er indicates a transcription-dependent change at the TATA element that probably reflects TBP binding (*19*). Finally, our results are consistent with and support the physiological relevance of biochemical experiments indicating that TBP binds very weakly to nucleosomal templates in the absence of a transcriptional activator protein.

The lag between synthesis of TBP^{m3} and its ability to support basal transcription takes several hours, the approximate length of a single cell cycle. It is possible that, in the absence of an activator, recruitment of TBP to the promoter might occur only at a specific time of the cell cycle. In this regard, DNA replication–coupled chromatin assembly has been associated with a variety of transcriptional events in vivo and in vitro (*20*). Alternatively, recruitment under these conditions might be stochastic but simply slow in comparison to the situation at the Gcn4-activated promoter.

Our results do not distinguish between potential mechanisms by which activation domains increase recruitment of TBP to promoters in vivo. Acidic activation domains and the Swi-Snf complex, which is involved in the activation process, cause changes in chromatin structure that occur in the absence of a functional TATA element and transcription (*21*). Perhaps these structural changes increase the accessibility of TBP to the promoter. Increased recruitment also may reflect direct interactions of activation domains to TBP or associated factors (TAFs) that are components of the TFIID complex (*22*). In this view, different classes of activation domains might function in a fundamentally similar manner even though they may interact with different components of the TFIID complex. These models are not incompatible and indeed may be synergistic in explaining how activation domains increase recruitment of TBP to the promoter. Finally, although our results strongly implicate TBP recruitment as a critical step in the transcriptional activation process in vivo, they do not exclude the importance of other steps implicated from in vitro experiments such as recruitment of TFIIB or other general factors (*18*).

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Microtubule Severing by Elongation Factor 1α

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An activity that severs stable microtubules is thought to be involved in microtubule reorganization during the cell cycle. Here, a 48-kilodalton microtubule-severing protein was purified from *Xenopus* eggs and identified as translational elongation factor 1α (EF-1α). Bacterially expressed human EF-1α also displayed microtubule-severing activity in vitro and, when microinjected into fibroblasts, induced rapid and transient fragmentation of cytoplasmic microtubule arrays. Thus, EF-1α, an essential component of the eukaryotic translational apparatus, appears to have a second role as a regulator of cytoskeletal rearrangements.

An activity that severs stable microtubules, first detected in mitotic extracts from *Xenopus laevis* eggs (*1*), is thought to participate in the microtubule rearrangements that occur between interphase and mitosis (*1–3*). Microtubule severing also occurs during flagellar excision in the green algae *Chlamydomonas* (*4*). Two microtubule-severing factors have been purified: p56, which severs microtubules slowly in an adenosine triphosphate (ATP)–independent manner (*3*), and katanin, a heterodimeric protein that severs and disassembles microtubules in an ATP-dependent manner (*5*).

We purified from *Xenopus* egg extracts a third protein of molecular weight 48,000 that can rapidly sever taxol-stabilized fluorescent microtubules in vitro (Fig. 1) (*6*). Phosphocellulose chromatography of the extracts generated two peaks of microtubule-severing activity. The activity in flow-through fractions had been previously purified and designated p56 (*3*). Immunoblot analysis with an antibody to p56 indicated that the activity in the adsorbed fraction was not due to p56. The adsorbed fraction severed microtubules only in the

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presence of ATP. This dependence on ATP may be attributable to an ATP-sensitive microtubule-binding factor or factors in the fraction, because subsequent chromatography on hydroxylapatite resulted in separation of an ATP-sensitive microtubule-bundling activity and an ATP-independent microtubule-severing activity (*7*). Further chromatographic analyses revealed that the microtubule-severing activity co-eluted with a 48-kD polypeptide, p48 (Fig. 1A), which we ultimately purified to near homogeneity (Fig. 1B).

