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Intramolecular signal transduction in c-Jun

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The DNA-binding activity of c-Jun is determined by the phosphorylation state of a cluster of threonine and serine residues located near its COOH-terminus. We have analyzed the events that lead to c-Jun activation via dephosphorylation of these sites in response to phorbol esters. Our results indicate that COOH-terminal dephosphorylation is an indirect consequence of a separate phosphorylation event targeted to the NH_2-terminus of c-Jun. Thus, the activation of c-Jun DNA-binding potential, caused by COOH-terminal dephosphorylation, may not require the regulation of the kinase/phosphatase system that brings about this change, but rather an alteration in the accessibility of the COOH-terminal phosphoacceptor sites of c-Jun.

Key words: c-Jun/protein phosphorylation/signal transduction/transcription factor AP-1

Introduction

The biological function of the proto-oncogene product c-Jun as a transcription factor has been reported to be regulated by two distinct phosphorylation systems. On the one hand, phosphorylation of the NH_2-terminally located residues serine 63 and serine 73 (human amino acid sequence) increases in response to growth factor or UV signaling and enhances the transcriptional activity of c-Jun (Binetruy *et al.*, 1991; Smeal *et al.*, 1991; Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993). This inducible phosphorylation event is controlled by well-defined signaling pathways culminating in the activation of MAP-type kinases (MAPK) such as ERK, JNK or SAPK, all of which potentially use c-Jun as a substrate, albeit with varying efficacy (Pulverer *et al.*, 1991; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994). Although much is known about the regulation of c-Jun NH_2-terminal phosphorylation, the ensuing transcriptional activation is poorly understood.

In contrast, phosphorylation of the COOH-terminal residues threonine 231, serine 243 and serine 249 (referred to here as 'COOH-terminal cluster') by CKII or GSK3 precludes DNA binding (Boyle *et al.*, 1991; Lin *et al.*, 1992; Papavassiliou *et al.*, 1992a). These inhibitory phosphate groups are lost after phorbol ester (TPA) or serum stimulation. The repression of c-Jun DNA binding by COOH-terminal phosphorylation seems mechanistically straightforward, conceivably involving electrostatic repulsions between the phosphate groups on c-Jun and the DNA-binding site. However, the regulation of COOH-terminal phosphorylation is obscure. Neither CKII nor GSK3 are down-regulated by growth factors or *Ras*, and the existence of an activated phosphatase (Karin, 1994) remains hypothetical. Conversely, previous analyses have suggested that dephosphorylation can occur as a consequence of DNA binding and does not require the activation of signal-transduction cascades (Baker *et al.*, 1992; Papavassiliou *et al.*, 1992b).

These results raised the possibility that the dephosphorylation of the COOH-terminal cluster is not triggered by a modulation of the cognate enzymatic activities, but by a signal-induced primary event that changes c-Jun's properties as substrate for a constitutively acting kinase or phosphatase. To investigate this hypothesis we sought to explore the nature of such a primary event. For two reasons the phosphorylation of c-Jun in the NH_2-terminal region by MAPK-type enzymes might be a good candidate: first, the activation of MAPK-type enzymes by inducers of c-Jun is well documented, and second, such enzymes appear to cause a major conformational change in c-Jun, as deduced from the different electrophoretic mobility of NH_2-terminally phosphorylated and non-phosphorylated forms of the protein (Pulverer *et al.*, 1991). Here we present evidence that phosphorylation in NH_2-terminal residues of c-Jun stimulates the dephosphorylation of the COOH-terminal sites, and consequently increases the DNA-binding activity of the transcription factor. This finding integrates prior observations on what were believed to be two separate mechanisms of c-Jun regulation and provides an illustrative example of intramolecular signaling.

Results

To study the functional consequences of signal-induced phosphorylations in the NH_2-terminal region of c-Jun, known or suspected kinase substrate sites were converted into alanine, to generate non-phosphorylatable mutants of c-Jun. In parallel, the same residues were changed to aspartic acid to introduce a negative charge at these sites in an attempt to generate 'pseudo-phosphorylated' mutant c-Jun proteins (Figure 1). Two clusters of potential phosphorylation sites were subjected to this manipulation: the first comprises serines 63 and 73, which have been shown to become phosphorylated in response to growth factor and UV stimulation (Pulverer *et al.*, 1991; Smeal *et al.*, 1991; Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993); the second cluster spans the sequence $T_{89}TTPTPT_{95}$, with threonines 91 and 93 conforming to MAPK-target site specificity (Marshall, 1994) (Figure 1). Threonine 91 and/or

