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Jun Is Phosphorylated by Several Protein Kinases at the Same Sites That Are Modified in Serum-Stimulated Fibroblasts

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c-*jun* is a member of the family of immediate-early genes whose expression is induced by factors such as serum stimulation, phorbol ester, and differentiation signals. Here we show that increased Jun synthesis after serum stimulation is accompanied by a concomitant increase in phosphorylation. Several serine-threonine kinases were evaluated for their ability to phosphorylate Jun in vitro. $p34^{cdc2}$, protein kinase C, casein kinase II, and $pp44^{mapk}$ phosphorylated Jun efficiently, whereas cyclic AMP-dependent protein kinase and glycogen synthase kinase III did not. The sites phosphorylated by $p34^{cdc2}$ were similar to those phosphorylated in vivo after serum induction. The major sites of phosphorylation were mapped to serines 63, 73, and 246. Phosphorylation of full-length Jun with several kinases did not affect the DNA-binding activity of Jun homodimers or Fos-Jun heterodimers. Comparison of the DNA binding and in vitro transcription properties of wild-type and mutated proteins containing either alanine or aspartic acid residues in place of Ser-63, -73, and -246 revealed only minor differences among homodimeric complexes and no differences among Fos-Jun heterodimers. Thus, phosphorylation of Jun did not produce a significant change in dimerization, DNA-binding, or in vitro transcription activity. The regulatory role of phosphorylation in the modulation of Jun function is likely to be considerably more complex than previously suggested.

Long-term cellular phenotypic responses to environmental cues are mediated by a complex network of messenger molecules that link cell surface events to alterations in gene expression. In recent years, many participants in this signal transduction cascade, including growth factors, receptors, G proteins, protein kinases, and transcription factors, have been shown to be encoded by proto-oncogenes (19, 75). The molecular dissection of proto-oncogene function has revealed that multiple layers of regulation exist at each of the critical stages of signal transduction. A general principle is emerging that, while a relatively small number of signaling pathways are involved in coordinating diverse biological responses such as proliferation, differentiation, or even cell death, these pathways operate in concert, and there is a substantial degree of cross-talk. Furthermore, although the number of regulatory molecules may be small, they operate in a combinatorial fashion, thereby generating a high degree of specificity.

The transcription factor (activator protein 1) (AP-1) was first described as a DNA-binding activity specific for basal enhancer elements in simian virus 40 and the human metallothionein II_A gene (53). Subsequently, it was shown to transduce signals generated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activation of protein kinase C (PKC) to TPA-responsive gene transcription (8, 54). Although the coupling mechanism was unclear, it was suggested that PKC activation led to posttranslational modification of preexisting AP-1 proteins. The discovery that the proto-oncogenes *fos* and *jun* encode components of AP-1 (reviewed in reference 28) has allowed an analysis of the molecular basis of transcriptional regulation in response to extracellular stimuli. However, the properties of Fos and Jun have revealed a hitherto unexpected level of complexity that is associated with stimulus-transcription coupling.

c-*fos* and c-*jun* are members of the set of genes known as immediate-early genes (29, 52). In the majority of cell types, although not all, the basal level of expression of these genes is relatively low. However, they can be induced rapidly and transiently by extracellular stimulation. In addition to TPA treatment, this occurs in response to a great variety of mitogenic and differentiation-inducing agents and even under conditions associated with depolarization of neurons (41, 64). In these circumstances, Fos and Jun are thought to function in coupling short-term signals elicited by cell surface stimulation to long-term changes in cellular phenotype by regulating gene expression. The situation is complicated by the existence of several *fos*- and *jun*-related genes that are also induced by cell surface stimulation (25, 62, 68, 77, 78, 98). These gene families contain a highly conserved leucine zipper and basic region motif (51) which is responsible for protein dimerization and DNA binding (36, 48, 70, 81, 90). Fos-related proteins form heterodimers with the Jun-related proteins and selected members of the activating transcription factor (ATF)-cyclic AMP response element binding (CREB) family, whereas Jun-related proteins can form homodimers as well as heterodimers with the Fos family and several of the ATF-CREB family (26, 27, 47, 48, 73, 74). The range of complexes present in any given cell type depends on the timing and duration of expression of these proteins in response to different stimuli. Thus, the composition, specificity, and activity of AP-1 are dynamic, changing over time in accordance with the fluctuating levels of available Jun and Fos family proteins.

In addition to the complex regulation of expression of these family members, accumulating evidence suggests that

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there are also several posttranscriptional control mechanisms. A critical cysteine residue present in the DNA-binding domain of all Fos and Jun family members must be reduced for high-affinity DNA-binding activity in vitro (6). Recently, a cellular protein (Ref-1) that is capable of reducing this cysteine residue has been identified (94, 95). Therefore, it is possible that the activity of AP-1 proteins is modulated through reduction-oxidation (redox) signaling. The relationship between Jun and AP-1 has led to the analysis of the role of TPA-mediated changes in phosphorylation in controlling Jun function. Indeed, several groups have suggested that there is a direct connection between phosphorylation or dephosphorylation and the activity of Jun homodimers (17, 72, 86). However, each of these studies invokes a different mechanism to explain the effects of TPA on Jun phosphorylation and function, none of which directly involve PKC.

We investigated the effects of phosphorylation on the activity of Jun homodimers and heterodimers. $p34^{cdc2}$ and $pp44^{mapk}$ phosphorylated Jun on the same sites that are phosphorylated in serum-stimulated fibroblasts. The major sites of phosphorylation, Ser-63, -73, and -246, were found to have only a modest effect on the DNA binding and in vitro transcription properties of Jun homodimers and had no effect on the activity of Fos-Jun heterodimers. In contrast, PKC phosphorylated Jun on peptides that were not phosphorylated in response to serum stimulation. Thus, a simple paradigm explaining altered Jun activity as a consequence of phosphorylation in response to extracellular signaling was not supported by our study. Instead, phosphorylation is likely to represent only one of a number of mechanisms that in combination modulate Jun function and specificity.

MATERIALS AND METHODS

Protein purification. Truncated *jun* genes corresponding to the sequence encoding amino acids 91 to 334, 187 to 334, 225 to 334, and 241 to 334 were derived from full-length *jun* as described previously (2). Truncated genes corresponding to Jun31-334 and Jun58-334 were constructed by using a similar strategy. Substitution of serine codons with alanine or aspartic acid codons was introduced into the full-length *c-jun* gene by polymerase chain reaction mutagenesis (1). Wild-type, mutated, and truncated Jun (rat) proteins were expressed in *Escherichia coli* as hexahistidine fusion proteins and purified from *E. coli* cell lysates by nickel affinity chromatography as previously described (1). Polyclonal antiserum raised against full-length Jun was used for immunoprecipitation of Jun proteins.

