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Activation of c-Jun transcription factor by substitution of a charged residue in its N-terminal domain

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ABSTRACT

C-Jun is a cellular transcription factor that can control gene expression in response to treatment of cells with phorbol esters, growth factors, and expression of some oncogenes. The ability of c-Jun to catalyze the transcription of certain genes is controlled, in part, by changes in the phosphorylation state of specific amino acids in c-Jun. One of the major sites that is phosphorylated during signal response is Ser73. Here we show that substitution of a negatively charged aspartic acid residue at 73 constitutively increased transcriptional activity of c-Jun. The Asp73 substitution also enhanced its availibility to bind to DNA in a whole cell extract without altering its intrinsic DNA binding activity since the intrinsic activity was unaltered for the c-Jun mutant proteins expressed in a bacterial system. The negatively charged Asp substitution may mimic the negative charge of a phosphorylated serine at 73. The substitution of an uncharged alanine at 73 resulted in lowered activities. The N-terminal end of c-Jun containing these substitutions was fused to the DNA-binding region of the bovine papilloma virus E2 protein, and was able to confer the same activation properties to the fusion protein at the heterologous E2 DNA-binding site. Ser73 lies in a region of c-Jun previously proposed to bind an uncharacterized inhibitor, perhaps related to a protein of approximately 17.5 kD that coprecipitates along with our c-Jun or the JunE2 fusion products.

INTRODUCTION

Transcription factors are the effector molecules that link second messenger pathways to gene expression pathways, allowing a range of cellular responses to extracellular stimuli. One transcription factor known to have this role is c-Jun , which has been shown to be positively regulated in response to treatment with phorbol esters, hormones, and growth factors (1, 2), as well as expression of oncogenes, including *ras*, *raf*, *mos* and *src*. (3–7). C-Jun was first identified as a constituent in a chromatographic fraction from human cell lysates termed activator protein 1 or AP-1 on the basis of specific DNA binding properties and ability to stimulate transcription *in vitro* (8–10). AP-1 has been shown to consist of a variety of related factors belonging to the Jun and Fos families, and together these factors are responsible for mediating transcriptional responses for a number of signaling events initiated by tyrosine kinase activation and phospholipid turnover (11). Target genes regulated by AP-1 were originally characterized as genes whose transcription is induced by TPA (phorbol ester), including certain cellular oncogenes (12), as well as collagenase I and stromelysin (13).

Two sets of clustered phosphorylation sites have been identified in c-Jun, and the phosphorylation state of specific amino acids within these clusters may serve to modulate the transcriptional activity of c-Jun. In a region at the N-terminal end, Ser63 and Ser73 (14,15) located within a previously described activation domain (16) are phosphorylated in response to, for example, *ras* expression, and their phosphorylation increases the transcriptional activity of c-Jun (17–20). At the C-terminal end, in proximity of the DNA binding domain, is a second cluster of potential phosphorylation sites, including Thr214, Ser 226, and Ser232 (14, 22), that when phosphorylated inhibit DNA binding of c-Jun. Dephosphorylation at these sites is observed in response to, for example, *ras* expression or TPA induction. There is a second mechanism controlling DNA-binding of the Jun and Fos family of proteins, reduction oxidation (redox) regulation also mediated through a C-terminal residue (21). A conserved cysteine at 252 under oxidative conditions will block DNA-binding, but the binding activity is restored by the addition of reducing agents *in vitro*.

Here we report that the substitution of an aspartic acid for the serine at position 73 in c-Jun, when over-expressed in certain cell lines, resulted in an apparent increase in the availability of the protein to bind to DNA, and activate transcription from a minimal promoter containing a Jun/Fos DNA-binding site. Since a likely explanation for the activation of c-Jun by the Asp substitution at 73 is that the negative charge of this acidic residue serves to mimic the negative charge of a phosphorylated serine, c-Jun73D is expected to have a similar higher level of activity as c-Jun does when phosphorylated at Ser73. Therefore, the increased activity of the mutant is in agreement with one of the

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properties expected of c-Jun phosphorylated at 73, and can be imparted stably to c-Jun by engineering this specific amino acid change. In contrast, the substitution of the uncharged residue, Ala, at 73 resulted in lowered activity in agreement with the expected activity of the unphosphorylated Ser73.

C-Jun belongs to the BZip group of transcriptional regulators which contain a basic region for binding to DNA (B) and a leucine zipper (Zip) to allow dimerization. These factors have individual domains for DNA binding, dimerization, and transcriptional activation that remain functional when separated from their original protein structures and fused with domains from other transcription factors in new arrangements (23, 2). Fusion proteins are useful tools to address probable mechanisms used by transcription activation domains, and it has already been shown that a fusion protein between the c-Jun activation domain and a heterologous DNA-binding domain are activated by signaling pathways that phosphorylate c-Jun. (17−19). The tentative conclusion from an earlier study was that transcriptional activation may involve subtle conformational changes in c-Jun.(24), although this is less likely to be the case if transcriptional activation can be effectively reconstituted in a fusion protein. In order to test if the amino acid substitutions at position 73 in c-Jun would function similarly in a fusion protein, the DNA-binding domain of the bovine papilloma virus E2 protein (16) was fused to the N-terminal activation domain of each of the mutant and wildtype c-Jun expression vectors. We show that in several cell types transcription activity can be modulated by amino acid substitutions at 73 in the fusion protein. This result was corroborated by an assay that measures available DNA-binding activity to the heterologous E2 DNA-binding site.

