collagenase promoter −73/+63 construct (Angel *et al.*, 1987b), which carries only one AP-1 binding site. The efficiency of transcription, however, is lower than with the 5 × TRE−CAT construct. Although the agents also induce the synthesis of Fos and Jun proteins at later time points, the immediate early activation can only be explained by post-translational modification of preformed Fos and/or Jun proteins.

Post-translational modification as the activating mechanism has been confirmed by two types of experiments: (i) potent inhibitors of protein synthesis, such as anisomycin or cycloheximide, do not inhibit the activation process (not shown); (ii) chimeric Jun−Gal4 proteins composed of the transactivation domain of Jun and the DNA binding domain of the yeast transcription factor Gal4 are able to stimulate transcription of reporter plasmids containing two Gal4 binding sites. This reporter plasmid does not contain AP-1 binding sites and its transcription in the absence of an appropriate Gal4−factor is therefore neither affected by phorbol ester nor by UV (Figure 2A, control experiment with a construct of Gal4 lacking the transactivation domain of Jun). In contrast, transactivation by the Jun−Gal4 hybrid is increased by both phorbol ester or UV 2- to 3-fold (Figure 2A). As shown below, this effect is specific since the activity of other transactivation domains, e.g. from growth hormone factor 1 (GHF1, Figure 2B; Theill *et al.*, 1989) or VP16 (data not shown; Webster *et al.*, 1988) is not affected. The activity of the RSV-LTR that drives the expression of Jun−Gal4 is not significantly affected by either stimulus (not shown). Since neither preexisting nor newly synthesized AP-1 can work on the reporter or dimerize with the exogenously expressed Jun−Gal4 chimeric proteins, the data prove that Jun is activated by post-translational modification.

Induced modification of Jun within < 15 min

A significant increase in transcriptional rate within 15−30 minutes after phorbol ester or UV treatment, as shown in Figure 1, is expected to depend on preceding changes in modification of AP-1. Since Jun−Gal4 responds to UV-induced signalling (Jun apparently not requiring other heterodimer partners), we have concentrated on the post-translational modification of Jun protein. The Jun protein has been shown to be phosphorylated (Curran *et al.*, 1987; Boyle *et al.*, 1991). Therefore, we have measured changes in modification of pre-existing Jun as early as 15 min after induction. HeLa cells were pre-labeled with either [^{35}S]-methionine or [^{32}P]orthophosphate for 3 h to label all of the pre-existing Jun at the time-point of induction of the cells with either phorbol ester or UV. An increase in apparent molecular weight, particularly after treatment of cells with UV, is complete at 15 min both with cell extracts labeled with [^{35}S]methionine (Figure 3A) or ^{32}P (not shown, but see data for exogenously expressed Jun in Figure 3B) demonstrating that the bulk of Jun proteins undergoes rapid post-translational modification. The dramatic changes could not be observed after treatment of cells with phorbol ester although the efficiencies of transcriptional activation of AP-1-dependent reporter plasmids were similar. It thus appears that although UV and phorbol ester are similarly effective in activating AP-1, UV induces more dramatic modifications of the Jun protein in these cells. Similar data have been obtained with the human fibroblast cell line GM637 (not shown) and F9 teratocarcinoma cells indicating that the rapid post-translational modification represents a general mechanism. In F9 cells Jun is not expressed spontaneously. Modification is seen upon transient transfection with a Jun expression clone (Figure 3B), demonstrating that also exogenous Jun can be efficiently modified.

Similar phosphopeptide patterns induced in c-Jun by UV and phorbol ester

Jun protein from [^{32}P]orthophosphate labeled HeLa cells resolved by one-dimensional PAGE (identical to the resolutions shown in Figure 3A and B, and marked by arrows as 1 and 2), was examined for phosphorylation changes of specific amino acid residues by two-dimensional tryptic phosphopeptide analysis (Figure 3C). In Jun from unstimulated HeLa cells, mono-, di- and triple phosphorylation of a peptide in the DNA binding domain (Thr231, Ser243, Ser249) (Boyle *et al.*, 1991; Lin *et al.*, 1992), is visible, represented by spots c, b and a, respectively. In addition, one phosphorylated peptide, X, representing phosphorylation of Ser73, is resolved. This serine is located in the transactivation domain (Angel *et al.*, 1989; Binétruy *et al.*, 1991; Pulverer *et al.*, 1991). Both phorbol ester and UV cause the reduction of the peptide spot a. The disappearance of the 'a' spot in phorbol ester treated cells (Boyle *et al.*, 1991) and in Ras-transformed 3T3 cells (Binétruy *et al.*, 1991; Smeal *et al.*, 1992) has been

