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Altering the specificity of signal transduction cascades: positive regulation of c-Jun transcriptional activity by protein kinase A

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Protein phosphorylation is commonly used to modulate transcription factor activity. However, all existing genetic evidence for stimulation of transcription factor activity by phosphorylation rests on loss-of-function mutations. To demonstrate conclusively that phosphorylation of a transcription factor potentiates its transactivation potential *in vivo*, we constructed a c-Jun mutant that is phosphorylated by the cAMP-sensitive protein kinase A (PKA) instead of the UV- and Ras-responsive protein kinase JNK. The transcriptional activity of this mutant is enhanced by PKA, but not by JNK activation. These results provide a positive and conclusive proof that phosphorylation of c-Jun on a critical site (Ser73) located in its activation domain is directly responsible for enhancing its transactivation function.

Key words: c-Jun/protein phosphorylation/signal transduction/transcriptional activation

Introduction

Most cellular responses to extracellular signals are mediated through changes in gene expression. Such control is frequently exerted through activation of protein kinases that phosphorylate nuclear transcription factors and thereby modulate their activity (Hunter and Karin, 1992; Jackson, 1992). It is assumed, but not proven, that the specificity of the biological response to a given signal is determined by the spectrum of transcription factors whose activity it modulates (Karin, 1991, 1994). Phosphorylation can modulate transcription factor activity through several different mechanisms (Hunter and Karin, 1992; Jackson, 1992). In the case of CREB, TCF-1, NF-IL6 and c-Jun (Gonzalez and Montminy, 1989; Smeal *et al.*, 1991; Marais *et al.*, 1993; Trautwein *et al.*, 1993), phosphorylation of the activation domain appears to potentiate their transactivating potential. In each case, evidence for regulation by phosphorylation rests on a correlation between increased phosphorylation and transcriptional activity and on loss-of-function mutations, in which substitution of the suspected regulatory site(s) by non-phosphorylatable residue(s) abolishes the response to the appropriate signal. Although strongly suggestive, such results are subject to alternative interpretations. For example, it is possible that the replacement of phosphoacceptors by other amino acids may inadvertently affect protein function in some other way and phosphorylation may only be a consequence of increased transcriptional activity. A better and formal proof for the regulatory role of phosphorylation can be provided by either gain-of-function mutations or by direct microinjection of a phosphorylated transcription factor into cells. Although the latter approach was successfully used to demonstrate reporter gene activation by microinjection of phospho-CREB and not by unphosphorylated CREB (Alberts *et al.*, 1994), it is a cumbersome approach that can be applied only to a small number of cells, thus precluding the possibility of biochemical analysis. Although more convenient and amenable to biochemical analysis, no gain-of-function mutations mimicking the effect of phosphorylation on transcription factors have been described. This is due to the difficulty in mimicking the effect of phosphorylation by the simple substitution of a phosphoacceptor with a negatively charged amino acid (Gonzalez and Montminy, 1989).

To solve this general problem, we chose c-Jun, a component of transcription factor AP-1, as a model. The transcriptional activity of c-Jun appears to be enhanced in response to phosphorylation at Ser63 and Ser73 in its activation domain (Pulverer *et al.*, 1991; Smeal *et al.*, 1991). Phosphorylation of Ser73 seems to be more critical than phosphorylation of Ser63, which usually occurs to a lower extent and is insufficient for stimulating the activating function of c-Jun (Smeal *et al.*, 1991). Substitution of these phosphoacceptors by non-phosphorylatable residues renders c-Jun non-responsive to signals generated by growth factors, transforming oncoproteins and UV irradiation, which normally stimulate Ser63 and Ser73 phosphorylation (Smeal *et al.*, 1991, 1992; Devary *et al.*, 1992). Although these results are consistent with a direct effect of phosphorylation on c-Jun, it was suggested that signals which stimulate c-Jun activity, including the v-Src and Ha-Ras oncoproteins, cause the phosphorylation of a putative repressor regulating c-Jun's activity (Baichwal *et al.*, 1991). Once phosphorylated, the repressor was suggested to dissociate from c-Jun. It is therefore important to test critically whether c-Jun is a direct target for the mitogenic signalling pathway activated by these oncoproteins. More evidence for the regulatory importance of c-Jun phosphorylation was recently provided by the identification of novel protein kinases, termed JNK, which phosphorylate Ser63 and Ser73 and whose activity is stimulated by the same signals that potentiate c-Jun's activity (Hibi *et al.*, 1993; Dérijard *et al.*, 1994). However, since the JNKs bind to c-Jun's activation domain, it is possible that their inactive form acts as a repressor and that extracellular stimuli increase c-Jun activity because they lead to dissociation of activated JNK from c-Jun (Hibi *et al.*, 1993).

