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Dimerization and DNA binding alter phosphorylation of Fos and Jun

(transcription factors/oncogenesis/signal transduction/posttranslational modification)

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ABSTRACT **Fos and Jun form dimeric complexes that bind to activator protein 1 (AP-1) DNA sequences and regulate gene expression. The levels of expression and activities of these proteins are regulated by a variety of extracellular stimuli. They are thought to function in nuclear signal transduction processes in many different cell types. The role of Fos and Jun in gene transcription is complex and may be regulated in several ways including association with different dimerization partners, interactions with other transcription factors, effects on DNA topology, and reduction/oxidation of a conserved cysteine residue in the DNA-binding domain. In addition, phosphorylation has been suggested to control the activity of Fos and Jun. Here we show that phosphorylation of Fos and Jun by several protein kinases is affected by dimerization and binding to DNA. Jun homodimers are phosphorylated efficiently by casein kinase II, whereas Fos-Jun heterodimers are not. DNA binding also reduces phosphorylation of Jun by casein kinase II, $p34^{cdc2}$ (cdc2) kinase, and protein kinase C. Phosphorylation of Fos by cAMP-dependent protein kinase and cdc2 is relatively insensitive to dimerization and DNA binding, whereas phosphorylation of Fos and Jun by DNA-dependent protein kinase is dramatically stimulated by binding to the AP-1 site. These results imply that different protein kinases can distinguish among Fos and Jun proteins in the form of monomers, homodimers, and heterodimers and between DNA-bound and non-DNA-bound proteins. Thus, potentially, these different states of Fos and Jun can be recognized and regulated independently by phosphorylation.**

The regulation of eukaryotic gene transcription requires the concerted action of many proteins that bind directly or indirectly to DNA regulatory elements. The molecular mechanisms that dictate the ordered assembly of these complexes are currently not well understood. This issue is particularly relevant for transcription factors that are encoded by gene families because these proteins may have similar structures and DNA-binding specificities, but distinct functions. The mammalian transcription factor AP-1 corresponds to a DNA-binding activity that is made up of multiple proteins including all of the members of the *fos* and *jun* gene families (1). These proteins bind to DNA as dimers formed by interaction of leucine zipper domains (2–6). Dimerization brings into close proximity a region of each subunit rich in basic amino acids that provides a bimolecular DNA contact surface (7–9). The three Jun family members bind to DNA either as homodimers or as heterodimers formed among themselves or with members of the Fos and activating transcription factor/cAMP responsive element binding protein (ATF/CREB) families (10–12). The four Fos family members bind to DNA as heterodimers with the Jun family and with selected members of the ATF/CREB family, but they do not form stable homodimers (12, 13). At present, more than 50 different complexes can be formed among the known subunits, and many of these complexes have been shown to exist *in vivo* (14).

In most cell types, members of the *fos* and *jun* gene families are expressed at relatively low levels, but they are induced transiently by a wide range of extracellular stimuli (15). They are thought to function as cellular immediate-early genes in coupling extracellular signals to phenotypic responses by regulating the expression of specific target genes. Although these genes exhibit cell type, stimulus, and temporal specificity of expression, a relatively large subset can be coexpressed in any given situation. Therefore, mechanisms must exist that permit the recognition and assembly of specific dimeric complexes with the appropriate DNA regulatory element.