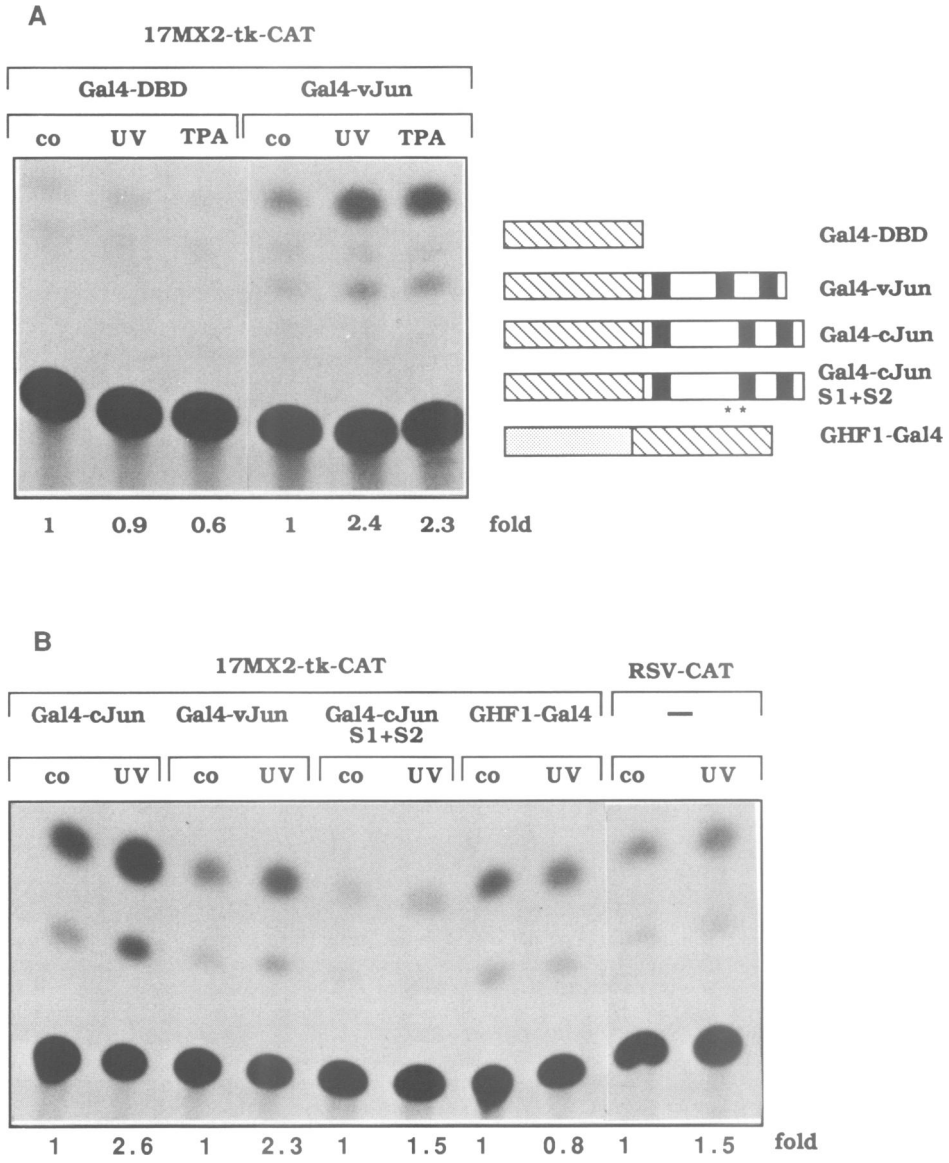

Fig. 2. A. Both UV and TPA induce the transactivation function of Jun. HeLa tk^- cells were grown and cotransfected with 8 μg of expression vectors carrying either the Gal4 DNA binding domain (lacking the transactivation domain of v-*jun*; Gal4-DBD) or the Gal4 DNA binding domain fused to the transactivation domain (Gal4−v-Jun), together with 4 μg of Gal4 reporter plasmid 17MX2-tk−CAT. 6 h after transfection the cells were treated with UV (30 J/m^2) or TPA (80 ng/ml) or left untreated (co) and CAT activity was determined 36 h later. On the right-hand side, the schematic organization of the various transactivator proteins is shown. Hatched boxes, DNA binding domain of Gal4; black boxes, transactivation domain of Jun; dotted box, transactivation domain of GHF1; the location of Ser63 and Ser73 mutated in Gal4−cJun S1 + S2 is marked by asterisks. **B.** UV-induced transactivation by Jun depends on Ser63 and Ser73. F9 teratocarcinoma cells were cotransfected with 0.5 μg of the expression vectors indicated together with 2 μg of the Gal4-dependent reporter plasmid 17MX2-tk−CAT. Other cultures received the reporter RSV−CAT (2 μg). 13 h after transfection, the cells were treated with UV (30 J/m^2) or left untreated (co) and CAT activity was determined 16 h later. The experiment shown has been repeated three times with essentially identical results. The difference in the induction factor between Jun wild type and the Jun double mutant is significant at the 5% level.

