Affinity-purified c-Jun Amino-terminal Protein Kinase Requires Serine/Threonine Phosphorylation for Activity*

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The addition of phorbol esters to U937 leukemic cells stimulates the phosphorylation of c-Jun on serines 63 and 73. To isolate the protein kinase which stimulates this phosphorylation, we have used heparin-Sepharose chromatography followed by affinity chromatography over glutathione-Sepharose beads bound with a fusion protein of glutathione S-transferase and amino acids 5–89 of c-Jun (GST-c-Jun). Using this procedure we purify a 67-kDa protein which is capable of phosphorylating GST-c-Jun as well as the complete c-Jun protein. By making mutations in serines 63 and 73 and then creating a fusion protein with GST (GST-c-Jun mut), we demonstrate that this protein kinase specifically phosphorylates these sites in the c-Jun amino terminus. Treatment of purified c-Jun amino-terminal protein kinase (cJAT-PK) with phosphatase 2A inhibits its ability to phosphorylate GST-c-Jun. This inactivated enzyme can be reactivated by phosphorylation with protein kinase C (PKC), although PKC is not capable of phosphorylating the GST-c-Jun substrate. Because v-Jun cannot be phosphorylated in vivo, we compared the ability of cJAT-PK to bind to GST-v-Jun or GST-c-Jun mut. The cJAT-PK bound 50-fold better to GST-c-Jun mut than GST-v-Jun suggesting that the Δ domain which is missing in v-Jun plays a role in binding the cJAT-PK. These results suggest that there is a protein kinase cascade mediated by protein phosphatases and PKC which regulates c-Jun phosphorylation.

Early events associated with phorbol esters in the induction of differentiation of human leukemic cells include the activation of transcription of the c-jun proto-oncogene (1–3), increase in c-Jun protein (1), and stimulation of transcription of genes located downstream from c-Jun DNA-binding sites (AP-1 enhancer elements) (1, 4). Because the c-Jun promoter also contains two AP-1-like sites (5, 6), it has been suggested that phorbol ester-induced modification of pre-existing Jun protein may regulate the transcription of this gene (5, 6). Recent experiments have shown that adding phorbol esters to U937 human leukemic cells stimulates the rapid phosphorylation of c-Jun protein (7, 8). In vitro mapping demonstrates that this phosphorylation occurs on serines 63 and 73 (7). By constructing a c-Jun plasmid containing a termination codon at amino acid 234 and, within this plasmid, mutating serines 63 and 73 to leucines, we have demonstrated that these 2 serines are the major sites of c-Jun phosphorylation in vivo (8). We further found that adding other protein kinase C (PKC) activators (diacylglycerol and bryostatin), okadaic acid, a phosphatase inhibitor, but not activators of cyclic AMP-dependent protein kinase stimulates the amino-terminal phosphorylation of c-Jun in these cells (8). To examine the role of this phosphorylation in regulating c-Jun-mediated transcriptional activation, we constructed a fusion protein containing the 84 NH2-terminal amino acids of c-Jun and fused it to the DNA-binding domain of the yeast GAL4 protein. Transfection of this plasmid along with a reporter gene demonstrates that this short segment of c-Jun protein is sufficient to mediate transcriptional activation by phorbol esters (8). However, mutation of serines 63 and 73 to leucines blocks this activation, suggesting that amino-terminal phosphorylation is necessary if transcriptional activation in U937 cells is to occur. Similarly, these sites in c-Jun have been shown to be phosphorylated by H-ras transfection of F9 cells and to mediate c-Jun transcriptional activity in these cells (9, 10). These data suggest that the protein kinases (PK(s)) which mediate the amino-terminal phosphorylation of c-Jun play a critical role in regulating transcription.