Several potential mechanisms have been identified that are likely to contribute to the specificity of AP-1 proteins. The many protein dimers exhibit distinct specificities and affinities for DNA sequences containing different AP-1 and cAMP responsive element binding sites (12, 16). Fos and Jun family proteins display different transcriptional properties in cotransfection (17, 18) and *in vitro* transcription (19) assays. DNA-binding activity is modulated by an unusual oxidation/reduction mechanism that involves a conserved cysteine residue located in the DNA-binding domain (20–22). Furthermore, Fos and Jun induce DNA bends of opposite orientation, suggesting that DNA topology may play a role in the assembly of higher order transcriptional complexes involving AP-1 proteins (9, 23). In addition to all of these properties, posttranslational modification by phosphorylation may have a significant effect. Both Fos and Jun are highly phosphorylated on serine and threonine residues. Multiple protein kinases including glycogen synthase kinase III, mitogen-activated protein kinase, casein kinase II (CKII), $p34^{cdc2}$ kinase (cdc2), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC) have been reported to phosphorylate either Fos or Jun on several sites *in vitro*. However, the relationship between phosphorylation and function is complex. Phosphorylation of Jun often has been reported to be increased (24–27) or decreased (28, 29) after treatment of cells with extracellular stimuli. Furthermore, it has been suggested that phosphorylation of Fos increases its ability to inhibit serum response element-mediated transcrip-

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Abbreviations: CKII, casein kinase II; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; DNA-PK, DNA-dependent protein kinase.

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tion (30). Cotransfection studies have suggested that phosphorylation of the C terminus of Jun decreased DNA-binding activity (28, 29), whereas phosphorylation of serine residues in the N terminus of Jun increased transcriptional activation (29). In contrast, we showed that phosphorylation of Jun *in vitro* had only modest effects on DNA binding and transcriptional activation (26). These discrepancies between cotransfection and *in vitro* studies imply that the effect of phosphorylation on Jun activity may only be understood in the context of higher order interactions that occur within cells. Thus, there is no simple relationship between phosphorylation and the function of Fos and Jun.

Here we have investigated the ability of Fos and Jun to be phosphorylated as monomers (in the case of Fos), homodimers (in the case of Jun), and heterodimers, in the presence and absence of the AP-1 binding site. The results show that dimerization and DNA binding have a dramatic effect on the ability of several protein kinases to phosphorylate Fos and Jun. However, the effect observed was dependent upon the specific protein kinase used. Thus, phosphorylation may serve a role in discriminating among the multiple states of Fos and Jun that exist within cells and may contribute to the specificity of assembly of higher order complexes.

MATERIALS AND METHODS

Proteins and Kinases. The sequences corresponding to the entire coding region of c-*fos* (rat) and c-*jun* (rat) were expressed in *Escherichia coli* as hexahistidine fusion proteins and purified by nickel affinity chromatography as described (7). PKA (catalytic subunit) was purchased from Sigma. Mitotically active $p34^{cdc2}$ (cdc2) was purified as described from nocodazole-treated HeLa cells. This preparation exhibited no detectable contamination with seven other classes of protein kinases (31). Purified PKC produced from baculovirus vector-infected insect cells was a generous gift of Sue Jaken (W. Alton Jones Cell Science Center, Lake Placid, NY). CKII was purified as described (32). DNA-dependent protein kinase (DNA-PK) was purified from HeLa cells as in ref. 33. $pp44^{mapk}$ was isolated from 208F rat fibroblasts after serum stimulation by using polyclonal antiserum obtained from John Blenis (Harvard Medical School, Boston). $pp44^{mapk}$ immunoprecipitation and kinase assays were performed as described (34). Oligonucleotides containing the SP-1 or AP-1 binding sites were as described (35).

Kinase Reactions. Phosphorylation reactions were performed at 30°C for 30–45 min in the appropriate reaction mixtures as described (25, 26, 34, 36). DNA-PK reactions in the presence of DNA included calf thymus DNA at 10 μg/ml. Each assay contained Fos or Jun at a concentration of 0.5 μM. All assays also included 1–5 mM dithiothreitol to maintain the proper redox conditions required for DNA binding by Fos and Jun (21). In assays that included AP-1 or SP-1 oligonucleotides, a concentration of 0.5 μM oligonucleotide was used. This represents a 2-fold molar excess relative to protein-dimer concentration in the reaction. For quantitation purposes, two different concentrations of protein dimers were analyzed in the absence and presence of DNA. In the first instance, the molar concentration of Fos and Jun was identical to that used when the proteins were assayed alone, as shown in the figures. In the second instance, the overall protein concentration was identical to that used when the proteins were assayed alone. Thus, in the latter case there was a 50% reduction in the molar concentration of Fos and Jun. Laser densitometric scanning was used to quantitate differences in phosphorylation. Tryptic phosphopeptide mapping was performed *in situ* on nitrocellulose filters (37). Briefly, Fos and Jun proteins in the form of monomers, homodimers, and heterodimers, bound to an AP-1 oligonucleotide or in the absence of a DNA binding site, were phosphorylated using [γ-^{32}P]ATP, separated by SDS/PAGE, and transferred electrophoretically to nitrocellulose filters. The labeled proteins were excised, digested with trypsin, subjected to performic acid oxidation, and separated on high-resolution Tris/*N*-tris(hydroxymethyl)methylglycine/urea gels as described (26). The radiolabeled phosphopeptides were visualized by autoradiography.