Incubation of taxol-stabilized microtubules (*8*) with purified p48 in the presence or absence of ATP induced rapid fragmentation of microtubules within 1 min (Fig. 1D), and the fragmented microtubules did not appear to undergo rapid endwise disassembly (Fig. 1D). The extent of fragmentation was dependent on the concentration of p48 (Fig. 1C). At lower concentrations of p48 (<20 μg/ml), both bundling and fragmentation of microtubules were observed (Fig. 1C). All these characteristics distinguish p48 from katanin, an adenosine

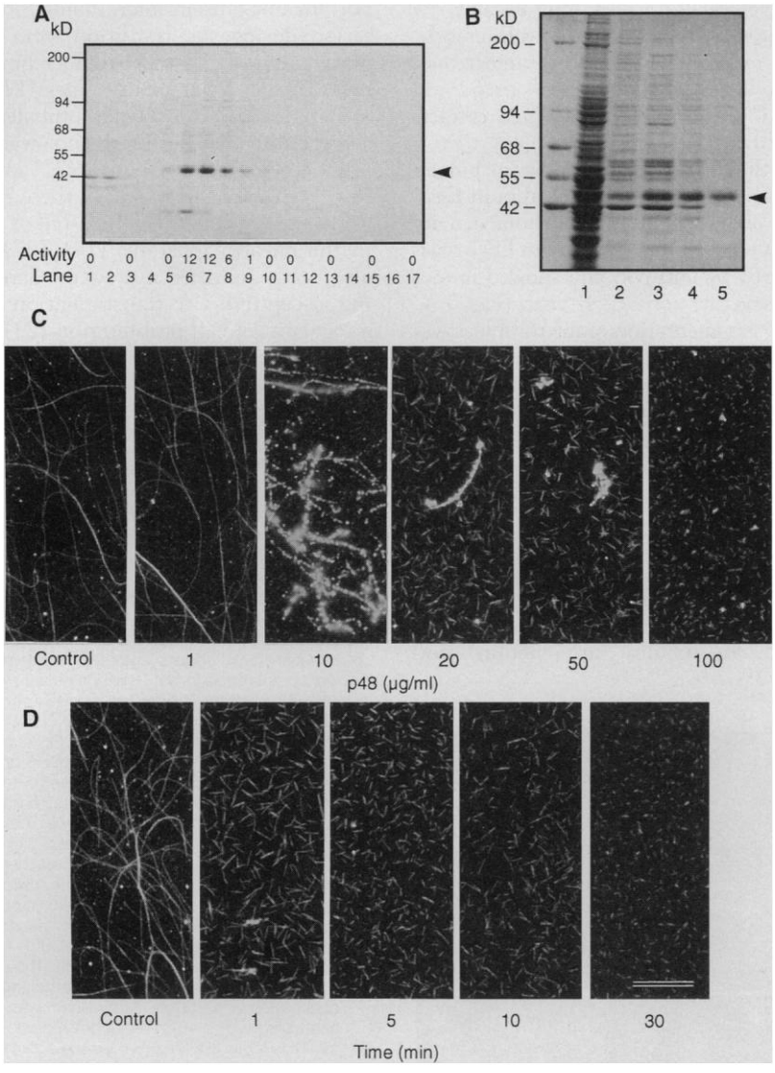

Fig. 1. A protein factor, p48, that rapidly severs stable microtubules was purified from *Xenopus* M-phase extracts by sequential chromatography on phosphocellulose, hydroxylapatite, Sephacryl S-300, and S-Sepharose. In phosphocellulose chromatography, p56, a previously identified severing factor (*3*), eluted in flow-through fractions; a second activity, eluting at ~0.35 M KCl, was subjected to further purification. (**A**) The SDS-PAGE profile and the activity of fractions from the S-Sepharose column are shown. The activity is expressed as the fold dilution at which each fraction still showed the same severing activity as that of purified p48 (10 μg/ml) (C). (**B**) SDS-PAGE of samples from each step of p48 purification. Lane 1, extracts; lane 2, phosphocellulose; lane 3, hydroxylapatite; lane 4, Sephacryl S-300; lane 5, S-Sepharose fractions. Arrowheads in (A) and (B) indicate p48. (**C** and **D**) Microtubule severing by purified p48. Taxol-stabilized rhodamine-labeled microtubules (100 μg/ml) were incubated at 20°C with control buffer or with increasing concentrations of purified p48 (S-Sepharose fraction) for 2 min (C) or with control buffer for 30 min or with purified p48 (30 μg/ml) for the indicated times (D). Microtubules were fixed and visualized as in (*3*). Scale bar, 10 μm.

