Inhibition of c-Jun DNA Binding by Mitogen-Activated Protein Kinase

Szu-yi Chou,*† V. Baichwal,‡ and James E. Ferrell, Jr.*

*Department of Zoology, University of Wisconsin—Madison, Madison, WI 53706; and

†Howard Hughes Medical Institute, Division of Biochemistry and Molecular Biology,
University of California, Berkeley, California 94720.

Submitted April 9, 1992; Accepted August 3, 1992

Here we demonstrate that partially purified *Xenopus* p42 mitogen-activated protein (MAP) kinase phosphorylates bacterially expressed human c-Jun at a single site, serine 243. Several lines of evidence argue that this phosphorylation is due to p42 MAP kinase itself rather than some contaminating species. Phosphorylation of serine 243 markedly decreases the binding of c-Jun to oligonucleotides containing the 12-O-tetradecanoylphorbol-13-acetate response element. These findings suggest that MAP kinase may play a role in the down-regulation of c-Jun or in the cycle of transcriptional initiation and elongation.

INTRODUCTION

A group of related protein kinases variously called MAP¹ kinases (denoting either mitogen-activated protein kinases or microtubule-associated protein-2 kinases) or ERKs (for extracellular signal-regulated kinases or early response kinases) has now been implicated in a wide variety of cellular responses. Members of this family become tyrosine-phosphorylated and activated in cells stimulated with diverse mitogens, including activators of cell surface protein tyrosine kinases and activators of protein kinase C (Cooper *et al.*, 1982, 1984; Bishop *et al.*, 1983; Gilmore and Martin, 1983; Kohno, 1985; Ray and Sturgill, 1987; Hoshi *et al.*, 1988; Rossomando *et al.*, 1989; Ahn *et al.*, 1990; Ferrell and Martin, 1990; Gotoh *et al.*, 1990b; Haystead *et al.*, 1990; Kyriakis and Avruch, 1990; Nel *et al.*, 1990; Tsao *et al.*, 1990; Anderson *et al.*, 1991; L'Allemain *et al.*, 1991; Tobe *et al.*, 1991; Funasaka *et al.*, 1992). MAP kinases are activated in other contexts as well. For example, in neuroendocrine cells, MAP kinases are activated in response to

nerve growth factor and secretagogues (Ely *et al.*, 1990; Gomez *et al.*, 1990; Gotoh *et al.*, 1990a; Tsao *et al.*, 1990; Miyasaka *et al.*, 1991; Schanan-King *et al.*, 1991). MAP kinases are also activated downstream from M-phase promoting factor during oocyte maturation (Cicirelli *et al.*, 1988; Cooper, 1989; Sanghera *et al.*, 1990; Ferrell *et al.*, 1991; Gotoh *et al.*, 1991a,b; Posada *et al.*, 1991; Posada and Cooper, 1992). MAP kinases may therefore be involved in mitogenesis, in activation and differentiation responses in neuronal cells, and in the G2-M transition in oocytes. It is not yet clear what specific roles MAP kinases play in these processes.

The two most thoroughly characterized MAP kinases are the 42-kDa ERK2 and 44-kDa ERK1 proteins. These MAP kinases appear to be very similar in a number of respects. Their predicted amino acid sequences are ~90% identical (Boulton *et al.*, 1990; Boulton *et al.*, 1991b). Both kinases are expressed in a wide variety of tissues and cell lines (Boulton and Cobb, 1991), and both are activated in response to diverse stimuli. The two kinases exhibit similar substrate preferences in vitro (Boulton *et al.*, 1991a; Gonzalez *et al.*, 1991; Gotoh *et al.*, 1991a; Rossomando *et al.*, 1991; Barrett *et al.*, 1992). And finally, both kinases require phosphorylation of tyrosine and threonine residues to be fully active (Anderson *et al.*, 1990; Gomez *et al.*, 1990; Haystead *et al.*, 1990; Ahn *et al.*, 1991; Boulton and Cobb, 1991; Posada and Cooper, 1992). The activating phosphorylations have been mapped to threonine 183 and tyrosine 185 in the mouse p42 MAP kinase (Her *et al.*, 1991; Payne *et al.*, 1991) and to the corresponding sites in *Xenopus*

† Present address: Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

¹ Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase or early response kinase; HPLC, high-pressure liquid chromatography; MAP, microtubule-associated protein 2 or mitogen-activated protein; MBP, myelin basic protein; MES, 2-(N-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRE, TPA response element; Tris, tris(hydroxymethyl)aminomethane.

p42 (Posada and Cooper, 1992). A 13 amino acid peptide containing these sites (TEYVATRWYRAPE) is perfectly conserved in the predicted protein sequences of ERK1, ERK2, and two more distant relatives, the *Saccharomyces cerevisiae* KSS1 and FUS3 gene products (Courchesne *et al.*, 1989; Elion *et al.*, 1989; Boulton *et al.*, 1990, 1991b; Gotoh *et al.*, 1991b; Her *et al.*, 1991; Posada *et al.*, 1991), raising the possibility that all of these kinases share a common mechanism for their activation.

A number of other candidates for inclusion in the MAP kinase family have been identified. These include p44^{mpk}, a sea star MAP kinase that lacks the canonical threonine phosphorylation site (Sanghera *et al.*, 1990; Posada *et al.*, 1991); p54 MAP kinase, an activity detected in the livers of cycloheximide-treated rats (Kyriakis and Avruch, 1990); the as yet uncharacterized product of the ERK3 gene (Boulton *et al.*, 1991b); and a 45-kDa putative ERK4 protein identified by antibody cross-reactivity (Boulton *et al.*, 1991b).

Many of the myriad stimuli known to activate the p42 and p44 MAP kinases are also known to cause rapid increases in the transcription of genes under the control of the 12-O-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) (reviewed by Karin, 1990). Although MAP kinases were originally identified as cytoplasmic enzymes, evidence recently has been presented that a substantial fraction of the p42 and p44 MAP kinases in HeLa cells resides in the nucleus (Chen *et al.*, 1992). We have found that ~10% of the p42 MAP kinase in immature stage VI *Xenopus* oocytes resides in the germinal vesicle (Shih and Ferrell, unpublished results). These observations make MAP kinases attractive candidates for the regulation of TRE-controlled transcription.

The transcription factor that activates TRE-controlled gene expression, AP-1, is a heterogeneous group of proteins; various members of the Jun family (c-Jun, JunB, JunD) can form homodimers and heterodimers that score as AP-1, as can heterodimers of Jun and Fos (c-Fos, FosB, Fra1) family proteins. Diverse stimuli can effect increases in AP-1 activity by inducing the transcription of fos and jun family genes. Recent work suggests that AP-1 also can be activated without new protein synthesis through changes in the phosphorylation state of c-Jun (Binetruy *et al.*, 1991; Boyle *et al.*, 1991a). Accordingly, two groups have examined the possibility that MAP kinases might phosphorylate c-Jun (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991). Both groups found c-Jun to be an excellent substrate for their purified MAP kinases in vitro, and both found that their MAP kinases phosphorylated sites known to be phosphorylated in vivo. However, the sites identified by the two groups are not the same. Pulverer *et al.* (1991) detected phosphorylation of serines 63 and 73; Alvarez *et al.* (1991) detected phosphorylation of serine 243. Neither group noted any phosphorylation of the sites identified by the

other group. This discrepancy has raised concerns about the identities and purities of the MAP kinases involved and about possible ambiguities in the phosphorylation site identifications.

We also have examined phosphorylation of the c-Jun protein by MAP kinase. In this article, we show that purified p42 MAP kinase efficiently phosphorylates bacterially expressed c-Jun and present evidence that this phosphorylation is due to p42 MAP kinase itself rather than to some contaminating species. We demonstrate that p42 MAP kinase phosphorylates c-Jun predominantly at serine 243 and that this phosphorylation markedly inhibits the binding of c-Jun to synthetic TREs. Finally, we show that a series of phosphotryptic peptide spots that have been thought to arise from phosphorylation of c-Jun in vivo at three closely clustered sites (threonine 239, serine 243, and serine 249) actually result from the phosphorylation of only two sites (probably serine 243 and either threonine 231 or 239).

MATERIALS AND METHODS

Preparation of *Xenopus laevis* Egg Extracts

Female frogs were induced to lay eggs by dorsal lymph sac injection of human chorionic gonadotropin (800 U; Sigma Chemical, St. Louis, MO). Eggs were dejellied in freshly prepared 2.5% cysteine-HCl (pH 8.0; unless otherwise indicated, buffer pHs were determined at room temperature), washed in 100 mM NaCl, and lysed in an equal volume of ice-cold *Xenopus* egg lysis buffer (40 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane [Tris], pH 7.0, 10 mM EDTA, pH 8.0, 5 mM NaF, 2 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM sodium orthovanadate, pH 10.0, 2% [vol/vol] aprotinin solution, 50 µg leupeptin per ml) by two strokes of a loose Dounce homogenizer. Lysates were centrifuged for 90 min at 27 000 rpm (140 000 × g) in an SW27 rotor (Beckman, Fullerton, CA). The cytosol was taken from the overlying lipid and underlying yolk and pigment for p42 MAP kinase purification.