RESULTS AND DISCUSSION

Full-length Fos and Jun proteins were expressed in *E. coli* as hexahistidine fusion proteins and were purified to apparent homogeneity as described (19) for use as substrates in phosphorylation assays. These proteins form dimers and bind to DNA with high efficiency, and they are capable of activating transcription from AP-1 sites *in vitro*. As demonstrated previously, Fos and Jun are excellent substrates for several different protein kinases *in vitro* (25, 26). In the case of Fos, efficient phosphorylation was observed with cdc2, PKC, PKA, and $pp44^{mapk}$ (Fig. 1) but not with CKII (Fig. 2). In the case of Jun, phosphorylation by PKA was inefficient (Fig. 1*C*), whereas PKC, cdc2, and CKII all phosphorylated Jun efficiently. $pp44^{mapk}$ phosphorylated Jun, although to a much lesser extent than phosphorylation of Fos (Fig. 1*D*). Under the conditions used in these experiments, Fos exists in solution primarily as a monomer and Jun as a homodimeric complex when assayed independently. When mixed together in solution, virtually all of the Fos and Jun combine to form heterodimers (38).

Dimerization and DNA Binding Reduce Phosphorylation of Fos and Jun by Selected Protein Kinases. To assess the influence of dimerization and DNA binding on phosphorylation of Fos and Jun, equimolar mixtures of each protein were preincubated in the presence and absence of an oligonucleotide containing an AP-1 binding site prior to phosphorylation. Phosphorylation of Fos and Jun by cdc2 was only modestly reduced by <2-fold following dimerization (Fig. 1*A*). Similarly, addition of an AP-1 oligonucleotide had little effect (less than a 2-fold reduction) on phosphorylation of Fos monomers (compare lanes 1 and 4). In contrast, DNA binding significantly reduced phosphorylation of Jun homodimers by ≈5-fold (compare lanes 2 and 5). These effects were less pronounced on Jun in the context of a heterodimeric complex (compare lanes 3 and 6). Phosphorylation of Fos and Jun by PKC was not affected by dimerization in the absence of DNA (Fig. 1*B*). Although the presence of DNA only modestly reduced phosphorylation of Fos monomers (1.2-fold) and Jun homodimers (2.5-fold) by PKC, phosphorylation of Jun, but not Fos, in a heterodimeric complex was reduced by ≈10-fold (Fig. 1*B*, lane 6). Therefore, a significant reduction in Jun phosphorylation resulted from a combination of dimerization and DNA binding.

Phosphorylation of Fos and Jun by some kinases was not affected by either dimerization or DNA binding. For example, phosphorylation of Fos and Jun by PKA and $p44^{mapk}$ was essentially unaffected by dimerization and DNA binding (Fig. 1 *C* and *D*).