Phosphorylation of Jun proteins. $p34^{cdc2}$ was purified from nuclear extracts from nocodazole-treated HeLa cells (61). This preparation exhibited no detectable contamination with seven other classes of protein kinases, including PKC, cyclic AMP-dependent protein kinase (PKA), casein kinase II (CKII), glycogen synthase kinase III (GSK-3), phosphorylase kinase, and calmodulin kinase II. The enzyme was effectively inhibited by an excess of synthetic peptide substrate specific for $p34^{cdc2}$, and the enzymatic activity was depleted by exposure to antibodies specific for $p34^{cdc2}$ (61). PKC was a generous gift from Sue Jaken (W. Alton Jones Cell Science Center, Lake Placid, N.Y.). CKII was purified as described previously (60). PKA was purchased from Sigma. GSK-3 was a generous gift from Jim Woodgett (Ludwig Institute, London) and from Balwant Khatra (Long Beach, Calif.). Phosphorylation reactions were performed at 30°C in the appropriate reaction mixtures as described previously (5). $pp44^{mapk}$ was isolated from 208F rat fibroblasts by using polyclonal antiserum obtained from John Blenis (Harvard Medical School, Boston, Mass.). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum or maintained for 36 h in 0.5% serum and then stimulated with 20% serum for 0 min, 5 min, or 2 h. $pp44^{mapk}$ immunoprecipitation and kinase assays were performed as described previously (22). $pp44^{mapk}$ reactions contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.0), 10 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, and 0.5% Nonidet P-40. For in vivo labeling of Jun, 208F rat fibroblast cells were grown in DMEM with low serum (0.5%) for 48 h. The cells were stimulated by the addition of 10% serum in the presence of phosphate-free or methionine-free medium containing $^{32}P_i$; Amersham (5 mCi/ml) or [^{35}S]methionine (150 μCi; Amersham) for 2 h. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, and the radiolabeled protein was immunoprecipitated with polyclonal antiserum prepared against full-length Jun.

Phosphopeptide maps. Phosphorylated proteins were digested in situ on nitrocellulose filters (57). Briefly, radiolabeled Jun was resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The proteins were transferred electrophoretically to nitrocellulose filters, and fragments containing the radiolabeled proteins were excised. Proteins were digested with 40 to 100 μg of trypsin in 50 mM ammonium bicarbonate for 2 to 12 h at 37°C. Fresh trypsin (40 to 100 μg) was then added, and digestion was continued for an additional 2 to 12 h. The soluble peptides were subjected to performic acid oxidation and were resolved on high-resolution tris-tricine-urea gels (80). The radiolabeled phosphopeptides were visualized by autoradiography.

HPLC of radioactive Jun tryptic phosphopeptides. Jun was phosphorylated with [γ-^{32}P]ATP (Amersham), digested with trypsin as described above, and subjected to high-pressure liquid chromatography (HPLC) with an octadecylsilanyl silica (TP-300 A pore size; Vydac) column (4.6 by 200 mm) on a Hewlett-Packard 1090M liquid chromatograph. The solvent system consisted of 0.1% (wt/vol) aqueous trifluoroacetic acid (solvent A) and 70% (vol/vol) acetonitrile in 0.085% (wt/vol) aqueous trifluoroacetic acid (solvent B). The sample was applied at a flow rate of 0.2 ml/min, and nonadsorbed material was eluted for 15 min. A gradient of 0 to 100% solvent B was run over 70 min, and the absorbance of the effluent was recorded at 214 and 280 nm. Fractions (75 to 100 μl) corresponding to absorbance peaks were collected in siliconized polypropylene tubes, and radioactivity was measured in a scintillation counter by Cerenkov radiation.

Mass spectrometry. Mass spectrometric analyses were performed on a Bio Ion 20 plasma desorption, time-of-flight mass spectrometer (Bio Ion Division, Applied Biosystems, Inc., Uppsala, Sweden) equipped with a ^{252}Cf source (87). Samples were prepared and analyzed essentially as described previously (18, 40). Briefly, an aliquot (10 μl of the fractions was applied to aluminized Mylar targets previously electrosprayed with 50 μg of nitrocellulose in acetone, spin dried, and washed with 20 μl of 0.1% (wt/vol) trifluoroacetic acid in water. Spectra were collected for 2 h at 16-kV positive accelerating potential.

Sequence analysis. For radioactive sequence analysis, samples were covalently attached to arylamine-derivitized polyvinylidene difluoride membranes (Sequalon AA; Millipore) by using water-soluble carbodiimide according to the instructions of the manufacturer. Sequencing and recovery of ^{32}P-labeled amino acid derivatives were performed by

repetitive Edman degradations on a protein sequencer (model 473; Applied Biosystems, Foster City, Calif.) with liquid trifluoracetic acid extraction of the membrane in the reaction cartridge as described previously (76).

DNA binding and in vitro transcription assays. Gel shift assays were performed with 200 to 600 pM protein and 20 pM radiolabeled oligonucleotide containing the AP-1-binding site. Protein dimerization, DNA binding, and electrophoresis were performed as described previously (2).

In vitro transcription assays to evaluate Jun activity were performed by adding Jun and a reporter DNA template with six tandem AP-1 sites to HeLa nuclear extract depleted for endogenous AP-1 activity as described previously (2).

RESULTS

Jun is phosphorylated on several sites in vivo and in vitro. A variety of extracellular signals influence *c-jun* expression and the degree of modification of its protein product. Jun was expressed at low levels in quiescent fibroblasts (Fig. 1A), and the protein was not extensively phosphorylated, as shown by immunoprecipitation analysis of extracts made from quiescent cells grown in medium containing ^{32}P. Serum stimulation induced a rapid increase in both the synthesis and extent of phosphorylation of Jun (+ serum, Fig. 1A). This contrasts with the report that phosphorylation of Jun decreased after TPA treatment of HeLa cells (17). However, in U937 cells, TPA treatment resulted in increased phosphorylation of Jun (72). Thus, Jun phosphorylation appears to be controlled in a cell-type and stimulus-specific manner.

To identify the kinases that modified Jun in response to serum stimulation, we tested several candidate serine-threonine kinases for their ability to modify purified Jun. Purified p34^{cdc2}, PKC, and CKII all efficiently modified Jun in vitro (Fig. 1B). However, PKA did not phosphorylate Jun efficiently (Fig. 1B) under conditions in which Fos was readily phosphorylated (5). The stoichiometry of phosphorylation obtained with p34^{cdc2}, PKC, and CKII was up to 0.3, 0.3, and 0.1 mol of phosphate per mol of Jun protein, respectively, in several independent experiments. In contrast to previous reports (17), we found that our Jun preparation was not phosphorylated efficiently using two independent sources of GSK-3. Levels of phosphorylation were less than 1/10th of those obtained with PKC or p34^{cdc2}. The reasons for the discrepancy between our findings and those of the prior study are unclear. One possible explanation is that the differences were due to the method of extraction and renaturation of Jun from *E. coli*. For our studies, Jun was expressed in *E. coli* as a hexahistidine fusion protein and purified to apparent homogeneity by nickel affinity chromatography as described previously (3). The purified protein was active in DNA binding and in vitro transcription assays (1–6). Furthermore, biophysical studies indicated that greater than 95% of the Fos and Jun molecules purified in this manner are capable of forming dimers and binding to DNA (71; unpublished data). Thus, by several independent criteria, the Jun proteins used in the present study were in an appropriate functional conformation. In addition, Fos, purified under the same conditions, was phosphorylated efficiently by GSK-3.