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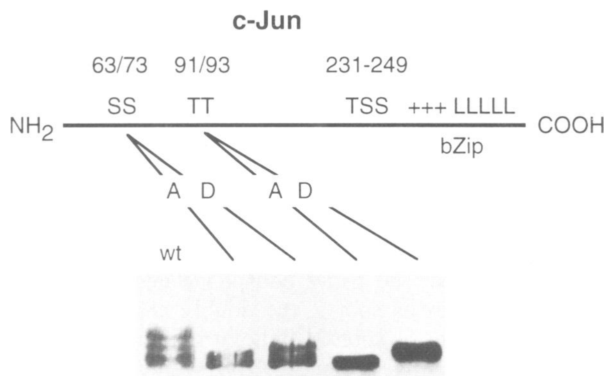

Fig. 1. Phosphorylation mutants of c-Jun. The top of the figure indicates the position of the various phosphorylation sites in c-Jun as well as the basic region (+++) and the leucine zipper (LLLLL). His-tagged wild-type c-Jun (wt) or the indicated phosphorylation point-mutant c-Jun proteins were transiently expressed in and purified from 293 cells. The recovered proteins were subjected to SDS−PAGE and visualized by Western blotting using polyclonal rabbit antiserum directed against full-length human c-Jun. Note that the complexity of the electrophoretic pattern decreases in c-Jun point mutants.

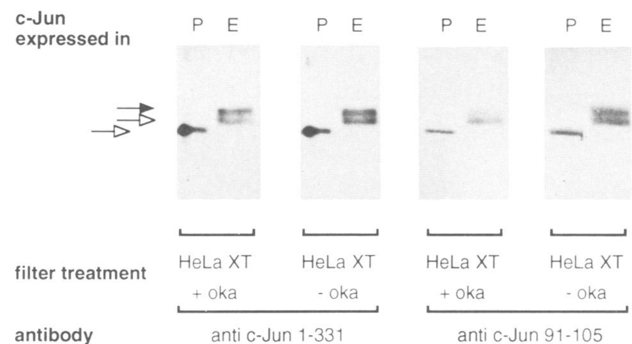

Fig. 2. c-Jun contains epitopes around amino acid 90 that are masked by phosphorylation. Purified recombinant wild-type c-Jun proteins expressed in bacteria (P) or eukaryotic cells 293 (E) were resolved by SDS−PAGE and electrotransferred onto nitrocellulose (NC) membrane. Four identical NC strips carrying the two proteins in adjacent lanes were incubated with a crude phosphatase preparation from HeLa cells (HeLa XT) in the presence (+) or absence (−) of okadaic acid. Subsequently, the strips were immunostained with either polyclonal anti-full-length-c-Jun antibody or with a polyclonal anti-peptide antibody directed against amino acids 91−105 of c-Jun. The running position of non-phosphorylated (white arrows) and of phosphorylated c-Jun species (black arrow) is indicated.

93 have been suggested as substrates for JNK (Hibi *et al.*, 1993; Dérijard *et al.*, 1994).

The appropriately engineered wild-type and mutant c-Jun coding sequences were fused at their 3′ end to six histidine codons, providing a tag for purification, and introduced into cytomegalovirus-derived plasmid expression vectors (Treier *et al.*, 1994). After transient transfection of these plasmids into 293 cells, wild-type and mutant c-Jun proteins were purified by Ni^{2+}-chelate affinity chromatography under denaturing conditions to prevent co-purification of proteins interacting with c-Jun. In the presented experiments 293 cells were employed because they are excellent recipients for transient transfection. Essentially the same results were obtained using HeLa cells, ruling out an artifactual influence of E1A which is the transforming agent in 293 cells, but is absent in HeLa cells (data not shown). Western-blot analysis using a polyclonal anti-c-Jun serum reveals that, similar to the endogenous protein (Radler-Pohl *et al.*, 1993), the over-expressed wild-type c-Jun gives rise to three or four differently migrating bands (Figure 1). As shown previously, the slower-migrating forms of this group represent phosphorylated species of c-Jun, which can be converted *in vitro* into the fastest-migrating band by phosphatase digestion (Pulverer *et al.*, 1991, and data not shown). All the tested point-mutated proteins lose at least some of the phospho-forms (Figure 1). c-Jun derivatives carrying mutations in the 63/73 site (c-Jun S 63/73 A and c-Jun S 63/73 D) give rise to the lowest band plus at least one phosphorylated form, whereas the mutants in the 91/93 cluster (c-Jun T 91/93 A and c-Jun T 91/93 D) appear as one predominant band. The electrophoretic mobility of the main band of the 91/93 mutant proteins, which cannot be altered by phosphatase treatment (data not shown), shows an interesting dependence on the amino acid replacing threonine. c-Jun carrying alanine instead of threonine (c-Jun T 91/93 A) in this position migrates with the mobility of the dephosphorylated wild-type protein, whereas the replacement of threonines 91 and 93 by aspartic acid residues causes a shift to lower electrophoretic mobility reminiscent of some of the phosphorylated forms. Judging from its electrophoretic properties, therefore, c-Jun T 91/93 D behaves as a 'pseudo-phosphorylated' protein.