To further address the mechanism of enhanced DNA-binding and transcriptional activities we created bacterial expression vectors to assess the potential role amino acid substitutions in c-Jun at position 73 play in altering the intrinsic DNA-binding activity of the protein. We found that all of the constructs had similar DNA-binding activity indicating that the mutations do not effect the intrinic DNA-binding activity. This result suggested a role for an additional component, a possibility consistent with the diminished binding of c-Jun73D to an inhibitory protein.

We describe our assay as measuring available DNA-binding activity, to distinguish it from assays designed to measure the inherent DNA-binding activity of proteins without interference from other potentially relevant pathways including the binding of specific inhibitors, or activators. Our whole cell extract includes additional nuclear and cytoplasmic components absent from the nuclear extract that may specifically inhibit or activate DNA-binding, for example, by binding to c-Jun. Inhibitors that binds to c-Jun have been postulated (25, 16, 26) and for one of these association with c-Jun may be controlled through the activation domain (16) containing Ser73. We observe a candidate protein for this inhibitor, of approximately 17.5 kD, that coprecipitates with our c-Jun and JunE2 fusion products.

RESULTS

Over-expression of c-Jun with single amino acid changes for serine 73

The availability of a cloned gene for c-*jun* makes possible its introduction and over-expression in mammalian cells, thereby allowing a range of functional studies to be conducted. We selected Hela cells, a human epitheloid carcinoma, for the transfection experiments partially because of earlier results showing evidence for a cell-type specific inhibitor present in Hela cells that interacts with an N-terminal activation domain of c-Jun (16). The finding that there is an inducible phosphorylation site at serine 73 (14, 15,22) in the middle of the A1 activation domain, suggested the possibility that interaction with an inhibitor might be regulated by the phosphorylation. Expression vectors using the cytomegalovirus early promoter to promote high levels of c-Jun in transfected cells were constructed. In addition to the wild-type c-*jun* containing a serine at position 73, two additional constructs were made containing either the codon for alanine at 73 (c-*jun*73A) or the codon for aspartic acid at 73 (c-*jun*73D). Duplicate plates of Hela cells were transiently transfected with each of the constructs and harvested 48 hrs post transfection. One of the plates from each transfection was labeled with ^{35}S-methionine while the duplicate plate was not labeled. Equivalent whole-cell extracts were prepared from both plates. Immunoprecipitation reactions were conducted with the labeled cell extract using an antisera directed against c-Jun. The levels of Jun proteins provided by transfection of all expression vectors were higher than the background levels of endogenous c-Jun activity (Fig.1, lane −) and were nearly equivalent for each of the constructs.

The availability of c-Jun with aspartic acid at 73 in whole cell extracts is increased as measured by binding to DNA

We conducted electrophoretic mobility shift assays (27, 28) on the unlabeled cell extracts prepared from the transiently transfected Hela cells to assay for effects of the amino acid

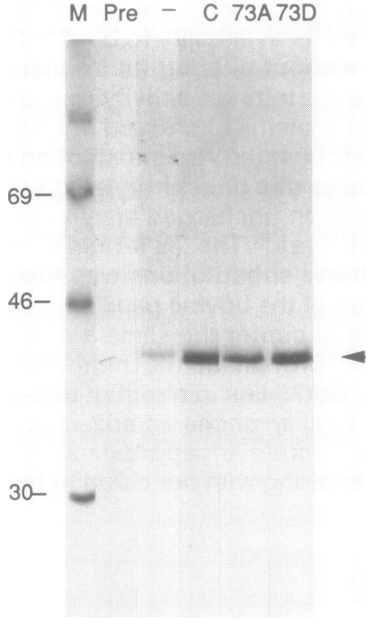

Figure 1. Transient expression of c-Jun and point-mutants in Hela cells. Expression vectors were constructed as described in materials and methods that allowed over-expression of c-Jun (lane C) in transiently transfected Hela cells as compared to nontransfected cell levels (lane−). Expression levels were measured by immunoprecipitation reactions using a rabbit antisera directed against c-Jun. Constructs containing a single amino acid change of the serine at position 73 of c-Jun to either an alanine (lane 73A) or an aspartic acid (lane 73D) also transiently introduced into Hela cells resulted in similar levels of expression of the mutant c-Jun proteins with wild-type c-Jun. The position of the immunoprecipitated Jun proteins is as expected relative to marker proteins (lane M) and are indicated by the arrow.