To compare the sites of in vitro phosphorylation of Jun with those phosphorylated after serum stimulation, we resolved tryptic phosphopeptides from in vitro- and in vivo-labeled proteins on tricine-urea gels (Fig. 1C). Phosphorylation of Jun by p34^{cdc2} generated four major phosphopeptides. CKII phosphorylated several peptides including

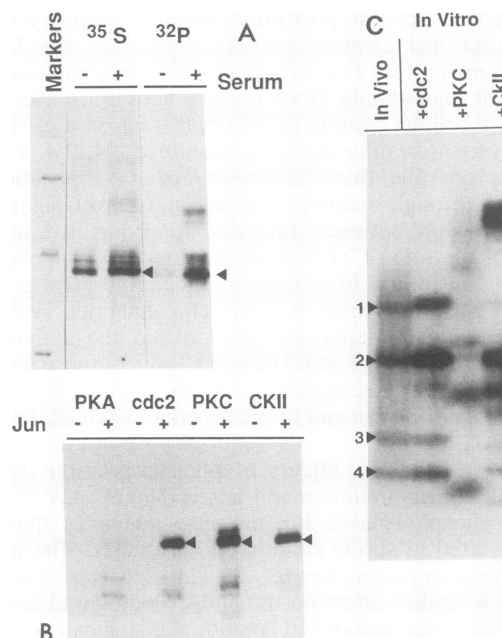

FIG. 1. Jun is phosphorylated at similar sites in vivo and in vitro. (A) Rat 208F fibroblast cells were grown in low serum (0.5%) for 48 h. Cell growth was stimulated by the addition of 20% serum in the presence of methionine-free or phosphate-free medium. The cells were labeled by the addition of [^{35}S]methionine (^{35}S) or $^{32}P_i$ (^{32}P) for 2 h and then lysed in RIPA buffer. The radiolabeled proteins were immunoprecipitated by the addition of anti-Jun antiserum, resolved on an SDS-9% polyacrylamide gel, and visualized by autoradiography. The arrowhead indicates the position of Jun. Markers are ^{14}C-labeled protein standards (Amersham). (B) In vitro phosphorylation assays were performed with Jun purified from *E. coli*. Purified PKA, p34^{cdc2} (cdc2), PKC, and CKII were incubated in the presence (+) or absence (−) of Jun (0.1 μg) in the appropriate reaction mixture (see Materials and Methods) containing 10 μM ATP supplemented with 1 μCi of [γ-^{32}P]ATP (Amersham). The reactions were stopped by the addition of SDS sample buffer. Radiolabeled proteins were resolved on an SDS-polyacrylamide gel and were visualized by autoradiography. The arrowheads indicate the position of Jun. (C) Jun was modified in vitro by p34^{cdc2} (cdc2), PKC, or CKII (as in panel B) or labeled in vivo (as in panel A). The proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto nitrocellulose. The radiolabeled proteins were excised and digested in situ with trypsin, as described in the Materials and Methods. The soluble phosphopeptides were resolved on a high-resolution tricine-urea gel and were visualized by autoradiography. Numbers 1 to 4 indicate the positions of the Jun phosphopeptides.

peptides 2 and 4, which were also phosphorylated by p34^{cdc2}. PKC phosphorylation of Jun generated two major phosphopeptides that did not comigrate with the peptides modified by p34^{cdc2} or CKII. In serum-stimulated rat fibroblasts, Jun contained four major phosphopeptides which corresponded to peptides 1 to 4 generated by p34^{cdc2} in vitro. The level of phosphorylation of Jun in serum-deprived rat fibroblasts was too low to determine the location of the sites. These findings suggest that p34^{cdc2} or a related kinase phosphorylates Jun at several sites as a consequence of serum stimulation.

Jun is composed of several functional domains that contribute to dimerization, DNA binding, and transcriptional regulation (2). To localize the phosphorylation sites, we tested a series of N-terminal truncations that lack portions of

TABLE 1. Mass spectrometric anslysis of major phosphorylation sites

Peptide (residues)	No. of phosphates	Measured $[M + H]^+$	Calculated $[M + H]^+$
Serine 63 and 73			
55–70	0	1,672.1	1,673.0
	1	1,755.1	1,753.0
57–70	0	1,472.3	1,472.7
	1	ND[a]	1,552.7
55–78	0	2,570.5	
	1	ND	2,650
	2	2,728.6	2,730.0
Serine 246			
225–255	0	3,476.6	3,478.6
	1	3,557.8	3,558.6
230–255	0	2,925.1	2,925.0
	1	3,002.5[b]	3,005.0

[a] ND, not detected.
[b] Calculated from measurement of the $[M + 2H]^+$. The $[M + H]^+$ was not of significant intensity.

these domains as substrates for phosphorylation by p34^{cdc2}, PKC, and CKII. Each of the truncated peptides contained the leucine zipper and basic region that compose the core motif required for dimerization and DNA binding (2). The truncated Jun polypeptides were all modified efficiently by p34^{cdc2}, PKC, and CKII (data not shown), suggesting that the DNA-binding core of Jun contains sites that can be phosphorylated by several kinases.

To determine the location of p34^{cdc2} phosphorylation sites more precisely, we used a combined approach (55) of peptide mapping of truncated proteins, HPLC, mass spectrometry, and sequencing. The major p34^{cdc2} phosphorylation sites were localized to the amino terminus at Ser-63 and -73 and in the carboxy terminus at Ser-246 of the rat c-Jun sequence. Mass spectrometric analysis of the major sites is shown in Table 1. Tryptic peptides of the expected molecular mass plus the mass of a hydrogen ion ($[M + H]^+$) together with a selection of the measured values are indicated. For Ser-63 and -73, single peptides representing residues 55 to 70 and 57 to 70 containing an additional phosphate group were identified. Furthermore, a peptide representing amino acids 55 to 78 that contained two phosphate groups was also identified. We reckoned that this peak corresponded to peptide 3 (Fig. 1C). Two phosphopeptides containing Ser-246 were identified (Table 1, 225-255, 230-255). The assignment of phosphorylation at Ser-246 was confirmed by sequence analysis (see Fig. 4). The identification of these major sites confirms previous reports. Ser-243 of c-Jun (human), which corresponds to Ser-246 in c-Jun (rat), was found to be phosphorylated in unstimulated HeLa cells and was phosphorylated by GSK-3 in vitro (17). In addition, phosphorylation of Ser-62 and -72 in c-Jun (human), which correspond to Ser-63 and -73 in c-Jun (rat), was observed in TPA-treated U937 cells (72). These sites were also phosphorylated by mitogen-activated protein (MAP) kinases pp54 and pp42/44 in vitro (72). Phosphorylation of Ser-63 and -73 was also detected in *ras*-transformed NIH 3T3 cells (86).