described previously and has been proposed to cause an increase in DNA binding. Both UV and phorbol ester cause hyperphosphorylation of the X peptide (Figure 3C), which resembles that seen in cells overexpressing either Ras, Sis, Src or Raf-1 (Binétruy *et al.*, 1991; Smeal *et al.*, 1992). Hyperphosphorylation in the transactivation domain appears to relate to the Ras (or phorbol ester-) dependent increase of AP-1-dependent transcription (Franklin *et al.*, 1992). The most retarded band of Jun that occurs predominantly after UV irradiation carries increased phosphate label in X and additional phosphate groups, the most prominent ones of which are located on the Y and V peptides (Figure 3C). Y and V may be different phosphorylation forms of the same tryptic peptide. Y has been mapped to Ser63 (Binétruy *et al.*, 1991). We have mapped the V spot tentatively to either Thr62 or Ser58 (data not shown). Similar data as obtained for the endogenous Jun in HeLa cells (Figure 3C), were obtained for transiently transfected c-Jun in GM637 or F9 cells (not shown). Jun proteins mutated in Ser63 and Ser73 neither become modified to the slow migrating form in the one-dimensional PAGE resolution (Figure 3B) nor generate phosphorylation of peptides X, Y and V after UV treatment (data not shown). One-dimensional resolution as a slow migrating peptide and phosphorylation appear to go parallel.