substitutions on DNA-binding activity. Oligonucleotide probes were employed that contained either the consensus binding site for the Jun−Fos complex, or a mutated binding site that changed three conserved base pairs in the consensus binding site (Fig. 2A). We minimized differences in binding due to redox regulation at Cys 252 in Jun by including 1mM DTT in both the extracts and in the assays.The amount of Jun/Fos DNA-binding activity was not detectable for the non-transfected Hela cell extracts under these conditions (lane-). A low signal level was detectable in the extracts from cells transfected with c-Jun and c-Jun73A, whereas a substantial signal is observed in the c-Jun73D transfected cell extracts. The amount of the specific DNA−protein complex can be indicative of the concentration, or the DNA-binding affinity, of a particular DNA-binding protein. The concentration of c-Jun73D is similar to the levels of c-Jun73A and wildtype c-Jun as shown in the previous figure, as expected since the same expression vector is used for all constructs. However, the protein levels shown in figure 1 do not address the possibility that the actual amount of factor available to bind to DNA may vary greatly due to sequestration of the factor, possibly by the binding of a putative repressor. The probability that the increased DNA-binding activity observed for c-Jun73D is due to a fundamental change in the DNA binding affinity constant seems less likely since dephosphorylation at Thr 214 and Ser 232, which lie within the DNA binding domain at the other end of the molecule, have already been shown to enhance DNA binding activity, whereas Ser 73 lies within a putative repressor binding domain (25, 16, 26). In addition, the activation of transcription mediated by c-Jun through phosphorylation of N-terminal sites in c-Jun occurs without an increase in DNA-binding activity when measured in nuclear extracts (19), consistent with no variation in the DNA-binding constant. Our results are unique from these because the increased binding activity we observe may be due to our assays being conducted with whole cell extracts that may contain a repressor, rather than with nuclear extracts.

To further substantiate that the changes in DNA-binding activity of Jun due to cotransfection of the mutant *jun* constructs is specific in these extracts to Jun DNA-binding, a control experiment was conducted utilizing the binding site for a distinctly different

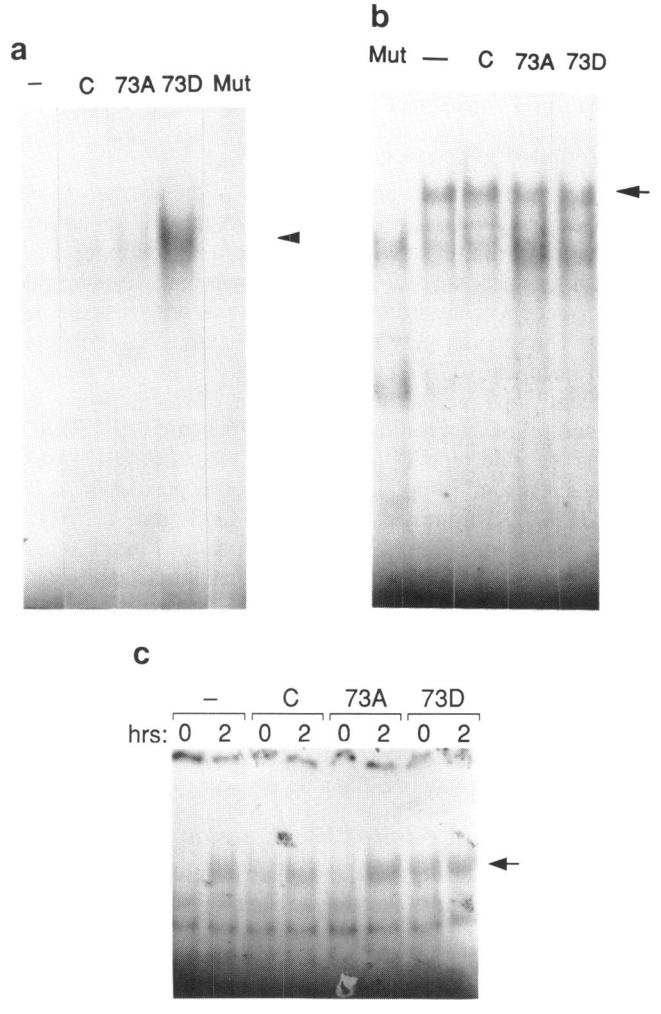

Figure 2. Availibility of Jun protein to bind to DNA is increased in c-Jun with a single amino acid substitution of aspartic acid at position 73. (**a**) Gel-shift assays were conducted using labeled oligonucleotide containing either the DNA-binding site homology for Jun, or a mutated binding site that will not bind Jun (Mut lane only). The probes were incubated in whole cell extracts prepared from untransfected cells (lane−) or cells transiently expressing c-Jun (lane C) or expressing the amino acid substitutions (lanes 73A and 73D). The lane containing the mutated probe (Mut) was incubated with the extract from cells transfected with the 73D construct. The arrow indicates the position of Jun specific complexes. (**b**) A second transcription factor is assayed for its DNA-binding activity in the same extracts and no variation is observed. Gel-shift assays were conducted with an oligonucleotide containing the DNA-binding site homology for Sp-1 and incubated in the same whole cell extracts as above. An oligonucleotide containing mutations in the binding site was included to determine which DNA−protein complexes were notspecific Sp-1 binding. The arrow indicates the position of the Sp-1 specific complexes. (**c**) Gel-shift assays for Jun were conducted in extracts from untreated cells (0) or cells treated for 2 hr with TPA (2) that were also transfected with each of the Jun mutants. The arrow indicates the position of Jun specific DNA complexes.

