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Phorbol esters stimulate the phosphorylation of c-Jun but not v-Jun: Regulation by the N-terminal δ domain

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ABSTRACT **c-Jun and its oncogenic counterpart v-Jun are completely conserved within the region from Ser-63 to Ser-73; these serines are sites for phorbol ester-inducible c-Jun phosphorylation. Using a U937 human leukemic cell line stably expressing v-Jun, we have demonstrated that phorbol esters stimulate the *in vivo* phosphorylation of c-Jun but not v-Jun. We developed an *in vitro* protein kinase assay to characterize the c-Jun protein kinase and to examine the determinants underlying this differential phosphorylation. Fusion proteins between glutathione *S*-transferase and the N terminus of c-Jun, v-Jun, or several c-Jun mutants were used as substrates. A c-Jun kinase activity was affinity-purified 5000-fold by using glutathione *S*-transferase–c-Jun-glutathione-Sepharose beads and was found to phosphorylate the N terminus of c-Jun but not v-Jun or c-Jun containing a 27-amino acid N-terminal deletion found in v-Jun. These effects were also observed *in vivo* as phorbol 12-myristate 13-acetate did not induce the phosphorylation of v-Jun or the c-Jun deletion mutant in U937 cell lines stably expressing these proteins. These findings indicate that the δ domain of c-Jun (amino acids 34–60), which is deleted in v-Jun, plays a critical role in regulating N-terminal c-Jun phosphorylation.**

The v-*jun* oncogene was originally isolated from avian sarcoma virus 17 and encodes a 65-kDa fusion protein containing viral gag sequences (1, 2). c-Jun is the normal cellular homolog of v-Jun and is a component of transcription factor AP-1 (3, 4). Both c-Jun and v-Jun are capable of forming heterodimers with Fos protein and activating transcription of AP-1-responsive genes (2–7). Although c-Jun is capable of cooperating with Ha-Ras to transform fibroblasts (8), v-Jun is 10-fold more potent than c-Jun in transforming chicken embryo fibroblasts (9). Deletion of a 27-amino acid region near the N terminus of c-Jun, termed the δ domain, and deletion of 3′ untranslated sequences stimulate the full oncogenic potential of c-Jun (9). *In vitro* transcription assays also indicate that v-Jun has a greater transcriptional activating capacity than c-Jun (10). Deletion of the N terminus of c-Jun, to create a protein similar to v-Jun, markedly enhances the ability of c-Jun to activate transcription (10, 11). These findings indicate that the δ domain of c-Jun, which is deleted in v-Jun, plays an important negative regulatory role in controlling both the transcriptional and transforming activity of c-Jun.

The N terminus of c-Jun is an important region for post-translational modification (12–14). Activation of protein kinase C with phorbol esters or bryostatin or inhibition of serine/threonine phosphatase activity with okadaic acid stimulates the N-terminal phosphorylation of c-Jun in U937 human leukemic cells (12, 13). *In vitro* mapping and *in vivo* phosphorylation studies demonstrate that this phosphorylation occurs on Ser-63 and Ser-73 (12, 13). Transfection of fibroblasts with activated Ha-Ras also stimulates the N-terminal phosphorylation of c-Jun (14). Deletion of this N-terminal region (amino acids 1–87) or mutation of Ser-63 and Ser-73 of c-Jun prevents the transcriptional activation of c-Jun by Ha-Ras (14) and phorbol 12-myristate 13-acetate (PMA) (12, 13), respectively. These findings suggest that N-terminal phosphorylation plays an important role in the regulation of the transcriptional activity of the c-Jun protein in response to a variety of stimuli.

Phorbol esters induce the macrophage-like differentiation of U937 human leukemic cells (15). This differentiation is accompanied by an increase in c-*jun* transcription (16–18), enhanced N-terminal c-Jun phosphorylation (12, 13), and increased transcription of genes containing an upstream AP-1 sequence (16, 17). Utilizing a U937 cell line stably expressing v-Jun, we demonstrate that phorbol esters stimulate the phosphorylation of c-Jun but not v-Jun. We have purified a c-Jun protein kinase activity from U937 cells that specifically phosphorylates the N terminus of c-Jun but not v-Jun or a c-Jun mutant in which the δ domain was deleted from c-Jun to resemble the deletion in v-Jun (3, 4). To determine the importance of this region in regulating *in vivo* c-Jun phosphorylation, we established a U937 cell line stably expressing this c-Jun deletion mutant. Phorbol esters stimulated an increase in phosphorylation of c-Jun but not the mutant c-Jun protein. These results demonstrate that the δ domain, which flanks the N-terminal phosphorylation sites of c-Jun, is critical for the constitutive and inducible phosphorylation of the N terminus of c-Jun.