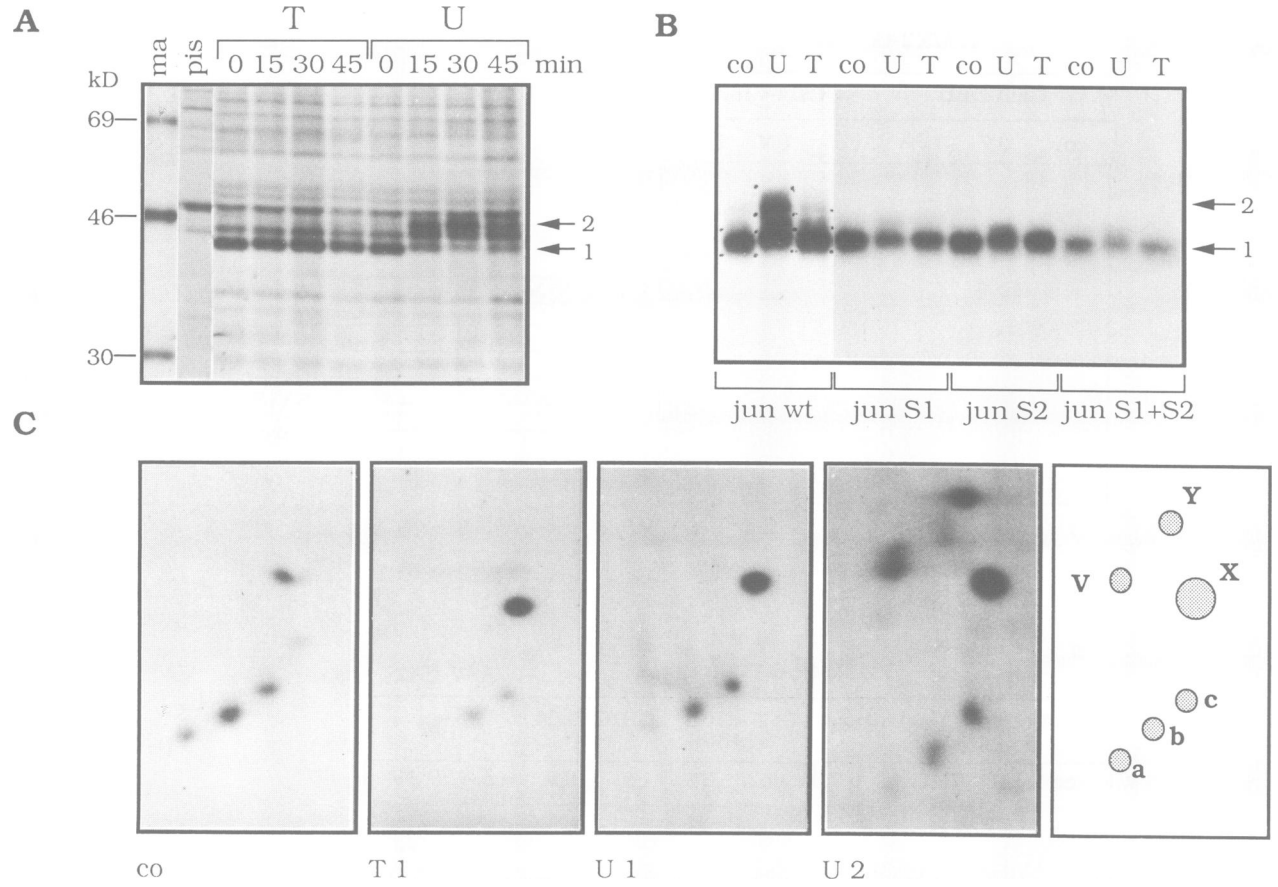

Fig. 3. Rapid posttranslational modification of Jun in response to TPA or UV. (**A**) HeLa *tk*$^-$ cells were labelled with [^{35}S]methionine and treated with TPA (T) or UV (U). c-Jun was immunoprecipitated with antibody 891 at various times after treatment as indicated, followed by SDS gel electrophoresis (SDS−PAGE) and fluorography. ma, ^{14}C-labelled marker proteins; pis, preimmune serum; arrows indicate non-shifted and shifted Jun proteins. (**B**) F9 mouse teratocarcinoma cells were transfected with 10 μg of c-*jun* wild type or mutant expression vectors per plate. Cells were labelled with [^{32}P]orthophosphate, treated with UV (U) or TPA (T) or left untreated (co), and Jun proteins were immunoprecipitated from cell extracts prepared 20 min after treatment, followed by SDS−PAGE. Lanes 1−3, RSV−c-*jun* wild type (jun wt); lanes 4−6, RSV−c-*jun* with ser → leu mutation at position 63 (jun S1); lanes 7−9, RSV−c-Jun with ser → leu mutation at position 73 (jun S2); lanes 10−12, RSV−c-Jun with leu at both positions, 63 and 73 (jun S1 + S2). (**C**) HeLa *tk*$^-$ cells were labelled with [^{32}P]orthophosphate and c-Jun was isolated by immunoprecipitation from cell extracts harvested at 20 min after stimulation with TPA (T) or UV (U). Jun was resolved by SDS−PAGE as shown in A and B, and bands migrating to the positions shown by arrows were further analysed separately (T1, U1, U2). Two-dimensional tryptic phosphopeptide mapping was performed with Jun from untreated (co) and treated cells (~200 c.p.m. per plate). ^{32}P-labelled peptides were visualized by autoradiography for 14 days with an intensifying screen at −70°C. A schematic representation of the phosphopeptides is also shown.

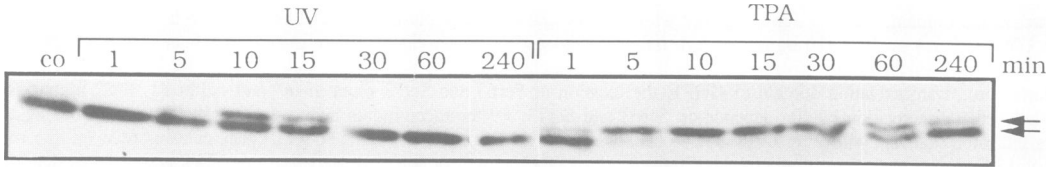

Fig. 4. UV- and TPA-induced MAP-2 kinase activation. Cell lysates were prepared from HeLa *tk*$^-$ cells at various times after either UV irradiation (30 J/m^2) or treatment with TPA (60 ng/ml) and from untreated cells (co). 30 μg of total cell lysate per lane were resolved on 10% SDS−PAGE, transferred to immobilon membrane and probed with rabbit antibodies directed against MAP-2 kinase. Arrows indicate the inactive (lower) and active (upper band) forms of MAP-2 kinase.

It is not ruled out, however, that another type of modification participates in forming the slow migrating form of c-Jun.

Our pre-label conditions clearly demonstrate that all these changes in phosphorylation are the result of direct post-translational modification immediately after the external stimulus. The data show that the signal transduction to Jun elicited by UV and phorbol ester is largely identical when reaching Jun. Although the V spot has not been described previously, it is visible in a previous publication using phorbol ester as the inducing agent (Pulverer *et al.*, 1991). Thus, the more dramatic shift to slower migration of Jun after UV that parallels the additional phosphorylation(s), may be the result of the strength of stimulation in a given cell. At least in HeLa, F9 and GM637 cells, UV appears to be a more potent modifier of c-Jun than phorbol ester.

Jun mutants in Ser63 and Ser73 cannot be activated by UV

In order to prove that the UV-induced modifications in the transactivation domain are relevant for the activation by UV,

we compared a wild type Jun−Gal4 fusion protein with an identical fusion of the Jun double mutant in Ser63 and Ser73. Figure 2B shows that for the transfection of the respective gene constructs into F9 cells, the double mutant Gal4−cJun S1 + S2 is barely UV inducible, while the wild type Gal4−Jun constructs respond to UV. The specific action of UV on c-Jun is also shown by the fact that other chimeric transcription factors carrying the transactivating domain of GHF1 (GHF1−Gal4; Figure 2B) or VP16 (Gal4−VP16, data not shown) do not respond to UV. Also, the RSV promoter (RSV-CAT) is not significantly activated by UV, demonstrating that the UV-induced transcription of the reporter carrying the Gal4 binding sites is not due to the enhanced synthesis of the chimeric Jun−Gal4 protein, but to its post-translational modification. Thus, modifications at Ser63 and Ser73 are indeed the mechanism of Jun activation by UV.

UV-induced cytoplasmic phosphorylations

Although the final steps of Jun phosphorylation/dephosphorylation appear to be largely identical between UV and phorbol ester treated cells, UV-induced Jun modification could still be a solely intranuclear event: e.g. the Jun modifying enzymes could be stimulated directly by a DNA damage-triggered 'signal', while after phorbol ester treatment the decisive stimulus to the modifying enzymes must come from outside the nucleus. From the activation of NFκB by UV (Stein *et al.*, 1989a) we expect, however, that DNA damage-induced signalling passes through the cytoplasmic compartments. We therefore explored whether putative cytoplasmic signalling components involved in the phorbol ester-dependent activation of Jun would also be affected shortly after UV treatment of cells.