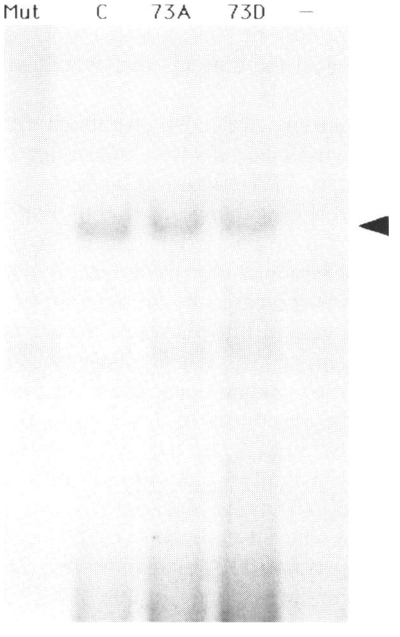

Figure 3. Inherent DNA-binding activity of c-Jun is unaltered in position 73 mutants expressed in bacteria. Mobility-shift assays were conducted using labeled oligonucleotides containing either the DNA-binding site homology for Jun or the mutated binding site that will not bind Jun (Mut lane). The probes were incubated with c-Jun, c-Jun73A, or c-Jun73D expressed in a bacterial system (see Materials and Methods), or a control fraction without c-Jun protein (−). Since Fos or other Jun specific binding proteins are absent in the bacterial system the DNA-binding activity observed is a measure of the inherent binding activity of each Jun protein. The arrow indicates the position of Jun specific complexes.

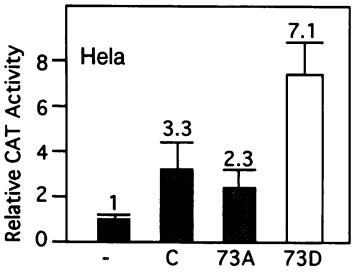

Figure 4. Relative transcriptional activity of c-Jun and position 73 mutants. Hela cells were transiently transfected with 2μg CAT (chloramphenicol acetyltransferase) reporter gene construct and 2 μg of c-Jun expression vector, or c-Jun vectors with the position 73 substitutions. TRE.colCAT, which contains a TRE (TPA responsive element) upstream of the HSV-TK promoter (1) was cotransfected to measure the transcriptional activation by each of the c-Jun constructs. CAT expression is shown as counts per minute of benzene-extractable [^{3}H]-mono-acetylated chloramphenicol (37) per μg extract protein. The data are the average of three transfections each done in duplicate.

transcription factor, Sp1. This factor binds to specific GC-rich DNA elements that occur in numerous gene promoters (29). An end-labeled oligonucleotide containing the consensus DNA-binding site for Sp1 was used in electrophoretic mobility assays of the same extracts used to assay Jun binding (figure 2B). A mutant probe was also included to indicate the positions of protein−DNA complexes that did not require the Sp1 consensus DNA-binding site. Only the top band, as indicated by the arrow, is unique to the Sp1 probe and is therefore the specific interaction of Sp1 and its consensus binding site. The amount of DNA-binding activity does not vary in the transfected cell extracts. Therefore the responses of Jun seen in Fig.2A are not due to any general change in the extracts that would non-specifically alter DNA-binding levels.

DNA-binding activity was also evaluated for the the Jun mutants after the transfected cells were treated for 2 hr with 50μM of the phorbol ester, TPA (figure 2C). Since phosphorylation levels of specific residues change, including Ser73, in response to TPA treatment we directly compared DNA-binding activity for each mutant before and after treatment. In each case DNA-binding activity was increased by stimulation of the cells with TPA, except in the case of Jun73D where the activity was already high before treatment, and was not significantly greater afterwards as measured in our assay conditions. Thus, the point mutant does mimic some of the responses of Jun to stimulation by TPA. Longer treatment with TPA, up to 6 hr, does result in marked stimulation of DNA-binding activity for both wild-type and mutant Jun, including Jun73D (data not shown), consistant with the observations of other investigators that DNA-binding activity is increased in response to TPA by dephosphorylation at residues within the DNA-binding domain of Jun (14,22).

Invariable intrinsic DNA-binding activity of c-Jun mutants expressed in bacteria

In some cases, amino acid substitutions can alter the enzymatic activity of a protein directly by causing changes in secondary structure. To test if this is the case for c-Jun DNA-binding activity in response to amino acid substitutions at position 73 we expressed each construct in a bacterial system (see Materials and Methods). Electrophoretic mobility shift assays were again conducted with the Jun consensus binding site oligonucleotides. DNA-binding activity for c-Jun, c-Jun73A, and c-Jun73D did not vary (Fig.3) indicating that the amino acid substitutions at 73 do not alter DNA-binding activity directly. Other proteins known to bind c-Jun, including Fos and other Jun specific binding proteins are not expressed in the bacterial system. These experimental results have direct bearing on the mechanistic interpretation of the results obtained in the eukaryotic expression system using whole cell extracts. If the increased DNA-binding activity of c-Jun 73D cannot be explained by a change in its intrinsic DNA-binding activity then it must require an additional component. Either the interaction with an activator or a repressor are possible, however Ser73 lies within a putative repressor binding domain (25,16,26) so we favor the possibility that c-Jun73D binds less efficiently to a repressor. Interestingly, a candidate repressor protein is identified in a subsequent figure (see Fig.5).

Jun-promoted expression levels

To determine if the DNA binding activity of the proteins encoded by the various *jun* constructs also correlated with the ability to catalyze transcription activity from a promoter utilizing the Jun DNA binding site, we examined the response of a Jun-dependent reporter gene to cotransfection with the various *jun* expression vectors. The reporter gene encodes the enzyme chloramphenicol acetyltransferase (CAT); CAT activity can be measured conveniently and is an accurate reflection of its expression level under control of the promoter (33). The plasmid Col.TRE CAT contains the wild type TPA-responsive element (TRE i.e., *jun* binding site) from the collagenase promoter, positioned in front of the HSV-TK promoter fused to the CAT gene (1, 34).