Mutational analysis of p34^{cdc2} phosphorylation sites. The preceding results identified several serine residues in Jun that are major sites for phosphorylation by p34^{cdc2}. To evaluate the functional implications of p34^{cdc2} phosphorylation, we substituted Ser-63, -73, and -246 in several combinations with alanine or aspartic acid residues (Fig. 2). Alanine residues are conservative substitutions for serine residues, whereas aspartic acid introduces a negative charge which can mimic serine phosphorylation under some circumstances. Phosphorylation of wild-type Jun protein (J) and proteins that contained single mutations at Ser-246 (JA246; JD246) generated two phosphorylated protein bands on SDS gels. (Fig. 3A). However, the upper band was absent in all Jun polypeptides with substitutions of Ser-63 and -73 (i.e., JA63,A73; JD63,D73; JA63,A73,A246; JA63,A73,D246; JD63,D73,A246; JD63,D73,D246). The upper band was not detected when wild-type Jun was phosphorylated with a fourfold dilution of p34^{cdc2}, suggesting that there is a critical threshold concentration of p34^{cdc2} that is required for phosphorylation of Ser-63 and -73. Tryptic phosphopeptide maps were generated for the wild-type and mutated proteins. The wild-type protein contained the four major phosphopeptides detected in serum-stimulated fibroblasts (Fig. 1C) and an additional fifth phosphopeptide (peptides 1 to 5, Fig. 3B). Both peptides 4 and 5 were generated by cleavage of peptide 2. Peptide 5 was only detected at high levels after treatment with elevated concentrations of trypsin. Proteins with substitutions of Ser-63 and -73 (JA63,A73; JD63,D73) were phosphorylated on peptides 1, 2, 4, and 5 but not on peptide 3. Conversely, the mutated proteins JA246 and JD246, which retain Ser-63 and -73, were phosphorylated on peptide 3. Substitution of Ser-246 with alanine (JA246; JA63,A73, A246; JD63,D73,A246) resulted in the loss of phosphorylation of peptide 5, whereas peptide 4 was not phosphorylated in proteins containing substitutions with aspartic acid at

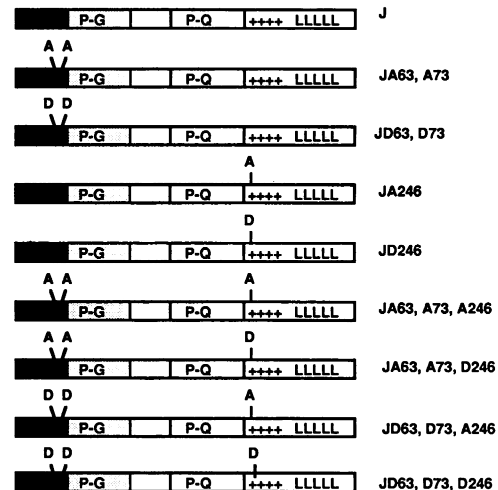

FIG. 2. Illustration of Jun phosphorylation site mutations. The leucine zipper of Jun (LLL) forms a dimerization interface that brings adjacent basic regions (+++) into contact with DNA. A proline-glutamine-rich region (P-Q) enhances the DNA-binding affinity of Jun homodimers, a proline-glycine-rich region (P-G) contains the activation domain, and the N terminus (black box) negatively regulates transcriptional activity. Serines 63 and 73, which are contained in the N terminus, and Serine 246, which is contained in the basic region, were identified as sites of phosphorylation by p34^{cdc2}. Mutations which replaced serines with alanine (A) or aspartic acid (D) at the N-terminal serines (JA63,A73; JD63,D73), at the C-terminal serine (JA246; JD246), or at all three serines (JA63,A73,A246; JA63,A73,D246; JD63,D73,A246; JD63,D73, D246) were introduced by PCR. The resulting mutated proteins were purified as hexahistidine fusion proteins from *E. coli* by nickel chelate chromatography.

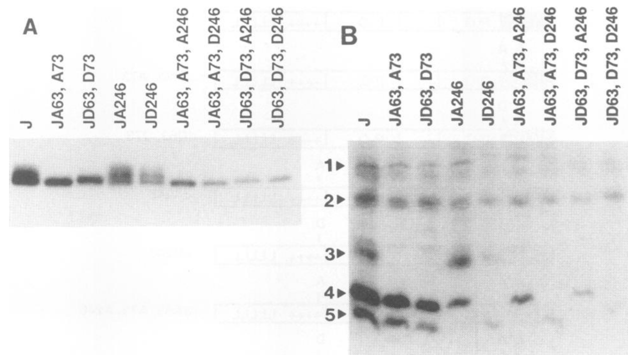

FIG. 3. p34^{cdc2} phosphorylation and peptide maps of mutated Jun proteins. (A) Wild-type and mutated Jun polypeptides (0.5 μg) (described in Fig. 2) were incubated with p34^{cdc2} in the appropriate reaction mixture containing 10 μM ATP supplemented with 5 μCi of [γ-^{32}P]ATP. The reactions were stopped by the addition of SDS sample buffer, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized by autoradiography. (B) Phosphorylated wild-type and mutated Jun proteins were digested with trypsin, and the resulting phosphopeptides were separated by electrophoresis as described in the legend to Fig. 1C. Phosphopeptides 1 to 5 are indicated with arrowheads.

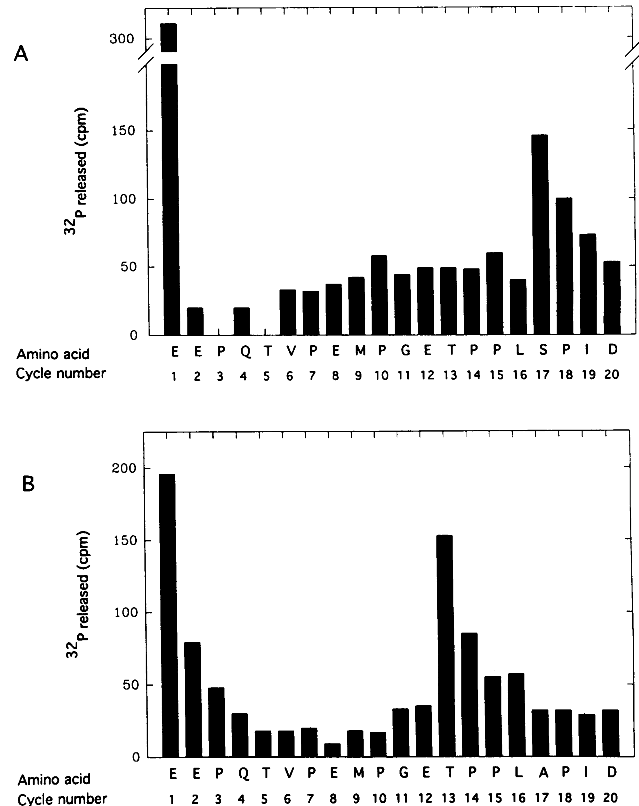

FIG. 4. Radioactive sequence analysis of phosphopeptide 4 labeled by p34^{cdc2}. Tryptic phosphopeptide maps were generated for JA63,A73 and JA63,A73,A246 phosphorylated by p34^{cdc2} as described in the legend to Fig. 3. Phosphopeptide 4 was excised and subjected to radioactive sequence analysis as described in the text (see Materials and Methods). The radioactivity released with each cycle of Edman degradation is presented in bar graphs for JA63,A73 (A) and JA63,A73,A246 (B). The amino acid sequence and each cycle number are listed under the bar graph. The large release of radioactivity on the first cycle represents free phosphate nonspecifically bound to the polyvinylidene difluoride membrane. The graphs were normalized by subtracting the average background counts, 47 cpm, from each cycle.

residue 246 (JD246; JA63,A73,D246; JD63,D73,D246). The presence of phosphopeptide 4 in mutated Jun peptides containing the Ala-246 substitution was not expected. The sites of phosphorylation within this peptide were determined by sequence analysis of JA63,A73 and JA63,A73,A246 (Fig. 4). In JA63,A73, the major peak of radioactivity was detected at cycle 17, which corresponds to Ser-246. In contrast, when Ser-246 is substituted with Ala (JA63,A73,A246), the major peak of radioactivity was released at cycle 13, which corresponds to Thr-242. Thus, the presence of an alanine residue at codon 246 in place of serine resulted in the recognition of a minor secondary phosphorylation site which was not detected in JA63,A73. Peptide 5 may be generated by an alteration in the trypsin cleavage pattern caused by the aspartic acid at Ser-246. In the wild-type Jun protein, peptide 5 was present in low and variable amounts and appeared to be a consequence of overdigestion with trypsin. Unfortunately, insufficient radioactivity was recovered from this peptide to allow sequence analysis. However, a peptide of the correct molecular mass was detected by mass spectrometry (Table 1). These mutagenesis studies confirm that Ser-63, -73, and -246 are major sites for phosphorylation of Jun by p34^{cdc2} in vitro.