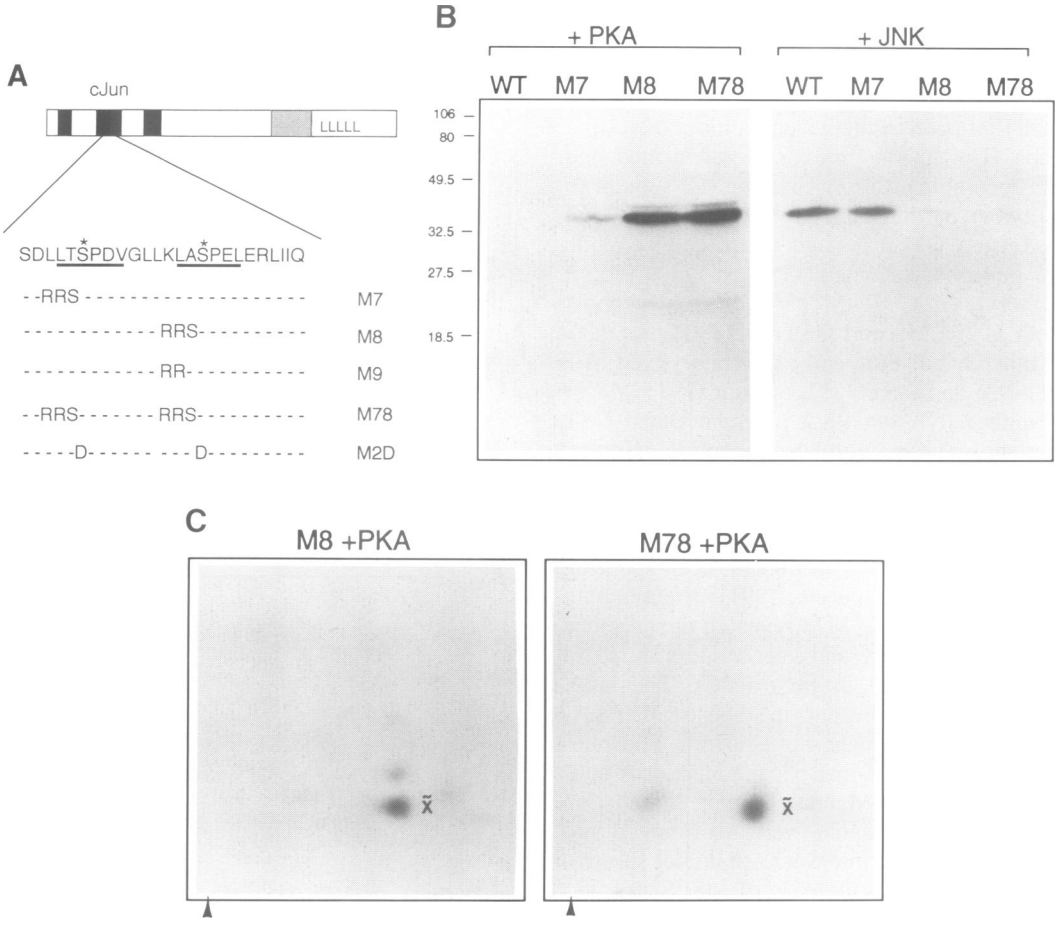

Fig. 1. Construction of an altered kinase specificity mutant of c-Jun phosphorylated by PKA. (**A**) A schematic representation of c-Jun and the sequence containing its N-terminal phosphorylation sites Ser63 and Ser73 (labeled by asterisks). The underlining denotes the similarity between the sequence surrounding Ser63 and Ser73, which may constitute part of the specificity site. The sequences of the different mutants are indicated. Black boxes, regions involved in transactivation; dotted box, basic region; LLLL, leucine zipper. (**B**) GST−c-Jun fusion proteins containing the first 93 amino acids of WT c-Jun, M7, M8, M78 were expressed in *Escherichia coli* and purified on glutathione−agarose beads. The purified proteins (1 μg each) were incubated with either PKA (C subunit, a gift from Dr S.Taylor) or activated JNK (purified from UV-irradiated HeLa cells) in the presence of [γ-^{32}P]ATP for 30 min at 37°C. The labeled proteins were resolved by SDS−PAGE and visualized by autoradiography. (**C**) The PKA-phosphorylated GST−M8 and GST−M78 were resolved by SDS−PAGE, eluted, digested by trypsin and subjected to 2-D phosphopeptide mapping. $\tilde{x}$ corresponds to the phosphopeptide containing the major PKA phosphorylation site Ser73. The arrowhead indicates the origin of the chromatogram.

We developed a novel experimental approach to determine whether the transcriptional activity of c-Jun, as a model activator, is directly regulated by its phosphorylation. We altered the sequences preceding Ser63 and Ser73 to convert them into phosphoacceptors for protein kinase A (PKA), a protein kinase that normally does not phosphorylate c-Jun. The transcriptional activity of this mutant is strongly enhanced by PKA, but not by JNK. The behavior of this altered-specificity mutant provides positive and conclusive evidence that the activation function of c-Jun is directly stimulated by phosphorylation of its activation domain. These results also demonstrate that the ability of a transcription factor to serve as a substrate for a given protein kinase renders its activity responsive to the signaling pathway affecting that kinase.

Results

Altering the kinase specificity of c-Jun

Activation of the Ras pathway increases the phosphorylation of c-Jun at Ser63 and Ser73 and stimulates its transactivating function (Binétruy *et al.*, 1991; Pulverer *et al.*, 1991; Smeal *et al.*, 1991,1992; Devary *et al.*, 1992). Substitution of Ser63 and Ser73 by Ala residues abolishes the stimulation of c-Jun activity (Smeal *et al.*, 1991, 1992; Devary *et al.