CAT activity was measured after incubations in the reaction mixture for 1hr and 2 hr and performed in triplicate and average values are shown (Fig. 4). Increased CAT expression resulted from the transfection of c-*jun* compared to vector alone (see figure legend), consistent with either limiting endogenous c-Jun activity or the titration of a cellular inhibitor by the presence of additional c-Jun molecules. CAT activity was lower for c-*jun*73A than either c-*jun* or c-*jun*73D. The performance of c-*jun*73D is consistent with the greater activity of c-Jun after phosphorylation at serine73.

Over-expression of c-Jun−BPV E2 fusion proteins

We also sought to determine if the relative activity of our mutants at position 73 would also function as heterologous fusion proteins, by fusing the N-terminal activation domains of the mutants, including position 73, to the DNA-binding domain of the bovine papilloma virus (BPV) E2 protein. If the modulation of available DNA-binding activity and transcriptional activity in the mutants are mediated by a mechanism other than direct alteration of DNA-binding affinity than the relative activities of the fusion protein mutants might mirror those of the c-Jun mutants. The same expression vector system using the cytomegalovirus early promoter to promote high levels of the fusion proteins in transfected cells was used. DNA sequence for the N-terminal 192 amino acids was ligated in the correct reading frame with the C-terminal sequence from the BPV-E2 encoding the DNA-binding domain. Duplicate plates of Hela cells were transiently transfected with each of the constructs and harvested 48 hrs post transfection. One of the plates from each transfection was labeled with ^{35}S-methionine while the duplicate plate was not labeled. Equivalent whole-cell extracts were prepared from both plates. Immunoprecipitation reactions were conducted with the labeled cell extract using an antisera directed against c-Jun (Fig.5). In immunoprecipitations of extracts prepared from cells transfected

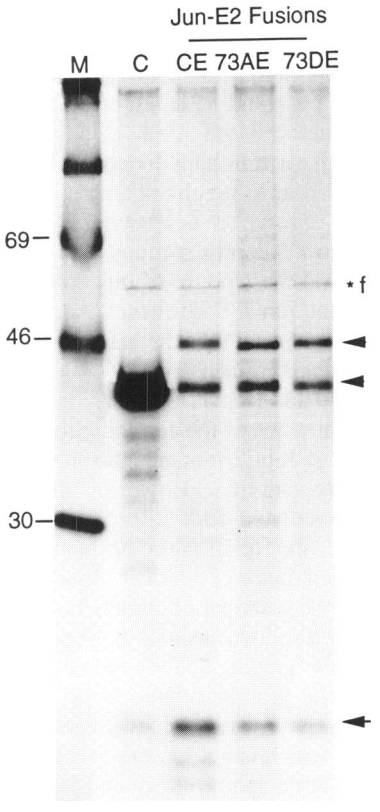

Figure 5. Expression of c-Jun/BPV-E2 fusion proteins. Expression vectors were constructed as described in materials and methods that allowed transient expression of fusion proteins containing the N-terminal end of c-Jun coupled to the C-terminal DNA-binding domain of the BPV-E2 protein. Expression levels in Hela cells were measured by immunoprecipitation reactions using a rabbit antisera directed against c-Jun. Although the antisera was more efficient in immunoprecipitating complete c-Jun protein (wild-type c-Jun transfected lane C, second arrow from top), the fusion proteins were also detectable (top arrow). Similar levels of expression were observed for each of the fusion proteins. Also visible are proteins coprecipitating with Jun and the Jun−E2 fusion product, including c-Fos with a M.W. of 55kD (labeled *f), and a previously unidentified protein of approximate M.W. 17.5kD (bottom arrow).

with the wild-type c-Jun construct (lane c) c-Jun overexpression is evident, while lower levels of endogenous c-Jun are observed in the other lanes. When the fusion protein constructs are transfected an additional protein of the expected size (approximately 46 kD) is immunoprecipitated. Equivalent levels of expression are seen for each of the constructs. The coprecipitation of an additional protein is observed that migrated as a small M.W. protein of approximate 17.5 kD. We noted that less of this coprecipitating protein was bound to the c-Jun73D protein, consistent with the possibility of diminished binding of a Jun inhibitor protein. We do not characterize this protein further here.

Availability for binding to DNA and promoted transcription levels for Jun- BPV E2 fusion proteins

The relative DNA-binding activity of the fusion proteins was evaluated by electrophoretic mobility shift assays on transfected cell extracts using an oligonucleotide probe containing the consensus DNA-binding site for E2. We sought to determine if the relative DNA-binding activities of the fusion proteins would also be regulated by the N-terminal sequence from the c-Jun