Fig. 2. Partial amino acid sequences of p48 (boxed) perfectly match the sequence of *Xenopus* EF-1α (*12*). Circles, unidentified amino acid; squares, amino acid with modification.

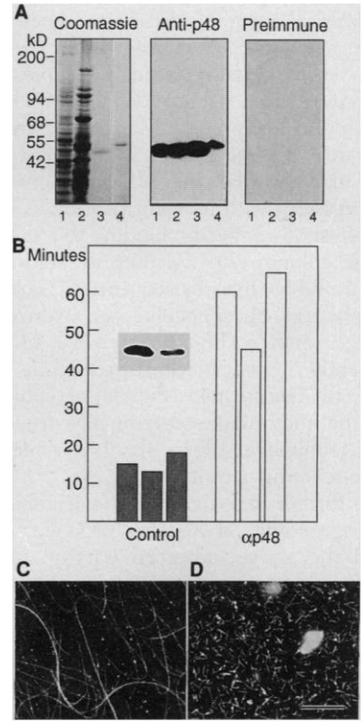

Fig. 3. Microtubule severing by EF-1α. (**A**) Reactivity of polyclonal anti-p48. Lane 1, *Xenopus* egg extracts (50 μg); lane 2, rabbit liver extracts (50 μg); lane 3, p48 purified from *Xenopus* eggs (0.5 μg) (see Fig. 1); lane 4, human recombinant EF-1α (0.5 μg). Proteins were subjected to electrophoresis and either stained with Coomassie blue (left) or immunoblotted with affinity-purified anti-p48 (middle) or preimmune serum (right). (**B**) *Xenopus* extracts were subjected to immunodepletion with control IgG or with anti-p48 (αp48). In each sample, the time required for taxol-stabilized microtubules to become ~3 μm long was measured. The results of three independent experiments are shown. The inset shows immunoblotting of mock-treated extracts (left) and immunodepleted extracts (right) with anti-p48. Immunodepletion removed ~70% of the p48 in the extracts. (**C** and **D**) Microtubule severing by human recombinant EF-1α. Taxol-stabilized fluorescent microtubules were incubated with control buffer (C) or purified human recombinant EF-1α (D) (150 μg/ml) for 2 min at 20°C. Scale bar, 10 μm.

triphosphatase that severs and disassembles microtubules in an ATP-dependent manner (*5*). The microtubule-severing activity of p48 was inhibited by the addition of free tubulin or of the microtubule-associated proteins p220 (*9*) or MAP2 (*7*, *10*). Furthermore, p48, like *Xenopus* egg extracts (*5*), did not sever taxol-stabilized microtubules previously treated with subtilisin (*7*), which is known to remove a small peptide from the COOH-terminus of tubulin. Therefore, p48 may bind to the COOH-terminal portion of tubulin.

Fifteen internal peptides from p48 were sequenced (Fig. 2) (*11*) and found to be identical to sequences in the form of EF-1α found in *Xenopus* oocytes (*12*) (Fig. 2). An essential component of the eukaryotic translational apparatus, EF-1α catalyzes the binding of aminoacyl transfer RNA to the ribosome (*13*). It is an abundantly expressed guanosine triphosphate (GTP)–binding protein and has previously been shown to associate with the mitotic apparatus (*14*) and with actin filaments (*15*).

Consistent with its identification as EF-1α, p48 showed specific binding to a GTP-agarose column (*7*). We then isolated EF-1α from rabbit liver by sequential chromatography on phosphocellulose, hydroxylapatite, and GTP-agarose. A 48-kD polypeptide that was recognized by an antibody to *Xenopus* p48 (Fig. 3A) co-eluted with the microtubule-severing activity (*7*). Thus, rabbit liver EF-1α also has a microtubule-severing activity.

To further investigate the microtubule-severing activity of *Xenopus* p48, we subjected the active, adsorbed fraction from the phosphocellulose column to chromatography on a protein A–Sepharose column linked to an antibody to p48 (anti-p48) (*16*). In the fractions showing microtubule-severing activity, p48 was the only detectable band on SDS-polyacrylamide gels (*7*).