MATERIALS AND METHODS

Stable Cell Lines and Plasmids. A v-Jun expression vector was prepared by cloning the full-length v-Jun cDNA (a gift of T. Bos, Eastern Virginia Medical School, Norfolk, VA) into the *Hin*dIII–*Not* I sites of pRC-CMV (Invitrogen, San Diego). Deletion of amino acids 34–60 from c-Jun (c-Jun AK-3) was performed by cloning c-Jun into M13 followed by site-directed mutagenesis (Amersham kit) with the oligonucleotide 5′-GGACCTTATGGCTACAGTAACCTCACCTCGCCCGACGTGGGG-3′. The mutated c-Jun cDNA was then cloned into the *Hin*dIII–*Xba* I sites of pRC-CMV. The c-Jun Ser → Leu double mutant (c-Jun, Δ63/73) was prepared as described (12). U937 cells (2–3 × 10^7 cells) were cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) bovine calf serum and transfected by electroporation with 30 μg of expression vector as described (12, 16). Cells were selected in medium containing G418 (1 mg/ml), and resistant clones were pooled and used after 1 month of continuous selection.

Cell Labeling and Immunoprecipitation. U937 cells (5–10 × 10^6 cells per ml) were labeled with either [^{32}P]orthophosphate (1 mCi/ml) or Tran^{35}S-label (0.1 mCi/ml; 1 Ci = 37 GBq) as described (19). Cells were washed with ice-cold phosphate-

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; GT, glutathione.

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buffered saline (PBS), and crude nuclei were isolated by hypotonic lysis (19). Native extracts were prepared by lysis in RIPA buffer (0.15 M NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS). Denatured lysates were prepared by boiling nuclei in 50 mM Tris·HCl, pH 8.0/0.5% SDS/5 mM dithiothreitol, followed by a 1:5 dilution with RIPA buffer lacking SDS. Lysates were clarified by centrifugation and Jun protein was immunoprecipitated with rabbit antisera raised against a bacterially expressed c-Jun—glutathionine *S*-transferase (GST) fusion protein at a 1:500 dilution as described (19).

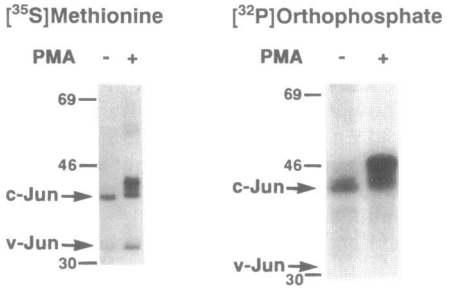
c-Jun Protein Kinase Assay. PCR was used to clone amino acids 5–89 of c-Jun, the corresponding region of v-Jun, the c-Jun deletion mutant (c-Jun AK-3), and c-Jun (Δ63/73), into either the pGEX-2T or pGEX-3X vector (Pharmacia). Bacteria containing the pGEX-Jun fusion plasmids were induced with isopropyl β-D-thiogalactoside. After sonication, the bacterial supernatant was mixed with glutathione (GT)-Sepharose beads according to the manufacturer's instructions. The beads were washed extensively with PBS containing 1% Triton X-100 (PBST) and once with 50 mM Tris·HCl (pH 7.5) prior to use as the *in vitro* protein kinase substrate. U937 cell extracts were prepared by lysis for 1 h at 4°C in cell lysis buffer (20 mM Hepes, pH 7.5/0.3 M NaCl/1 mM EGTA/2 mM $MgCl_2$/2 mM $MnCl_2$/1 mM dithiothreitol/0.5% Nonidet P-40/0.5 mM phenylmethylsulfonyl fluoride/5 mM benzamidine). The reaction mixture (50 μl) contained 10 μl of pGEX-Jun beads, 10 μg of cell extract, 10 mM $MgCl_2$, 20 μM ATP, 0.5 μl of [γ-^{32}P]ATP (30 Ci/mmol, Amersham), 50 mM Tris·HCl (pH 7.5), 5 mM NaF, and 1 mM sodium vanadate. The assay mixture was incubated at 30°C for 10 min. The beads were washed once with PBST and twice with a solution of 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% 2-mercaptoethanol. The beads were incubated with either 0.125 unit of thrombin for 1 h (pGEX-2T) or 2 μg of factor Xa overnight (pGEX-3X). The cleavage fragments were analyzed by SDS/PAGE on 15% gels.