CKII exhibited a surprisingly high degree of specificity for Jun homodimers in the absence of DNA (Fig. 2). Phosphorylation of Jun by CKII was severely reduced both by dimerization with Fos and by binding to the AP-1 site (>10-fold). The effect of DNA was specific to the AP-1 site because an oligonucleotide containing an SP-1 binding site had no effect on phosphorylation of Jun by CKII (lane 8). Similarly, all of the kinases evaluated were tested with Fos monomers, Jun homodimers, or Fos-Jun heterodimers in the presence of an oligonucleotide containing the SP-1 binding site. In all cases, the SP-1 oligonucleotide had no effect on the levels of phosphorylation when compared to reactions performed in

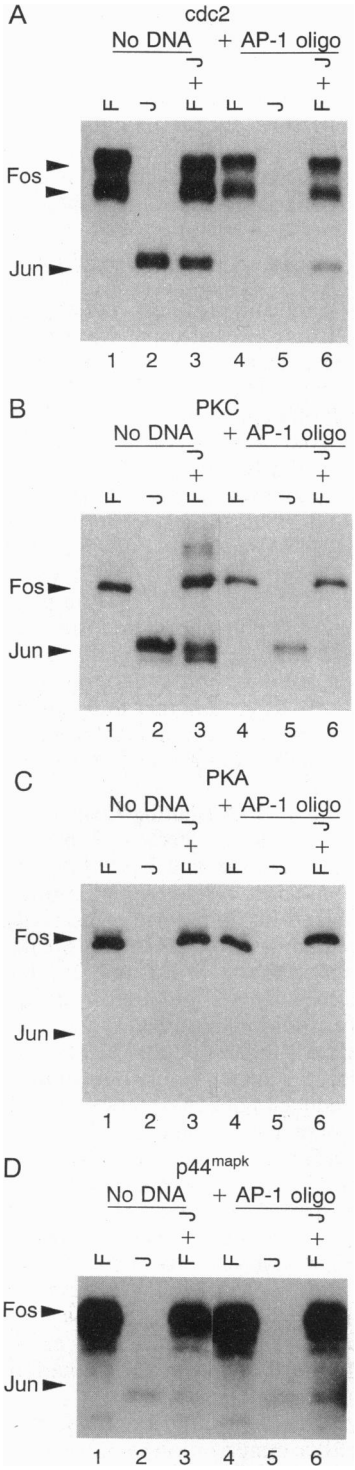

FIG. 1. Effect of dimerization and DNA binding on phosphorylation efficiency is kinase specific. Fos and Jun proteins at a concentration of 0.5 μM each were tested as substrates in the form of Fos (F) monomers (lanes 1 and 4) and Jun (J) homodimers (lanes 2 and 5) or as Fos-Jun (F + J) heterodimers (lanes 3 and 6) in the presence (lanes 4–6) or absence (lanes 1–3) of a 0.5 μM concentration of an oligonucleotide (oligo) containing the AP-1 binding site. Proteins were labeled with [γ-^{32}P]ATP by the indicated kinase, separated on an SDS/9% polyacrylamide gel, and visualized by autoradiography. (*A*) p34^{cdc2} kinase. (*B*) PKC. (*C*) PKA. (*D*) pp44^{mapk}.

the absence of DNA (data not shown and Fig. 2). Furthermore, the effects of the AP-1 oligonucleotide appear to be substrate specific as phosphorylation of histone H1 protein by cdc2 was not altered by the presence of AP-1 or SP-1

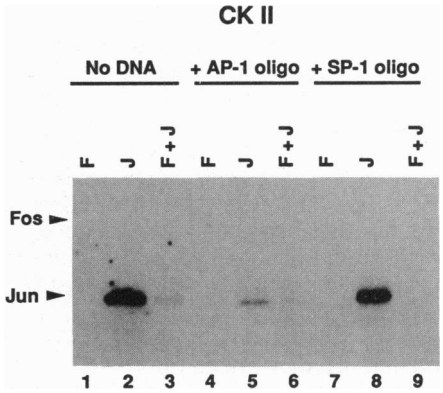

FIG. 2. Dimerization and DNA binding inhibit Jun phosphorylation by CKII. Fos and Jun proteins, at a concentration of 0.5 μM each, were tested as substrates for CKII in the form of Fos (F) monomers (lanes 1, 4, and 7), Jun (J) homodimers (lanes 2, 5, and 8), or Fos-Jun (F + J) heterodimers (lanes 3, 6, and 9). Phosphorylation reactions were performed in the absence of DNA (lanes 1–3), in the presence of an oligonucleotide (oligo) containing the AP-1 site (lanes 4–6), or in the presence of an oligonucleotide containing the SP-1 site (lanes 7–9). Labeled proteins were separated and visualized as in Fig. 1.