Adding phorbol esters to U937 cells also induces an increase in JunB protein. While this member of the Jun family forms a heterodimer with c-Fos and binds to AP-1 sites, it blocks transcriptional activation mediated by c-Jun (11–13). In contrast, adding phorbol esters to U937 cells do not activate phosphorylation of JunB (8). Also, the 89 NH2-terminal amino acids of JunB do not activate the transcriptional activity of the Gal4 DNA-binding domain in U937 cells (8). Although the amino terminus of c-Jun and JunB are highly similar (60%), serines 63 and 73 of c-Jun are followed by prolines, whereas, in JunB they are followed by a serine and aspartic acid residue, respectively. These data suggest that differential phosphorylation of Jun family members may determine their ability to function as either transcriptional activators or inhibitors.

In v-jun, the transforming gene isolated from avian sarcoma virus 17 (14), when compared to c-Jun the sequence surrounding serines 63 and 73 is identical. In comparison to c-Jun, however, v-Jun is deleted of 27 amino acids from position 34 to 60. This region of the c-Jun protein has been shown to bind a potential inhibitor of transcriptional activation (15, 16). It is clear that these 27 amino acids are important in the regulation of transformation, since removal of specific amino

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The abbreviations used are: PKC, protein kinase C; cJAT-PK, c-Jun amino-terminal protein kinase; GST, glutathione S-transferase; PMA, phorbol myristate acetate; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MAP, microtubule-associated protein.
acids within this region increases the transforming activity of c-Jun (17). Unlike c-Jun, v-Jun is not phosphorylated when U937 cells are treated with phorbol esters, suggesting that the amino-terminal PK might need to bind to the deleted region not found in v-Jun before it can phosphorylate serines 63 and 73 (22).

To better understand how the c-Jun amino-terminal kinase functions, we have isolated this kinase by using heparin-Sepharose chromatography followed by affinity chromatography using the 84 NH2-terminal amino acids of c-Jun linked to glutathione S-transferase. The purified PK has a molecular mass of 67 kDa. It will phosphorylate the amino terminus of c-Jun but not a JunB or an amino-terminal fragment of Jun with serines 63 and 73 mutated to leucines. It is active in the presence of magnesium and manganese. It is not recognized by antipeptide antibodies to pp42/44 (ERK 1 and 2). Dephosphorylation of this purified enzyme with phosphatase 2A blocks its ability to phosphorylate c-Jun, suggesting that it is activated by serine/threonine phosphorylation. The ability of this PK to phosphorylate c-Jun can be reactivated in vitro by phosphorylation with PKC. The c-Jun PK binds with a higher affinity to c-Jun than v-Jun, suggesting that the 27-amino acid deletion in the amino terminus plays a critical role in the enzyme-binding site.

**EXPERIMENTAL PROCEDURES**

**Isolation of the c-Jun Amino-terminal PK (cJAT-PK)—PMA-treated U937 cells (approximately 109) were washed twice with phosphate-buffered saline, and the cell pellet was resuspended in an equal volume of lysis buffer A (20 mM Hepes, pH 7.5, 1 mM EGTA, 2 mM MgCl2, 2 mM MnCl2, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 0.6 mM NaCl). U937 cells were lysed on ice for 1 h and centrifuged at 10000 g for 15 min at 4 °C. Glycerol was added to the supernatant to a concentration of 50%. An equal volume of lysis buffer B was added to the supernatant to bring the final concentration of NaCl to 0.3 M. This extract was passed over a 1-ml glutathione S-transferase/glycogen-Sepharose column (Pharmacia LKB Biotechnology Inc.). The column was washed with 3 ml of buffer A containing 0.3 M NaCl. The flow-through and wash were combined and loaded onto a heparin-Sepharose column (Pierce Chemical Co.). The column was washed with buffer A containing 0.3 M NaCl, and the PK activity was eluted with a 20-ml linear gradient of KC1 (0.3-1.5 M) in buffer A. The flow rate was approximately 0.25 ml/min, and 0.5-ml fractions were collected. Fractions with the highest amino-terminal c-Jun kinase activity were combined and dialyzed for 2 h against buffer A containing 0.3 M KC1. This material was loaded onto a 1-ml Jun (amino acids 5-89)/glutathione S-transferase/glycogen-Sepharose (see below for details) column and washed with 3 ml of buffer A containing 0.6 M KC1. The proteins were eluted with 3 ml of buffer A containing 80% ethylene glycol. Eluted proteins were dialyzed against buffer A for 2 h and concentrated with 50% polyethylene glycol. Glycerol was added to the supernatant to a concentration of 50%.