Affinity Purification of the c-Jun Protein Kinase. U937 cells (1×10^8 cells) were washed with PBS, and the pellet was lysed on ice for 1 h in an equal volume of cell lysis buffer containing 0.6 M NaCl. The cell lysate was centrifuged for 15 min at 10,000 × *g* and glycerol was added to a final concentration of 50% (vol/vol). The extract was passed through a 1-ml GST–GT-Sepharose column and the column was washed with 3 ml of lysis buffer. The two flow-through fractions were combined and passed over a 1-ml heparin-Sepharose column, and the column was washed extensively with lysis buffer. The c-Jun protein kinase was eluted with a linear gradient of 0.3–1.5 M KCl in lysis buffer. Fractions containing maximal c-Jun N-terminal kinase activity were dialyzed for 2 h against lysis buffer containing 50 mM KCl and loaded onto a 1-ml c-Jun–GST–GT-Sepharose column. The column was washed with 3 ml of lysis buffer containing 0.6 M KCl and material was eluted with lysis buffer containing 80% (vol/vol) ethylene glycol.

RESULTS

Phorbol Esters Stimulate the Phosphorylation of c-Jun but Not v-Jun. Although c-Jun and v-Jun exhibit a high degree of N-terminal amino acid homology (86% identity; Fig. 1), c-Jun contains a stretch of 27 amino acids (the δ domain) that is deleted in v-Jun. Ser-63 and Ser-73 of c-Jun, which flank the δ domain, are conserved within v-Jun and are sites for constitutive and PMA-inducible c-Jun phosphorylation (12, 13). To determine whether these residues are similarly phosphorylated within v-Jun, U937 human leukemic cells were transfected with a pRC-CMV expression vector containing full-length v-Jun and a neomycin selection marker. After selection with G418 the cells were pooled and labeled with either Tran^{35}S-label or [^{32}P]orthophosphate. Immunoprecipitation of Jun protein from ^{35}S-labeled cells demonstrated that this cell line stably expresses v-Jun protein (Fig. 1). Whereas PMA stimulated the synthesis of both c-Jun and v-Jun, only c-Jun displayed a retarded mobility upon SDS/PAGE (Fig. 1). We (12) and others (13) have shown that the retarded mobility of c-Jun after PMA treatment of U937 cells is due to the enhanced phosphorylation of Ser-63 and Ser-73. To directly assess the phosphorylation state of v-Jun, Jun protein was immunoprecipitated from ^{32}P-labeled cells. Whereas c-Jun was constitutively phosphorylated and became hyperphosphorylated in response to PMA treatment, no phosphorylation of v-Jun was observed even after PMA treatment (Fig. 1). Similar results were obtained using a transient transfection protocol that yielded equivalent levels of v-Jun and c-Jun protein (data not shown).

***In Vitro* c-Jun Kinase Activity Parallels *in Vivo* c-Jun Phosphorylation in Response to PMA Treatment.** To further characterize the phosphorylation of c-Jun, we developed an *in vitro* assay for c-Jun protein kinase activity. The N-terminal 84 amino acids of c-Jun (residues 5–89) were cloned into the pGEX-2T vector creating a c-Jun–GST fusion protein containing a protease cleavage site between c-Jun and GST. The fusion protein was bound to GT-Sepharose beads and used as a substrate in the *in vitro* kinase assay. U937 cell extracts were assayed for c-Jun protein kinase activity using the c-Jun–GST beads as substrate and analyzed by SDS/PAGE after cleaving the c-Jun fragment with thrombin. Extracts from untreated U937 cells stimulated low-level phosphorylation of the c-Jun cleavage product (Fig. 2*A*). Treating the cells with PMA for 30 min increased the c-Jun kinase activity as detected by the enhanced phosphorylation of the low molecular weight c-Jun fragment. No phosphorylation of

