Phosphorylation of Mnk1 by Caspase-activated Pak2/γ-PAK Inhibits Phosphorylation and Interaction of eIF4G with Mnk*

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The mitogen-activated protein kinase-interacting kinase 1 (Mnk1) is phosphorylated by caspase-cleaved protein kinase Pak2/γ-PAK but not by Cdc42-activated Pak2. Phosphorylation of Mnk1 is rapid, reaching 1 mol/mol within 15 min of incubation with Pak2. A kinetic analysis of the phosphorylation of Mnk1 by Pak2 yields a $K_a$ of 0.6 μM and a $V_{max}$ of 14.9 pmol of 32P/min/μg of Pak2. Two-dimensional tryptic phosphopeptide mapping of Mnk1 phosphorylated by Pak2 yields two distinct phosphopeptides. Analysis of the phosphopeptides by automated microsequencing and manual Edman degradation identified the sites in Mnk1 as Thr22 and Ser27. Mnk1, activated by phosphorylation with Erk2, phosphorylates the eukaryotic initiation factor (eIF) 4E and the eIF4G components of eIF4F. Phosphorylation of Mnk1 by Pak2 does not activate Mnk1, as measured with either eIF4E or eIF4F as substrate. Phosphorylation of Erk2-activated Mnk1 by Pak2 has no effect on phosphorylation of eIF4E but reduces phosphorylation of eIF4G by Mnk1 by up to 50%. Phosphorylation of Mnk1 by Pak2 inhibits binding of eIF4G peptides containing the Mnk1 binding site by up to 80%. When 293T cells are subjected to apoptotic induction by hydrogen peroxide, Mnk1 is phosphorylated at both Thr22 and Ser27. These results indicate a role for Pak2 in the down-regulation of translation initiation in apoptosis by phosphorylation of Mnk1.

Within the framework of translational initiation, one of the major points of regulation involves the recruitment of the mRNA to the 43S pre-initiation complex. Recruitment is mediated by members of the group four initiation factors (eIF4),1 the most prominent member of which is the cap-binding component of eIF4F. eIF4F (via eIF4E) binds to the m7G-cap of mRNAs along with eIF4B and eIF4H, positioning eIF4A to unwind mRNA secondary structures5 to the AUG start codon (reviewed in Refs. 1–7). Unwinding of the secondary structural elements presumably facilitates the binding of the 43S pre-initiation complex to the eIF4-mRNA complex.

Both eIF4E and eIF4G are phosphoproteins (reviewed in Refs. 8 and 9). While the phosphorylation of eIF4G has not been well characterized, the site in eIF4E phosphorylated in vitro and in vivo has been identified as Ser209 (10). eIF4E is phosphorylated at this site in vitro by the mitogen-activated protein kinase-interacting kinases 1 and 2 (Mnk1 and -2) and by protein kinase C (11–15). Phosphorylation of eIF4E and eIF4G is stimulated in vivo by insulin, progesterone, tumor necrosis factor α, interleukin-1β, and phorbol ester (PMA) (15–20). eIF4G is phosphorylated in vitro by protein kinase C, multifunctional S6 kinase, and the p21-activated protein kinase Pak2/γ-PAK (11, 21, 22). Two gene products of eIF4G have been identified, eIF4G1 and -II. Raught et al. (23) have identified three sites that are phosphorylated in response to serum within a putative hinge region (amino acids 1035–1190) in the C terminus of human eIF4G1 and one site (Ser754) in the N terminus. Two unidentified serum-repressed phosphorylation sites in eIF4G were also observed. Tuazon et al. (21) showed the rate of phosphorylation/dephosphorylation of eIF4E is significantly greater in the eIF4E complex than with purified eIF4E, suggesting that regulation of eIF4E by phosphorylation occurs primarily on eIF4F. Phosphorylation of eIF4F by protein kinase C or the eIF4G subunit of eIF4F by multifunctional S6 kinase stimulates translation in a reconstituted protein-synthesizing system dependent on eIF4F (22). However, overall the role of phosphorylation of eIF4E and 4G is not well understood.