*, 1992). These loss-of-function results suggest, but do not absolutely prove, that Ser63 and Ser73 are important targets for the Ras signaling pathway and their phosphorylation potentiates c-Jun activity. To obtain a formal genetic proof of this critical point, we altered the sequence surrounding these Ser residues so that they would become phosphoacceptors for a nuclear protein kinase that does not phosphorylate c-Jun. We chose PKA for this purpose because (i) its activity is regulatable (Walsh *et al.*, 1968), (ii) upon activation it translocates to the nucleus (Nigg *et al.*, 1985), (iii) it does not phosphorylate c-Jun (Boyle *et al.*, 1991) and (iv) the determinants of its site selectivity are known (Kemp and Pearson, 1990).

PKA phosphorylates Ser within the motif RRXS (Kemp and Pearson, 1990; where X is any small residue). We therefore changed the residues preceding Ser63 and Ser73 to conform with that consensus (Figure 1A). In mutants M7 and M8, the sequence preceding either Ser63 or Ser73,

respectively, was altered, whereas in M78, the sequences preceding both residues were altered. We compared the ability of these mutants and WT c-Jun to serve as substrates for either PKA or JNK, by preparing glutathione *S*-transferase (GST) fusion proteins containing their first 93 amino acids. The fusion proteins were purified and incubated with recombinant PKA catalytic (C) subunit (a gift from S.Taylor) or activated JNK (both JNK1 and JNK2, purified from UV irradiated HeLa cells). While PKA did not phosphorylate WT GST−c-Jun(1–93), it weakly phosphorylated GST−M7 and efficiently phosphorylated both GST−M8 and GST−M78 (Figure 1B). JNK, on the other hand, efficiently phosphorylated WT GST−c-Jun (1–93) and GST−M7, but not GST−M8 or GST−M78. Neither JNK nor PKA phosphorylated GST itself (data not shown). Tryptic phosphopeptide mapping revealed the presence of a single major phosphopeptide, x̃, in digests of PKA-phosphorylated GST−M8 (Figure 1C; the faint spot above x̃ is due to alkylation of this peptide, a modification that occasionally occurs during the mapping procedure; Smeal *et al.*, 1991). The mapping of PKA-phosphorylated GST−M78 revealed the presence of the same phosphopeptide, x̃, and a second less abundant phosphopeptide (not labeled in Figure 1C). The latter phosphopeptide was also present in digests of PKA-phosphorylated GST−M7 (data not shown). Phosphoamino acid analysis indicated that phosphopeptide x̃ contained only phosphoserine. Manual Edman degradation revealed that 50% of the phosphate was incorporated into Ser72 and 50% incorporated into Ser73 (data not shown). These results indicate that in the case of M8, Ser73 was successfully converted to a PKA phosphorylation site, whereas the M7 substitution did not convert Ser63 to a high affinity PKA site. Furthermore, the double mutant M78, while being an efficient PKA substrate (K_m = 0.6 μM), is no longer phosphorylated by JNK.