oligonucleotides (data not shown). Taken together, these results indicate that dimerization and DNA binding alter the substrate specificity of different protein kinases for Fos and Jun.

DNA Binding Enhances Phosphorylation of Fos and Jun by DNA-PK. One concern about the above results is that DNA binding could simply reduce phosphorylation of Fos and Jun nonspecifically because of steric hindrance. Therefore, we examined the phosphorylation of Fos and Jun by a protein kinase that is activated in the presence of DNA, DNA-PK (33). DNA-PK phosphorylates simian virus 40 large tumor antigen, SP-1, and several other DNA-binding proteins (33, 39). Recently, DNA-PK has been demonstrated to consist of a complex that includes the human autoimmune antigen Ku, corresponding to a 70-kDa and an 80-kDa subunit. The active kinase represents a complex of Ku proteins bound to double-stranded DNA ends in association with the 350-kDa protein kinase catalytic subunit (40, 41). DNA-PK is activated in the presence of nonspecific DNA (usually calf thymus oligonucleotides). Therefore, we assayed the ability of DNA-PK to phosphorylate Fos and Jun in the presence and absence of calf thymus oligonucleotides (Fig. 3). Very low levels of phosphorylation were observed in the absence of DNA, and a significant increase in phosphorylation of Fos and Jun was observed in the presence of calf thymus DNA.

Dimerization did not significantly reduce phosphorylation of Fos and Jun by DNA-PK. A dramatic stimulation of phosphorylation was obtained in the presence of an oligonucleotide containing an AP-1 binding site (>30-fold). DNA binding enhanced phosphorylation of Fos monomers, Jun homodimers, and Fos-Jun heterodimers. This effect was specific to the AP-1 site as no enhanced phosphorylation was observed in the presence of an oligonucleotide containing an Sp-1 site (data not shown). The increased phosphorylation of Fos monomers in the presence of the AP-1 site was somewhat surprising and may reflect a low-affinity DNA-binding activity of Fos. Thus, DNA binding can result in either inhibition or stimulation of phosphorylation depending on the protein kinase used. The dramatic increase in phosphorylation of Fos and Jun by DNA-PK in the presence of an AP-1 oligonucleotide is likely a consequence of the simultaneous occupation of a single DNA molecule by both enzyme and substrate (41).

Phosphopeptide Mapping Analysis. To determine whether specific regions of Fos and Jun were particularly sensitive to

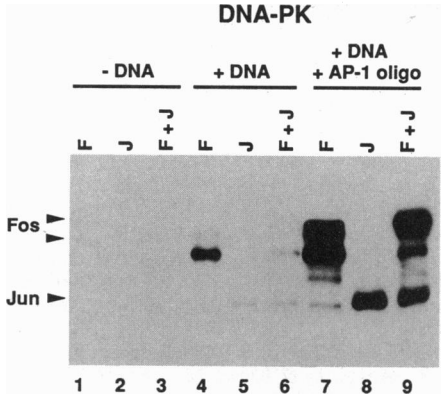

FIG. 3. DNA binding enhances phosphorylation of Fos and Jun by DNA-PK. Fos and Jun proteins, at a concentration of 0.5 μM, were phosphorylated by DNA-PK in the form of Fos (F) monomers (lanes 1, 4, and 7), Jun (J) homodimers (lanes 2, 5, and 8), or Fos-Jun (F + J) heterodimers (lanes 3, 6, and 9). Reactions were performed in the absence of DNA (lanes 1–3), in the presence of calf thymus oligonucleotides at a concentration of 10 μg/ml (lanes 4–6), or in the presence of calf thymus oligonucleotide plus an oligonucleotide (oligo) containing the AP-1 site at a concentration of 0.5 μM (lanes 7–9). Labeled proteins were separated and visualized as in Fig. 1.