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C-JUN   MTAKMETTFYDDALNASFLPSESGPYGYSN   30
        : :::: ::: :::::::: : ::: ::: :
V-JUN   MSAKMEPTFYEDALNASFAPPESGGYGYNN

C-JUN   PKILKQSMTLNLADPVGSLKPHLRAKNSDL   60
           :
V-JUN   GDI----------------------------

C-JUN   LTSPDVGLLKLASPELERLIIQSSNGHITT   90
        ::::::::::::::::::::::::::::::
V-JUN   LTSPDVGLLKLASPELERLIIQSSNGHITT
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FIG. 1. PMA induces the phosphorylation of c-Jun but not v-Jun in U937 cells. U937 cells stably expressing v-Jun were labeled with either Tran^{35}S-label (0.1 mCi/ml) for 2 h or [^{32}P]orthophosphate (1 mCi/ml) for 6–12 h. Cultures were split and one-half was treated with 0.1 μM PMA for the last hour of labeling. Jun protein was immunoprecipitated from native RIPA lysates and resolved by SDS/PAGE on a 10% gel. A comparison between the N-terminal sequences of c-Jun (4) and v-Jun (4) is shown. The double dots indicate sequence identity and the dashed line indicates the 27-amino acid region of c-Jun that is deleted in v-Jun. Molecular masses in kDa are shown.

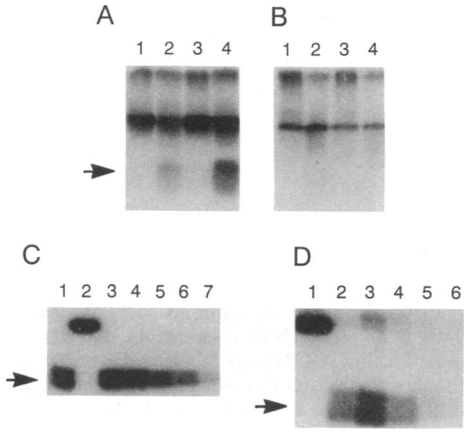

FIG. 2. PMA enhances N-terminal c-Jun protein kinase activity measured *in vitro*. (*A* and *B*) Whole-cell extracts (10 μg) from untreated or PMA-treated U937 cells (0.5 μM PMA for 30 min) were assayed for *in vitro* c-Jun kinase activity. Extracts were incubated with GT-Sepharose beads containing either c-Jun–GST (pGEX-2T-Jun) (*A*) or GST (pGEX-2T) (*B*) for 10 min at 30°C, washed, and cleaved with thrombin. Phosphorylated N-terminal c-Jun cleavage fragments are indicated by the arrows. (*A* and *B*) Lanes: 1 and 2, control; 3 and 4, PMA; 1 and 3, no thrombin; 2 and 4, thrombin added. (*C* and *D*) Beads containing c-Jun–GST were incubated with increasing concentrations of whole-cell extract from PMA-treated cells for 10 min (*C*) or 10 μg of extract for various times (*D*) and assayed for substrate phosphorylation. (*C*) Lanes: 1, 3–7, thrombin added; 2, no thrombin; 1 and 2, 64 μg of extract; 3, 32 μg; 4, 16 μg; 5, 8 μg; 6, 4 μg; 7, 2 μg. (*D*) Lanes: 1, no thrombin; 2–6, thrombin added; 1 and 2, 16 min; 3, 8 min; 4, 4 min; 5, 2 min; 6, 1 min.

such a cleavage product was observed when GST beads in the absence of the c-Jun fusion were used as substrate (Fig. 2*B*). The kinetics of this assay were linear for up to 8 min at protein concentrations up to 32 μg (Fig. 2 *C* and *D*).