Purification of p42 MAP Kinase

Our basic strategy was to purify the phosphotyrosyl protein p42 using antiphosphotyrosine immunoblotting as an assay. In the later stages of the purification, we also assessed myelin basic protein kinase activity.

A cytosolic extract was prepared from 100 ml of packed eggs from 10 frogs, yielding 1.9 g of protein. Precipitation with 30–45% ammonium sulfate resulted in a purification of p42 of 3.7-fold and a yield of 85%. The ammonium sulfate precipitate was dissolved in enough S-Sepharose loading buffer (25 mM 2-(N-morpholino)ethanesulfonic acid [MES], pH 6.5, 1 mM EDTA, 1 mM sodium orthovanadate) to yield a conductance of <5 mmho. Protein was loaded onto a 50-ml S-Sepharose column (Pharmacia, Piscataway, NJ), washed with S-Sepharose loading buffer, and eluted with a 0- to 250-mM NaCl gradient. p42 eluted at a NaCl concentration of ~60 mM. This step provided a 36-fold purification and 40% yield of p42, for a cumulative purification of 130-fold and a cumulative yield of 35%. Peak fractions were pooled and diluted with 25 mM MES (pH 6.5) to a conductance of <5 mmho, and MgCl₂ was added to yield a final concentration of 5 mM. The sample was loaded onto a 10-ml Orange-A agarose column (Amicon, Danvers, MA), washed with Orange-A agarose loading buffer (25 mM MES, pH 6.5, 5 mM MgCl₂), and eluted with a gradient of Orange-A agarose elution buffer (500 mM NaCl, 25 mM MES, pH 6.5, 1 mM EDTA). p42 MAP kinase eluted

as a broad peak centered at 180 mM NaCl. The most highly purified fraction, designated OA27, was purified ~340-fold, with a purity of p42 MAP kinase of ~10% as assessed by Coomassie staining of one- and two-dimensional polyacrylamide gels. This fraction was used for many of the kinase reactions described below. A second Orange-A agarose fraction was adjusted to pH 7.4 (at 4°C) with Tris base, loaded onto a 2 ml phenyl Sepharose (Pharmacia) column, and eluted with a decreasing NaCl gradient (150–0 mM) and increasing ethylene glycol gradient (0–60%). Peak fractions contained three silver-stainable proteins, at 67, 42, and 20 kDa, in roughly equal proportions. The intensity of the 42-kDa, but not the intensities of the 67- or 20-kDa proteins, correlated with myelin basic protein kinase activity across the column profile. Phenyl Sepharose-purified p42 MAP kinase was used for a few of the kinase reactions described below. A third partially purified Orange-A agarose fraction was dissolved in 5 M guanidine hydrochloride and S-pyridylethylated (Charbonneau, 1989) and subjected to reverse-phase C₄ high-pressure liquid chromatography (HPLC). p42 MAP kinase eluted as a single peak at an acetonitrile concentration of 43%. Its purity in three preparations varied from ~60 to 90% as estimated by silver staining. Two minor impurities migrated at 67 and 20 kDa. HPLC-purified p42 was lyophilized and digested with 50 ng trypsin in 400 mM ammonium bicarbonate buffer overnight at 37°C. Peptides were separated by reverse-phase HPLC. Sequences were obtained for two tryptic peptides.

Expression and Purification of Human c-Jun

Human c-Jun was provided by A. Admon and R. Tjian (Howard Hughes Medical Institute, University of California, Berkeley). This c-Jun was expressed in bacteria using methionine 5 as the translational start site and was renatured and purified as described (Bohmann and Tjian, 1989). (Several conventions have been used for the amino acid numbering of c-Jun. Here, we have taken the first in frame methionine of human c-Jun to be residue 1. This residue is position 10 in the numbering convention of Bohmann *et al.* [1987]. The phosphorylation site we refer to as serine 243 corresponds to serine 246 in the mouse c-Jun sequence [Alvarez *et al.*, 1991]. The sites we refer to as serines 63 and 73 are designated serines 62 and 72 by Pulverer *et al.* [1991].)

Cell Cultures and Radiolabeling

MG63 human osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For radiolabeling, cells were grown to 80% confluence in 100-mm dishes, washed twice with Tris-buffered saline, and overlaid with 3 ml of labeling medium (phosphate-free DMEM containing 3 mCi/ml carrier-free [³²P]orthophosphate) for 3 h. During the final 15 min of labeling, TPA was added in dimethyl sulfoxide to a final concentration of 100 ng/ml. Radiolabeled cells were washed four times with ice-cold Tris-buffered saline and were lysed in 200 µl of boiling hot lysis buffer (0.5% sodium dodecyl sulfate [SDS], 50 mM Tris, pH 7.4, 30 mM 2-mercaptoethanol, 5 mM dithiothreitol [DTT]).

Antibodies and Immunoprecipitation

For immunoprecipitation of c-Jun, lysates were diluted in 800 µl of immunoprecipitation buffer (150 mM NaCl, 50 mM β-glycerophosphate, 50 mM Tris, pH 7.4, 2 mM EDTA, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 1% aprotinin solution, 100 µg leupeptin per ml, 200 µM PMSF, 1 mM sodium orthovanadate). DNA was sheared through a 20-gauge needle to reduce viscosity. Lysates were clarified by adding 100 µl of a 10% suspension of protein A agarose beads and centrifuging at 12 000 × g for 30 min at 4°C. Supernatants were mixed with anti-Jun antiserum (1:200) and 100 µl of a 10% suspension of protein A agarose beads and incubated for 12 h at 4°C. Immune complexes were collected by centrifugation, washed four times with immunoprecipitation buffer, and dissolved in Laemmli sample buffer. Proteins were resolved by electrophoresis on 10%

polyacrylamide gels. Labeled proteins were detected by autoradiography of unfixed wet gels using an intensifying screen.

Anti-*Xenopus* p42 MAP kinase anti-peptide antiserum 1913.2 was provided by J. Posada and J. Cooper (Fred Hutchinson Cancer Research Center; Posada and Cooper, 1992) and used for immune complex kinase assays. Purified MAP kinase (20 µl of fraction OA27, containing ~60 ng p42 MAP kinase) was mixed with 2 µl antiserum and 80 µl of 10% protein A agarose in 400 µl buffer A (150 mM NaCl, 50 mM Tris, pH 7.4, 50 mM β-glycerophosphate, 1% Triton X-100, 0.5% NP-40, 1% aprotinin, 100 µg leupeptin/ml, 1 mM sodium orthovanadate, and 200 µM PMSF) for 1 h at 4°C. Pellets were washed three times with buffer A and twice with kinase assay buffer (50 mM Tris, pH 7.4, and 10 mM MgCl₂). Pellets were used for c-Jun kinase and myelin basic protein kinase assays as described below.

Three other anti-peptide antisera were used in immunoblotting experiments. The first was antiserum 837, provided by T. Boulton and M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX), which was raised against a 16 amino acid peptide from the C-terminus of ERK1 (IFQETARFQPGAPEAP). This antiserum recognizes ERK1 and ERK2 proteins, including the ERK2-like *Xenopus* p42 MAP kinase (Boulton and Cobb, 1991). A second antiserum, DC3, was raised by the Multiple Peptide Systems custom antibody service against a 12 amino acid peptide from the *Xenopus* p42 MAP kinase sequence (IFETARFQPGY) conjugated to keyhole limpet hemocyanin through an N-terminal cysteine residue.

Kinase Assays

Kinase reactions were carried out for 20 min at room temperature in 50 mM β-glycerophosphate (pH 7.0), 10 mM MgCl₂, 1 mM sodium orthovanadate containing 1 µCi [γ-³²P]ATP (made with the Promega [Madison, WI] GammaPrep kit, specific activity nominally 3000–6000 Ci/mmol), 1–5 µl purified p42 MAP kinase (3–15 ng p42 MAP kinase protein), and 1 µg of myelin basic protein (MBP, Sigma) or 1 µg of purified bacterially expressed human c-Jun, in a reaction volume of 10 µl. In some reactions, we used MAP kinase immunoprecipitated from various cells, or bacterially expressed human ERK1 (a gift from Tom Geppert, University of Texas Southwestern Medical Center), in place of purified *Xenopus* p42 MAP kinase. Reactions were terminated by the addition of excess EDTA followed by Laemmli sample buffer. Reaction products were separated by electrophoresis on 15% polyacrylamide SDS gels and were detected by autoradiography and quantified by Cerenkov counting. The protein kinase inhibitors staurosporine and K-252a were purchased from Kamiya Biomedical Company (Thousand Oaks, CA) and added to kinase reactions as concentrates in dimethyl sulfoxide.