To confirm that the 91/93 threonine residues are phosphorylated *in vivo*, we exploited a property of an antibody which is directed against a peptide encompassing amino acids 91−105 of human c-Jun. The Western blot in Figure 2 demonstrates that this antibody does not recognize the slower-migrating, phosphorylated form of wild-type c-Jun as efficiently as a polyclonal antiserum raised against the entire c-Jun protein. Dephosphorylation of c-Jun on the filter by an okadaic acid-sensitive phosphatase from HeLa cells, presumably PP-2A, enhances immunostaining of the slower-migrating form by the peptide antibody, but not of the lower band or a bacterially expressed control c-Jun protein. Evidently, some epitopes recognized by the peptide antibody can be masked by a phosphate group(s) that is present only in the electrophoretically retarded form of c-Jun, providing good evidence that these sites are targets for phosphorylation *in vivo*.

The effect on c-Jun function and signal responsiveness of mutations in the phosphoacceptor sites serine 63 and serine 73 (Pulverer *et al.*, 1991; Smeal *et al.*, 1991) as well as threonine 91 and/or threonine 93 was investigated by assessing the specific DNA-binding activity of the corresponding proteins in an electrophoretic mobility-shift assay (Figure 3). 293 cells were transfected with plasmids encoding either the wild-type or the mutant His-tagged c-Jun proteins. Following a time of 32 h post-transfection, cells were treated or mock-treated with TPA for 1 h, and equivalent amounts of each affinity-purified c-Jun species (as measured by Western blotting) were incubated with a ^{32}P-labeled TRE probe. DNA−protein complexes were then visualized by non-denaturing gel electrophoresis and subsequent autoradiography. As expected, the DNA-binding activity of c-Jun is much higher when it is isolated

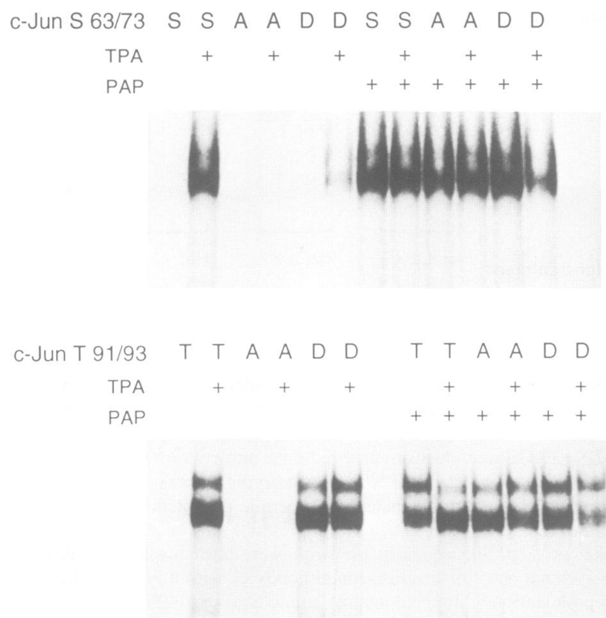

Fig. 3. TPA-inducible DNA-binding activity of c-Jun and mutant derivatives. His-tagged wild-type and mutant c-Jun proteins carrying alanine or aspartic acid substitutions of the 63/73 or of the 91/93 phosphorylation sites were transiently expressed in TPA-treated or non-treated 293 cells, as indicated above the autoradiographs. Aliquots of the different recombinant c-Jun proteins were digested with potato acid phosphatase (+PAP) or mock-digested and the DNA-binding activity of the resulting c-Jun preparations was analyzed by an electrophoretic mobility-shift assay using a probe containing the collagenase TRE. Under these assay conditions c-Jun−TRE complexes appear as a doublet band. The empty lanes (right of the top panel, middle of the bottom panel) show negative controls carried out with material purified from mock-transfected cells.

from TPA-treated cells than from control cells (Figure 3). This induction of c-Jun DNA-binding activity by TPA is lost in the c-Jun S 63/73 A mutant protein and severely diminished but still detectable in c-Jun S 63/73 D. When the threonine residues at positions 91 and 93 are mutated, inducibility is abolished completely; in the case of c-Jun T 91/93 A the DNA-binding activity remains low regardless of TPA treatment of the expressing cells, whereas the DNA-binding activity of c-Jun T 91/93 D is high even in material obtained from uninduced cells. Thus c-Jun T 91/93 D represents a constitutive gain-of-function mutant of c-Jun.