To confirm that EF-1α (p48) contributes to the microtubule-severing activity of the *Xenopus* extracts, we did an immunodepletion experiment with anti-p48 (*17*). Immunodepletion reduced the amount of p48 by ~70% (Fig. 3B) and reduced the microtubule-severing activity of the *Xenopus* extracts. Taxol-stabilized microtubules that were ~20 to 30 μm long became ~3 μm long within 10 to 20 min in the mock-treated extracts, whereas the same reduction in length required a 45- to 60-min incubation in the EF-1α–depleted extracts (Fig. 3B).

We then expressed recombinant human EF-1α as a histidine-tagged protein in *Escherichia coli* and purified it to homogeneity (Fig. 3A) (*18*). The recombinant EF-1α was recognized by anti-p48 and showed microtubule-severing activity in vitro (Fig. 3, C and D). Fragmentation of microtubules was clearly observed when polymerized tubulin (100 μg/ml) was incubated with recombinant human EF-1α (15 μg/ml) for 2 min at 20°C (*7*).

We also tested the activity of recombinant human EF-1α in permeabilized rat fibroblasts (*19*). Treatment of the permeabilized cells with EF-1α resulted in rapid fragmentation of cytoplasmic microtubule arrays emanating from centrosomes (Fig. 4, A and C). Double staining with an antibody to tubulin (anti-tubulin) and with anti-p48 showed that EF-1α co-localized with the remaining microtubules (Fig. 4, C and D), which suggests that it bound to severed microtubules. Microinjection of the recombinant EF-1α into fibroblasts (*20*) also resulted in rapid destruction of cytoplasmic microtubule networks (Fig. 4, E through G). Thus, EF-1α can sever microtubules in intact cells as well as in cell-free extracts.

EF-1α, an essential component of the translational machinery, appears to have a distinct role as a microtubule-severing protein. Microtubule severing may be important not only in microtubule reorganization during the transition from interphase to mitosis, but also in detachment of microtubules from centrosomes (*21*) and in depolymerization of microtubules near the spindle poles during the poleward flux of kinetochore microtubules (*22*) in mitosis. EF-1α may participate in these events, as it is present at the centrosphere region in the mitotic apparatus (*14*). EF-1α has also been identified as a factor determining susceptibility to transformation and as a regulator of cell proliferation (*23*). How these various activities of EF-1α are controlled throughout the cell cycle remains to be determined.