Utilizing this *in vitro* kinase assay, we examined several characteristics of this PMA-induced c-Jun kinase activity. PMA stimulated c-Jun protein kinase activity in a dose-dependent manner with an EC_{50} value of 10 nM (Fig. 3*A*, bottom band). The effect of PMA on c-Jun kinase activity was transient, with maximal activation occurring within 30 min of treatment and returning to near control levels by 6 h (Fig. 3*B*). To determine whether PMA stimulated c-Jun phosphorylation with a similar time course *in vivo*, cells were pulse-

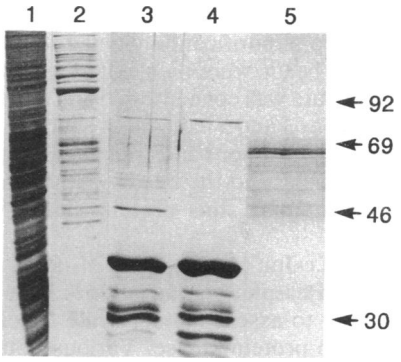

FIG. 4. Affinity purification of a c-Jun protein kinase. The protein kinase was purified from 1 × 10^8 U937 cells. Lanes: 1, GST–GT-Sepharose flow-through; 2, eluate from a heparin-Sepharose column; 3, c-Jun protein kinase bound to a c-Jun–GST–GT-Sepharose column; 4, proteins remaining on the c-Jun–GST–GT-Sepharose beads after elution with 80% ethylene glycol; 5, concentrated eluate from the c-Jun–GST–GT-Sepharose column. Samples were resolved by SDS/PAGE on a 10% gel and silver stained. Molecular masses in kDa are shown.

labeled with Tran^{35}S-label and then treated with PMA in the presence of unlabeled methionine. After 15 min, a majority of the c-Jun protein migrated as a highly retarded 46-kDa c-Jun species (Fig. 3*C*). The fraction of c-Jun migrating at 46 kDa was greatly diminished by 1 h and was barely detectable by 2 h. We have shown (12) that this retarded mobility reflects c-Jun phosphorylation; thus, PMA-induced c-Jun kinase activity measured *in vitro* directly parallels the induction of c-Jun phosphorylation *in vivo* (compare Fig. 3 *B* and *C*).

Affinity Purification of a c-Jun Protein Kinase. A c-Jun protein kinase was purified from U937 cells treated with PMA for 30 min to maximize c-Jun kinase activity (Fig. 3*B*). Fold purity at each step of purification was monitored by both *in vitro* kinase activity and SDS/PAGE followed by silver staining (Fig. 4). Whole-cell extracts of PMA-treated U937 cells were chromatographed over GST–GT-Sepharose beads. The flow-through fraction (lane 1) was then bound to heparin-Sepharose beads and material was eluted with a linear KCl gradient, resulting in an ≈200-fold purification (lane 2). The eluate was bound to c-Jun–GST–GT-Sepharose beads and washed extensively with lysis buffer containing 0.6 M KCl (lane 3). The beads were then washed with 1.5 M KCl