Tryptic Peptide Mapping

³²P-labeled c-Jun was electrophoretically eluted from slices of unfixed gels. The eluted protein was precipitated with 15% trichloroacetic acid using bovine serum albumin as a carrier. Precipitates were washed once with ice-cold ethanol, oxidized with 50 µl of freshly prepared performic acid, diluted with 500 µl water, and lyophilized. The lyophilized sample was digested with 1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Boehringer Mannheim) as described (Boyle *et al.*, 1991a,b). Tryptic digests were loaded onto thin-layer cellulose plates (E. M. Sciences, Gibbstown, NJ) alongside two marker dyes: phosphotyramine, synthesized as described (White and Backer, 1991), which acts as a neutral marker at pH 1.9, and Nε-2,4-dinitrophenyllysine (Sigma), which bears a fractional positive charge at this pH. Samples were subjected to electrophoresis at pH 1.9 for 25 min at 1000 V, anode to the left, as described (Boyle *et al.*, 1991a,b). The plates were allowed to air dry and then were subjected to chromatography in phosphochromatography buffer (n-butanol:pyridine:acetic acid:water, 75:50:15:60). Radiolabeled peptide spots were detected by autoradiography.

V8 Mapping of Tryptic Phosphopeptides

Phosphotryptic peptide spots were scraped, eluted into water by vortexing, lyophilized, and digested with 10 µg *Staphylococcus* V8 protease

(Boehringer Mannheim) in 50 mM ammonium bicarbonate for 12 h at 37°C. Samples were diluted in water, lyophilized twice, and subjected to two-dimensional peptide mapping (pH 1.9 followed by phosphochromatography buffer) as described above.

Phosphoamino Acid Analysis

Samples were hydrolyzed with 6 M HCl at 110°C in screw cap microcentrifuge tubes for 1 h. Partial acid hydrolysates were lyophilized, mixed with authentic phosphoamino acids, and subjected to two-dimensional thin-layer electrophoresis at pH 1.9 followed by pH 3.5 (Boyle *et al.*, 1991b). Phosphoamino acid standards were detected with ninhydrin. Radiolabeled phosphoamino acids were detected by autoradiography.

Synthetic Peptides

PPLSPID (~98% pure) and PPLAPID (~95% pure) were purchased from Peptide Technologies Corp. (Washington, DC). TPPLSPIDME was synthesized by Operon Technologies (Alameda, CA) and was phosphorylated nonenzymatically essentially as described (Schieven and Martin, 1988). The peptide (10 µg) was mixed with 10 µCi [γ -³²P]ATP (nominally 3000–6000 Ci/mmol), 10 mM MnCl₂, and 10 mM Tris (pH 7.4) in a reaction volume of 13 µl and was heated to 90°C for 2 h. A portion of the reaction products (1 µl) was subjected to thin-layer electrophoresis at pH 1.9 for 25 min at 1000 V. Radiolabeled spots were detected by autoradiography. Most of the radiolabel was found in a putative orthophosphate spot, which migrated farther toward the anode than the starting material. A small amount of radiolabel migrated toward the cathode, co-migrating with the neutral marker phosphotyramine, as would be expected for phosphorylated TPPLSPIDME. This spot was eluted and subjected to phosphoamino acid analysis as described above. The hydrolysate was found to contain ³²P-phosphoserine but no detectable ³²P-phosphothreonine. The phosphorylated TPPLSPIDME product was also subjected to thin-layer electrophoresis at pH 1.9 and pH 3.5 and to thin-layer chromatography in phosphochromatography buffer. In each case, the radiolabel migrated as a single major spot.

Phosphatase Treatment

Phosphotryptic peptides were dephosphorylated by treatment with calf intestinal alkaline phosphatase (Promega, used at a 1:1000 dilution) in phosphatase buffer (50 mM Tris, pH 9.0, 1 mM MgCl₂, 1 mM ZnCl₂, 1 mM spermidine) for 10 min at room temperature.

Gel Shift Assays

A synthetic oligonucleotide based on the TPA-response element of the collagenase promoter (5'-CGCTTGATGACTCAGCCGAA-3' plus its complement) was purchased from Promega. The oligonucleotide was labeled with [γ -³²P]ATP and T4 polynucleotide kinase. c-Jun was preincubated with p42 MAP kinase (6 ng) and/or nonradioactive MgATP (1 mM) for 2 h at room temperature. The labeled oligonucleotide (0.1 ng) was mixed with 100 ng phosphorylated or nonphosphorylated c-Jun in 12 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9), 4 mM Tris (pH 7.9), 60 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 100 mg/ml bovine serum albumin, 100 µg/ml poly(dI-dC), 12.5% glycerol, essentially as described (Boyle *et al.*, 1991a). In some experiments, unlabeled excess TRE oligonucleotide or AP-2 oligonucleotide (Promega) were added as specificity controls. Oligonucleotide-Jun complexes were allowed to form for 30 min on ice and were analyzed on a non-denaturing 5% polyacrylamide gel containing 0.25× Tris-borate EDTA buffer.

Glutaraldehyde Cross-Linking

³²P-labeled c-Jun samples were treated with 0.001% glutaraldehyde at room temperature for 1 h to cross-link Jun-Jun dimers, as described

(Turner and Tjian, 1989). Samples were subjected to electrophoresis on 10% polyacrylamide SDS gels. The bulk c-Jun protein was detected by silver staining, and phosphorylated c-Jun was detected by autoradiography.

RESULTS

A 42-kDa phosphotyrosyl protein was purified from *Xenopus laevis* eggs by a combination of ion exchange chromatography, dye affinity chromatography, and hydrophobic interaction chromatography. The 42-kDa phosphotyrosyl protein copurified with a protein kinase activity that phosphorylated microtubule-associated protein-2 and MBP over the Orange-A agarose and phenyl Sepharose column steps and was the only detectable protein band to do so.

Sequences were obtained for two internal tryptic peptides derived from HPLC-purified p42 (FQPGY-end and IEVXAAALAXPYLXQY). Both sequences are found in the predicted protein sequences of two *Xenopus* ERK2-like cDNA clones (Gotoh *et al.*, 1991b; Posada *et al.*, 1991). Purified *Xenopus* p42 was recognized by antisera 837 and DC3, both of which recognize p42 ERK2 proteins. These findings establish our purified p42 as an ERK2-like member of the MAP kinase family.

Purified p42 MAP kinase avidly phosphorylated MBP and microtubule-associated protein-2 and was much less active (1–5% as active) toward casein, phosphorylase, and histone. p42 MAP kinase was relatively insensitive to staurosporine (no detectable inhibition at 500 nM) but sensitive to the structurally similar inhibitor K-252a (90% inhibition at 200 nM). The substrate specificity, sensitivity to inhibitors, and kinetic properties of our purified *Xenopus* p42 MAP kinase are consistent with those reported by Gotoh *et al.* (1991a) and Barrett *et al.* (1992) for *Xenopus* p42 MAP kinases, with those reported by Rossomando *et al.* (1991) and Gotoh *et al.* (1991a) for mouse and rat p42 MAP kinases, and with those reported by Boulton *et al.* (1991a) for rat ERK1 MAP kinase.

Phosphorylation of c-Jun by p42 MAP Kinase

We assessed bacterially expressed human c-Jun as a potential substrate for p42 MAP kinase in vitro. As shown in Figure 1, c-Jun was efficiently phosphorylated by partially purified MAP kinase (OA27). Several observations argue that the observed Jun kinase activity is due to p42 MAP kinase rather than some contaminating kinase: both the Jun kinase and MBP kinase activities were inhibited by K-252a (~90% inhibition of both activities at 200 nM; 100% inhibition at 200 µM; Figure 1) but not by staurosporine (500 nM); both the Jun kinase and MBP kinase activities were inhibited by synthetic peptides derived from the sequence around the Jun phosphorylation site (14 mM PPLAPID, Figure 1; vide infra); and both the Jun kinase and MBP kinase activities were immunoprecipitated by antiserum

Figure 1. Phosphorylation of myelin basic protein (MBP) and recombinant c-Jun by partially purified MAP kinase. Kinase reactions were carried out with no kinase protein (lane 1); 6 ng p42 MAP kinase (lane 2); MAP kinase plus staurosporine (500 nM, lane 3); MAP kinase plus K-252a (200 μ M, lane 4); MAP kinase plus PPLAPID (14 mM, lane 5); mock-immunoprecipitated MAP kinase, starting with 60 ng p42 MAP kinase and 4 μ l rabbit IgG (lane 6); MAP kinase immunoprecipitated with 4 μ l antiserum 1913.2 (lane 7). Autoradiogram was exposed for 5 h at -70°C with an intensifying screen.