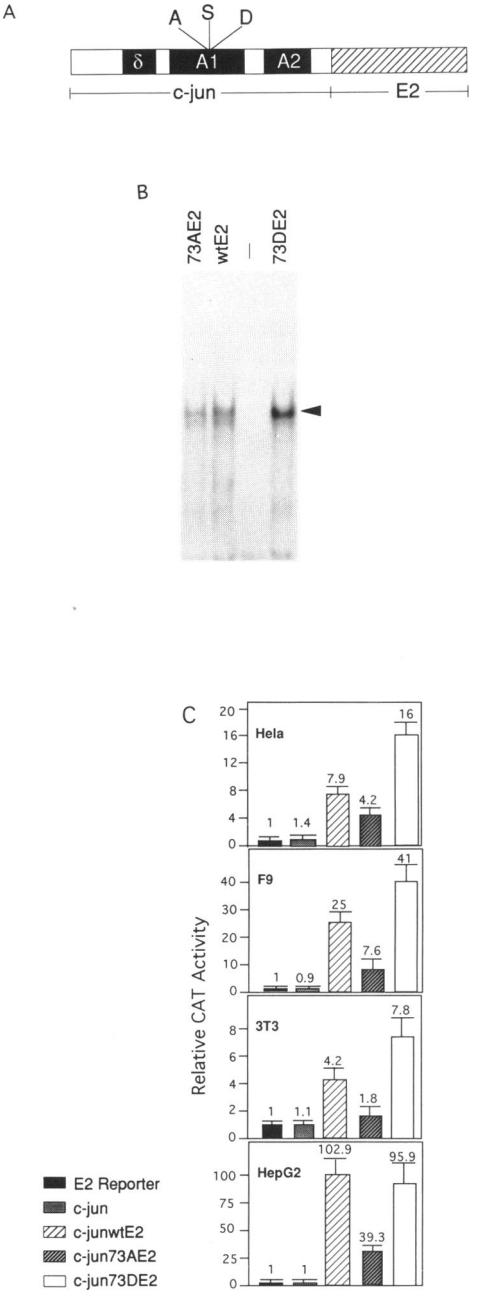

Figure 6. (a) Diagram of c-Jun/BPV-E2 fusion proteins. The N-terminal of c-Jun containing both activation domains A1 and A2 and either the wild-type serine at position 73 or alanine or aspartic acid substitutions, were fused to the DNA-binding region of the BPV-E2 protein. (b) Availibility of c-Jun/BPV-E2 fusion proteins to bind to DNA is increased when the c-Jun portion contains a single amino acid substitution of aspartic acid at position 73. Gel-shift assays were conducted using labeled oligonucleotide containing the DNA-binding site homology for the BPV-E2 protein, and were incubated in whole cell extracts prepared from cells either not transfected (lane −) or transiently expressing fusion proteins. The availability of DNA-binding activity for the fusion protein either containing c-Jun (lane wtE2) or expressing the amino acid substitutions (lanes 73AE2 and 73DE2) was the greatest for the aspartic acid substitution (lane 73DE2). (c) Relative transcriptional activity of c-Jun/BPV-E2 fusion proteins. Hela, F9, 3T3, and HepG2 cells were transiently transfected with 2μg CAT (chloramphenical acetyltransferase) reporter gene construct alone , or with 2 μg of c-Jun or Jun/BPV-E2 expression vectors. E2CAT, which contains an E2 DNA-binding site was cotransfected to measure the transcriptional activation by each of the c-Jun constructs. CAT expression is shown as counts per minute of benzene-extractable [^{3}H]-mono-acetylated chloramphenicol (37) per μg extract protein. The data are the average of three transfections each done in duplicate.

constructs even when functioning with the DNA-binding domain of an unrelated protein. In addition, because there is no known cellular protein with the same consensus DNA-binding site as E2, the assay evaluates the role of the c-Jun N-terminal domain without interference from endogenous c-Jun. Thus, this experiment may also improve the resolution of relative activities of the position 73 mutations. As predicted, the c-Jun73AE2 fusion protein showed reduced available DNA binding activity in this system, presumably due to the absence of complicating effects from endogenous c-Jun and its putative inhibitor. The c-Jun73DE2 fusion protein had greater binding activity than c-Jun73AE2 and the wild-type construct c-JunwtE2 (Fig. 6B). As for the previous binding assays, the concentration of DTT in the extract and during the assays is maintained at 1 mM, in order to minimize differences due to redox regulation, since BPV E2 DNA binding domain is also subject to redox regulation (30).

To confirm that the DNA-binding activity of the mutants was also reflected in their transcriptional activity, CAT assays were conducted. C-JunE2 fusion constructs were co-transfected with E2CAT, which contains the E2 DNA-binding site positioned in front of the HSV-TK promoter fused to the CAT gene (1,17). Hela cells were transfected, as previously, as well as additional cell lines F9, 3T3, and HepG2 in order to illustrate the generality of the effect. CAT activity was measured after incubations in the reaction mixture for 1hr and 2 hr and performed in triplicate and the averaged values are shown (Fig. 6C). CAT expression was increased by c-*jun* E2 transfection, as compared to either transfection with the E2 reporter alone or a c-*jun* vector that does not contain the E2 DNA-binding site. In agreement with the DNA binding data, CAT activity was lower for c-*jun*73AE2, and greater for c-*jun*73DE2 than for c-*jun*.E2. The performance of the c-*jun* E2 mutants is also consistent with the regulation of c-Jun transcriptional activity by the control of phosphorylation at serine73. Data obtained with both c-Jun constructs and the C-Jun/E2 fusion constructs show the same trends when measuring relative available DNA-binding activity and CAT activity.