**Assay of Amino-terminal c-Jun Kinase Activity—Preparation of c-Jun-glutathione S-transferase fusion protein bound to glutathione-Sepharose beads to create a substrate for the cJAT-PK polymerase chain reaction was used to clone amino acids 5-89 of c-Jun, JunB, and c-Jun with serines 63 and 73 mutated to leucines (c-Jun mut). This c-Jun PK binds with a higher affinity to c-Jun than v-Jun, suggesting that the 27-amino acid deletion in the amino terminus plays a critical role in the enzyme-binding site.

**Measurement of PK Activity—cJAT-PK activity was measured in a 20-μl reaction containing 0.2 μg of c-Jun/glutathione S-transferase/glutathione-Sepharose beads, 5 μl of protein extract, and 10 μl of kinase buffer, 20 mM Hepes, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl2, 2 mM MnCl2, 5 mM NaF, 1 mM Na vanadate, and 50 μM [γ-32P]ATP. The reaction was run at 30 °C with an added buffer containing 15 μM KC1 and twice with PBST buffer without salt. A slurry suspension of beads (50% v/v) in glycerol was stored at -20 °C.

**RESULTS AND DISCUSSION**

Because c-Jun is phosphorylated both on amino- and carboxy-terminal serines (7, 8, 20), it is necessary to develop an assay which would recognize only the amino-terminal PK. To accomplish this, 34 amino acids (5-89) which include serines 63 and 73 in the X and Y peptides (7, 8) (22), were fused to glutathione S-transferase (GST) which contained a thrombin cleavage site at its carboxyl terminus. The fusion protein was expressed in E. coli and the bacteria lysate was mixed with glutathione-Sepharose beads. To assay PK activity, extracts or column eluates were incubated with the fusion protein beads, [γ-32P]ATP, and Mg2+ at 30 °C for 10 min. The beads were pelleted, washed, and cleaved with thrombin. The supernatant was then run on a 15% SDS gel to identify the phosphorylated Jun peptide. In partially purified or fully purified extracts from cells no phosphorylation of the GST protein was seen (data not shown).

To isolate the c-Jun amino-terminal PK, U937 cells were first treated with PMA (0.15 μM) for 30 min to activate the PK in vivo and lysed (see “Experimental Procedures”) (22). This high salt extract of the cells was passed over a GST/glutathione-Sepharose column, and the flow-through bound to a heparin-Sepharose column. The active enzyme was eluted with a linear KC1 gradient and the fractions with the highest activity were combined, dialyzed, and loaded onto a substrate affinity column containing the amino-terminal c-Jun GST fusion protein bound to glutathione-Sepharose beads (Fig. 1A, lane 1). The column was washed extensively, and the PK was eluted in the same buffer containing 80% ethylene glycol (Fig. 1A, lane 2). The enzyme was 5000-fold purified with a yield of 8-15 μg/2 × 106 cells. Silver staining of an SDS gel containing an aliquot of the final column eluate demonstrates a major band at 67 kDa. When increased protein is loaded
Carried out as described under "Experimental Procedures." Aliquots from each stage of purification were run on a 10% SDS gel which was silver stained. Lane 1 contains the proteins bound to the GST-Jun affinity column. Lane 2 contains the concentrated ethylene glycol-elimuted cJAT-PK. B, reconstitution of cJAT-PK. The ethylene glycol extract from GSTc-Jun beads was concentrated and run on an SDS gel which was sliced. Reconstitution of cJAT-PK activity was carried out as described under "Experimental Procedures" and aliquots of the reconstituted material were assayed for cJAT-PK activity. C, the effect of increasing enzyme concentration on phosphorylation of the GSTc-Jun fusion bands. Increasing amounts of enzyme were added to 20 μg of GSTc-Jun beads in a 10-min PK assay. The reaction was terminated and run on an SDS gel. The autoradiogram of this gel is shown in the insert, and the counts/min in the c-Jun amino terminus are plotted. D, time course of cJAT-PK phosphorylation of GSTc-Jun fusion protein. 2 units of enzyme were added to GSTc-Jun beads for varying periods of time in a cJAT-PK assay as described under "Experimental Procedures." At each time point the reaction was stopped with 1 ml of PBST. The insert is the autoradiogram of c-Jun amino-terminal peptide. The SDS gel was cut and counted.