the effects of dimerization and DNA binding on phosphorylation, peptide mapping analysis was performed. In the case of Fos, several peptides have been shown to be substrates for a variety of protein kinases (24, 25). Although several phosphorylated regions of Fos have been identified, the exact amino acids involved have not yet been identified. The regions of Fos phosphorylated by cdc2 and DNA-PK were unaffected by dimerization and DNA binding (Fig. 4*A* and data not shown). Similarly, no specific effect was found using PKC and PKA (data not shown). The major Fos peptide phosphorylated by DNA-PK comigrated with the major peptide phosphorylated by cdc2. This peptide lies in an N-terminal domain of Fos, amino acids 58–139, that has been associated with a reduction in transcriptional activity *in vitro* (19). The same peptide is phosphorylated in serum-stimulated fibroblasts.

In Fig. 4*B*, peptide maps of Jun phosphorylated in the presence and absence of DNA by cdc2, CKII, and DNA-PK are presented. There was insufficient label in Jun phosphorylated by CKII in the presence of Fos or DNA to prepare a peptide map. DNA binding resulted in a reduction in all of the cdc2 phosphorylation sites (Fig. 4*B*). Increased amounts of the samples in lanes 2 and 4 were loaded on the gel to identify phosphorylated peptides. Interestingly, in the presence of the AP-1 site, phosphorylation of the same peptide was decreased using cdc2 and CKII and increased using DNA-PK. This peptide lies in a region of Jun immediately N-terminal of the DNA-binding domain. Thus, the effect of DNA binding on phosphorylation is not a simple consequence of steric hindrance. DNA-PK also phosphorylated one peptide that was phosphorylated by CKII and an additional peptide of unknown origin (Fig. 4*B*). The exact amino acids phosphorylated in Jun by DNA-PK have not yet been determined.