Jun is phosphorylated by pp44^{mapk} on Ser-246. p34^{cdc2} is one of a number of kinases that are activated by mitogenic signals elicited by serum stimulation. The MAP family of kinases is also responsive to such signaling cascades. pp44^{mapk}, also termed ERK-1 for extracellular regulated kinase, is activated during the G_2/M transition period in the meiotic maturation of *Xenopus* oocytes and during mitosis at the G_0/G_1 transition after treatment of fibroblasts with polypeptide growth factors and serum (reviewed in references 14 and 88). Therefore, we investigated the ability of pp44^{mapk} to phosphorylate Jun. pp44^{mapk} isolated by immunoprecipitation from fibroblasts 5 min after serum stimulation was capable of phosphorylating purified Jun (J, Fig. 4A). This is consistent with a previous report of Jun phosphorylation by pp42/44^{mapk} (23). However, Jun is expressed at its highest levels approximately 2 h after serum stimulation. While maximal induction of pp44^{mapk} activity was observed 5 min after serum stimulation (Fig. 5A, 5 min), elevated levels of pp44^{mapk} activity were still present 2 h after serum addition, and pp44^{mapk} activity was present in normally cycling cells (Fig. 5A, 2 hr and C). Little or no pp44^{mapk} activity was observed in extracts from serum-deprived cells (0 min). Thus, pp44^{mapk} may phosphorylate Jun in serum-stimulated fibroblasts.

Previously, it was reported that phosphorylation of Ser-63 and -73 by pp54 MAP kinase resulted in a decrease in the mobility of Jun on SDS gels (72). Phosphorylation of wild-type Jun (J) and mutated proteins containing substitutions at Ser-246 (JA246, JD246) with pp44^{mapk} also produced a faint upper band which was not present after phosphorylation of mutated proteins containing substitutions of Ser-63 and -73 (JA63,A73; JA63,A73,A246) (Fig. 5A). However, the level of phosphorylation of Ser-63 and -73 was significantly less than that of Ser-246. In tryptic phosphopeptide maps generated from wild-type and mutated proteins phosphorylated with pp44^{mapk}, the major phosphopeptide observed in wild-type Jun (J) and in a mutated protein containing a substitu-

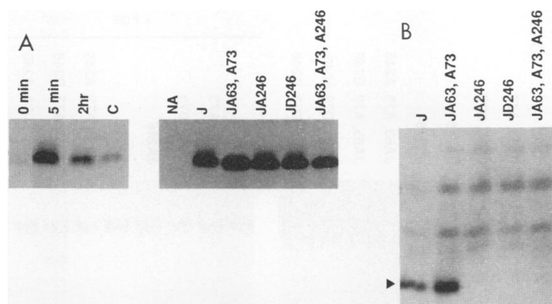

FIG. 5. pp44^{mapk} phosphorylation and peptide maps of mutated Jun proteins. (A) pp44^{mapk} was isolated as an immune complex from 208F rat fibroblasts grown in DMEM with 10% serum (C) or from 208F rat fibroblasts grown for 36 h in DMEM with 0.5% serum, unstimulated (0 min), or subsequently stimulated by DMEM containing 20% serum for 5 min (5 min) or 2 h (2 hr). Kinase assays were performed on 500 ng of purified wild-type Jun protein with 50 μM ATP supplemented with 5 μCi of [γ-^{32}P]ATP (see Materials and Methods). Wild-type (J) and mutated (JA63,A73; JA246; JD246; JA63,A73,A246), Jun proteins were phosphorylated after 5 min of serum stimulation. In lane NA, no Jun protein was added to the pp44^{mapk} immune complex. Labeled proteins were resolved and visualized as in Fig. 1B. (B) Wild-type and mutated Jun proteins were phosphorylated with pp44^{mapk} as described above. The labeled proteins were digested with trypsin and resolved on a tricine-urea gel as described in the legend to Fig. 1C. The major phosphopeptide is indicated with an arrowhead.

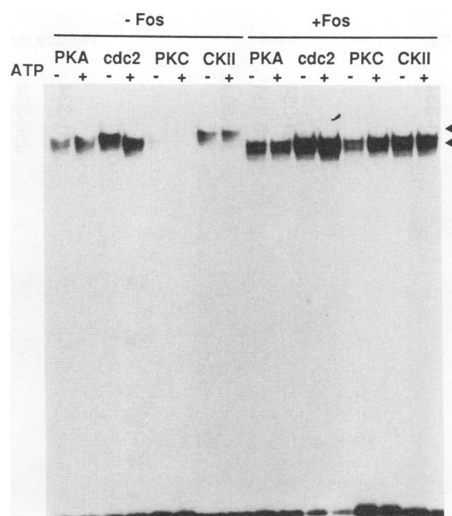

FIG. 6. Effect of phosphorylation on DNA binding. Jun was treated with PKA, p34^{cdc2}, PKC, or CKII in the presence (+) or absence (−) of 0.5 mM ATP in the appropriate reaction mixture (see Materials and Methods). The reactions were stopped by dilution into DNA binding buffer (3). The phosphorylated Jun proteins were incubated in the presence (+Fos) or absence (−Fos) of Fos together with a radiolabeled oligonucleotide containing the AP-1-binding site. The protein-DNA complexes were resolved on a low-ionic-strength polyacrylamide gel and were visualized by autoradiography. The upper arrowhead indicates Jun homodimers and the lower arrowhead indicates Fos-Jun heterodimers binding to DNA.

tion of Ser-63 and -73 (JA63,A73) corresponded to peptide 4 from the p34^{cdc2} phosphopeptide map, which contains Ser-246 (Fig. 5B). This phosphopeptide was not detected after phosphorylation of proteins with a mutation at Ser-246 (JA246; JD246; JA63,A73,A246). These findings are in agreement with a previous report that Jun Ser-246 was the major phosphorylation site for a growth factor-stimulated kinase (7). This kinase activity was subsequently identified as pp42^{mapk} and pp44^{mapk} (16, 69). In contrast to the situation with p34^{cdc2} kinase, substitution of Ser-246 with Ala does not result in phosphorylation of Thr-242 by pp44^{mapk}. Although JA246, JD246, and JA63,A73,A246 were phosphorylated relatively efficiently by pp44^{mapk} (Fig. 5A), there was no alteration in the specificity of the secondary sites of phosphorylation. Thus, p34^{cdc2} and pp44^{mapk} have overlapping specificities for phosphorylation sites on Jun. Furthermore, the results of our study and others (7), in contrast to Pulverer et al. (72), suggest that the specificities of pp42^{mapk} and pp44^{mapk} are distinct from that of pp54.