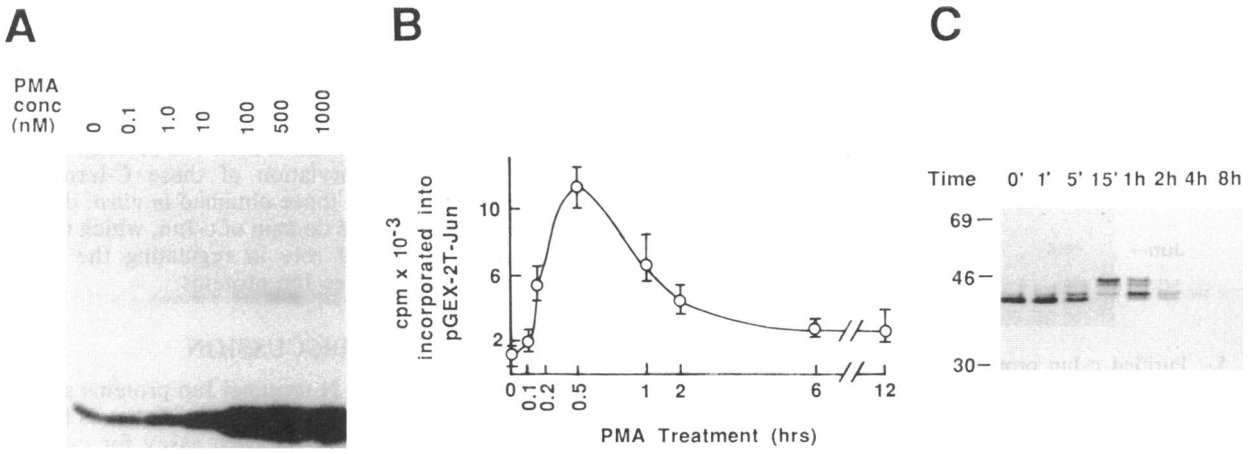

FIG. 3. PMA-induced N-terminal c-Jun kinase activity measured *in vitro* parallels c-Jun phosphorylation *in vivo*. (*A*) U937 cells were treated for 30 min with PMA. Whole-cell extracts were then assayed for c-Jun kinase activity by using c-Jun–GST–GT-Sepharose beads as substrate. (*B*) The time course of PMA-induced N-terminal c-Jun kinase activation was examined by treating cells with 0.5 μM PMA for various times. Whole-cell extracts were assayed for *in vitro* c-Jun kinase activity. The phosphorylated c-Jun fragment was excised from the gel, radioactivity was measured, and the results were plotted. (*C*) Time course of PMA-induced c-Jun phosphorylation was determined *in vivo* by pulse–chase analysis. Cells were labeled with Tran^{35}S-label for 2 h, washed in complete medium containing unlabeled methionine, and treated with 0.1 μM PMA. Cells were harvested at various times, and c-Jun was immunoprecipitated from denatured nuclear lysates.

with little loss of bound material (data not shown). Silver staining at this step of purification yielded several major and minor bands. The beads were then eluted with 80% ethylene glycol, and the eluate was concentrated and dialyzed (lane 5). Comparison of lanes 3 and 5 indicates a major band migrating at ≈67 kDa and other minor bands ranging from 46 to 66 kDa that bound prominently to the beads. Although not homogenous by silver staining, this protein kinase was purified 5000-fold.

The δ Domain of c-Jun (Amino Acids 34–60) Is Required for N-Terminal c-Jun Phosphorylation *in Vitro*. We utilized our *in vitro* kinase assay to assess the substrate specificity of this semipurified c-Jun protein kinase. Various N-terminal Jun–GST fusion proteins were prepared by a PCR and cloning into the pGEX-3X vector and used as substrates in the *in vitro* kinase assay. These include a c-Jun fusion in which the *in vivo* N-terminal phosphorylation sites Ser-63 and Ser-73 were mutated to leucines, c-Jun(Δ63/73) and a v-Jun construct containing the N terminus of v-Jun corresponding to amino acids 5–89 of c-Jun. The Jun–GST fusion proteins and the subsequent factor Xa cleavage fragments could be detected by Coomassie blue staining (Fig. 5*A*). Although the N-terminal fragments of v-Jun and the c-Jun deletion mutant did not bind this dye very well (see arrows), significant cleavage of the GST fusion protein was demonstrated by the appearance of free GST. Better visualization of the N-terminal fragments can be observed upon silver staining (data not shown). The apparent difference in mobility of the c-Jun and c-Jun(Δ63/73) fragment is a gel artifact and these two fragments normally exhibit identical electrophoretic mobilities. By using the kinase assay described above, the purified c-Jun