MAP-2 kinase. From the comparison of phorbol ester-induced c-Jun phosphorylation *in vivo* with MAP-2 kinase action on bacterially expressed or *in vitro* translated c-Jun, it has been inferred that MAP-2 kinase could be involved in signal transduction to Jun (Pulverer *et al.*, 1991). Figure 4 shows that in HeLa cells, MAP-2 kinase is transiently modified in response to both phorbol ester and UV, as shown by the appearance of a slow migrating MAP-2 kinase band in Western analysis. The modification occurs within 10 min and disappears rapidly, while phorbol ester induced MAP-2 kinase modification persists longer (Figure 4). UV-induced MAP-2 kinase activation is also observed in primary human fibroblasts and in F9 cells (data not shown). The modification of MAP-2 kinase required for its activation (and reduced migration in SDS−polyacrylamide gels, Figure 4), has been shown to be due to phosphorylation at serine/threonine and tyrosine residues (Anderson *et al.*, 1990; Ahn *et al.*, 1991; Payne *et al.*, 1991). This suggested that either, in addition to a protein serine/threonine kinase, at least one protein tyrosine kinase or a dual specificity protein kinase (Ahn *et al.*, 1991) would be activated upon UV irradiation.

Requirement for Raf kinase in the UV response. An important protein kinase involved in the response to growth factors and phorbol ester, which was proposed to affect MAP-2 kinase activity, is Raf-1 kinase (Morrison *et al.*, 1989; Rapp, 1991; Howe *et al.*, 1992). To determine whether Raf-1 kinase activation is essential in the UV-induced activation of AP-1, AP-1-specific transactivation by UV (and phorbol ester) was measured in the presence of a '*trans*-dominant negative' mutant of Raf-1 kinase, Raf-C4 (Bruder *et al.*, 1992). This mutant blocks specifically endogenous Raf-1 activation. Cotransfection of an expression vector encoding the dominant-negative mutant into HeLa cells interferes with the UV as well as phorbol ester dependent transcription of the collagenase (−300/+63) promoter−CAT reporter (Table I, panel A). Comparing cells that have been transfected with cells that have received the empty control expression vector and with cells that have been transfected with an expression construct encoding Raf-1 wild type, Raf-1 enhances basal level expression as well as UV-induced and phorbol ester-induced transcription. Raf-C4 interferes with basal, UV- and phorbol ester-induced transcription. The Raf mutant appears to affect induced transcription more efficiently than basal transcription. The effect on basal transcription is most likely due to interference with serum factor-induced signal transduction. In HeLa cells even reduced concentrations of serum cause constantly elevated levels of active AP-1 (as compared with primary cells, data not shown). The induction of the collagenase −300/+63 promoter-CAT reporter by UV and TPA critically depends on the presence of an intact AP-1 binding site since collagenase promoter gene constructs lacking this site are not inducible (Stein *et al.*, 1989a; Table I, panel D). Also the expression of a reporter plasmid carrying multiple AP-1 binding sites fused to TATA and the CAT gene was repressed by the dominant negative c-Raf mutant (Table I, panel B).

Table I. A dominant-negative mutant of Raf-1 kinase inhibits UV- and TPA-induced transcription of AP-1 dependent reporter genes

Expression vectors (reporter plasmids)	co	TPA (-fold)	UV (-fold)
A (−300/+63coll−CAT)			
kRSPA	56	19950 (356)	746 (13)
cRaf-1	109	33900 (311)	2088 (19)
cRaf-C4	26	4462 (171)	67 (3)
B (5×TRE-TATA−CAT)			
kRSPA	1050	51300 (49)	11025 (11)
cRaf-1	3375	73500 (22)	34125 (10)
cRaf-C4	285	10425 (37)	2925 (10)
C (−517/−42colltkCAT)	210	19900 (95)	570 (2.7)
D (−517/−42colltkCAT−mTRE)	150	120 (0.8)	130 (0.9)

HeLa tk^- cells were cotransfected with 5 μg of the reporter −300/+63 coll CAT (A), 5×TRE-TATA-CAT (B), −517/−42colltkCAT (C) or −517/−42colltkCAT-mTRE (D) and 10 μg of the expression vectors encoding either wild type c-Raf-1 or the dominant-negative mutant c-Raf-C4 or the empty vector kRSPA. −517/−42colltkCAT is −517/−42 (in pBLCAT2, Angel *et al.*, 1987b) in which pBLCAT2 has been changed into pBLCAT4 (Jonat *et al.*, 1990). In the gene construct −517/−42colltkCAT−mTRE the AP-1 binding site (−74 ATG AGT CAG −66) was mutated to the sequence −74 AGT ACT CAG −66. The effect of a mutation in the AP-1 binding site was tested in the thymidine kinase promoter background in order to see at least basal level transcription with the mutant. The AP-1 site is absolutely required for any induction of the collagenase promoter. Transfected cells were treated with 30 J/m^2 UV, 60 ng/ml TPA or left untreated (co). Cells were harvested 48 h (A and B) or 25 h (C and D) after treatment. CAT activity is shown (pmol acetylated chloramphenicol/mg protein × h) and induction factors are indicated in parentheses. The experiment shown has been repeated twice with essentially identical results.