The TPA-dependent DNA-binding activity of c-Jun can be regulated by inhibitory phosphorylations on the COOH-terminal cluster (Boyle *et al.*, 1991; Papavassiliou *et al.*, 1992a). This offers two explanations to account for the DNA-binding phenotype elicited by mutations in the NH_2-terminally located phosphorylation sites described above: either these mutations modulate DNA binding directly, overriding the function of phosphorylation at the COOH-terminal cluster, or they act indirectly by influencing the COOH-terminal phosphorylations. If the latter is true, the DNA-binding capacity of all mutant proteins should be restored by *in vitro* dephosphorylation, as previously shown for wild-type c-Jun protein from uninduced cells (Papavassiliou *et al.*, 1992a). Figure 3 demonstrates that the second hypothesis is correct. After potato acid phosphatase treatment of the various purified c-Jun mutant proteins (+ PAP lanes), DNA binding was high in every case, regardless of the nature of the mutation or the induction state of the expressing cells. Taken together, these data suggest that phosphorylation of serines 63/73 and of threonine 91 and/or threonine 93 influences the phosphorylation state of the COOH-terminal cluster and hence the DNA-binding activity of c-Jun.

To test this idea directly, we generated tryptic phosphopeptide maps of wild-type and mutant forms of c-Jun expressed in TPA-treated or control 293 cells. The COOH-terminal cluster is located on a single tryptic fragment of 26 amino acids. The triply phosphorylated form of this peptide migrates as spot a, the doubly and singly phosphorylated forms as spots b and c, respectively (Figure 4, upper panel) (Boyle *et al.*, 1991). Figure 4 shows the reduction of phosphorylation in the 26-amino acid peptide of wild-type c-Jun after TPA stimulation, evident by the decrease of label in spot a and the concomitant increase in spots b and c. Phosphopeptide mapping of c-Jun T 91/93 A and of c-Jun T 91/93 D in comparison reveals several important differences. Whereas the phosphorylation of the COOH-terminal cluster, as reflected in the intensity of spots a, b and c, decreases in response to TPA in wild-type c-Jun, no change is evident when either c-Jun T 91/93 A or c-Jun T 91/93 D are analyzed. The phosphorylation state of c-Jun T 91/93 A corresponds to that of c-Jun in the uninduced state, while c-Jun T 91/93 D is constitutively hypophosphorylated, closely resembling c-Jun in the TPA-stimulated form. These results are consistent with the DNA-binding data described above and support the idea that the phosphorylation state of the COOH-terminal cluster is dependent on, and changes in response to, the phosphorylation of threonine 91 and/or threonine 93.

The data presented so far indicate (i) that the activation of DNA binding by COOH-terminal dephosphorylation of c-Jun is governed by a separate signal-dependent phosphorylation in the NH_2-terminal region and (ii) that the kinase/phosphatase system specific for the COOH-terminal cluster is itself not regulated (at least not in response to TPA). To confirm the latter point, we directly measured c-Jun COOH-terminal kinase activity from TPA-treated and control cells *in vitro*. This was done by incubating a bacterially expressed fragment of c-Jun that spans the COOH-terminal 150 amino acids, including the COOH-terminal cluster but not the NH_2-terminally located phosphorylation sites, with the respective cell extracts in the presence of [γ-^{32}P]ATP. The incorporation of radioactive phosphate into the truncated c-Jun substrate was monitored by immunoprecipitation and autoradiography (Figure 5). Consistent with the results presented above, no change in the activity of the c-Jun COOH-terminal kinase was detected in this assay. These data support the notion that the TPA-induced COOH-terminal dephosphorylation of c-Jun is triggered by an intramolecular rather than an intermolecular signal.