Effect of phosphorylation on DNA-binding activity. To investigate the functional consequences of phosphorylation by p34^{cdc2}, PKC, and CKII on DNA-binding activity, we performed gel shift assays using phosphorylated Jun. Treatment of Jun with p34^{cdc2}, PKC, or CKII had no detectable effect on the DNA-binding activities of either Jun homodimers or Fos-Jun heterodimers (Fig. 6). The loss of DNA-binding activity in the PKC lanes is a result of the presence of phospholipid in the PKC buffer which inhibits DNA-binding activity. These experiments were performed several times, using a wide range of protein concentrations and DNA binding conditions. In contrast, Boyle et al. (17) reported that GSK-3 phosphorylation of Ser-246 in a truncated Jun protein inhibited the DNA-binding activity of homodimers in vitro. One reason for the discrepancy between our results and those of Boyle et al. (17) may be that the stoichiometry of phosphorylation was too low to reveal differences in the DNA-binding activities of the modified and unmodified proteins. This is unlikely, however, because modification of Jun by p34^{cdc2} resulted in a shift in the mobility of the DNA-protein complexes, suggesting that the DNA-bound protein complex was phosphorylated (Fig. 6). Because the truncated proteins were phosphorylated more efficiently than the full-length proteins, we also tested the effect of phosphorylation of truncated Jun proteins with p34^{cdc2} on DNA-binding activity. No significant difference was found in the DNA-binding activity of several phosphorylated and nonphosphorylated truncated proteins either in the form of homodimers or as heterodimers with Fos (data not shown).

Effects of mutating Ser-63, -73, and -246 on DNA-binding activity. It is conceivable that phosphorylation of Jun by p34^{cdc2} at the N-terminal and C-terminal sites could have produced opposing effects on DNA-binding activity. To investigate the effects of phosphorylating Ser-63, -73, and -246 independently, we investigated the DNA-binding activities of the truncated and serine-substituted Jun polypeptides shown in Fig. 2 by gel shift analysis (Fig. 7A and 8A). Figure 7A shows that a Jun polypeptide truncated at amino acid 91 (J91-334) exhibited a higher apparent affinity for DNA than full-length Jun (J). Although the primary dimerization and DNA-binding domains of Jun are located in the C-terminal region, we noted previously that the N-terminal 90 amino acids had a negative effect on DNA-binding activity. Analysis of a series of polypeptides truncated at the N terminus revealed that a Jun polypeptide lacking 30 N-terminal amino acids (J31-334) and full-length Jun exhibited similar affinities for DNA. In contrast, a Jun polypeptide lacking 57 N-terminal amino acids (J58-334) exhibited an increased apparent affinity for DNA. Therefore, an N-terminal region of Jun

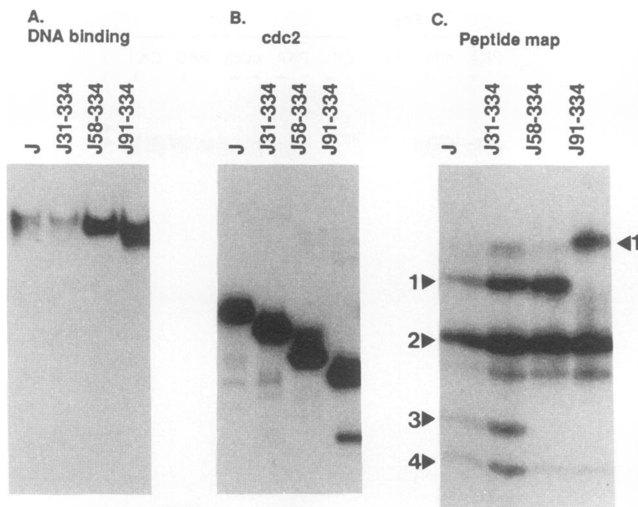

FIG. 7. Deletion of amino acids 30 to 57 enhances DNA binding. (A) DNA-binding activities of Jun proteins containing N-terminal deletions were compared. Full-length Jun (J) and deletions of the N-terminal 30 (J31-334), 57 (J58-334), and 90 (J91-334) amino acids were isolated from *E. coli* as described in the Materials and Methods. Purified proteins were incubated with a radiolabeled oligonucleotide containing the AP-1 site as described in the Materials and Methods. The protein-DNA complexes were resolved on a low-ionic-strength polyacrylamide gel and visualized by autoradiography. (B) Purified full-length and truncated proteins were phosphorylated with p34^{cdc2} as described in the legend to Fig. 3A, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized by autoradiography. (C) Radiolabeled full-length and truncated Jun proteins described in the legend to Fig. 6B were digested with trypsin for phosphopeptide maps as in Fig. 1C. Peptides 1 to 4 are indicated with arrowheads.

corresponding to amino acids 31 to 57 exerts a negative influence on Jun DNA-binding activity. Interestingly, this is the region of Jun that is deleted in v-*jun* (67). Moreover, it has been suggested that this region (termed the δ region) negatively regulates Jun transcription in vitro and in vivo (10, 15).

To examine the effect of N-terminal sequences on phosphorylation, we treated the truncated Jun proteins with p34^{cdc2} (Fig. 7B). Peptide mapping analysis of these truncated polypeptides demonstrated that J31-334 can be phosphorylated at Ser-63 and -73 (peptide 3) as well as at Ser-246 (peptides 2 and 4) by p34^{cdc2} (Fig. 7C). Although J57-334 contains Ser-63 and -73, it was not phosphorylated at these sites, as illustrated by the loss of phosphopeptide 3 in the trypsin digest. The decreased mobility of peptide 1 in J91-334 is caused by the addition of six histidine residues to the N terminus. The sites of phosphorylation in this peptide may be Thr-91 and/or Thr-93, which are good candidate p34^{cdc2} phosphorylation sites. Collectively, these data suggest that a conformational change caused by N-terminal truncation prevents recognition and modification of Ser-63 and -73 by p34^{cdc2}. Furthermore, this N-terminal region appears to have an allosteric effect on the activity of the C-terminal DNA-binding domain.

To determine the effects of phosphorylation of individual sites on Jun DNA-binding activity, we compared the activities of full-length proteins containing point mutations at serines 63, 73, and 246. A representative analysis of the DNA-binding activities of these mutated proteins in homodimeric and heterodimeric forms is shown in Fig. 8A.