1913.2, but not by control antiserum (Figure 1). In addition, the Jun kinase and MBP kinase activities copurified when Orange-A agarose-purified p42 MAP kinase was fractionated by anion exchange chromatography on a Mono Q column.

We then set out to determine what site or sites in c-Jun were phosphorylated by p42 MAP kinase and to compare them with those phosphorylated in vivo. In vivo ^{32}P -labeled human c-Jun was obtained by immunoprecipitation from ^{32}P -labeled TPA-treated MG63 cells; in vitro ^{32}P -labeled c-Jun was obtained by reaction of bacterially expressed c-Jun with Orange-A agarose-purified MAP kinase (Figure 2A). Tryptic digestion of in vitro phosphorylated c-Jun typically yielded two major radiolabeled spots, designated spots b and c in Figure 2B. The relative proportions of spots b and c varied from digest to digest, with spot b always predominating. Both spot b and spot c moved toward the cathode with respect to a neutral marker, phosphotyramine. Thus both spots b and c represent peptides that are positively charged at pH 1.9. Spot c migrated about twice as far from the neutral marker as did spot b, implying that the charge to mass ratio (or more precisely, the charge to mass^{2/3} ratio) (Boyle *et al.*, 1991b) of the spot c peptide is about twice that of the spot b peptide.

Phosphotryptic peptide maps were also obtained for c-Jun phosphorylated by immunoprecipitated p42 MAP kinase, Mono Q-purified p42 MAP kinase, and phenyl Sepharose-purified p42 MAP kinase; by p42 and p44 MAP kinases immunoprecipitated from TPA-stimulated NIH 3T3 cells; by bacterially expressed human p44/ERK1 MAP kinase; and for c-Jun phosphorylated by Orange-A agarose-purified MAP kinase using saturating ATP concentrations. In each case, the in vitro phosphorylated c-Jun yielded one or two major spots, spot b or spots b and c. The stoichiometry of c-Jun phos-

phorylation ranged from <0.01 under our standard phosphorylation conditions to 0.8–1.1 when saturating ATP concentrations were used.

Tryptic digestion of in vivo ^{32}P -labeled human c-Jun typically yielded five phosphopeptide spots, labeled a, b, c, x, and y (Figure 2C). Essentially identical patterns have been reported by others (Boyle *et al.*, 1991a; Binetruy *et al.*, 1991; Pulverer *et al.*, 1991). Spot a co-migrated with the neutral marker in the electrophoresis dimension. The other four spots moved farther toward the cathode than did the neutral marker and hence are positively charged at pH 1.9. Mixing experiments established that the spots b and c derived from in vitro labeled c-Jun co-migrated with the spots b and c derived from in vitro labeled c-Jun (Figure 2D). Trace amounts of spots x and y could occasionally be detected in tryptic digests of in vitro phosphorylated c-Jun.

Identification of Spots a, b, and c

Exhaustive digestion of human c-Jun with trypsin would yield 28 peptides and 5 individual amino acids. Boyle *et al.* (1991a) have mapped spots a, b, and c to a single tryptic peptide comprising residues 227 through 252 (EEPQTVPEMPGETPPLSPIDMESQER), which we will refer to as tryptic peptide 13 (TP13; Figure 2, E and F). This peptide spans the A2 domain, a proline- and glutamic acid-rich stretch of sequence just N-terminal to the basic DNA binding domain and the leucine zipper region (Figure 2F). From analysis of deletion mutants, it appears that the A2 region participates in transcriptional transactivation in vitro and in vivo (Bohmann and Tjian, 1989; Baichwal and Tjian, 1990).

There are a number of ways that phosphorylation within TP13 could yield multiple tryptic phosphopeptide spots. It has been hypothesized that spot a represents TP13 phosphorylated at three sites, spot b at two sites, and spot c at one site (Boyle *et al.*, 1991a). These assignments would explain the qualitative pattern of spots a, b, and c; however, two sets of observations now argue that they are not correct. First, the predicted charges of triply, doubly, and singly phosphorylated TP13 are not compatible with the directions in which spots a, b, and c migrate with respect to the neutral marker. Triply phosphorylated TP13 should bear a charge of -1 at pH 1.9: $+1$ contributed by its N-terminal amino group, another $+1$ by its arginine side group, and -1 by each of its three phosphates (Boyle *et al.*, 1991b). Doubly phosphorylated TP13 should be neutral at pH 1.9, and singly phosphorylated TP13 should bear a charge of $+1$. However, we have found that spot a migrates with the neutral marker, not toward the anode, and spot b migrates toward the cathode rather than with the neutral marker (Figure 2). A second argument can be made on the basis of the recent identification of spot x as monophosphorylated tryptic peptide 8 and spot y as a monophosphorylated tryptic peptide 7 (Fig-

ure 2, E and F) (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991). Monophosphorylated TP13 would be predicted to migrate 47% as far as monophosphorylated TP8 and 64% as far as monophosphorylated TP7. The observed migration of spot b, rather than that of spot c, is consistent with the predicted migration of monophosphorylated TP13 (Figure 2, B, D, and E). Spot c migrates twice as far as would be expected of monophosphorylated TP13, raising the possibility that it bears a charge of +2 rather than +1.

One straightforward way that TP13 could acquire an additional positive charge would be by addition of a flanking peptide through incomplete tryptic digestion. The resulting peptide would be TP12+13 or TP13+14 (LQALK-EEPQTVPEMPGETPPLSPIDMESQER or EEPQTVPEMPGETPPLSPIDMESQER-IK). The monophosphorylated forms of either peptide would bear the requisite charge of +2 and would be expected to move farther in the chromatography dimension than monophosphorylated TP13 (predicted R_f values are 0.405, 0.423, and 0.413 for monophosphorylated TP13, TP12+13, and TP13+14). Either or both of the partially digested peptides could contribute to spot c.

In an analogous manner, spot b could represent incompletely digested peptide with two phosphates and/or completely digested TP13 with one phosphate. Spot

Figure 2. Tryptic phosphopeptide maps of c-Jun phosphorylated in vitro and in vivo. (A) Immunoprecipitation of c-Jun. Lysates from ^{32}P -labeled cells were immunoprecipitated with control antiserum (lane 1) or anti-Jun antiserum (lane 2). The c-Jun band was identified by its comigration with in vitro labeled c-Jun (lanes 3 and 4). The autoradiogram was exposed for 12 h at -70°C with an intensifying screen. (B–D) Two-dimensional tryptic phosphopeptide maps. In each case, ^{32}P -labeled c-Jun protein was eluted from a polyacrylamide gel, digested with trypsin, and subjected to electrophoresis at pH 1.9, anode to the left, followed by chromatography in phosphochromatography buffer. Origins are denoted by o's. The neutral marker phosphotyramine coelectrophoresed with spot a; the positively charged marker ε-DNP lysine migrated ~25% farther than spot x. Samples were tryptic digests of: (B) c-Jun phosphorylated in vitro by p42 MAP kinase; (C) in vivo labeled c-Jun; or (D) a mix of in vitro and in vivo labeled c-Jun. Autoradiograms were exposed for 5 d at -70°C with intensifying screens. (E) Predicted peptide maps for the various phosphopeptide species. The black spots represent limit tryptic peptides. The light grey spots represent TP12+13 with one (right) or two (left) phosphates. The dark grey spots represent TP13+14 with one (right) or two (left) phosphates. (F) Schematic diagram of the c-Jun protein sequence showing functional domains as defined by analysis of deletion mutants. δ denotes a region from amino acids 31–57 that is deleted from v-Jun; A1 denotes the upstream transactivation domain, which stretches from amino acids 83–187; A2 is the proline and glutamic acid rich downstream transactivation domain, which stretches from amino acids 229–248; + denotes the lysine and arginine-rich DNA binding domain, which runs from amino acids 254–273; and L denotes the leucine zipper, which extends from amino acids 274–308. Five tryptic peptides are shown: TP7, which includes the serine 63 phosphorylation site and corresponds to spot y; TP8, which includes the serine 73 phosphorylation site and corresponds to spot x; TP13, which includes the serine 243 phosphorylation site and an unidentified threonine phosphorylation site; and TP12 and TP14, two peptides that flank TP13.

a could represent TP13 with two phosphates. In principle, spot a could also represent TP12+13 or TP13+14 with three phosphates. In that case, we would expect to detect some triply phosphorylated completely digested TP13, which would yield a fourth spot to the left and down from spot a. We have never detected such a spot nor has such a spot been reported by others (Binetruy *et al.*, 1991; Boyle *et al.*, 1991a; Pulverer *et al.*, 1991). Therefore, we consider it likely that c-Jun is singly and doubly phosphorylated within TP13 in vivo but not triply phosphorylated.