Differential phosphorylation of M78 and WT c-Jun in vivo

To examine the regulation of WT c-Jun and M78 phosphorylation *in vivo*, we constructed a mammalian expression vector encoding M78. This expression vector, as well as the WT c-Jun expression vector (Binétruy *et al.*, 1991), were used to express the two proteins in F9 cells. Co-transfection of an oncogenic Ha-Ras^{Leu61} expression vector or UV irradiation were used to activate JNK (Hibi *et al.*, 1993), while co-transfection of a PKA C subunit expression vector was used to increase PKA activity (Gonzalez and Montminy, 1989; Chrivia *et al.*, 1993). While phosphorylation of WT c-Jun was strongly stimulated by co-expression of Ha-Ras^{Leu61} or UV irradiation, it was not responsive to co-expression of PKA (Figure 2). A different pattern was displayed by M78. Its phosphorylation was strongly stimulated by PKA, but not by Ha-Ras^{Leu61} or UV irradiation. Phosphopeptide mapping revealed that co-expression of PKA stimulated phosphorylation of M78 on the same site that was phosphorylated by PKA *in vitro*, giving rise to phosphopeptide x̃ (Figure 3). These results were confirmed by mixing experiments (data not shown). In addition, like WT c-Jun, M78 was phosphorylated at C-terminal sites (Boyle *et al.*, 1991; Lin *et al.*, 1992) giving rise to phosphopeptides b and c. As expected, WT c-Jun isolated from Ha-Ras^{Leu61} expressing cells was

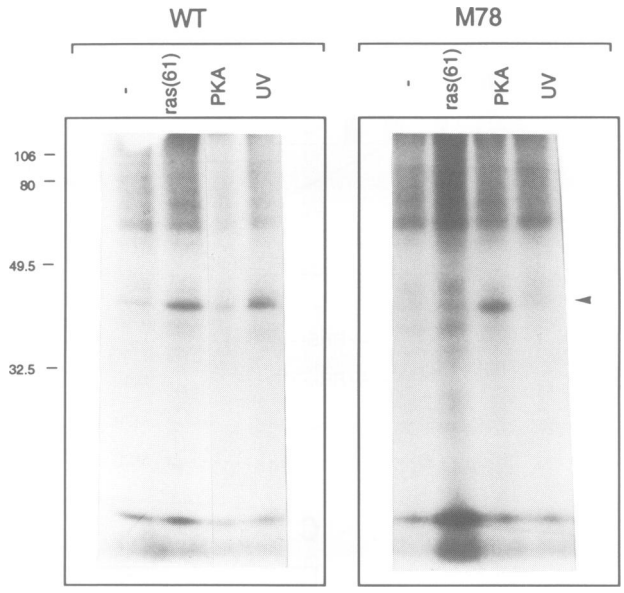

Fig. 2. Differential *in vivo* phosphorylation of WT c-Jun and M78. F9 cells were transiently co-transfected with 4 μg RSV−c-Jun or RSV−M78 expression vectors. Where indicated, these plasmids were co-transfected with 8 μg of either pZipNeoRas(Leu61) or pCMV-PKA. Alternatively, the cells were exposed or not exposed to UVC irradiation (40 J/m^2) 30 min prior to harvesting. Twenty four hours after transfection, the cells were metabolically labeled with [^{32}P]orthophosphate for 4 h, harvested in RIPA buffer, and c-Jun was isolated by immunoprecipitation and resolved by SDS−PAGE as previously described (Smeal *et al.*, 1991, 1992).

phosphorylated at Ser63 and Ser73, as evidenced by the presence of phosphopeptides X and Y (Smeal *et al.*, 1991).