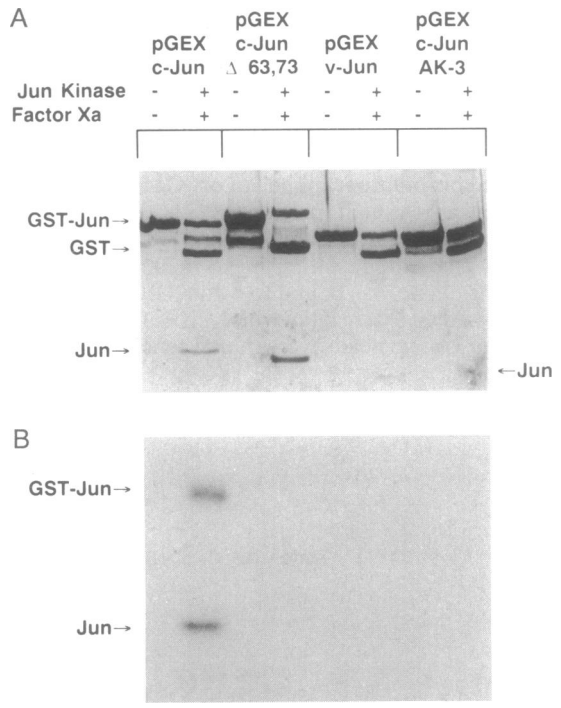

FIG. 5. Purified c-Jun protein kinase does not phosphorylate v-Jun or c-Jun lacking the δ domain. Purified c-Jun protein kinase was eluted from a c-Jun–GST–GT-Sepharose column with 80% ethylene glycol. This enzyme was used to phosphorylate GT-Sepharose beads coupled to GST fusions containing c-Jun, c-Jun with Ser-63 and Ser-73 mutated to leucines [c-Jun(Δ63/73)], v-Jun, or c-Jun lacking the δ domain (c-Jun AK-3). The Jun fragments were cloned into the pGEX-3X vector and thus products expressed were cleaved with factor Xa. (*A*) Coomasie blue stained cleaved material resolved by SDS/PAGE on a 15% gel. The arrows denote the various cleavage fragments. (*B*) Autoradiograph of the gel shown in *A* that had been dried and exposed to Kodak XAR-5 film overnight.

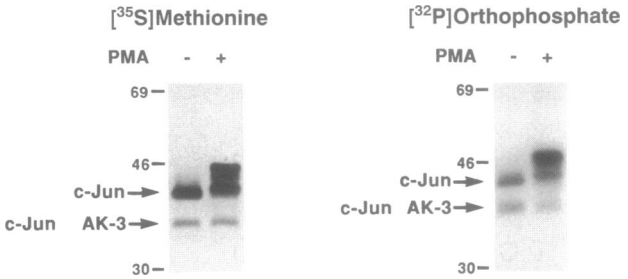

FIG. 6. c-Jun δ domain is required for PMA-inducible c-Jun phosphorylation *in vivo*. U937 cells stably expressing the c-Jun deletion mutant (c-Jun AK-3) were labeled with either Tran^{35}S-label or [^{32}P]orthophosphate. Cultures were split and one-half was treated with 0.1 μM PMA for 1 h. Jun protein was immunoprecipitated from denatured nuclear lysates.

protein kinase was able to phosphorylate the c-Jun fusion protein but not the c-Jun(Δ63/73) mutant or the v-Jun fusion (Fig. 5*B*; compare lanes 2, 4, and 6, respectively). Because the N termini of c-Jun and v-Jun differ by a 27-amino acid deletion (the δ domain), we investigated the role of this deletion in regulating phosphorylation. By using site-directed mutagenesis, this deletion was introduced into the N terminus of c-Jun (c-Jun AK-3). In contrast to the unmutated c-Jun fusion, the c-Jun deletion mutant lacking the δ domain was not phosphorylated by our purified c-Jun protein kinase *in vitro* (Fig. 5*B*, lane 8).

Deletion of the c-Jun δ Domain Abolishes PMA-Inducible N-Terminal c-Jun Phosphorylation *in Vivo*. To investigate whether the δ domain of c-Jun regulates phosphorylation *in vivo*, we established a U937 cell line containing exogenous c-Jun in which the δ domain was deleted (c-Jun AK-3). Immunoprecipitation of Jun protein from cells labeled with Tran^{35}S-label indicated that this cell line stably expressed the c-Jun deletion mutant, which migrated at 36 kDa (Fig. 6 *Left*). Although PMA induced the characteristic retarded mobility of endogenous c-Jun protein, the mobility of the c-Jun deletion mutant was not affected by PMA treatment. To determine whether the deletion mutant was phosphorylated in the absence of an effect on electrophoretic mobility, Jun protein was immunoprecipitated from cells labeled with [^{32}P]orthophosphate. As in Fig. 1, PMA induced the hyperphosphorylation of endogenous c-Jun (Fig. 6 *Right*). The c-Jun deletion mutant was constitutively phosphorylated in untreated cells; PMA did not enhance this phosphorylation, but rather a slight reduction in phosphorylation was observed. PMA has been shown to induce the site-specific dephosphorylation of residues proximal to the C-terminal DNA-binding domain of c-Jun (20). Thus, this PMA-induced decrease in phosphorylation of the c-Jun deletion mutant may reflect the dephosphorylation of these C-terminal sites. These data, along with those obtained *in vitro*, demonstrate that the 27-amino acid δ domain of c-Jun, which is deleted in v-Jun, plays a critical role in regulating the N-terminal phosphorylation of these Jun proteins.