In Figure 2E we summarize the predicted migrations of the various peptide species. Migrations in the electrophoretic dimension are calculated from the peptides' charge to mass^{2/3} ratios. Chromatographic behavior is predicted as described by Boyle *et al.* (1991b). Actual chromatography R_f values are said to be less bunched together than the predicted values (Boyle *et al.*, 1991b). The observed tryptic phosphopeptide maps (Figures 2, B–D) are in good agreement with the predicted map (Figure 2E). Experiments with in vitro phosphorylated c-Jun described below corroborate the identities of spots b and c.

Identification of the p42 MAP Kinase Phosphorylation Site as Serine 243

The two spots observed in the peptide maps of in vitro phosphorylated c-Jun (Figure 2B) could represent a variety of different species. Spot b could represent doubly phosphorylated TP12+13 or TP13+14, singly phosphorylated TP13, or a mixture. To distinguish among these possibilities, spot b was eluted and partially dephosphorylated with alkaline phosphatase. If spot b included some doubly phosphorylated peptide, we would expect some of its radiolabel to be shifted to the right and up the chromatography plate after phosphatase treatment to the position of spot c. If spot b included only singly phosphorylated TP13, we would expect it to be converted to unlabeled peptide and labeled inorganic phosphate. As shown in Figure 3, A–C, spot b yielded inorganic phosphate but did not yield spot c. Phosphatase treatment also converted spot c to inorganic phosphate without yielding other peptide spots (Figure 3D). These results suggest that spot b represents singly phosphorylated TP13 and spot c represents singly phosphorylated TP12+13 or TP13+14.

To test this hypothesis, a mixture of spots b and c was subjected to a second tryptic digestion. The resulting products contained spot b but not spot c, implying that trypsin had converted spot c to spot b (Figure 3, D–F). Thus, both spots b and c represent singly phosphorylated c-Jun peptides, with spot b representing singly phosphorylated TP13 and spot c representing singly phosphorylated TP12+13 or TP13+14. None of the c-Jun molecules appear to have been phosphorylated twice.

There are four potential phosphorylation sites within TP13: threonine 231, threonine 239, serine 243, and serine 249. The singly phosphorylated c-Jun molecules could all be phosphorylated at the same site or could be phosphorylated at a mixture of sites. To distinguish between these possibilities, we characterized spots b and c further. On partial acid hydrolysis, spots b and c each yielded phosphoserine but no phosphothreonine (Figure 4A), which rules out threonine 231 and threonine 239 as possible p42 MAP kinase phosphorylation sites. To determine whether MAP kinase phosphorylated serine 243, serine 249, or a mixture of the two sites, spot b was exhaustively digested with *Staphylococcal* V8 protease. The V8 peptide containing serine 243 (TPPLSPIDME) would be more hydrophobic than the parent peptide TP13 (the predicted R_f value for monophosphorylated TPPLSPIDME, is 0.458, compared with 0.405 for monophosphorylated TP13), whereas the V8 peptide containing serine 249 (SQE or possibly SQER) would be substantially less hydrophobic than TP13 (predicted R_f values of 0.267 and 0.253 for monophosphorylated SQE and SQER). As shown in Figure 4B, V8 treatment of spot b yielded a neutral peptide that was more hydrophobic than the parent peptide. This observation is consistent with the identification of serine 243 as the sole p42 MAP kinase phosphorylation site.

To test this identification, we phosphorylated the synthetic peptide TPPLSPIDME nonenzymatically with manganese and [γ -³²P]ATP and compared its migration with that of the spot b V8 product. As shown in Figure 4, B–D, the synthetic standard co-migrated with the spot b V8 product. These findings establish serine 243 as the site in c-Jun phosphorylated by p42 MAP kinase in vitro.

As further corroboration of this identification, we tested whether p42 MAP kinase could phosphorylate synthetic peptides derived from this sequence. As shown in Figure 5, p42 MAP kinase phosphorylated the seven amino acid peptide PPLSPID, yielding authentic phosphoserine on partial acid hydrolysis. Both PPLSPID and PPLAPID acted as low affinity inhibitors of p42 MAP kinase, with roughly millimolar K_i values. These findings are consistent with the proposed phosphorylation site identification. Similar affinities have been reported for peptide substrates derived from the sequences around MAP kinase phosphorylation sites in MBP and the epidermal growth factor (EGF) receptor (Gonzalez *et al.*, 1991; Mukhopadhyay *et al.*, 1992).

Effect of Phosphorylation on DNA Binding

We examined the effect of serine 243 phosphorylation on the binding of c-Jun to a TRE-containing oligonucleotide by a gel shift assay. c-Jun was incubated with a ³²P-labeled TRE in the presence of excess poly(dI-dC),

Figure 3. Digestion of spots b and c with alkaline phosphatase and trypsin. c-Jun was phosphorylated by p42 MAP kinase in vitro and subjected to tryptic digestion and two-dimensional peptide mapping. Spots b and c were eluted from the plate and subjected to further treatments. In each panel, o denotes the origin; the anode is to the left; the neutral marker phosphotyramine migrated about halfway between the origin and spot b; and ϵ -DNP lysine migrated $\sim 25\%$ farther than spot c. (A–C) Treatment of spot b with alkaline phosphatase. (A) Peptide map of spot b partially dephosphorylated by phosphatase treatment. P_i denotes inorganic phosphate. (B) Untreated spot c. (C) A mixture of phosphatase-treated spot b and untreated spot c. (D) Treatment of spot c with alkaline phosphatase. Note that the samples for C and D were loaded at different origins on one cellulose plate. The left-hand inorganic phosphate spot corresponds to the left-hand (C) origin, and the right-hand inorganic phosphate spot corresponds to the right hand (D) origin. (E–G) Treatment of spots b and c with trypsin. A mixture of spots b and c was subjected to a second trypsin digest (F) or a mock digest (E). (G) A mixture of the once- and twice-digested spots b and c. Each autoradiogram was exposed for 2 d at -70°C with an intensifying screen.

and the resulting complexes were resolved on a non-denaturing polyacrylamide gel. As shown in Figure 6A, incubation of the ^{32}P -labeled TRE with c-Jun resulted in the formation of a new species, a putative c-Jun/TRE complex, whose electrophoretic mobility was substantially lower than that of the free probe. Such a complex was not seen in the absence of added c-Jun protein (lane 7). Addition of excess unlabeled TRE competed with labeled TRE for c-Jun binding (lanes 1–3 and 8), but excess unlabeled AP-2 oligonucleotide did not (lanes 4–6). Preincubation of the c-Jun with p42 MAP kinase or ATP alone had no effect on complex formation (lanes 10 and 11), but preincubation of c-Jun with both kinase and ATP markedly inhibited complex formation (lane 12). These findings suggest that phosphorylation of serine 243 prevents c-Jun from binding to the TRE, either by inhibiting dimer formation or by inhibiting interaction of the dimer with the TRE.

c-Jun homodimers can be trapped by glutaraldehyde cross-linking and detected as 80- to 90-kDa species on

SDS polyacrylamide gels (Turner and Tjian, 1989). c-Jun phosphorylated in vitro by p42 MAP kinase was found to be capable of forming dimers (Figure 6B). This observation suggests that phosphorylation of serine 243 does not prevent the c-Jun dimerization but rather inhibits the interaction of c-Jun dimers with the TRE.

DISCUSSION

We have purified p42 MAP kinase from *Xenopus* eggs and have established its identity as a member of the ERK2 subfamily of MAP kinases by peptide sequencing. Partially purified p42 MAP kinase efficiently phosphorylates bacterially expressed human c-Jun. The Jun kinase and MAP kinase activities appear to be identical: the two activities copurify and coimmunoprecipitate. Both activities are inhibited by K-252a and by peptides based on the c-Jun phosphorylation site, and both activities are relatively insensitive to staurosporine.