Minor bands are seen between 46 and 69 kDa. When U937 cells which are not treated with PMA are used for purification no cJAT-PK activity was evident.

To identify whether the 67-kDa protein was the c-Jun PK, the PK activity was reconstituted from a SDS gel. After electrophoresis, the gel was washed twice with a urea containing buffer. Slices of the gel were then extracted overnight with a buffer containing 0.5 mM EGTA, and each extract was assayed for c-Jun PK activity. The only PK activity evident was in lane 5 (Fig. 1B) which correlates to a position at approximately the 69-kDa marker protein. This experiment demonstrates that the purified cJAT-PK is the major band seen on silver stain of the affinity column eluate.

Using purified cJAT-PK, the time and concentration dependence of phosphorylation was examined. Half-maximal phosphorylation of the GST-c-Jun fusion protein at 30°C was achieved in 8 min (Fig. 1D) and the rate of incorporation appeared linear over 10 min using 2 units (1 unit of cJAT kinase equals 660 cpm/10 min) of enzyme. Using a 10-min period of incubation, the phosphorylation of the GST-c-Jun fusion protein was linear to 2 units of enzyme (Fig. 1C). This amino-terminal c-Jun kinase can be activated either in the presence of magnesium or manganese with maximal stimulation occurring at 2 and 1 mM, respectively. Because it is possible that the Jun peptide fused to GST is abnormally folded and becomes a substrate for this PK, we examined whether this PK can phosphorylate the entire c-Jun protein, using bacterially expressed c-Jun. After expression of this c-Jun protein in bacteria, it was solubilized in urea (7), followed by the removal of the urea by dialysis. This bacterially expressed c-Jun protein is phosphorylated by cJAT-PK. (Fig. 2B). To determine whether this cJAT-PK phosphorylates identical serines (63 and 73) to those modified in vivo, we constructed a GST: amino-terminal Jun fusion protein in which serines 63 and 73 were mutated to leucines (22). If serines 63 and 73 were the only sites of phosphorylation, then this mutant should not be phosphorylated by the cJAT-PK. Because we have shown that addition of phorbol esters to U937 cells does not lead to an increase in JunB phosphorylation, we constructed a GST fusion protein encoding the 89 NH2-terminal amino acids of JunB. These two fusion proteins were expressed in bacteria and bound to glutathione beads. In comparison to the amino terminus of c-Jun, neither the amino terminus of c-Jun with serines 63 and 73 mutated to leucine (Fig. 2A, lane 1) nor the amino terminus of JunB (Fig. 2A, lane 2) could function as substrates for this purified PK (Fig. 2A, compare lanes 1 and 2 with 3). This result suggests that the enzyme which we have purified functions similarly in vitro to the PK which is activated by phorbol ester treatment of U937 cells phosphorylating the amino terminus of c-Jun on serines 63 and 73 but not the amino terminus of JunB.