M78 transcriptional activity is stimulated by PKA

Next we examined the transcriptional activity of the various c-Jun proteins and their response to PKA and JNK. The different expression vectors were co-transfected into F9 cells along with the AP-1 responsive reporter -73Col-CAT (Angel *et al.*, 1987). Measurement of CAT activity indicated that the transcriptional activity of WT c-Jun was stimulated 10-fold by co-expression of Ha-Ras^{Leu61}, an activator of JNK1 (Hibi *et al.*, 1993; Dérijard *et al.*,1994), but <2-fold in response to co-expression of PKA (Figure 4). On the other hand, M78, whose basal activity was ~70% of that of WT c-Jun, was stimulated 9.5-fold by PKA, but was unresponsive to Ha-Ras^{Leu61}. A similar pattern of regulation was exhibited by M8, whose activity increased 5.5-fold upon PKA co-expression but was unresponsive to Ha-Ras^{Leu61}. M7, on the other hand, was more similar to WT c-Jun and its activity was stimulated 7.5-fold by Ha-Ras^{Leu61}, but only marginally by PKA. Since M8 contains Ser residues at positions 72 and 73 (Figure 1A), and both of these residues are phosphorylated by PKA, we generated an additional c-Jun mutant, M9, which contains Ala at position 72 and Ser at position 73 (Figure 1A). Like M8, the activity of M9 was stimulated 5-fold by co-expression of PKA, but was only marginally effected by activated Ha-Ras (Figure 4). These results indicate that phosphorylation of Ser73 is sufficient for potentiating c-Jun transcriptional activity regardless of the protein kinase executing this reaction. These results concur with the previous finding that substitution of Ser63

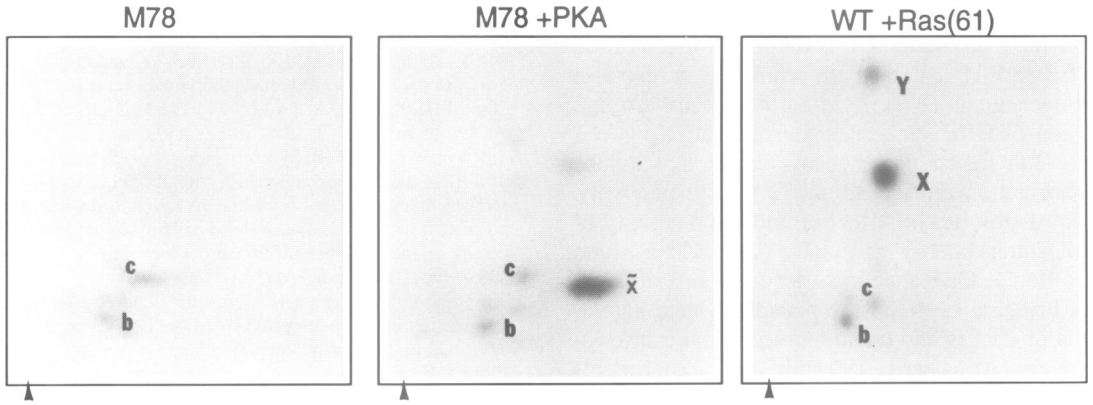

Fig. 3. Phosphopeptide maps of *in vivo* labeled M78 and WT c-Jun. ^{32}P-labeled M78 and WT c-Jun were isolated as described in text from either non-stimulated cells (M78), PKA co-transfected cells (M78+PKA) or Ha-Ras^{Leu61} co-transfected cells (WT+Ras). Proteins isolated by immunoprecipitation and resolved by SDS−PAGE were eluted, trypsinized and subjected to 2-D phosphopeptide mapping. Phosphopeptides b and c represent phosphorylation of c-Jun C-terminal sites (Boyle *et al.*, 1991), while phosphopeptides X and Y are due to phosphorylation of c-Jun at Ser73 and Ser63, respectively (Smeal *et al.*, 1991). Phosphopeptide x̃ is due to phosphorylation of M78 at Ser73, as indicated in Figure 1.

by Ala has only a slight effect on stimulation of c-Jun activity by oncogenic Ha-Ras (Smeal *et al.*, 1991). We also examined a mutant, M2D, in which Ser63 and Ser73 were replaced by Asp residues. Despite the introduction of two negatively charged residues into these positions, the activity of M2D was only 2.2-fold higher than that of WT c-Jun. Immunoprecipitation of ^{35}S-labeled proteins indicated that all of the mutants were expressed as efficiently as WT c-Jun (data not shown).