DISCUSSION

Characterization of the changing pattern of post translational modification of c-Jun in response to signal transduction pathways has been an area of current interest. Significantly, it has been shown that the ability of c-Jun to catalyze the transcription of genes containing the Jun/Fos DNA-binding homology is altered by phosphorylation of the c-Jun protein at serine and threonine residues (17, 19, 20, 22) and by reduction−oxidation at a conserved cysteine residue (21). Sites of phosphorylation are clustered in two domains of c-Jun. We have made amino acid substitutions designed to mimic changes in the phosphorylation state of a major signal responsive phosphorylation site at Ser73 that lies in a transcriptional activation domain. *In vivo*, Ser73 is phosphorylated to a greater extent than Ser63, a second neighboring site also contained in the transcriptional activation domain (15,14).

The other domain of c-Jun important for phosphorylation mediated changes in activity is the DNA-binding domain which contains several amino acids that are dephosphorylated in response to treatment with phorbol ester, including Thr214, Ser226 and Ser232 (14). Although the mechanism for how phosphorylations at these sites might interfere with DNA-binding are not known, one interpretation is that the negatively charged phosphodiester backbone of DNA and the negatively charged phosphorylated amino acids near the basic DNA binding region of c-Jun repel. In addition, phosphorylation at one of these sites, Ser226, also effects redox regulation at Cys252 (31), which *in vivo* is regulated by Ref-1 (21,32), a protein that is able to physically associate with Jun. Perhaps physical association of another protein with the activation domain of Jun containing Ser63 and Ser73 plays a role in regulating Jun activity. It is of some interest that we observe a unique small M.W. protein of approximately 17.5 kD coprecipitating with Jun or JunE2 fusion proteins (fig.5), and the amount of this coprecipitating protein is diminished for the Jun 73D construct. Additional experiments will be required to determine if the association of Jun with this protein can modulate Jun activity.

Our results in F9 and HepG2 cells may be contrary to earlier speculation of an absence of the Jun inhibitor in these cells (16) since transfection of JunE2 mutants into these cells also shows differential activity (fig.6C). However, the presence of the inhibitor was postulated by inference from competition experiments (16), and no protein or cloning data has been published for this inhibitor. Therefore, it is clearly too early to establish rules for the behavior of the inhibitor. A key difference between the earlier experiments and those presented here is that earlier the role of the δ region (an N-terminal domain of 27 amino acids deleted in the closely related viral homolog v-Jun) in determining Jun activity was being evaluated. Here we evaluate the role of Ser73, which is not located in the δ region. Thus the rules for how this site might effect an interaction with a Jun inhibitor have not previously been determined.

Some insights into the role of Ser73 phosphorylation in the control of c-Jun transcriptional activity may be derived from the situation of another member of the BZip proteins, CREB, that regulates cyclic AMP (cAMP) responsiveness. One of the three phosphorylation sites in the N-terminal transcriptional activation domain of CREB, Ser133, is directly phosphorylated by protein kinase A (PKA). This causes a significant increase in CREB transcriptional activity (35,36) without changing the inherent DNA-binding affinity of the factor, perhaps in a way similar to c-Jun N-terminal phosphorylation that also increases transcriptional activity but does not increase DNA-binding activity (19). In addition, a similar series of experiments have been conducted for both c-Jun (17−19) and CREB (35,37) where the N-terminal activation domains were fused to heterologous DNA binding domains and the resultant chimeras retain the ability to be transcriptionally activated under conditions that cause phosphorylation of the activation domain. An important difference between CREB and c-Jun is that substitution of Ser133 in CREB with acidic residues simply results in an inactive protein (35), whereas we show here that a substitution of Asp for Ser73 activates both c-Jun and its chimera with BPV E2. The fact that c-Jun tolerates the acidic amino acid substitution, but CREB does not, may be fortuitous. The possibility that CREB may also interact with another protein via its N-terminal domain, as has also been suggested for c-Jun, is indicated by the fact that overexpression of the regulatory subunit of PKA not only reduces phosphorylation of Ser133 but also reduces observable DNA binding at the CREB site (38) without affecting the DNA binding affinity (37). This data indicates a similar phenomena to our own observation that an uncharged amino acid substitution, alanine, similar in charge to the unphosphorylated serine, reduces available DNA binding of c-Jun73A. Likewise, substitution of the negatively charged Asp73, similar in charge to the phosphorylated serine, increases available DNA binding of c-

Jun73Asp, although the DNA binding affinity of c-Jun does not change in response to phosphorylation of Ser 63 and Ser73 (19).

The fact that serine 73 lies within a region defined as an inhibitor binding site (16) suggests a model whereby TPA-induced phosphorylation modulates c-Jun activity by controlling association of an inhibitor. An inhibitory activity (IP-1) of Jun/Fos (a.k.a. AP-1) has recently been isolated that is present in both cytoplasmic and nuclear fractions (39). This inhibitor is thought to interact with Fos and/or Jun through the leucine zipper. In contrast, the inhibitor described by Baichwal and Tjian (16) may require only the N-terminus of c-Jun, therefore excluding the leucine zipper. It remains to be determined if there is a single inhibitor or perhaps several different inhibitors of Jun.