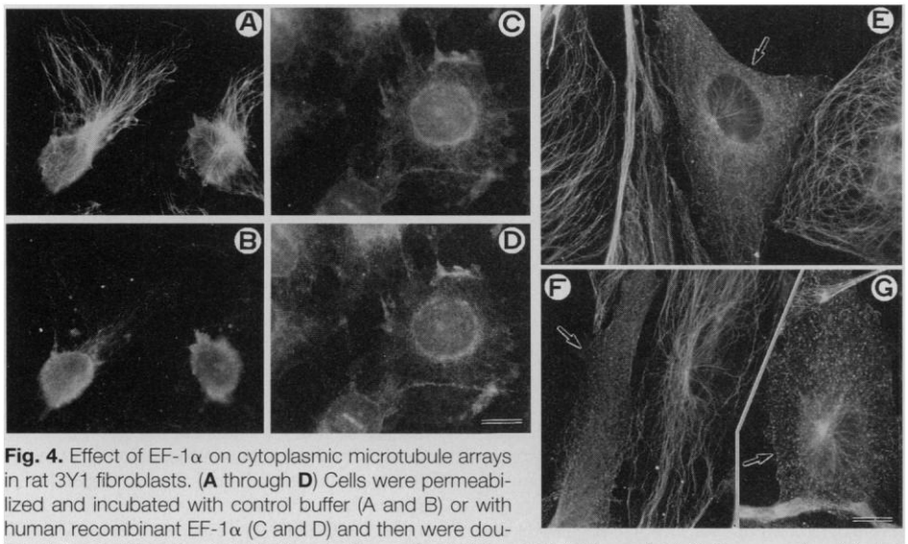

Fig. 4. Effect of EF-1α on cytoplasmic microtubule arrays in rat 3Y1 fibroblasts. (**A** through **D**) Cells were permeabilized and incubated with control buffer (A and B) or with human recombinant EF-1α (C and D) and then were double-stained with anti–α-tubulin (A and C) or anti-p48 (B and D). (**E** through **G**) Cells were microinjected with human recombinant EF-1α (200 μg/ml), and the overall concentration of EF-1α was estimated to be changed by a factor of about 2. Cells were incubated for 10 min at 37°C and then fixed and stained with anti–α-tubulin. Arrows indicate the cells injected with EF-1α; the others are uninjected cells. Scale bars, 10 μm.

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8. Rhodamine-labeled taxol-stabilized microtubules

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11. Purified p48 (150 μg) from *Xenopus* eggs was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The transferred p48 was reduced and *S*-carboxymethylated in situ (*24*) and digested with *Achromobacter* protease I. Peptides released from the membrane were fractionated by reversed-phase high-performance liquid chromatography (HPLC) on a Wakosil-II 5C18 AR column (Wako Pure Chemical Industries, Osaka, Japan) and subjected to amino acid sequence analysis with a Shimadzu gas-phase sequencer, model PSQ-10 (Shimadzu, Kyoto, Japan). Molecular mass determination was carried out on a Sciex API-III mass spectrometer (Perkin-Elmer Sciex, Thorn Hill, Ontario, Canada) with an ionspray ion source and a quadrupole mass analyzer.
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16. The anti-p48 serum was raised in rabbits against purified *Xenopus* p48 (S-Sepharose fraction; see Fig. 1). The antibody was affinity-purified and used for immunoaffinity purification of p48 as described (*25*).
17. Affinity-purified anti-p48 was concentrated and dialyzed against phosphate-buffered saline (PBS). This antibody solution (15 μl) or PBS (15 μl) was added to 50 μl of M-phase extracts from *Xenopus* eggs. After incubation of the mixtures for 1.5 hours at 4°C, protein A beads (10 μl) were added and the incubation at 4°C continued for 1 hour with gentle shaking. The samples were then centrifuged at 10,000*g* for 15 s at 4°C and the supernatants were assayed for microtubule-severing activity.
18. The open reading frame (ORF) of human EF-1α (*26*) was amplified by PCR with the 5′ primer 5′-CGCGGGATCCAATGGGAAAGGAAAAGAC-3′ and the 3′ primer 5′-GGAGGGATCCTATTCATTTAGCCTTCTG-3′, which produce Bam HI sites at both ends of the EF-1α ORF. The resultant Bam HI fragment was cloned into pET-16b (Novagen, Madison, WI) and used to transfect the BL21 (DE3) pLysS strain of *Escherichia coli*. The expression of histidine-tagged EF-1α was induced for 10 hours at 30°C in the presence of 0.7 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted and lysed in a solution containing 5 mM imidazole, 500 mM NaCl, 20 mM tris-Cl (pH 7.9), 0.1% NP40, 1 mM PMSF, 1% aprotinin, 10 mM $MgCl_2$, and deoxyribonuclease I (20 μg/ml). After sonication and centrifugation at 39,000*g* for 20 min, the supernatant was applied to ProBond resin (Invitrogen), and the recombinant EF-1α was eluted with a gradient of 60 to 500 mM imidazole and dialyzed against 80PME.
19. Rat 3Y1 cells on glass cover slips were treated with 80PME containing 0.5% Triton X-100, 10 mM EGTA, and 5 μM taxol for 2 min at 37°C. After washing the cells with 80PME containing 10 mM EGTA and 0.4 M NaCl, we incubated them with 80PME or human recombinant EF-1α (150 μg/ml) for 10 min at 20°C. The cells were then washed with 80PME and fixed with 1% glutaraldehyde in PBS for 15 min. After blocking with PBS containing 3% bovine serum albumin and goat immunoglobulin (1 mg/ml), the cells were incubated with an antibody to α-tubulin (anti-α-tubulin) (DM1A, 1:150 dilution) and anti-p48 (1:5 dilution) and then washed with PBS. Immunoreactive proteins were detected by rhodamine isothiocyanate (RITC)–coupled goat antibody to mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)–coupled goat antibody to rabbit IgG, respectively.
20. Microinjection was done as in (*27*). FITC-labeled goat IgG was included in the EF-1α solution or in control buffer to distinguish injected from uninjected cells. The cells were fixed with 3.7% formaldehyde in PBS for 10 min. After blocking, they were stained with anti–α-tubulin (DM1A, 1:100 dilution) and RITC goat antibody to mouse IgG (anti-mouse IgG).
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Inhibition of Ras-Induced DNA Synthesis by Expression of the Phosphatase MKP-1