Discussion

We document here by several independent lines of evidence that c-Jun is phosphorylated on threonine 91 and/or 93. Phosphorylation at these sites functions as an important part of a molecular switch that modulates c-Jun activity. It is so far the only phosphorylation site on c-Jun (or, to our knowledge, any other transcription factor) that upon conversion into a negatively charged residue

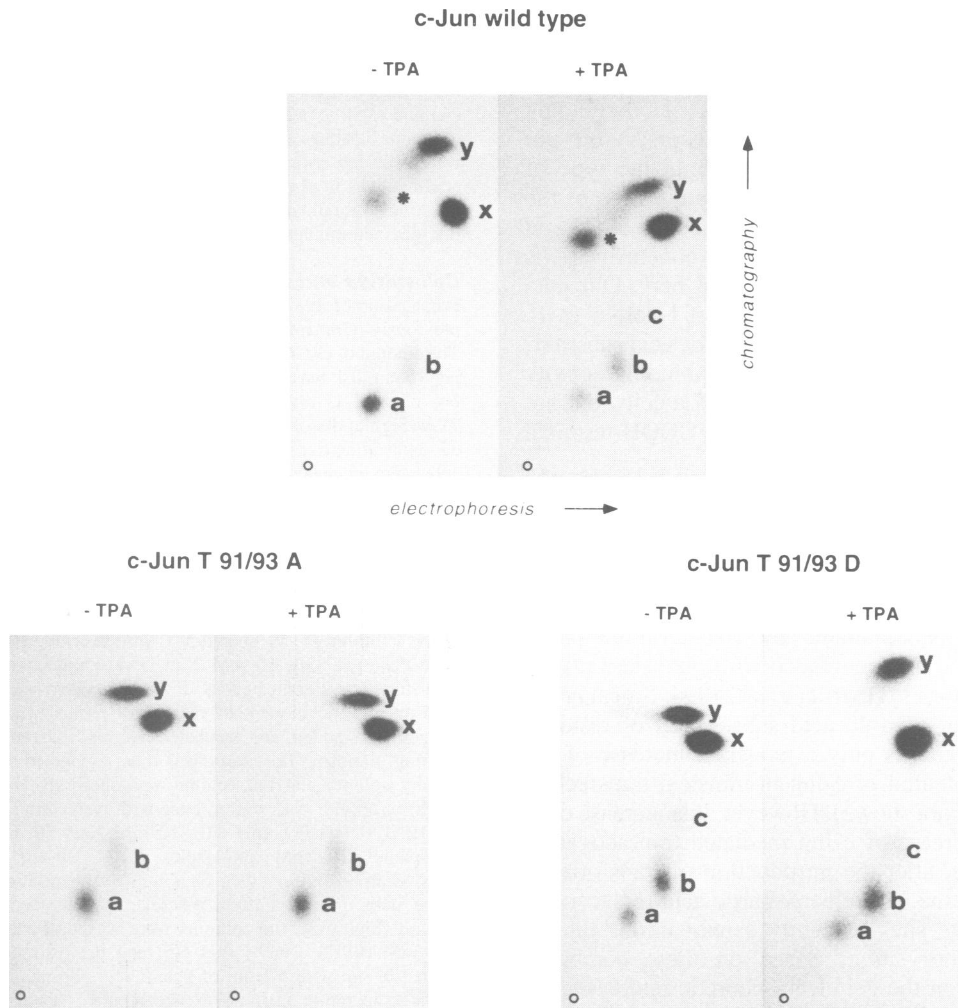

Fig. 4. Phosphopeptide maps of wild-type c-Jun, c-Jun T 91/93 A and c-Jun T 91/93 D. The three His-tagged proteins were transiently expressed in 293 cells with or without TPA induction and labeled *in vivo* with $^{32}P_i$. Equal amounts of purified c-Jun cross-reactive material were subjected to tryptic digestion and to two-dimensional separation by electrophoresis and ascending thin-layer chromatography. The a, b and c spots represent tri-, di- and monophosphorylated forms, respectively, of the COOH-terminal peptide containing threonine 231, serine 243 and serine 249. The x and y spots originate from two NH_2-terminal peptides phosphorylated at serine 73 and serine 63, respectively. The asterisk marks a spot which is missing in the phosphopeptide maps of the mutants and most likely originates from the peptide encompassing threonine 91 and 93 (an unambiguous assignment of this spot by Edman degradation was not possible, because threonines 91 and 93 are the 13th and 15th residues of a tryptic peptide). The position of sample application is marked by a small black circle. The autoradiographs with equal exposure times are shown.

generates a strong gain-of-function phenotype, in this case constitutive DNA binding. Remarkably, the effect of the mutation on DNA binding is indirect, and is mediated via dephosphorylation of residues located at the other end of c-Jun. A related mechanism was reported for the mitotic activation of c-*Src*, where the removal of a COOH-terminally located inhibitory tyrosine phosphate is triggered by NH_2-terminal cdc2 phosphorylation (Shenoy *et al.*, 1992).