We found that p42 MAP kinase phosphorylates c-Jun predominantly at one site, serine 243. The phos-

Figure 4. Phosphoamino acid analysis and V8 peptide mapping of spots b and c. c-Jun was phosphorylated by p42 MAP kinase in vitro and subjected to tryptic digestion and two-dimensional peptide mapping. Spots b and c were eluted from the plate and subjected to further treatments. (A) Phosphoamino acid analysis of spots b and c. The first dimension (pH 1.9) is horizontal, with the anode to the left; the second dimension (pH 3.5) is vertical, with the anode toward the top. Autoradiograms were exposed for 3 d at -70°C with intensifying screens. (B) V8 digestion of spot b. The V8 product co-migrated with the neutral marker phosphotyramine in the electrophoresis dimension. The location of the parent spot b was determined in parallel; its approximate position is shown in dotted lines. (C) Migration of synthetic phosphorylated TPPLSPIDME. (D) Mix of V8-digested spot b and synthetic phosphorylated TPPLSPIDME. Autoradiograms were exposed for 2 d at -70°C with intensifying screens for B–D. The anode is to the left; the origin is denoted by an o.

phorylation of this one residue gives rise to two phosphotryptic peptide spots, spots b and c, which represent the limit tryptic digestion product (tryptic peptide 13) and a partial digestion product (tryptic peptide 12 + 13 or tryptic peptide 13 + 14). We have detected at most trace amounts of spots x and y in the peptide map of in vitro phosphorylated c-Jun and conclude that p42 MAP kinase phosphorylates serine 63 and serine 73 relatively inefficiently in vitro. We have obtained similar results with MAP kinases immunoprecipitated from NIH 3T3 cells and with recombinant human ERK1; thus, our result is not peculiar to the frog enzyme or to ERK2 family members.

Other MAP Kinases? Other Sites?

Davis and co-workers previously examined the phosphorylation of bacterially expressed mouse c-Jun by two partially purified MAP kinase preparations (Alvarez *et al.*, 1991; Northwood *et al.*, 1991). One preparation was termed EGF receptor threonine 669 kinase; it now appears to be identical to human p44/ERK1 (Gonzalez *et al.*, 1991). The other preparation was mouse p42 MAP

kinase. The site in c-Jun phosphorylated by both of these preparations was identified as serine 243 based on the observation that wild-type c-Jun served as a substrate, but a mutant with residues 242–244 deleted did not. Our studies provide direct evidence in support of the same conclusion. The sequence around serine 243, P-L-S-P, satisfies an emerging consensus for p42 and p44 MAP kinase phosphorylation sites, P-X-S/T-P (Erickson *et al.*, 1990; Alvarez *et al.*, 1991; Gonzalez *et al.*, 1991; Izumi and Maller, 1991; Takishima *et al.*, 1991; Mukhopadhyay *et al.*, 1992). Other nonpolar residues may be able to substitute for the upstream proline (Haycock *et al.*, 1992).

Pulverer *et al.* (1991) also have examined the phosphorylation of c-Jun by two partially purified MAP kinase preparations. One preparation was a 54-kDa MAP kinase whose substrate specificity differs from those of p42 and p44 MAP kinases (Mukhopadhyay *et al.*, 1992); the other was a mixture of p42 and p44 MAP kinases partially purified from a rat hepatoma cell line. They found that both of their preparations phosphorylated human c-Jun (expressed in bacteria, in reticulocyte lysates, or in wheat germ lysates) at two N-terminal sites, serines 63 and 73, and that neither preparation phosphorylated serine 243. It is difficult to reconcile their p42/p44 MAP kinase findings with our results and with the results of Alvarez *et al.* (1991). Possibly their p42/p44 MAP kinase preparation contained other activities that were responsible for the serine 63 and serine 73 phosphorylation. It is also possible that the substrate specificities of rat hepatoma p42 and p44 MAP kinases differ from those of human p44 MAP kinase and mouse and *Xenopus* p42 MAP kinases or that different c-Jun

Figure 5. Phosphorylation of PPLSPID by p42 MAP kinase. Kinase reactions were carried out with or without p42 MAP kinase (30 ng) and with or without PPLSPID (3 μg). Reaction products were separated by thin-layer chromatography on cellulose plates with phosphochromatography buffer. Most of the radiolabel remained in ATP (R_f 0.13); a small amount was detectable in a putative peptide spot (R_f 0.46) when kinase and ATP were present, but not when either alone were present. The region around R_f = 0.46 was scraped from each lane, eluted, and divided into aliquots. One aliquot was rechromatographed (A) and the other was subjected to acid hydrolysis and phosphoamino acid analysis (B). Autoradiograms were exposed for 4 d at -70°C with intensifying screens.

Figure 6. Effect of serine 243 phosphorylation on c-Jun function in vitro. (A) Binding of c-Jun to a TRE-containing oligonucleotide. The left-hand autoradiogram assesses the specificity of the Jun-TRE interaction. Each lane (1–7) contains ^{32}P -labeled TRE-oligonucleotide. Lanes 1–6 contain c-Jun protein. Lanes 2 and 3 contain excess unlabeled TRE oligonucleotide (5 $\times$ and 10 $\times$ excesses relative to the labeled TRE). Lanes 5 and 6 contain excess unlabeled AP-2 oligonucleotide (5 $\times$ and 10 $\times$). The right-hand autoradiogram shows the effect of preincubating the c-Jun protein with MgATP and/or p42 MAP kinase on complex formation. Lane 8 contains a 10 $\times$ excess of unlabeled TRE-oligonucleotide. Exposure time is 20 min at room temperature with an intensifying screen for both autoradiograms. (B) c-Jun dimer formation. ^{32}P -labeled c-Jun protein was treated with or without glutaraldehyde (0.001%, 1 h, room temperature) to cross-link dimers. Monomers and dimers were resolved by SDS polyacrylamide electrophoresis. The autoradiogram was exposed for 12 h at -70°C with an intensifying screen.

preparations behave differently as MAP kinase substrates.

c-Jun Phosphorylation in Vivo

Previously, it had been thought that c-Jun is phosphorylated at five sites in vivo: serines 63 and 73 near the N-terminus (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991) and three sites clustered in the proline-rich transactivation A2 region, threonine 239, serine 243, and serine 249 (Boyle *et al.*, 1991a). Identification of the three sites in the proline-rich region depended on the assumption that the tryptic phosphopeptide spots a, b, and c represent triply, doubly, and singly phosphorylated forms of TP13. However, the present work demonstrates that TP13 becomes doubly phosphorylated in vivo but not triply phosphorylated.

Which two sites are phosphorylated in vivo? Although our work does not address this question directly, it may be useful to reconsider previous in vivo data in light of the present findings. One site must be a serine residue and the other a threonine residue, because both phosphoserine and phosphothreonine are recovered from spots a and b (Boyle *et al.*, 1991a). The most likely serine residue is serine 243, the site phosphorylated by p42 MAP kinase in vitro. This identification is based on the observation that substitution of phenylalanine for

serine at position 243 yields a mutant Jun protein that lacks phosphopeptide spots a, b, and c (Boyle *et al.*, 1991). This identification remains to be confirmed by direct approaches. The second site could be either of the two threonine residues within tryptic peptide 13, threonine 231 and threonine 239. It is possible that more than two sites are phosphorylated within tryptic peptide 13, although from the present work it appears that no more than two sites are phosphorylated in any individual c-Jun molecule.

Consequences of Serine 243 Phosphorylation

Phosphorylation of c-Jun at serine 243 markedly diminishes the binding of c-Jun to TRE-containing oligonucleotides. This observation is compatible with Boyle *et al.*'s (1991) finding that glycogen synthase kinase-3 (which in hindsight probably phosphorylated c-Jun at 2 sites, serine 243 and threonine 239) also inhibits c-Jun binding to TREs. Serine 243 is mutated to phenylalanine in v-Jun, and the phenylalanine 243 c-Jun mutant appears to be more effective than wild-type c-Jun at activating the transcription of TRE-containing reporter constructs in vivo (Boyle *et al.*, 1991).

A number of mechanisms can be envisioned for how serine 243 phosphorylation inhibits c-Jun DNA binding. We schematically show one such mechanism in Figure

Figure 7. Possible competition between the DNA phosphate backbone and the proline/glutamic acid rich A2 region for binding to the c-Jun DNA binding domain.

7. The DNA binding domain of c-Jun probably forms an α -helix that binds in the major groove of DNA, with the positively charged lysine and arginine side groups interacting with the phosphate backbone. The 20 amino acids from residues 254 through 273 include 11 basic amino acids and 1 acidic amino acid, for a net charge of +10. The A2 domain in which serine 243 resides is rich in proline residues and acidic residues, with six glutamic acid residues and one aspartic acid residue between residues 227 and 251 for a net charge of -7. It is possible DNA and the A2 domain compete for interaction with the positively charged DNA binding domain, either intramolecularly (Figure 7) or intermolecularly. When serine 243 and the nearby threonine residue are not phosphorylated, the DNA binding domain-DNA interaction might predominate; when the residues are phosphorylated, the additional negative charges could tip the balance in favor of the DNA binding domain-A2 domain interaction.