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Mitogen-activated protein kinases (MAP kinases) are common components of signaling pathways induced by diverse growth stimuli. Although the guanidine nucleotide-binding Ras proteins are known to be upstream activators of MAP kinases, the extent to which MAP kinases directly contribute to the mitogenic effect of Ras is as yet undefined. In this study, inhibition of MAP kinases by the MAP kinase phosphatase MKP-1 blocked the induction of DNA synthesis in quiescent rat embryonic fibroblast REF-52 cells by an activated mutant of Ras, V^{12}Ras. These results suggest an essential role for activation of MAP kinases in the transition from the quiescent to the DNA replication phase of the eukaryotic cell cycle.

Mitogen-activated protein kinases or extracellular signal–regulated kinases ($p42^{mapk}$ or ERK2 and $p44^{mapk}$ or ERK1, hereafter referred to as MAP kinase) are protein serine-threonine kinases that are activated by various mitogenic stimuli (*1*). The binding of a growth factor to its receptor tyrosine kinase induces formation of multiprotein complexes in the plasma membrane that trigger conversion of Ras from an inactive guanosine diphosphate (GDP)–bound form to an active guanosine triphosphate (GTP)–bound state (*2*). Activated Ras then initiates a cascade of sequential phosphorylation events in which the serine-threonine kinase Raf phosphorylates and activates MAP kinase kinase (also known as MEK) (*3*), a dual specificity kinase, that in turn phosphorylates both threonine and tyrosine regulatory sites in MAP kinase (*4*), thus leading to activation of its kinase function. Once activated, MAP kinase can phosphorylate other serine-threonine kinases such as $p90^{rsk}$ and transcription factors including $p62^{TCF}$ or Elk-1 (*5*). Expression of oncogenic V^{12}Ras, in which Gly^{12} is mutated to Val and the resulting protein is locked in the GTP-bound form, leads to constitutive activation of MAP kinase (*6*). However, whether activation of MAP kinase is an obligatory step for the mitogenic effect of Ras has yet to be established.

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MAP kinase phosphatase 1 (MKP-1) is encoded by the mitogen-inducible gene *3CH134* (*7*). MKP-1 inactivates $p42^{mapk}$ and $p44^{mapk}$ by dephosphorylating both phosphothreonine and phosphotyrosine regulatory sites in the enzymes (*8*). MKP-1 appears to be highly specific for the dephosphorylation of MAP kinase, because transient expression of the phosphatase in COS cells leads to selective dephosphorylation of MAP kinase from the spectrum of phosphotyrosine-containing proteins. Furthermore, we have demonstrated that a catalytically inactive mutant phosphatase in which Cys^{258} is changed to Ser forms a specific complex with its substrate, the phosphorylated form of MAP kinase. The selectivity of MKP-1 toward MAP kinase has also been reported for CL100, the human homolog of MKP-1 (*9*, *10*), and the related phosphatases PAC1 in lymphocytes (*11*) and MSG5 in yeast *Saccharomyces cerevisiae* (*12*). In addition, MKP-1 or CL100 does not dephosphorylate $p34^{cdc2}$, another serine-threonine kinase regulated by phosphorylation of threonine and tyrosine residues (*9*, *13*).

Recently, members of the Jun kinase (JNK) (*14*) or stress-activated protein kinase (SAPK) (*15*) family have been identified. These enzymes are distantly related to MAP kinase, and they are also regulated by threonine and tyrosine phosphorylation. We therefore examined whether MKP-1 had the capacity to dephosphorylate these enzymes. In parallel in vitro assays, approx-