In conclusion, these results demonstrate that Raf-1 kinase

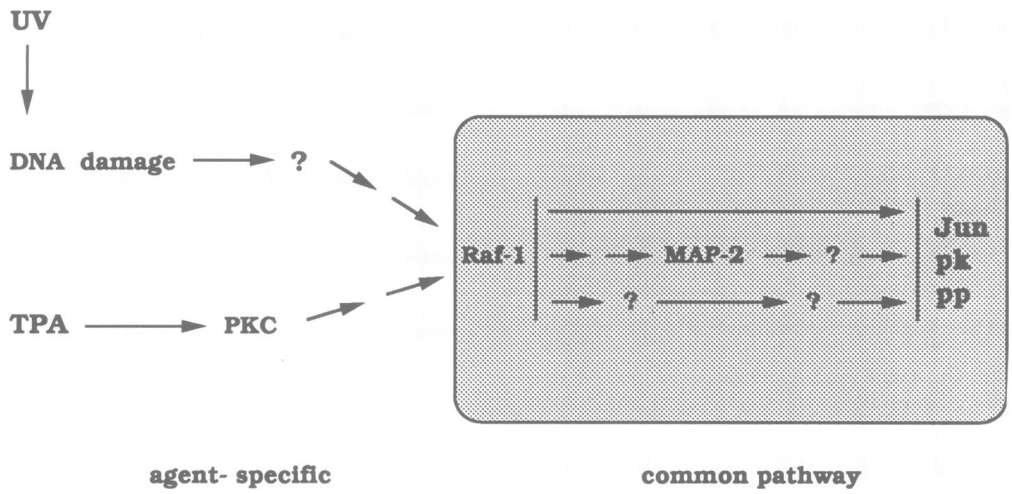

Fig. 5. Scheme of UV-induced Jun activation. pk, protein kinases; pp, protein phosphatases.

is required for UV- and phorbol ester-induced signal transduction to AP-1. UV-induced signalling must pass through a cytoplasmic protein kinase cascade in order to elicit changes in a nuclear transcription factor. The signal transduction pathways elicited by either UV or phorbol ester must have merged at or prior to Raf-1 kinase.

Discussion

The UV response involves a large number of changes in macromolecule synthesis. One of the earliest changes is the elevated rate of c-*jun* transcription (Krämer *et al.*, 1990; Devary *et al.*, 1991; Stein *et al.*, 1992). Even prior to c-*jun* transcription, pre-existing Jun must be activated since Jun participates in its own promoter control (Angel *et al.*, 1988b) and both Jun- and AP-1-like binding sites in the promoter have been shown to mediate the UV response (Stein *et al.*, 1989b, 1992; van Dam *et al.*, 1990, 1993; Devary *et al.*, 1991). UV-induced Jun activation represents also the necessary initial step in the control of Jun (AP-1) dependent genes such as collagenase. In this paper we have addressed the question of the mechanism(s) responsible for UV induction of Jun activation. The decisive conclusion is that the UV-induced activation of Jun involves obligatory steps outside the nucleus, that is components of cytoplasmic signal transduction pathways to the nucleus. Raf-1 kinase serves an obligatory role. This is surprising since Raf-1 kinase is known to be activated by growth factors at a membrane-proximal site (Rapp, 1991). Further, UV activates MAP-2 kinase, which is known to involve phosphorylation of this protein at tyrosine. Obviously, UV elicits a signal transduction pathway as suggested in Figure 5. The UV signal feeds into the pathway that is known to transfer signals from the plasma membrane to the nucleus. Prior to the merging point UV induced signalling appears to differ from that elicited by phorbol esters or growth factors.

As has been shown earlier (Schorpp *et al.*, 1984; Stein *et al.*, 1989a) the primary event occurs in the nucleus where DNA absorbs UV energy and suffers from UV induced DNA lesions. The nuclear origin of signalling in the UV induction of genes is supported by the following arguments: in addition to UV, Jun (AP-1) is also activated by other carcinogenic agents such as alkylating agents, mitomycin C, 4-nitroquinoline-*N*-oxide, H_2O_2 and gamma irradiation (for references see Herrlich *et al.*, 1992). The common denominator of action is DNA damage. Further, AP-1 activation appears to correlate with DNA lesion density. In cells from patients with *Xeroderma pigmentosum* a much lower dose of UV is required to obtain a certain level of enhanced transcription of AP-1-dependent genes than in wild type human fibroblasts (Miskin and Ben-Ishai, 1981; Schorpp *et al.*, 1984; Mai *et al.*, 1989; Stein *et al.*, 1989a). Since repair time is required to generate a difference in DNA lesion density between *Xeroderma* and wild type cells, the data cannot strictly exclude additional primary events in the first few minutes of AP-1 activation. However, action spectra of UV-induced gene expression are also compatible with DNA as the primary target (Stein *et al.*, 1989a). Taking DNA damage as primary event, our data imply reverse flow of 'information' prior to the obligatory components of signal transduction in the cytoplasm detected in this paper. Such reverse flow of signals is not without precedence. Completion of DNA replication appears to signal to the rest of the cell that mitosis is to be entered (Hartwell and Weinert, 1989; Enoch and Nurse, 1991).