The data presented in this study offer an appealingly simple model for the post-translational regulation of c-Jun in response to extracellular signals. Upon phosphorylation of c-Jun on serines 63 and 73 and on threonine 91 and/or 93 most likely by a MAPK-related enzyme (Dérijard *et al.*, 1994), dephosphorylation of the COOH-terminal cluster ensues. This may involve a structural change of c-Jun which alters its accessibility for kinase(s) and/or phosphatase(s). As a result, the DNA-binding capacity of c-Jun is increased. Our results show that the integrity of both the 63/73 and the 91/93 phosphorylation sites is

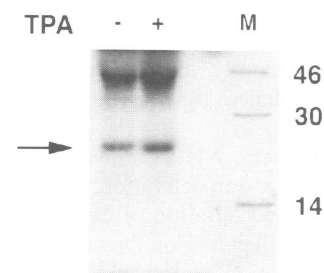

Fig. 5. c-Jun COOH-terminal kinase activity in extracts from uninduced and TPA-stimulated cells. A bacterially expressed fragment of human c-Jun encompassing the COOH-terminal 150 amino acids was incubated with whole-cell extracts prepared from equal numbers of TPA-treated (+) or mock-treated (−) HeLa cells, in kinase buffer containing [γ-^{32}P]ATP. Phosphorylated proteins were immunoprecipitated and analyzed by SDS−PAGE and autoradiography. The location of the truncated c-Jun form is indicated by the arrow. Lane M contains ^{14}C-methylated protein molecular size standards.

required for the TPA-induced dephosphorylation of the COOH-terminal cluster, while conversion of threonines 91 and 93 alone to aspartic acid residues is enough to create a constitutively dephosphorylated, DNA-binding competent protein. The reason for the necessary but not sufficient function of serines 63 and 73 in the type of mutagenesis assay described here and the question of the kind of regulatory interrelationship between the 63/73 and the 91/93 phosphorylation sites cannot conclusively be resolved from the experiments presented here. Our data provide an explanation for the findings of Hoeffler *et al.* (1994) who report that c-Jun with an aspartic acid substituting serine 73 has a slightly higher DNA-binding activity than the wild type when expressed in HeLa cells, but not when expressed in bacteria where no COOH-terminal phosphorylation would take place.

Several observations indicate that the activation of c-Jun DNA-binding potential by the mechanism proposed here is not the only effect of NH_2-terminal c-Jun phosphorylation. Binetruy *et al.* (1991) concluded from experiments with Jun fusion proteins that also the transactivating function of the molecule is stimulated by NH_2-terminal phosphorylation. Indeed, in accordance with published results from other laboratories (Hoeffler *et al.*, 1994; Smeal *et al.*, 1994) we find that aspartic acid substitution of residues 63, 73, 91 or 93 causes only a moderate increase of the transactivation potential of c-Jun in transient transfection experiments (data not shown). However, this increase does not match the increase of c-Jun mediated transactivation seen, for example, after the introduction of transforming alleles of *ras* into the test cells. Aspartic acid replacement of kinase substrate sites evidently cannot mimic all the effects of phosphorylation. Based on these combined observations and on the gel electrophoretic retardation of NH_2-terminal phospho-forms of c-Jun, we suggest that MAPK-type phosphorylations cause a conformational change in the protein that modifies its biochemical properties in a pleiotropic manner. Such phosphorylation-induced changes might alter the accessibility of the COOH-terminal cluster to a kinase and/or phosphatase in a way that leads to its dephosphorylation, thus promoting the DNA-binding potential of c-Jun. In contrast to previous models (Karin, 1994), such a scenario implies that dephosphorylation at the COOH-terminus may be catalyzed by a constitutive protein kinase/phosphatase system whose activity is unregulated. Consistently, our *in vitro* experiments do not show any evidence for a regulated c-Jun COOH-terminal kinase.

If, as suggested here, NH_2-terminal phosphorylation is the primary event of c-Jun activation, it may seem surprising that the extent and duration of these phosphorylations vary dramatically, depending on the cell type and inducing agent. One possible explanation might be that the activation of DNA binding by dephosphorylation of the COOH-terminal cluster of c-Jun, once triggered, does not require the persistence of NH_2-terminal phosphorylations (hit-and-run mechanism).