Mnk1 and -2 are activated by the MAP kinases Erk1 and -2 and p38 (18, 19). Activation of Mnk occurs upon phosphorylation at two sites, Thr197 and Thr202. An additional residue, Thr332 in mouse Mnk2 and Thr344 in human Mnk1, has been identified as a phosphorylation site for Erk2 (14, 15, 24). Ser22 has also been shown to be phosphorylated in vitro, but the protein kinase has not been identified (24). Support for phosphorylation of eIF4E by Mnk1 and -2 in vivo comes from studies utilizing kinase-inactive and constitutively active mutants of Mnk1 (15). Kinase-inactive mutants of Mnk1 expressed in 293 cells inhibit the mitogen-induced phosphorylation of eIF4E, while expression of active Mnk1 increases the basal level of eIF4E phosphorylation. Additional support comes from studies using the pharmacological inhibitor PD98059 that specifically inhibits MEK activation and SB203580 a specific

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§ The abbreviations used are: eIF, eukaryotic initiation factor; Mnk1 and -2, mitogen-activated protein kinase-interacting kinases 1 and 2; Erk, extracellular signal-regulated kinase MAPK; GST, glutathione S-transferase; Pak2, p21-activated protein kinase; PMA, phorbol ester; HA, hemagglutinin; GTP-S, guanosine 5'-O-(thiotriphosphate); HPLC, high-performance liquid chromatography.

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Inhibitor of p38. Individually, the inhibitors partially block, and together completely inhibit, PMA-induced phosphorylation of eIF4E in 293T cells (20), suggesting that PMA-induced phosphorylation of eIF4E is mediated indirectly by protein kinase C through the Erk or p38 kinase signaling pathways or via a separate pathway. The N-terminal 23 amino acids of Mnk1 contain a binding site for eIF4G (15, 25). Binding of Mnk1 to eIF4G recruits Mnk1 to eIF4E, leading to enhanced phosphorylation of eIF4E (25).

Pak2 is a ubiquitous isofrom of a family of serine/threonine protein kinases related to the yeast protein kinase Ste20 (26–29). Pak2 activity is stimulated in response to various forms of moderate stress, including ionizing radiation, DNA damaging agents, hyperosmolarity, heat shock, and serum starvation following activation and binding of the small G-protein Cdc42(GTP) reviewed in Ref. 29. In response to UV light, high doses of ionizing radiation, and Fas-ligand, Pak2 is constitutively activated during early apoptosis via cleavage by caspase 3 (30, 31) and functions to promote apoptosis (32). Pak2 is involved in the induction of cytostasis as shown by expression of Pak2 in mammalian cells (33) and by injection of active Pak2 into one blastomere of two-cell frog embryos resulting in cleavage arrest of Pak2 in mammalian cells (33) and by injection of active Pak2 into one blastomere of two-cell frog embryos resulting in cleavage arrest at mitotic metaphase (34). This induction is due in part to phosphorylation of c-Myc by Pak2, which reduces transcription of growth related genes (35). Pak2 also inhibits translation when added to rabbit reticulocyte lysate or when transfected into 293T cells. Pak2 has been shown to phosphorylate two subunits of eIF3, eIF4B and eIF4G (11). Thus, we examined whether Mnk1, which is activated by the stress-activated kinase p38, could be a substrate for Pak2.

In these studies, mouse Mnk1 was phosphorylated by caspase 3 cleaved Pak2 but not by Cdc42-activated Pak2. Two phosphorylation sites for Pak2 were identified, Ser27 and Thr28. Phosphorylation of Mnk1 by Pak2 was independent of the phosphorylation and activation by Erk2. Mnk1 phosphorylated eIF4E alone and both the 4E and 4G subunits in eIF4F. Phosphorylation of Mnk1 by Pak2 did not alter phosphorylation of eIF4F but decreased the phosphorylation of eIF4G, which resulted from reduced binding of Mnk1 to eIF4F. Phosphorylation of Mnk1 by Pak2 was observed in 293T cells under apoptotic conditions, where Pak2 was cleaved and activated by caspase 3.

EXPERIMENTAL PROCEDURES

Materials—Active Erk2 was purchased from Alexis Biochemicals. Factor Xa and S-protein-agarose were from Novagen. Trypsin (1-tosylamido-2-phenethyl chloromethyl ketone-treated) was from Sigma. Thin-layer chromatography sheets without fluorescent indicator were from Whatman. Soybean trypsin inhibitor (0.04 mg/ml) was added to the supernatant of SDS-sample buffer containing 10 mM ATP, EDTA, and EGTA. Following SDS-PAGE on 10% gels, Mnk1 was detected by ECL following Western blotting with anti-GST antibodies conjugated to horseradish peroxidase.