Because phorbol ester treatment of U937 cells activates PKC, we examined whether the c-Jun amino-terminal PK was activated by serine/threonine phosphorylation and whether dephosphorylated c-Jun kinase could be reactivated by PKC. To examine whether the c-Jun PK could be inactivated by dephosphorylation, it was incubated with phosphatase 2A for 30 min (Fig. 3, lane 3) or 60 min (Fig. 3, lane 4) at 37°C. This treatment completely inactivates the cJAT-PK (Fig. 3, compare lanes 3 and 4 with 1 and 2), suggesting that it is activated by serine/threonine phosphorylation. However, it can be reactivated after phosphatase treatment, by PKC stimulated with calcium and phospholipids (Fig. 3, compare lanes 3 and 4 with 5 and 6). However, PKC alone is not capable of stimulating the phosphorylation of the GSTc-Jun peptide (Fig. 3, lane 9). Also, a pseudosubstrate inhibitor of PKC (21), while not affecting cJAT-PK directly (Fig. 3, lane 2), inhibited the ability of the PKC with and without calcium and phospholipids to reactivate the cJAT-PK (Fig. 3, compare lanes 5 and 6 with 7 and 8). These results demonstrate that PKC mediates phosphorylation of the cJAT-PK.

The cJAT-PK is capable of phosphorylating c-Jun but not
The assay preincubated with GST beads alone is shown in the supernatant. However, when the enzyme was incubated was increased from 2 to 16 pg there was a 60% decrease (Fig. 4, lane 1) or presence of the PKC pseudosubstrate peptide (1 μM) (lane 2) using GST:v-Jun fusion beads. This same amount of cJAT-PK was incubated with protein phosphatase 2A for 30 (lane 3) or 60 min (lane 4) prior to PK assay. In lane 3, the cJAT-PK was treated with protein phosphatase 2A which was inactivated with NaF and then incubated with (lane 6) and without (lane 5) calcium and phospholipids. The assay in lanes 5 and 6 was carried out in the presence of 1 μM PKC pseudosubstrate prior to assay, yielding the results in lanes 7 and 8, respectively. In lane 9 GST:c-Jun beads were phosphorylated with the same amount of PKC used in lanes 5 and 6 in the presence of calcium and phospholipids.

FIG. 3. cJAT-PK is inactivated by protein phosphatase 2A and reactivated by PKC. 3 units of cJAT-PK were assayed for 10 min in the absence (lane 1) or presence of the PKC pseudosubstrate peptide (1 μM) (lane 2) using GST:c-Jun fusion beads. This same amount of cJAT-PK was incubated with protein phosphatase 2A for 30 (lane 3) or 60 min (lane 4) prior to PK assay. As in lane 3, the cJAT-PK was treated with protein phosphatase 2A which was inactivated with NaF and then incubated with (lane 6) and without (lane 5) calcium and phospholipids. The assay in lanes 5 and 6 was carried out in the presence of 1 μM PKC pseudosubstrate prior to assay, yielding the results in lanes 7 and 8, respectively. In lane 9 GST:c-Jun beads were phosphorylated with the same amount of PKC used in lanes 5 and 6 in the presence of calcium and phospholipids.

FIG. 4. The amino terminus of v-Jun binds cJAT-PK less efficiently than the amino terminus of c-Jun. In a 20-μl reaction, 2 units of cJAT-PK were preincubated with various amounts of Sepharose beads containing GST:v-Jun or GST:c-Jun mut for 15 min at 4 °C. The samples were pelleted, and 10 μl of the supernatant were assayed using GST:v-Jun beads for cJAT-PK activity. The results of the assay preincubated with GST beads alone is shown in lane 1. The enzyme in lane 2 was preincubated with 2 μg of GSTv-Jun, lane 3 with 4 μg, lane 4 with 8 μg, and lane 5 with 16 μg. The assay of lane 6 was done without competitor and using GSTc-Jun mut beads as a substrate. The enzyme in lanes 7 was preincubated with 16 μg of GST:c-Jun mut, lane 8 with 8 μg, lane 9 with 4 μg, and lane 10 with 2 μg.