Materials and methods

Plasmids

The vectors used for the expression of His-tagged wild-type c-Jun protein in 293 cells (plasmid MT35) and of wild-type c-Jun protein in *Escherichia coli* (plasmid pHJmet5) have been described elsewhere (Bohmann and Tjian, 1989; Treier *et al.*, 1994). Plasmids encoding the His-tagged c-Jun S 63/73 A and c-Jun S 63/73 D proteins were generated from MT35 by replacing the codons for serines (S) 63 and 73 with codons for alanine (A) and aspartic acid (D), respectively. In His-tagged c-Jun T 91/93 A threonine residues (T) at amino acid positions 89, 90, 91, 93 and 95 were replaced with alanine. His-tagged c-Jun T 91/93 D has aspartic acid residues in place of threonines 91 and 93. All mutant clones were constructed employing a polymerase chain reaction-based oligonucleotide-directed mutagenesis system (Landt *et al.*, 1990).

Cell culture and transient transfections

Propagation of 293 cells and transfections were performed as described previously (Graham and van der Eb, 1973; Papavassiliou *et al.*, 1992b). The amount of CsCl-purified wild-type or mutant MT35 DNA transfected per 10 cm dish was 3 μg, adjusted to 20 μg with carrier DNA (pUC18). For phorbol 12-myristate 13-acetate (TPA) treatment of the cultures, TPA (Sigma; dissolved in dimethyl sulfoxide) was added to the medium 32–34 h after transfection to a final concentration of 100 ng/ml, and cells were incubated for an additional 60 min prior to harvesting. Control cultures received the solvent alone.

Purification of recombinant c-Jun proteins

After a time of 33–35 h post-transfection, mock-treated or TPA-treated c-Jun-expressing 293 cells were washed three times with ice-cold phosphate-buffered saline (PBS) and harvested in 1 ml per 10 cm plate of lysis buffer [6 M guanidine hydrochloride, 100 mM Na_2HPO_4–NaH_2PO_4 (pH 8.0), 10 mM NaF, 5 mM imidazole]. This lysate was centrifuged and sonicated as described (Papavassiliou *et al.*, 1992a), 0.16 ml of a nickel–chelate resin (Ni^{2+}–nitrilotriacetic acid–agarose; Qiagen) was added, and incubated overnight at room temperature on a rotating platform. The slurry was then applied to a Bio-rad chromatography column and the column was successively washed (2 ml per wash) once with lysis buffer, once with lysis–dilution buffer [100 mM Na_2HPO_4–NaH_2PO_4 (pH 8.0), 10 mM NaF, 50 mM (Na_2)β-glycerolphosphate, 10 mM imidazole, 0.1% NP-40] (3:1), once with lysis–dilution buffer (1:1), once with lysis–dilution buffer (1:3), and twice with dilution buffer. Wherever protein dephosphorylation was desired (Figure 3), the columns were washed further with 2×5 ml of 100 mM PIPES–NaOH (pH 6.5) and the resin-bound proteins were treated *in situ* with 5 U/ml of potato acid phosphatase (PAP) for 1 h at room temperature in the presence (control samples) or absence of a specific combination of phosphatase inhibitors (Papavassiliou *et al.*, 1992a).

The columns were subsequently washed with 3×5 ml of dilution buffer. Bound His-tagged protein was eluted with 0.5 ml of 100 mM Na_2HPO_4–NaH_2PO_4 (pH 5.9), 10 mM NaF, 50 mM (Na_2)β-glycerolphosphate, 250 mM imidazole and 0.1% NP-40. The eluate was then dialyzed for 12 h at 4°C against 2000 volumes of 50 mM Na_2HPO_4–NaH_2PO_4 (pH 7.5), 7.5% glycerol, 0.2 mM phenylmethyl sulfonyl fluoride (PMSF), 0.5 μg/ml aprotinin, 0.2 μg/ml pepstatin and 0.2 μg/ml leupeptin. For electrophoretic mobility-shift assays (Figure 3), the eluates were concentrated to 0.05 ml by lyophilization and their c-Jun protein concentration was quantified by comparative Western blotting. The yield of both wild-type and mutant c-Jun proteins was in the order of 1 μg per plate of 293 cells. The purification of bacterially expressed c-Jun protein has been described elsewhere (Bohmann and Tjian, 1989).

SDS–PAGE and Western blotting

Aliquots of the various affinity-purified c-Jun proteins were boiled in 1× SDS sample buffer, subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 10% gels, electrotransferred onto nitrocellulose (NC) membrane, probed with anti-c-Jun polyclonal antibody raised in rabbits against the bacterially expressed full-length protein, and visualized using anti-rabbit IgG antibody conjugated to alkaline phosphatase. The anti-peptide c-Jun antibody employed for the experiment presented in Figure 2 was a rabbit, affinity-purified polyclonal antibody raised against the c-Jun NH_2-terminal peptide T P T P T Q F L C P K N V T D (amino acids 91–105 of the human sequence) (Oncogene Science).