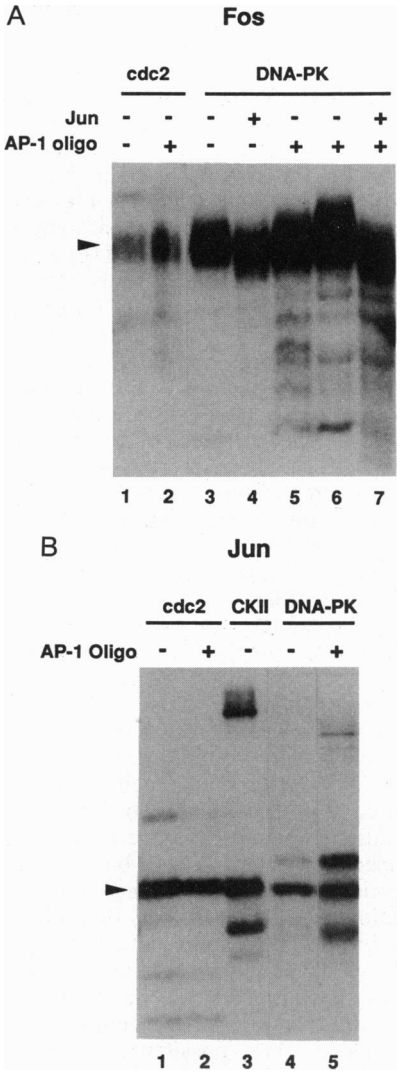

FIG. 4. Dimerization and DNA binding do not affect the sites of phosphorylation. (*A*) Fos was phosphorylated with [γ-^{32}P]ATP by cdc2 or DNA-PK in the absence and presence of Jun and/or an oligonucleotide (oligo) containing an AP-1 site as in Figs. 1 and 3. The proteins were separated by SDS/PAGE and transferred onto nitrocellulose. Labeled Fos proteins were excised and digested with trypsin, and peptides were resolved on tricine/urea gels (35). Lanes 1–3, 5, and 6, Fos monomers; lanes 4 and 7, Fos-Jun heterodimers. Phosphorylation reactions in lanes 2 and 5–7 include an oligonucleotide containing an AP-1 site. Lanes 1, 3, and 4 do not include an AP-1 oligonucleotide. All DNA-PK phosphorylations were performed in the presence of calf thymus oligonucleotides at 10 μg/ml. The arrowhead points to the major phosphopeptide. (*B*) Jun homodimers were phosphorylated by cdc2 (lanes 1 and 2), CKII (lane 3), and DNA-PK (lanes 4 and 5) in the presence (lanes 2 and 5) or absence (lanes 1, 3, and 4) of an oligonucleotide containing the AP-1 site as in Figs. 1–3. Peptide maps were generated as described in *A*. Both DNA-PK reactions contained calf thymus DNA at 10 μg/ml. The arrowhead points to the major phosphopeptide.

CONCLUSIONS

Most *in vitro* studies of transcription factor phosphorylation have utilized purified enzyme substrates incubated in the absence of DNA. While these have been useful in delineating phosphorylation sites and potential kinases involved, it has proven difficult to establish clear relationships between phosphorylation and function. One possible reason for this is that the majority of transcription factors function in the context of protein complexes. Furthermore, in many situations transcription factors that are involved in signal transduction appear to be bound to DNA prior to stimulation (42). Fos and Jun provide a unique opportunity to test the hypothesis that protein complex formation and DNA binding affect the specificity of phosphorylation. Both proteins are available in pure form, and it is known that they can be phosphorylated independently by a number of kinases. Here we show that dimerization and DNA binding can have a profound influence, both negatively and positively, on the ability of these proteins to be phosphorylated by several protein kinases.

This does not appear to be a simple consequence of steric hindrance because several regions of the proteins outside of the dimerization and DNA-binding domains are affected. Furthermore, DNA binding can either result in increased or decreased phosphorylation of the same peptide *in vitro*. Interestingly, a recent study demonstrated decreased phosphorylation of the C terminus of c-Jun in cells transfected with a vector containing AP-1 binding sites when compared to c-Jun from cells transfected with a control vector (43). These observations imply that different states of Fos and Jun, monomers, homodimers, heterodimers, DNA-bound, and non-DNA-bound, can be recognized independently in the cell.

It is remarkably difficult to determine which protein kinases actually phosphorylate Fos and Jun under physiological circumstances. Cotransfection studies can only indirectly evaluate the effect of phosphorylation by removing a target serine or threonine. Interpretation of these experiments is further complicated by alteration in phosphorylation of adjacent sites. For example, mutation of Ser-243 prevents phosphorylation of Ser-249 by CKII (44) and results in phosphorylation of a cryptic site at Thr-239 by cdc2 (26). Furthermore, several kinases can apparently phosphorylate the same peptides. In the case of Jun, the same peptide has been phosphorylated by glycogen synthase kinase III (28), cdc2, pp44^{mapk} (26), DNA-PK (Fig. 4*B*), and CKII (26, 44). It is likely that several of these kinases may be simultaneously expressed within the cell. Changes in AP-1 activity may be the contribution of only a small proportion of the total Fos and Jun population present in the cell. However, analysis of phosphorylated proteins isolated by immunoprecipitation gives information about the average state of Jun phosphorylation irrespective of whether it is bound to DNA, to Fos, or to other proteins. Given the dramatic effect of Fos on Jun phosphorylation by CKII, it is likely that other dimerization partners can influence phosphorylation. Thus, it is not possible to make global statements about the role of phosphorylation on Fos and Jun function. The exact kinase involved, the dimeric state of the proteins, whether they are bound to DNA and, indeed, their possible association with other transcription factors must all be taken into account. This level of complexity may be a general feature of transcription factors that function as components of nuclear signal transduction processes.

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