Discussion

Phosphorylation of several transcription factors at specific phosphoacceptor sites located within their activation domains correlates with increased transcriptional activity, whereas substitution of the phosphoacceptors by non-phosphorylatable residues prevents this increase (Gonzalez and Montminy, 1989; Smeal *et al.*, 1991; Marais *et al.*, 1993; Trautwein *et al.*, 1993). It was therefore proposed that in each of these cases phosphorylation of the trans-activation domain is directly responsible for stimulating its activity (Hunter and Karin, 1992; Jackson, 1992). However, these loss-of-function mutations do not provide a formal genetic proof that phosphorylation of any of these factors is the primary regulatory event leading to increased transcriptional activity. This basic problem was addressed by construction of a c-Jun mutant that is phosphorylated by a protein kinase (e.g. PKA) which does not affect the WT protein. As shown above, this mutant, M78, is phosphorylated efficiently both *in vitro* and *in vivo* by PKA, but is no longer phosphorylated by either JNK1 or JNK2, the protein kinases thought to be responsible for stimulating c-Jun transcriptional activity (Hibi *et al.*, 1993). In both cases, phosphorylation occurs at the same phosphoacceptor sites, with Ser73 being the primary site and Ser63 the secondary site. Most importantly, the transcriptional activity of WT c-Jun is strongly enhanced (10-fold) by signals that activate JNK (e.g. Ha-Ras; Dérijard *et al.*, 1994) but not by PKA, whereas M78 activity is augmented by PKA (9.5-fold) but not by JNK activation. These results provide a definitive proof that phosphorylation of c-Jun itself, at Ser73 (and Ser63), rather than the concomitant phosphorylation of another

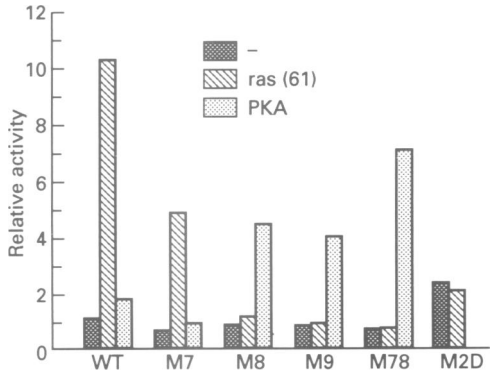

Fig. 4. Effects of PKA and Ras on the transcriptional activity of WT c-Jun and the altered kinase specificity mutants. F9 cells were co-transfected with 2.5 μg of the AP-1 responsive reporter -73Col-CAT along with the RSV expression vectors (1 μg each) encoding either WT c-Jun, M7, M8, M9, M78 or the Asp substitution mutant M2D. Where indicated, the co-transfections include pZipNeo (2 μg, solid boxes), pZipNeoRas^{Leu61} (2 μg, striped boxes) or pCMV-PKA (5 μg, dotted boxes). Twenty four hours after transfection, the cells were harvested and CAT activity determined. The results shown are the averages of three experiments and are reported as activity relative to that of WT c-Jun co-transfected with pZipNeo, which is given an arbitrary value of 1.0. This unit of activity represents 8-fold activation above basal activity observed with 'empty' RSV expression vector.

protein, is directly responsible for enhancing its transcriptional activity. This experimental approach, we believe, should be applicable for confirming the role of phosphorylation in controlling the activity of other transcription factors, provided that their phosphorylation sites are known.

It was postulated that phosphorylation of activation domains enhances their activity by stabilizing their interaction with the basal transcriptional machinery (Hunter and Karin, 1992; Jackson, 1992). We recently found that the N-terminal activation domain of c-Jun can bind to the newly discovered nuclear protein CBP (Chrivia *et al.*, 1993) in a phosphorylation-dependent manner (Arias *et al.*, 1994). This interaction is indeed mediated by the N-terminal activation domain, because it occurs only after phosphorylation of c-Jun by JNK rather than its phosphorylation by CKII, which phosphorylates c-Jun on C-terminal sites which affect its DNA binding activity (Lin