It is not clear at which location and by which mechanism UV activates Raf-1 kinase. PKC appears to have no essential role in the UV response, because cells that have been pretreated with phorbol esters and that are refractory to a second treatment with phorbol esters still respond to UV by the increased transcription of the immediate early genes c-*fos* and c-*jun* (Büscher *et al.*, 1988; Devary *et al.*, 1991; H.J.Rahmsdorf, unpublished observations).

Raf-1 kinase is apparently an obligatory bottle neck shared by UV, phorbol ester and other growth factors (Rapp, 1991; Kyriakis *et al.*, 1992). As shown in Figure 5, Raf-1 kinase does supposedly act on MAP-2 kinase kinase, which in turn activates MAP-2 kinase (Howe *et al.*, 1992). Ultimately Jun is reached. Jun is both dephosphorylated (in the DNA binding domain) and phosphorylated (in the transactivation domain). Thus, at least two enzymes must be addressed by the UV-induced signal transduction pathway (see Figure 5). The experiments described here on one hand show that UV-induced AP-1 activation needs to pass through Raf-1, and on the other hand that UV causes both an enhancement and a decrease in the phosphorylation of Jun. It is therefore plausible to assume that the Raf-1-dependent pathway causes both types of changes of Jun. In Figure 5, we have not

specified the identity of the Jun modifying enzymes. Candidates are several protein kinases (Baker *et al.*, 1992). They range from a putative nuclear form of Raf-1 to the isoforms of MAP-2 kinase (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991), to casein kinase II (Lin *et al.*, 1992) and to a newly identified protein kinase of 65 kDa (Adler *et al.*, 1992). One of the targets of the protein kinase cascade will be a phosphatase that has not yet been identified and that removes phosphates in the DNA binding domain of Jun.

The merging of signal transduction at and beyond Raf-1 kinase does, however, not imply that the programs of genes addressed are strictly identical. Although the modifications elicited at Jun are largely identical between UV induction and treatment with phorbol ester, there is clearly a difference in magnitude. At present, we do not know whether TPA and UV affect all possible modifiers of Jun in a similar manner. The strong hyperphosphorylation of c-Jun by UV on one hand and on the other hand the weaker activation of MAP-2 kinase, suggest that UV affects protein kinases that are not addressed by TPA. Further, UV may, in addition to the Raf-1 kinase pathway, stimulate one or more other signalling pathways that are UV specific and end at other transcription factors. Nevertheless, in the case of a promoter containing protein binding sites for multiple transcription factors, including AP-1, rapid induction of AP-1 might still be required for cooperation with these other transcription factors specifically addressed by UV. Interestingly, Fos is modified in a phorbol ester-specific way the significance of which is not yet clear (S.Gebel, unpublished observations). Thus, there will be different sets of active transcription factors and even of members of the AP-1 family. By screening cDNA libraries, genes have indeed been detected that appear to belong exclusively to either phorbol ester, UV- or other DNA damage-induced programs (Devary *et al.*, 1991; Holbrook and Fornace, 1991).

In conclusion, the peculiarities of the UV response have led to the detection of an information flow from the nucleus to the cytoplasm and back to the nucleus. This exchange may be important in general to link cellular reactions to the state of the genome. Feeding into the cytoplasmic signal transduction pathway by reverse signalling may exploit the already existing pleiotropic circuitry such that several transcription factors can be addressed simultaneously.

Materials and methods

Plasmid constructs

The 5 × TRE-TATA−CAT reporter construct has been described previously (Jonat *et al.*, 1990). The −300/+63coll−CAT construct was constructed by amplifying by PCR the DNA between a primer binding to the CAT gene and a primer binding to positions −300 to −285 of −517/+63coll−CAT5 (Jonat *et al.*, 1990) and subcloning the amplified DNA into −517/+63coll−CAT5 cut with *Hin*dIII and *Bam*HI according to Scharf (1990). The Gal4 reporter plasmid carrying two Gal4 binding sites upstream of the HSV *tk* promoter (17MX2−*tk*−CAT) and the RSV-LTR-driven expression vector encoding the DNA binding domain of Gal4 (Gal4-DBD) have been described (Webster *et al.*, 1988; Schmitz and Baeuerle, 1991). To construct RSV−Gal4−v-Jun, RSV−v-Jun−Mut (Angel *et al.*, 1989), which encodes the transactivation domain of Jun, was digested with *Xba*I followed by a 'fill-in' reaction and ligation of a pentameric *Xho*I linker. After digestion with *Hin*dIII and *Xho*I, the 0.4 kb *Hin*dIII−*Xho*I fragment of Gal4-ER 147/282 (Webster *et al.*, 1988) was inserted between the RSV and Jun sequences. The expression vectors encoding wild type (Angel *et al.*, 1988a) or Jun proteins containing single or double point mutations at positions 63 and 73 (Pulverer *et al.*, 1991) or wild type and mutant Raf-1 kinase (Bruder *et al.*, 1992) were as described. In order to construct Gal4−c-Jun and Gal4−c-Jun S1 + S2, the sequences from wild type and c-*jun* mutated at Ser 63 and Ser73 were amplified from RSV−c-Jun expression vectors (Pulverer *et al.*, 1991) using the 5′ primer 5′-ATG ACT GCA AAG ATG GAA AC-3′ corresponding to amino acid codons 1−6 and the 3′ primer 5′-CTC GCT GTG CAG GCT GGCG-3′ corresponding to amino acid codons 161−166 with an *Xho*I site at the 5′ end and a *Bgl*II site at the 3′ end. The PCR fragments were subcloned into pBluescript (Stratagene) for sequencing. The c-*jun* sequences were then excised with *Xho*I and *Bgl*II and ligated into the *Xho*I and *Bgl*II cut RSV−Gal4−v-Jun (see above). GHF1−Gal4 was constructed by amplification of the DNA binding domain of Gal4 by PCR using the primers A: 5′-CGGCCTGCAGATCTTGATGAAGCTACTGTCTTCTATC-3′ and B: 5′-GCTAGTGCGGCCGCTAAATCGATACAGTCAACTGTCT-3′ and RSV−Gal4−v-Jun as a template. The amplified fragment was digested with *Pst*I and *Not*I and cloned into *Pst*I and *Not*I digested RSV−GHF-c-Jun (P.Angel, unpublished) to replace the DNA binding domain of c-Jun.