v-Jun (22). These two proteins are identical over the region constituting the phosphorylation sites of serines 63 and 73. However, they differ in that 27 amino acids (34-60) are deleted in v-Jun when compared to c-Jun. To determine whether the 27 amino acids in c-Jun (the δ domain, 15, 16) play a role in mediating the binding of c-JAT PK to the substrate, we have evaluated the ability of increasing equivalent amounts of GST:v-Jun amino-terminal fusion protein or GST:c-Jun amino terminus containing serines 63 and 73 mutated to leucines to bind the cJAT-PK. The enzyme was incubated with the beads containing either fusion protein, and the beads were then spun out. The supernatant was assayed for GSTc-Jun PK activity (see “Experimental Procedures”). As the amount of GST:v-Jun beads used in the preincubation was increased from 2 to 16 μg there was a 60% decrease (Fig. 4, lanes 2-5) in the amount of cJAT PK activity assayed in the supernatant. However, when the enzyme was incubated with an identical concentration of beads bound with GST:c-Jun containing serine to leucine mutations at positions 63 and 73 (GST:c-Jun mut) at all concentrations tested, the beads bound greater than 95% of the kinase activity (Fig. 4, lanes 7-10). At the smallest amount of beads used in the preincubation (Fig. 4, compare lanes 10, c-Jun mut and 2, v-Jun), GSTc-Jun mut bound 50-fold more enzyme activity than the equivalent v-Jun beads. Similar results were obtained with GST:c-Jun beads. Beads containing only the GST fusion did not bind this PK activity (Fig. 4, lane 1) and if GST:c-Jun mut beads were used as a substrate no phosphorylation occurred (Fig. 4, lane 6). This experiment suggests that the 27-amino acid NH2-terminal amino acids found in c-Jun and not v-Jun functions to mediate the binding of the cJAT-PK.

Previous results have demonstrated that serines 63 and 73 can be phosphorylated by pp42/44 and pp54 MAP kinases in vitro (7). Using a partially purified preparation of these kinases, we find that they are capable of phosphorylating the GST:c-Jun fusion protein (data not shown). These kinases can be inactivated by phosphatase 2A, but they cannot be reactivated by PKC to phosphorylate GST:c-Jun fusion (data not shown). However, the material eluted from the heparin-Sepharose and the GSTc-Jun affinity column does not contain pp42,44 enzymes as demonstrated by Western blotting using a monoclonal antibody specific for pp42,44 MAP kinase. We have also found that the M07e human leukemia cell line, when treated with phorbol esters has little cJAT-PK activity but contains equivalent amounts of activated pp42 MAP kinase to that seen in U937 cells (data not shown). This suggests that pp42 is not the cJAT-PK. The molecular weight of the protein kinase eluted from SDS gels is considerably different from the pp54 MAP kinase. However, the pp54 has been purified from rat, and the human kinase could be somewhat larger. Although the cJAT-PK is also capable of phosphorylating MAP-2 (data not shown), it is not one of the MAP kinases previously described to phosphorylate c-Jun. Since higher molecular weight MAP-2 phosphorylating enzymes have been discovered, it is possible that this cJAT-PK is related to the MAP family of enzymes (18).

Our results suggest that cJAT-PK can be inactivated by serine/threonine dephosphorylation and reactivated by PKC. This suggests that there is a PK cascade leading to c-Jun phosphorylation. The existence of such a cascade would be strengthened by the demonstration both in vitro and in vivo of PKC phosphorylation of the c-JAT protein kinase. Previous in vitro results from our laboratory have suggested that serine/threonine dephosphorylation may play a role in regulating the c-Jun PK. Treatment of U937 cells with okadaic acid, a serine phosphatase inhibitor, increases the phosphorylation of c-Jun protein and enhances the phosphorylation stimulated by PKC activators (8).

The fact that the 27-amino acid domain deleted in v-Jun but present in c-Jun appears to play an important role in both transformation and transcription mediated by the c-Jun protein. This region has been shown to bind a potential inhibitor of transcriptional activation (15). In addition, mutations within this domain increase the transforming activity of c-Jun (17). Our data suggest that the absence of this domain greatly inhibits the binding of the cJAT-PK to the c-Jun protein. Could the c-JAT PK be either a transcriptional inhibitory protein or a regulator of the transcriptional inhibitor by phosphorylating serines 63 and 73? Further experiments will be necessary to examine these possibilities.

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