MATERIALS AND METHODS

Cell culture and transfections

Transformed human embryonic kidney cells, 293 cells, and human Hela cells, were grown in F12-Dulbecco's modified Eagle medium (DMEM) (1:1) supplemented with 5% fetal calf serum. Each 60-mm culture dish was incubated with a calcium phosphate/DNA coprecipitate containing 2 μg of DNA for each expression vector. 1 μg of the reporter plasmids TRE.col CAT was included in the CAT assay experiments. The coprecipitates were removed after 3–5 hours. The cells were shocked for 45 seconds with 50% glycerol, and replenished with fresh medium. Cells were harvested 48 hours post transfection.

Plasmids

Expression vectors that utilized the CMV IE promoter and the SV40 poly A site (40) were used to construct vectors that expressed each of the *jun* genes. Briefly, c-*jun* was subcloned from the plasmid pHJ40, which contained the gene in a bluescript SK vector (Stratagene). This plasmid contains a BamHI site 19 nucleotides upstream of the initiating AUG. The 1074 bp BamHI to StyI fragment of c-*jun* was inserted into the BamHI/SmaI site in the CMV expression vector. Point mutations of serine73 into either alanine (c-*jun*73A) or aspartic acid (c-*jun*73D) were constructed by oligonucleotide-directed mutagenesis. The PCR-amplified DNA fragments containing the point mutants were inserted into the avaI/pstI site of the wild-type c-*jun* expression vector. The validities of the mutations were confirmed by DNA sequencing. The Jun-E2 expression vectors containing point mutations at position 73 were constructed by cleaving the mutant containing c-*jun* expression vectors with Eag I to include the first 192 amino acids of c-Jun and ligating to the Eag I site in the BPV-E2 gene. The Jun-E2 fusion protein and the CAT reporter plasmid containing the E2 DNA binding site have been described previously. (16)

Immunoprecipitations

Transfected cells extracts were subjected to immunoprecipitation essentially as described (41) for figure 1. For the remaining figures, including the gel shift experiments, the extraction buffer contained 0.5% NP-40, but did not contain deoxycholate or SDS so as not to dissociate proteins noncovalently attached to Jun. Briefly, unless indicated otherwise, cells were placed in methionine-free DMEM for 1 hour then labeled for 3 hours using 50.0 μCi of L-[^{35}S]methionine (800μCi/ml, Amersham) per plate. After washing twice with PBS (phosphate buffered saline), extracts were prepared from 60-mm plates of either Hela cells or 293 cells by solubilization in 600 μl of immunoprecipitation buffer (41) containing 1 mM DTT. Extracts were pretreated with 5μl of preimmune serum for 20′ on ice and were then precleared with formaldehyde fixed Staphlococcus aureus (pansorbin, Calbiochem). 5μl of protein A-purified rabbit antisera against c-Jun, or 5μl of rabbit antisera against fos, was incubated with each extract for 2 hours. Antibody–antigen complexes were immunoprecipated with pansorbin. Pellets were resuspended in sample buffer and loaded on 11% SDS polyacrylamide gels.

Mobility-shift assays

60mm plates of transfected 293 cells were treated as above. To insure accurate quantitative assessment of DNA-binding activity, extract volumes were kept proportional to the numbers of cells extracted. Protein concentrations of the extracts were measured and were found not to vary substantially; typical measurements were 2 mg/ml. 2μg of each extract was used in mobility-shift assays as previously described (42) except that the final concentration of KCl in all reactions was 70 mM. The Jun/Fos binding-site probe was a double-stranded oligonucleotide containing the sequence CTCGAGCCGTGACTCAGCGCG, the consensus binding sequence is underlined. In the mutant probe the consensus sequence was changed to GTACTCC. For the Sp1 specific probe the consensus sequence was changed to GGGCGG. All probes were prepared by labeling the 5′ end of the specific oligonucleotides with γ-ATP^{32} and polynucleotide kinase.

Prokarotic expression

A prokaryotic expression vector (pGEX 1λT, Pharmacia) was used to express each of the *jun* constructs as a fusion protein with glutatione S-transferase (GST), thus allowing easy purification by affinity chromatography using glutathione sephrose 4B (Pharmacia). Each of the *jun* constructs, c-*jun*, c-*jun* 73A, and c-*jun* 73D were excised from the eukaryotic expression vectors utilizing 5′ XbaI and 3′EcoRI cleavage sites. A linker was designed to place the *jun* inserts in the correct reading frame with GST from the BamHI site in pGEX1λT, and contained a 5′ BamHI site, an internal XhoI site for miniscreening, and a 3′ XbaI site for ligation to the *jun* fragment. JM109 bacterial cells transformed with each of the *jun* constructs were incubated at 37°C for 4 hrs to achieve logarithmic growth phase before the addition of 100μM isopropyl-β-D-thiogalactoside (IPTG). After a 6 hr incubation extracts were prepared essentially as described by the manufacturer (Pharmacia), and mobility-shift assays were conducted as described above.

CAT assays

CAT assays were conducted by the procedure of Nordeen *et al.*, (43), except that cell pellets from each 60 mm plate of cells were resuspended in 100 μl of 250 mM Tris–HCl pH 7.8. Extracts were subjected to three freeze/thaw cycles using dry ice and a 37°C H_20 bath, and were spun in a microfuge for 2′. The supernatants were assayed for CAT activity. Immunoprecipitation of Jun was conducted on transfected cells to check that expression levels were the same for each construct.

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