Cell culture, transfections and analysis of gene expression

HeLa *tk*$^-$ cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. F9 cells were grown in F12-DMEM (1:1) supplemented with 10% fetal calf serum and 10^{-4} M β-mercaptoethanol. HeLa *tk*$^-$ and F9 cells were transfected with expression vectors and reporter plasmid constructs using the DEAE-Dextran or the calcium phosphate method, respectively, as described previously by Angel *et al.* (1987b) and Oehler and Angel (1992). After removing excess DNA, cells were kept in DMEM containing 0.5% FCS for 24 h (HeLa) or complete F9 medium for 12−16 h (F9), and then treated with the agents specified in Figure legends. UV irradiation was performed as described by Büscher *et al.* (1988). Expression of the transfected reporter gene constructs was determined either by measuring CAT enzyme activity (Gorman *et al.*, 1982) or by analyzing the amount of CAT transcripts using the RNase protection procedure as described previously by Angel *et al.* (1987a). Preparation of poly(A)$^+$ RNA and Northern blot analysis was performed as described by Rahmsdorf *et al.* (1987).

Labelling and immunoprecipitation

After starvation in medium containing 0.5% FCS for 24 h, HeLa *tk*$^-$ cells were supplied with fresh medium lacking methionine or phosphate. Cells were incubated in the presence of 0.15 mCi/ml [^{35}S]methionine or 1 mCi/ml [^{32}P]phosphate for 3 h. F9 cells transiently transfected with the appropriate Jun expression vectors, were labeled accordingly starting at 12−16 h after removal of excess DNA−calcium phosphate coprecipitate. After the labelling period the cells were either left untreated or TPA was added at a final concentration of 80 ng/ml. For UV irradiation, the labelling medium was removed and the cells were washed twice with PBS (37°C) followed by irradiation (30 J/m^2) and addition of the original labelling medium. After treatment, the labelling medium was removed and both non-treated and stimulated cells were lysed in RIPA buffer (Hunter and Sefton, 1980). After preclearing the extracts with non-immune rabbit serum, c-Jun was immunoprecipitated with a c-Jun-specific polyclonal antibody raised against amino acids 47−59 of c-Jun (antibody 891, provided by A.Darling, Glasgow). Immunoprecipitates were washed three times with RIPA buffer and once with PBS, resuspended in 1 × Laemmli sample buffer (Laemmli, 1970) and resolved on 10% SDS−PAGE.

Two-dimensional phosphopeptide mapping

c-Jun protein labelled with [^{32}P]orthophosphate and resolved on 10% SDS−PAGE was transferred to nitrocellulose membrane by Western Blotting. The membrane was exposed to X-ray film and the Jun-specific bands were excised from the membrane and subjected to trypsin digestion as described (Hunter and Sefton, 1980). After repeated washing, tryptic digests were applied to thin layer cellulose plates for two-dimensional peptide mapping by electrophoresis at pH 1.9 at 1000 V for 25 min in the first dimension followed by chromatography in 37.5% butanol−25% pyridine−7.5% acetic acid at pH 3.9 in the second dimension for 4−5 h (Hunter and Sefton, 1980).

Western blotting for MAP-2 kinase detection

HeLa *tk*$^-$ cells were lysed and processed for one-dimensional PAGE as described by Leevers and Marshall (1992). Proteins were blotted and blots were blocked in 5% skimmed milk in PBS at 37°C for 1 h and probed with antiserum 121 for 1 h (anti-ERK-2 directed against the 42 kDa form of MAP-2 kinase, diluted 1:15000 in PBS, 5% skimmed milk, 0.3% Tween). The blots were washed three times for 5 min in PBS containing 0.3% Tween and incubated for 1 h with goat anti-rabbit serum coupled to horse radish peroxidase for MAP-2 kinase detection (diluted 1:5000 in hybridization buffer). The blots were extensively washed and developed using the ECL detection system (Amersham).

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