DISCUSSION

The cloning of various N-terminal Jun proteins as Jun–GST fusion proteins coupled to GT-Sepharose beads has allowed (*i*) the development of an *in vitro* assay for c-Jun protein kinase activity, (*ii*) the identification of potential c-Jun protein kinase substrates, (*iii*) the rapid examination of regions critical for c-Jun phosphorylation, and (*iv*) the affinity purification of a c-Jun protein kinase. Utilizing this assay, we have demonstrated that PMA rapidly and transiently activates the c-Jun protein kinase, which parallels PMA-induced c-Jun phosphorylation *in vivo*. A c-Jun protein kinase was

highly purified by affinity chromatography and was shown to phosphorylate both an N-terminal c-Jun fusion protein and bacterially expressed full-length c-Jun (data not shown). The major visible band at this stage of purification migrates at 67 kDa, although other bands are also seen, including a 46-kDa band that was eluted from the beads but was not prominent in the concentrated material. Recent studies have shown that pp42 and pp54 mitogen-activated protein kinases phosphorylate the N terminus of c-Jun (13). However, we did not detect pp42 by immunoblot analysis of our final purification step with anti-pp42 (ERK-2) antibodies (data not shown). By using partially purified pp42 and pp54 mitogen-activated protein kinases, we have been able to demonstrate that these kinases and our affinity-purified c-Jun kinase exhibit similar substrate specificities. Like our purified material, pp42 and pp54 are capable of phosphorylating c-Jun but not v-Jun or the c-Jun deletion mutant lacking the δ domain (data not shown). A 46-kDa protein kinase [epidermal growth factor receptor threonine (ERT) kinase] has been isolated that phosphorylates Ser-246 within the C terminus of c-Jun (21). Although the ERT kinase consensus sequence Pro-Leu-Ser/Thr-Pro is similar to that found flanking Ser-63 and Ser-73 of c-Jun, no N-terminal c-Jun phosphorylation was reported (21). Further purification and analysis of the N-terminal c-Jun protein kinase will be necessary to determine whether this is an uncharacterized protein kinase or a kinase that has been implicated in the phorbol ester-mediated signal transduction pathway.

In vitro and *in vivo* studies indicate that the δ domain of c-Jun (amino acids 34–60), which is deleted in v-Jun, plays an important role in negatively regulating c-Jun transcriptional activity (10, 11, 22). Deletion of the δ domain markedly enhances the ability of c-Jun to stimulate transcription (10, 11, 22). v-Src and EJ-Ras have been shown to stimulate c-Jun transcriptional activity by relieving the repressor function associated with the c-Jun δ domain (23). Recent reports indicate that both Ha-Ras and PMA stimulate c-Jun transcriptional activity by the phosphorylation of c-Jun at Ser-63 and Ser-73 located proximal to the δ domain (12–14). Our data suggest that the δ domain is required for this N-terminal c-Jun phosphorylation. It is possible that the δ domain provides a binding site for the c-Jun kinase. This is suggested by our ability to affinity purify the c-Jun kinase on Sepharose beads coupled to the N terminus of c-Jun, which suggests a direct interaction between the kinase and the N-terminal 85 amino acids of c-Jun. Activation of the c-Jun protein kinase may displace a transcriptional inhibitory factor bound to the δ domain by phosphorylating Ser-63 and Ser-73. Alternatively, the amino acids within the δ domain might themselves act to repress transcription from the A1 or A2 activation domains (10, 22) or the recently identified A3 activation domain located within the N terminus of c-Jun (12). In this case, the phosphorylation of Ser-63 and Ser-73 may change the tertiary structure of c-Jun to allow the activation of transcription.

The observation that c-Jun is rapidly dephosphorylated after PMA treatment (Fig. 3*C*) suggests that the activation of transcription by N-terminal phosphorylation may be a transient event. Whereas phosphorylation enhances the transcriptional activity of c-Jun (12, 13), v-Jun is not phosphorylated. Two mutational differences between c-Jun and v-Jun appear to correlate directly with their ability to be phosphorylated. Deletion of the δ domain abolishes N-terminal phosphorylation and a single point mutation proximal to the DNA-binding domain abolishes C-terminal phosphorylation of c-Jun (20). Both of these mutations have been found to enhance the oncogenic potential of c-Jun (9). Thus, phosphorylation may play an important regulatory role in controlling the normal cellular function of the c-Jun protein.

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