HeLa cell extracts as a source of phosphatase

A quantity of 8×10^6 logarithmically growing HeLa cells (in five 10 cm plates) was harvested in 4 ml of ice-cold Tris-buffered saline (TBS) supplemented with 1 mM dithiothreitol, 2 mM EDTA, 2 mM PMSF, 4 μg/ml E-64, 5 μg/ml aprotinin, 2 μg/ml pepstatin and 2 μg/ml

leupeptin, and crude cellular extracts were prepared by forcing the cell suspension five times through a 21 g syringe needle. After removal of cellular debris (10 000 *g*, 10 min, 4°C), the supernatant was made 1 mM in $MnCl_2$ [1.25 μM okadaic acid (Sigma; dissolved in ethanol) was also included in control reactions] and added to plastic bags containing preblocked (in TBS + 0.2% Tween 20) c-Jun-bearing NC strips, at 0.25 ml per cm^2 filter. Sealed bags were incubated for 1 h in a 30°C agitating water bath, after which the strips were washed extensively with PBS + 0.2% Tween 20 and processed for immunostaining using the antibodies described above.

Electrophoretic mobility-shift assays

DNA-binding reactions contained: 1–2.5×10^4 c.p.m. (~5 ng) of a 134-bp 5′ ^{32}P end-labeled DNA restriction fragment bearing the AP-1-binding site (TRE, sequence 5′-TGAGTCA-3′) of the human collagenase promoter, equivalent amounts (in 18 μl) of the concentrated c-Jun preparations (see above), 25 ng of poly(dI-dC), 5 mM dithiothreitol, 3.75% NP-40 and 160 μg of bovine serum albumin in a total reaction volume of 40 μl. After a 20 min incubation at room temperature, reaction mixtures were loaded on 5% non-denaturing polyacrylamide gels containing 36 mM Tris, 25.85 mM boric acid and 1 mM EDTA (pH 8.0), and protein−DNA complexes were resolved by electrophoresis at 150 V for ~3.5 h at room temperature. Specificity of binding was determined by competition experiments using an excess of the unlabeled TRE probe described above.

Metabolic labeling of recombinant c-Jun proteins

After a time of 32 h following transfection with the indicated c-Jun expression vectors, 293 cell cultures (in 10 cm dishes) were rinsed twice with and incubated in 10 ml of phosphate-free Dulbecco's modified Eagle's medium (DMEM) for 30 min. $^{32}P_i$ (Amersham) was then added to 1.4 mCi/ml (1 Ci = 37 GBq). After a 3 h labeling period (the last hour in the presence or absence of TPA, see above), the cells were washed three times with ice-cold TBS and ^{32}P-labeled His-tagged c-Jun proteins were purified from guanidine hydrochloride lysates as detailed above. The concentration of c-Jun in the dialyzed eluates was equalized by comparative Western blotting.

Tryptic phosphopeptide analysis of recombinant c-Jun proteins

Tryptic phosphopeptide mapping of equivalent amounts of affinity-purified and immunoprecipitated ^{32}P-labeled wild-type and mutant c-Jun proteins was carried out as described previously (Papavassiliou *et al.*, 1992b).

In vitro kinase assay

Whole-cell extracts from TPA-treated (100 ng/ml for 1 h) or mock-treated HeLa cells were prepared as described above, except that the harvesting buffer included 0.025% Triton X-100, 60 mM $(Na_2)\beta$-glycerol-phosphate, 60 mM (Na_2)*p*-nitrophenyl-phosphate and 0.3 mM Na_3VO_4. For each extract, 10 μl were mixed with ~1 μg of a bacterially expressed fragment of human c-Jun spanning the COOH-terminal 150 amino acids, in a total reaction volume of 30 μl containing (final concentrations) 100 mM Tris−HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM $(Na_2)\beta$-glycerol-phosphate, 20 mM (Na_2)*p*-nitrophenyl-phosphate, 0.1 mM Na_3VO_4, 1 mM $MnCl_2$, 2 mM dithiothreitol and 150 μCi of [γ-^{32}P]ATP (New England Nuclear). After 15 min at 30°C the reaction was terminated by raising the volume to 1 ml with modified RIPA buffer (Papavassiliou, 1994), and immunoprecipitations using a polyclonal rabbit antiserum directed against full-length human c-Jun were performed as described previously (Papavassiliou, 1994). Phosphorylated c-Jun proteins were eluted with 30 μl of 1.5× SDS sample buffer and resolved on 15% SDS−polyacrylamide gel, followed by autoradiography.

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