Whatever the mechanism for the inhibition of c-Jun DNA binding, it may have appeared early in evolution. The yeast GCN4 protein has a stretch of sequence just N-terminal to its DNA binding domain that closely resembles the sequence around serine 243 in c-Jun (Hope and Struhl, 1986). In particular, there is perfect conservation of the P-L-S-P sequence. It will be of interest to determine whether this site is phosphorylated in GCN4 in vivo and whether phosphorylation of this site inhibits GCN4 DNA binding.

Analysis of deletion mutants has implicated the c-Jun A2 region in transactivation (Bohmann and Tjian, 1989; Baichwal and Tjian, 1990). It is possible that phosphorylation of serine 243 and the nearby threonine residue would affect the interaction of c-Jun with other proteins in the transcriptional initiation complex. Whether and how these interactions are affected remains to be determined.

Why Turn Off c-Jun DNA Binding in Response to Mitogens?

We had initially expected that p42 MAP kinase might positively regulate c-Jun, because p42 MAP kinase ac-

tivity and AP-1 activity are increased in response to similar stimuli. However, p42 MAP kinase exerts a negative effect on c-Jun in vitro, inhibiting its binding to DNA. A number of reasons can be imagined why turning off c-Jun DNA binding might be important in vivo; here we will consider three possibilities. 1) Dissociation of c-Jun from the TRE might be an essential step in a transcriptional initiation-elongation cycle. Formation of the initiation complex might require c-Jun to be dephosphorylated at serine 243; elongation of the RNA transcript might require release of various components, including c-Jun, from the initiation complex. This release could be brought about by serine 243 phosphorylation. The observation that the phenylalanine 243 c-Jun mutant induces AP-1 activity in vivo argues against this model (Boyle *et al.*, 1991a); however, it is possible that the mutant acts indirectly, for example, by titrating away some negative regulatory factor from endogenous AP-1 proteins. 2) p42 MAP kinase may help to restore activated AP-1 back to its basal less active state. c-Jun appears to undergo a rapid net dephosphorylation of the A2 region in response to phorbol esters in vivo (Boyle *et al.*, 1991a). Perhaps this dephosphorylation is followed by p42 MAP kinase-mediated phosphorylation, which limits the duration of AP-1 activation. 3) p42 MAP kinase may be activated simultaneously with the phosphatase that dephosphorylates serine 243 to limit the extent of AP-1 activation.

Coordination with RNA Polymerase II?

The sequence around the serine 243, P-L-S-P, is reminiscent of the heavily phosphorylated C-terminal domain of RNA polymerase II, which is composed of repeats of the sequence Y-S-P-T-S-P-S. This raises the possibility that activation of p42 MAP kinase, or some other nuclear kinase with a similar substrate specificity (Cisek and Corden, 1989), could carry out coordinated changes in the associations between the various components of the transcriptional initiation complex.

ACKNOWLEDGMENTS

We thank Mike Wu, for help preparing *Xenopus* extracts; Arie Admon, for providing purified recombinant c-Jun protein and for help with

the peptide sequencing; Richard Turner, for providing c-Jun protein; Tom Geppert, for providing purified recombinant human ERK1 protein; Dave Dailey and Moon Young Lim, for help with the protein purification; Melanie Cobb, Jon Cooper, and Jim Posada, for providing antisera; and Steve Martin, John Gerhart, Bob Tjian, Tom Martin, and Tony Stretton, for support, advice, and encouragement. We also thank Shian-Jiun Shih and members of the Ferrell laboratory for critical reading of this manuscript. This work was supported by NIH grant GM-46383 and ACS institutional grant IN-35-32. J.E.F. is a Searle Scholar and a Special Fellow of the Leukemia Society of America.

REFERENCES

- Ahn, N.G., Seger, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K., and Krebs, E.G. (1991). Multiple components in an epidermal growth factor-stimulated protein kinase cascade. In vitro activation of a myelin basic protein/microtubule-associated protein 2 kinase. *J. Biol. Chem.* 266, 4220–4227.
- Ahn, N.G., Weiel, J.E., Chan, C.P., and Krebs, E.G. (1990). Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* 265, 11487–11494.
- Alvarez, E., Northwood, I.C., Gonzalez, F.A., Latour, D.A., Seth, A., Abate, C., Curran, T., and Davis, R.J. (1991). Pro-Leu-Ser/Thr-Pro is a primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of *c-myc* and *c-jun* proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J. Biol. Chem.* 266, 15277–15285.
- Anderson, N.G., Kilgour, E., and Sturgill, T.W. (1991). Activation of mitogen-activated protein kinase in BC₃H1 myocytes by fluroaluminate. *J. Biol. Chem.* 266, 10131–10135.
- Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990). Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 343, 651–653.
- Baichwal, V.R., and Tjian, R. (1990). Control of c-Jun activity by interaction of a cell-specific inhibitor with regulatory domain δ : differences between v- and c-Jun. *Cell* 63, 815–825.
- Barrett, C.B., Erickson, E., and Maller, J.L. (1992). A purified S6 kinase kinase from *Xenopus* eggs activates S6 kinase II and autophosphorylates on serine, threonine, and tyrosine residues. *J. Biol. Chem.* 267, 4408–4415.
- Binetruy, B., Smeal, T., and Karin, M. (1991). Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature* 351, 122–127.
- Bishop, R., Martinez, R., Nakamura, K.D., and Weber, M.J. (1983). A tumor promoter stimulates phosphorylation on tyrosine. *Biochem. Biophys. Res. Commun.* 115, 536–543.
- Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* 238, 1386–1392.
- Bohmann, D., and Tjian, R. (1989). Biochemical analysis of transcriptional activation by Jun: differential activity of c- and v-Jun. *Cell* 59, 709–717.
- Boulton, T.G., and Cobb, M.H. (1991). Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357–371.
- Boulton, T.G., Gregory, J.S., and Cobb, M.H. (1991a). Purification and properties of ERK1, an insulin-stimulated MAP2 protein kinase. *Biochemistry* 30, 278–286.
- Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H., and Yancopoulos, G.D. (1991b). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65, 663–675.
- Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* 249, 64–67.
- Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M., and Hunter, T. (1991a). Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. *Cell* 64, 573–584.
- Boyle, W.J., van der Geer, P., and Hunter, T. (1991b). Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201, 110–149.
- Charbonneau, H. (1989). Strategies for obtaining partial amino acid sequence data from small quantities (<5 nmol) of pure or partially purified protein. In: *A Practical Guide to Protein Purification for Microsequencing*, ed. P.T. Matsudaira, San Diego: Academic Press, 15–30.
- Chen, R.-H., Sarnecki, C., and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* 12, 915–927.
- Cicirelli, M., Pelech, S., and Krebs, E. (1988). Activation of multiple protein kinases during the burst in protein phosphorylation that precedes the first meiotic cell division in *Xenopus* oocytes. *J. Biol. Chem.* 263, 2009–2019.
- Cisek, L.J., and Corden, J.L. (1989). Phosphorylation of RNA polymerase by the murine homologue of the cell-cycle control protein cdc2. *Nature* 339, 679–684.
- Cooper, J.A. (1989). Related proteins are phosphorylated at tyrosine in response to mitogenic stimuli and at meiosis. *Mol. Cell. Biol.* 9, 3143–3147.
- Cooper, J.A., Bowen-Pope, D.F., Raines, E., Ross, R., and Hunter, T. (1982). Similar effects of platelet-derived growth factor and epidermal growth factor on the phosphorylation of tyrosine in cellular proteins. *Cell* 31, 263–273.
- Cooper, J.A., Sefton, B.M., and Hunter, T. (1984). Diverse mitogenic agents induce the phosphorylation of two related 42,000-dalton proteins on tyrosine in quiescent chick cells. *Mol. Cell. Biol.* 4, 30–37.
- Courchesne, W.E., Kunisawa, R., and Thorner, J. (1989). A putative protein kinase overcomes pheromone-induced arrest of cell cycling in *S. cerevisiae*. *Cell* 58, 1107–1119.
- Elion, E.A., Grisafi, P.L., and Fink, G.R. (1989). FUS3 encodes a cdc2+ / CDC28-related kinase required for the transition from mitosis into conjugation. *Cell* 60, 649–664.
- Ely, C.M., Oddie, K.M., Litz, L.S., Rossomando, A.J., Kanner, S.B., Sturgill, T.W., and Parsons, S.J. (1990). A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen stimulated protein in fibroblasts. *J. Cell Biol.* 110, 731–742.
- Erickson, A.K., Payne, D.M., Martino, P.A., Rossomando, A.J., Shabanowitz, J., Weber, M.J., Hunt, D.F., and Sturgill, T.W. (1990). Identification by mass spectroscopy of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. *J. Biol. Chem.* 265, 19728–19735.
- Ferrell, J.E., Jr., and Martin, G.S. (1990). Identification of a 42-kilodalton phosphotyrosyl protein as a serine(threonine) protein kinase by renaturation. *Mol. Cell. Biol.* 10, 3020–3025.
- Ferrell, J.E., Jr., Wu, M., Gerhart, J.C., and Martin, G.S. (1991). Cell cycle tyrosine phosphorylation of p34^{cdc2} and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. *Mol. Cell. Biol.* 11, 1965–1971.