et al., 1992). Although the exact biochemical function of CBP remains to be determined, it is required for activation of both CREB and AP-1-dependent promoters in response to extracellular signals (Arias *et al.*, 1994). Furthermore, overexpression of CBP stimulates transactivation by WT CREB and c-Jun in the presence of signals that induce their phosphorylation on Ser133 and Ser73, respectively. CBP overexpression has no effect on the basal activity of the non-phosphorylatable mutants $CREB^{Ala133}$ and $c\text{-}Jun^{Ala63/73}$. Hence, CBP appears to act as a transcriptional co-activator bridging between the phosphorylated activation domains of CREB and c-Jun and the basal transcriptional machinery (Arias *et al.*, 1994). In other experiments we found that the M78 mutant c-Jun can also bind CBP, but only after its phosphorylation by PKA (T.Smeal and J.Arias, unpublished results). Therefore, phosphorylation of Ser73, regardless of the actual protein kinase involved, confers upon c-Jun or M78 the ability to bind CBP. These results suggest a simple model according to which phosphorylation of either CREB or c-Jun results in a considerable increase in their affinity to CBP which then mediates their interaction with the basal transcription machinery. At this point, it is not clear whether phosphorylation activates CBP binding by altering the conformation of the c-Jun activation domain or by providing additional critical contacts. Since the substitution of Ser73 and Ser63 by Asp residues does not result in a considerable increase in c-Jun activity, it appears that phosphorylation of these residues provides more than just negative charges.

Another important conclusion of the present experiments is the possibility of changing the biological specificity of signal transduction pathways by altering the specificity of the protein kinase–substrate interaction lying at their nuclear end. Normally, AP-1 target genes, such as collagenase, are not induced in response to PKA activation (Angel and Karin, 1991; Karin and Smeal, 1992). However, in cells co-transfected with the PKA substrate M78, the collagenase reporter is efficiently induced in response to PKA activation. These findings provide strong support to the notion that the specificity of the biological response to a given extracellular stimulus is dictated by the spectrum of transcription factors that are phosphorylated by the protein kinases that act at the nuclear end of the signaling cascades activated by that stimulus.

Materials and methods

Tissue culture and transfections

F9 cells were grown in F12-DMEM (1:1) medium supplemented with 10% FCS and 10^{-4} M β-mercaptoethanol. Cells were transfected with calcium phosphate–DNA co-precipitates, after 8 h the co-precipitates were removed and the cells were harvested after 24 h for CAT analysis or were metabolically labeled and harvested after 16 h as indicated (Boyle *et al.*, 1991; Smeal *et al.*, 1991). For immunoprecipitations, cells were harvested in RIPA buffer and protein complexes were immunopurified as described previously (Smeal *et al.*, 1991).

Plasmid constructs, kinase assays and phosphopeptide maps

Single-stranded template of M13mp18 containing the *Xho*II to *Pst*I *c-jun* cDNA fragment used for site-directed mutagenesis was kindly provided by T.Deng. Site-directed mutagenesis was performed as described previously (Smeal *et al.*, 1991) and its results were confirmed by sequencing. GST–c-Jun fusion proteins were constructed, expressed and purified as previously described (Hibi *et al.*, 1993).

The PKA phosphorylation reactions were done in 20 mM HEPES pH 7.5, 2 mM EGTA, 2 mM EDTA, 60 mM β-glycerophosphate, 20 mM PNPP, 20 mM $MgCl_2$, 100 μM Na_3VO_4 and 5 μM ATP containing 5 μCi of [γ-^{32}P]ATP. JNK phosphorylation reactions were performed in 20 mM HEPES pH 7.8, 2 mM DTT, 20 mM β-glycerophosphate, 20 mM PNPP, 20 mM $MgCl_2$, 100 μM Na_3VO_4 and 5 μM ATP containing 5 μCi of [γ-^{32}P]ATP. Both reactions were performed at 37°C for 30 min. The PKA was the recombinant C subunit (Slice and Taylor, 1989) and JNK included both the JNK1 and JNK2 isoforms that were extensively purified from UV irradiated HeLa cells by liquid chromatography. The details of the JNK purification procedure will be published elsewhere and are available upon request.

Phosphorylated proteins were immunoprecipitated with c-Jun C-terminal antibodies, subjected to SDS–PAGE, eluted, trypsinized and subjected to 2-D phosphopeptide mapping (Boyle *et al.*, 1991). Manual Edman degradation was performed as previously described (Lin *et al.*, 1992).

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