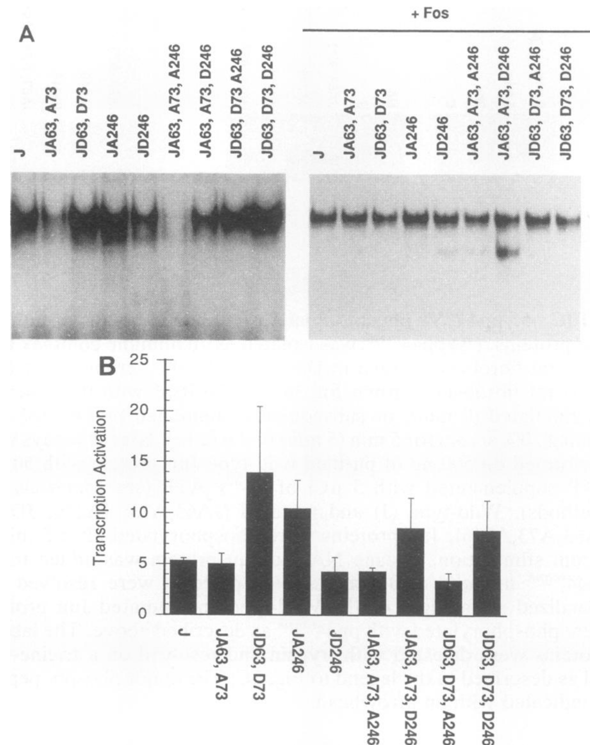

FIG. 8. Functional effect of point mutations at p34^{cdc2} phosphorylation sites in Jun. (A) Wild-type and mutated proteins described in the legend to Fig. 2 were assayed for DNA-binding activity in a gel shift assay as homodimers or Fos-Jun heterodimers (+Fos), using a radiolabeled oligonucleotide containing the AP-1-binding site as described in the legend to Fig. 6. Considerable experimental variability was observed. The figure is representative of the average of more than 50 independent experiments. The Jun homodimer gel shifts are overexposed compared with the Fos-Jun heterodimers to emphasize subtle differences. (B) Wild-type and mutated proteins were added to HeLa nuclear extracts which were depleted of AP-1 activity. Jun activity was assayed by the ability to transcribe a DNA template containing six tandem AP-1 sites in its promoter as described previously (2). Bars represent the average results of six experiments. Experimental variation is shown with error bars. Transcription activation represents the fold activation above the basal level of activity of the depleted HeLa extract.

While the results obtained included a substantial degree of variability, the following trends were noted in approximately 50 independent DNA binding assays done under a variety of conditions. The DNA-binding affinity of Jun homodimers containing substitutions of aspartic acid for serines 63 and 73 (JD63,D73) was enhanced two- to threefold compared with wild-type Jun (J) or with a mutated protein containing alanine residues at Ser-63 and -73 (JA63,A73). Although an aspartic acid substitution at serine 246 (JD246) might be predicted to inhibit the DNA-binding activity of Jun homodimers according to the results of Boyle et al. (17), no significant effect was observed. However, while substitutions with alanine at either the N-terminal serines (JA63,A73) or the C-terminal serine (JA246) did not alter DNA binding, a consistent decrease in DNA binding was observed with a mutated protein in which all three serines were replaced with alanine residues (JA63,A73,A246). This suggests that the point mutations in Jun may not act simply to mimic or prevent phosphorylation but might have an indirect effect on DNA-binding activity.

AP-1 DNA-binding activity in vivo is composed primarily of heterodimeric complexes between Fos and Jun family members (35, 49, 74). Therefore, it is important to determine the effects of Jun phosphorylation on heterodimer formation and on the DNA-binding activity of heterodimeric complexes. Using Jun proteins containing point mutations in association with wild-type Fos, no influence of Ser-63, -73, and -246 on dimerization and DNA-binding activity was observed (+Fos, Fig. 8A). This is consistent with the results of experiments done with full-length phosphorylated Jun in association with Fos (Fig. 6).

Effect of Ser-63, -73, and -246 on transcriptional activity. It is possible that phosphorylation of Jun alters its transcriptional activity rather than its ability to bind to DNA. For Ser-63 and -73, two previous studies addressed this question using cotransfection experiments with mutated *jun* genes. However, the results obtained were not consistent. Pulverer et al. (72) showed that mutation of Ser-63 and -73 to Leu residues abolished Jun transactivation activity, whereas Smeal et al. (86) reported that mutation of Ser-63 and -73 to Ala had no effect on transactivation, but inhibited the stimulation of Jun activity by a cotransfected *ras* gene. Previously, we used an in vitro transcription approach to define the domains of Jun involved in transactivation (2). Therefore, we used this assay to determine the effects of point mutations at Ser-63, -73, and -246 on transcriptional activity (Fig. 8B). The levels of transcriptional activation obtained with wild-type and mutated proteins closely paralleled the modest differences observed in their DNA-binding activities. Replacement of the N-terminal serines with aspartic acid (JD63,D73) resulted in the greatest increase in transcriptional activation, but also resulted in the largest experimental variability. Overall, the differences observed were less than twofold (Fig. 8B). For heterodimeric complexes with wild-type or mutated Jun in combination with full-length Fos, no differences in transcriptional activity were detected. Thus, Ser-63, -73, and -246 do not appear to play a major role in the transcriptional activity of Jun. In a previous study, we found that deletion of amino acids 1 to 90 resulted in an increase in the transcriptional activity of Jun (2). Therefore, Ser-63 and -73 lie N terminal to the major activation domain in Jun.

DISCUSSION

Protein phosphorylation is a general regulatory mechanism in eukaryotic cells (19, 42, 91). The activity and specificity of several enzymes have been shown to be increased or decreased by serine, threonine, and tyrosine phosphorylation. Recently, phosphorylation has also been implicated as a regulatory mechanism in the control of gene transcription. For example, the nonphosphorylated form of the large subunit of RNA polymerase II preferentially associates with the preinitiation complex in class II promoters. Phosphorylation of a heptad repeat contained in the C-terminal domain of this protein is associated with the transition from initiation to elongation of transcription (56). Phosphorylation has also been implicated in the regulation of DNA binding by transcription factors. The POU homeodomain proteins Pit-1 and Oct-1 show decreased DNA-binding activity after phosphorylation (46, 83). The effects of phosphorylation are more complex, however, for transcription factor SRF (serum response factor). Phosphorylation by CKII changes the SRF-DNA exchange rate, but does not alter the equilibrium DNA-binding affinity of SRF (59). Modulation of activity by phosphorylation has also been proposed for the CREB protein, which is involved in regulating expression of genes containing CRE sites in response to PKA activation. Phosphorylation of CREB is associated with increased activity in vivo and in vitro (63). Although the increase in CREB activity induced by phosphorylation was originally attributed to changes in dimerization and DNA binding (96), later studies reported that phosphorylation by PKA, but not PKC, may change the protein conformation and affect allosteric interactions with the transcriptional machinery (37). It has also been suggested that phosphorylation changes the role of CREB from a transcriptional repressor to a transcriptional activator by allowing it to interact with other transcriptional factors (50). An additional report suggested that the stimulation of CREB activity after membrane depolarization and Ca^{2+} influx is mediated by phosphorylation of CREB by Ca^{2+}-calmodulin-dependent protein kinase I and II at Ser-133, which is the major site of phosphorylation for these kinases and PKA (84). Thus, different signal transduction pathways impact on the CREB transcription effect through the same phosphorylation site. While it is generally agreed that phosphorylation influences CREB activity, the molecular events involved are still unclear and may depend on the exact circumstance and cell type.