- Funasaka, Y., Boulton, T., Cobb, M., Yarden, Y., Fan, B., Lyman, S.D., Williams, D.E., Anderson, D.M., Zakut, R., Mishima, Y., and Halaban, R. (1992). c-Kit-kinase induces a cascade of protein tyrosine phosphorylation in normal human melanocytes in response to mast cell growth factor and stimulates mitogen-activated protein kinase but is down-regulated in melanomas. *Mol. Biol. Cell* 3, 197–209.
- Gilmore, T., and Martin, G.S. (1983). Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. *Nature* 306, 487–490.
- Gomez, N., Tonks, N.K., Morrison, C., Harmar, T., and Cohen, P. (1990). Evidence for communication between nerve growth factor and protein tyrosine phosphorylation. *FEBS Lett.* 271, 119–122.
- Gonzalez, F.A., Raden, D.L., and Davis, R.J. (1991). Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J. Biol. Chem.* 266, 22159–22163.
- Gotoh, Y., Moriyama, K., Matsuda, S., Okumura, E., Kishimoto, T., Kawasaki, H., Suzuki, K., Yahara, I., Sakai, H., and Nishida, E. (1991b). *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. *EMBO J.* 10, 2661–2668.
- Gotoh, Y., Nishida, E., Matsuda, S., Shiina, N., Kosako, H., Shiokawa, K., Aikawa, T., Ohta, K., and Sakai, H. (1991a). In vitro effects on microtubule dynamics of purified *Xenopus* M phase activated MAP kinase. *Nature* 349, 251–254.
- Gotoh, Y., Nishida, E., Sakai, H. (1990b). Okadaic acid activates microtubule-associated protein kinase in quiescent fibroblastic cells. *Eur. J. Biochem.* 193, 671–674.
- Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M., and Sakai, H. (1990a). Microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc. Natl. Acad. Sci. USA* 89, 2365–2369.
- Haycock, J.W., Ahn, N.G., Cobb, M.H., and Krebs, E.G. (1992). ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. *Proc. Natl. Acad. Sci. USA* 89, 2365–2369.
- Haystead, T.A.J., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H., Krebs, E.G. (1990). Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. The role of protein phosphatase 2A in attenuation of the signal. *J. Biol. Chem.* 265, 16571–16580.
- Her, J.-H., Wu, J., Rall, T.B., Sturgill, T.W., and Weber, M.J. (1991). Sequence of pp42/MAP kinase, a serine/threonine kinase regulated by tyrosine phosphorylation. *Nuc. Acids Res.* 19, 3743.
- Hope, I.A., and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator protein, GCN4 of yeast. *Cell* 46, 885–894.
- Hoshi, M., Nishida, E., and Sakai, H. (1988). Activation of a Ca^{2+} -inhibitable protein kinase that phosphorylates microtubule-associated protein 2 in vitro by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. *J. Biol. Chem.* 263, 5396–5401.
- Izumi, T., and Maller, J. (1991). Phosphorylation of *Xenopus* cyclins B1 and B2 is not required for cell cycle transitions. *Mol. Cell. Biol.* 11, 3860–3867.
- Karin, M. (1990). The AP-1 complex and its role in transcriptional control by protein kinase C. In: *Molecular Aspects of Cellular Regulation*, Vol. 6. ed. P. Cohen and G. Foulkes, Amsterdam: Elsevier/North-Holland Biomedical Press, 143–161.
- Kohn, M. (1985). Diverse mitogenic agents induce rapid phosphorylation of a common set of cellular proteins at tyrosine in quiescent mammalian cells. *J. Biol. Chem.* 260, 1771–1779.
- Kyriakis, J.M., and Avruch, J. (1990). pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem.* 265, 17355–17363.
- L'Allemain, G., Pouyssegur, J., and Weber, M.J. (1991). p42/mitogen-activated protein kinase as a converging target for different growth factor signalling pathways: use of pertussis toxin as a discrimination factor. *Cell Regul.* 2, 413–425.
- Miyasaka, T., Sternberg, D.W., Miyasaka, J., Sherline, P., and Saltiel, A.R. (1991). Nerve growth factor stimulates protein tyrosine phosphorylation in PC-12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* 88, 2653–2657.
- Mukhopadhyay, N.K., Price, D.J., Kyriakis, J.M., Pelech, S., Sanghera, J., and Avruch, J. (1992). An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. *J. Biol. Chem.* 267, 3325–3335.
- Nel, A.E., Hanekom, C., Rheeder, A., Williams, K., Pollack, S., Katz, R., and Landreth, G.E. (1990). Stimulation of MAP2 kinase activity in T lymphocytes by anti-CD3 or anti-Ti monoclonal antibody is partially dependent of protein kinase C. *J. Immunol.* 144, 2683–2689.
- Northwood, I.C., Gonzalez, F.A., Wartmann, M., Raden, D.L., and Davis, R.J. (1991). Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. *J. Biol. Chem.* 266, 15266–15276.
- Payne, D.M., Rossomando, A.J., Martino, P., Erickson, A.K., Her, J.-H., Shabanowitz, J., Hunt, D.F., Weber, M.J., and Sturgill, T.W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.* 10, 885–892.
- Posada, J., and Cooper, J.A. (1992). Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. *Science* 255, 212–215.
- Posada, J., Sanghera, J., Pelech, S., Aebersold, R., and Cooper, J.A. (1991). Tyrosine phosphorylation and activation of homologous protein kinase during oocyte maturation and mitogenic activation of fibroblasts. *Mol. Cell. Biol.* 11, 2517–2528.
- Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E., and Woodgett, J.R. (1991). Phosphorylation of c-jun mediated by MAP kinases. *Nature* 353, 670–674.
- Ray, L.B., and Sturgill, T.W. (1987). Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. *Proc. Natl. Acad. Sci. USA* 84, 1502–1506.
- Rossomando, A., Payne, D.M., Weber, M., and Sturgill, T. (1989). Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. *Proc. Natl. Acad. Sci. USA* 86, 6940–6943.
- Rossomando, A.J., Sanghera, J.S., Marsden, L.A., Weber, M.J., Pelech, S.L., and Sturgill, T.W. (1991). Biochemical characterization of a family of serine/threonine protein kinases regulated by tyrosine and serine/threonine phosphorylations. *J. Biol. Chem.* 266, 20270–20275.
- Sanghera, J., Haddon, H., Bader, S., and Pelech, S. (1990). Purification and characterization of a maturation-activated myelin basic protein kinase from sea star oocytes. *J. Biol. Chem.* 265, 52–57.
- Schanan-King, C., Nel, A., Williams, L.K., and Landreth, G. (1991). Nerve growth factor stimulates the tyrosine phosphorylation of MAP2 kinase in PC12 cells. *Neuron* 6, 915–922.
- Schieven, G., and Martin, G.S. (1988). Nonenzymatic phosphorylation of tyrosine and serine by ATP is catalyzed by manganese but not magnesium. *J. Biol. Chem.* 263, 15590–15593.
- Takishima, K., Griswold-Prenner, I., Ingebritsen, T., and Rosner, M.R. (1991). Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase. *Proc. Natl. Acad. Sci. USA* 88, 2520–2524.
- Tobe, K., Kadowaki, T., Tamemoto, H., Ueki, K., Hara, K., Koshio, O., Momomura, K., Gotoh, Y., Nishida, E., Akanuma, Y., Yazaki, Y.,

and Kasuga, M. (1991). Insulin and 12-O-tetradecanoylphorbol-13-acetate activation of two immunologically distinct myelin basic protein/microtubule-associated protein 2 (MBP/MAP2) kinase via de novo phosphorylation of threonine and tyrosine residues. *J. Biol. Chem.* 266, 24793–24803.

Tsao, H., Aletta, J.M., and Greene, L.A. (1990). Nerve growth factor and fibroblast growth factor selectively activate a protein kinase that phosphorylates high molecular weight microtubule-associated pro-

teins. Detection, partial purification, and characterization in PC12 cells. *J. Biol. Chem.* 265, 15471–15480.

Turner, R., and Tjian, R. (1989). Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers. *Science* 243, 1689–1694.

White, M.F., and Backer, J.M. (1991). Preparation and use of anti-phosphotyrosine antibodies to study structure and function of insulin receptor. *Methods Enzymol.* 201, 65–79.