The influence of phosphorylation on the activity of Jun also appears to be complex. Here we showed that several different protein kinases, whose activity can be increased by extracellular stimuli, are capable of phosphorylating Jun efficiently in vitro. Several reports have also described phosphorylation of Jun by other protein kinases at the same sites that are phosphorylated by $p34^{cdc2}$ (17, 72, 86). Thus, Jun may be a common target for signaling by a number of protein kinases. In serum-stimulated fibroblasts, the major phosphopeptides generated were identical to those obtained with purified $p34^{cdc2}$ in vitro. This enzyme is generally believed to function at the G_2/M transition of the cell cycle and possibly during G_1 at start, the commitment to DNA replication (11). However, it is possible that $p34^{cdc2}$ or a related kinase is transiently activated in response to serum stimulation. Although an increase in Jun synthesis occurs during serum stimulation, which is coincidental with a transition from G_0 to G_1, Jun is present at lower levels at all stages of the cell cycle (20, 49). Furthermore, it is expressed in many noncycling, differentiated cells such as neurons (44, 64). The kinases used here, which exhibit increased activity after mitogenic stimulation, may also be active in other cellular contexts. For example, $pp44^{mapk}$ showed its highest activity immediately after serum stimulation, but also displayed activity 2 h after stimulation and in normally proliferating fibroblasts (Fig. 4A). MAP kinases have also been reported to be stimulated during T-cell signaling by CD4 receptor activation, which is thought to act through $p56^{lck}$ (33). In addition, MAP kinases are activated in chromaffin cells in response to secretagogues (32) and in neurons after glutamate activation of the *N*-methyl-D-aspartate (NMDA) receptor (9). Thus, it is possible that the kinases that act on Jun in vivo are dictated by the differentiation state of the host cell type.

The conclusions of our study differ from those of previous reports which suggested a clear functional link between Jun phosphorylation and the DNA-binding or transcriptional activities of Jun homodimers. Replacement of Ser-246 with Ala or Asp (Fig. 8A) or phosphorylation of Ser-246 in full-length Jun (Fig. 6) or in truncated proteins did not significantly alter DNA binding by gel shift analysis. This contrasts with the suggestion that phosphorylation of Ser-

246 reduced Jun DNA-binding activity (17). In this previous study, phosphorylation of a truncated Jun polypeptide decreased DNA-binding activity, although the investigators did not demonstrate recovery of DNA-binding activity by phosphatase treatment. Boyle et al. (17) suggested that phosphorylation of Ser-246 affected Jun function in vivo because transactivation by a mutated protein containing a Phe substitution for Ser-246, which could not be phosphorylated, was increased. However, it is possible that this mutation, which is one of the alterations found in the viral *jun* oncogene, alters Jun conformation or affects its interactions with other proteins, irrespective of phosphorylation. Substitution of Ser-246 with Ala would also prevent phosphorylation but might not affect the structure of Jun adversely.

Phosphorylation of the N-terminal Ser-63 and -73 residues has been reported either to affect the basal transcriptional activity of Jun (72) or to mediate stimulation of Jun function by cotransfected *ras* (86). Phosphorylation of these sites was associated with MAP kinase activation after TPA treatment of U937 cells (72). This treatment results in macrophage differentiation of U937 cells (92). Replacement of Ser-63 and -73 with Leu residues resulted in a significant decrease in the transactivation function of Jun in unstimulated F9 cells (72). However, Leu is not a conservative substitution for Ser because the Leu side chain is significantly bulkier than that of Ser. Comparisons of amino acid substitutions in evolutionarily related proteins reveal that Ala is a more conservative substitution for Ser than Leu (34). In contrast to Pulverer et al. (72), we find that Jun containing Ala or Asp substitutions for Ser-63 and -73 exhibited DNA binding and in vitro transcription properties similar to those of wild-type Jun. This agrees with the finding of Smeal et al. (86) that Ala substitutions had no effect on Jun transactivation function in F9 and NIH 3T3 cells. Interestingly, these mutations caused a loss in the augmentation of Jun activity conferred by a cotransfected *ras* gene (86). It was not determined whether this *ras* effect was mediated through changes in DNA-binding activity or transactivation activity of Jun or through the interaction of Jun with an additional cellular factor. Recent reports demonstrated that *ras* is required for the activation of $pp42^{mapk}$ and $pp44^{mapk}$ in response to nerve growth factor and TPA treatment of PC-12 cells (89, 93). Thus, *ras* may play a role in the regulation of kinases during signal transduction.

The discrepancies between the results reported here and those of previous studies (17, 72, 86) may in part be reconciled by the explanation that each study used different cell types and extracellular stimuli. For example, TPA treatment of HeLa cells was associated with decreased phosphorylation of Ser-246 (17), whereas serum stimulation of fibroblasts resulted in increased phosphorylation of Ser-246. It is quite possible that in these varying circumstances, Jun is phosphorylated by distinct protein kinases and that the consequence of phosphorylation is cell type specific. Thus, a simple unified model for the control of Jun function by phosphorylation may not be tenable.

Phosphorylation is only one of several mechanisms that may control the functional activity of Jun. TPA stimulation is known to induce PKC activity as well as increase AP-1 transcriptional activity, but no causal relationship between the two events has yet been established. Although PKC phosphorylated Jun (Fig. 1B), the phosphopeptide maps suggested that it did not modify any of the previously identified phosphorylation sites (Fig. 1C). In addition to activating PKC, phorbol esters generate a pro-oxidant state in the cell (21). Alterations in the redox state of Jun and Fos proteins have been shown to dramatically affect their DNA-binding activities (6). Therefore, redox regulation presents an alternative mechanism for TPA regulation of AP-1 activity which is independent of phosphorylation signaling.

Jun was first identified as a 39-kDa protein that was coimmunoprecipitated in association with Fos (30). It is now clear that transcription factor AP-1 consists of an array of heterodimeric complexes involving several members of the Fos and Jun families (47). Indeed, to date, Jun has been shown to be capable of forming heterodimers with nine other leucine zipper proteins (12, 25, 38, 39, 43, 58, 65, 66, 68, 74, 79). Thus, it is likely that Jun functions primarily as a heterodimer with high-affinity DNA binding properties as opposed to being a low-affinity homodimer. In this regard, the significance of two- to threefold effects of phosphorylation on the dimerization and DNA binding properties of Jun homodimers may be modest. Phosphorylation had no detectable effect on the ability of Jun to form heterodimers and to bind to DNA in association with Fos. Thus, the role of Jun phosphorylation does not appear to be in mediating dimerization and/or DNA binding.

AP-1 sites frequently occur in the context of complex promoter regions surrounded by binding sites for a number of other regulatory factors (24, 85). In many circumstances, Jun can act in concert with several other transcription factors. It is possible that phosphorylation influences the multiple protein-protein interactions that occur within these natural complex elements. The assay systems used here would not be sensitive to these higher-order associations. Furthermore, modification of Jun may be dependent on the other factors with which it is complexed, such as Fos, Fos-related proteins, Jun-related proteins, the glucocorticoid receptor (31, 45, 82, 97), CREB-ATF proteins, and MyoD (13). Recently, we obtained an indication of this complexity in that the state of Jun—homodimer, heterodimer, bound to DNA, or free—can substantially influence its ability to serve as a substrate for protein kinases (unpublished data). Taken together, the in vitro and in vivo data suggest that an additional cellular component that is not present in the in vitro assay systems is required for the effects of Jun phosphorylation to be evident. Thus, phosphorylation may influence complex higher-order protein-protein interactions involving Jun that dictate the specificity of a multicomponent signal transduction cascade.

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