Expression, Purification, and Phosphorylation of Mnk1—Mnk1 was expressed in TN5B-4 insect cells, purified, and activated by cleavage with caspase 3 for 30 min, followed by autophosphorylation, as described (36). Alternatively, Pak2 was activated by Cdc42(GTP-S) (33). Phosphorylation of Mnk1 by Erk2 or Pak2 was carried out in buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 30 mM β-mercaptoethanol, and 0.2 mM [γ-32P]ATP (specific activity: 500–1000 cpm/pmol) at 30 °C for the times indicated. Mnk1/GST-Mnk1 (1 μg) was phosphorylated with 0.1 μg of Pak2 or Erk2 in 25 μl, unless otherwise indicated. Reactions were terminated by the addition of 25 μl of SDS-sample buffer containing 10 mM ATP, EDTA, and EGTA. Following SDS-PAGE on 10% gels and staining with Coomassie Blue, the proteins were visualized by phosphorimaging or autoradiography. To determine the Kmax and Vmax values, GST-Mnk1 (0.25–5 μg) was phosphorylated by 0.08 μg of GST-Pak2 for 10 min at 30 °C under the conditions described for phosphorylation of Mnk1.

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Mnk1 (2 μg) and GST-Mnk1 (20 μg) phosphorylated by Pak2 were subjected to tryptic digestion as described previously (11). Following lyophilization, the samples were resuspended in 20 μl of water, and 1–2 μl was removed for phosphoamino acid analysis (11). The remainder of the protein was analyzed by two-dimensional phosphopeptide mapping on thin-layer cellulose plates (11). The radiolabeled peptides visualized by autoradiography were scraped from the plates, eluted twice with 100 μl of 0.1% trifluoroacetic acid, lyophilized, and 1% trifluoroacetic acid. The eluates were pooled and lyophilized, and the phosphopeptides were used directly for manual and automated sequencing.

Phosphopeptide 1 was purified further by reverse phase HPLC using a Waters Delta-Pak C18 narrow bore reverse phase column (2.1 mm × 150 mm) running on a Waters LC625 HPLC system. Samples (500 μl) were loaded onto the column equilibrated in 98% solvent A (0.06% trifluoroacetic acid in water) and 2% solvent B (80% acetonitrile, 0.052% trifluoroacetic acid in water) at a flow rate of 0.2 ml/min, and the peptides were eluted with a three-step linear gradient. Elution was monitored at 210 nm, and radioactivity was quantified by Cerenkov counting. Fractions containing radioactivity were concentrated to less than 50 μl in a SpeedVac.

Manual and Automated Sequencing—Radiolabeled phosphopeptides obtained after peptide mapping or HPLC were brought to 65% acetonitrile and 0.1% trifluoroacetic acid in 20 μl, covalently attached to SequeIon-AA membrane discs, and subjected to manual Edman degradation as described (39). The membrane and the dried trifluoroacetic acid extracts were monitored for 32P release by Cerenkov counting after each cycle. Duplicate samples were subjected to automated microsequencing on a 492 Procise Sequencer.

Binding of Mnk1 to IF4E Peptides—Peptides of human eIF4E fused to the S-peptide of Rnase A, thioredoxin, and His6 tags were expressed from E. coli and purified as described (40). The samples described for these experiments, S-eIF4E (877–1078), S-4G (1078–1560), and S-4G (1317–1456) were incubated with GST-Mnk1 in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, 2 mM 2-mercaptoethanol, 0.1% Tween 20, and 2 mM EDTA, in a volume of 20 μl on ice for 1 h. Following incubation, 60 μl of buffer containing 1% nonfat milk and 20 μl of S-4G agarose were added, and the samples were incubated overnight at 4 °C. The beads were washed four times with buffer containing milk and twice with buffer, and the bound proteins were analyzed by SDS-PAGE on 10% gels. Mnk1 was detected by ECL following Western blotting with anti-GST antibodies conjugated to horseradish peroxidase.

Phosphorylation of GST-Mnk1 in 293T Cells—HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 10-cm plates to ~20–25% confluence. The cells were transfected with wild type GST-Mnk1 (1 μg), HA-tagged Pak2 (2.5 μg), and constitutively active HA-Cdc42L61 (1 μg) using FuGENE 6.

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reagent. The control was transfected with GST-Mnk1 kinase-inactive T402A and dominant-negative HA-Cdc42N17. After incubation for 36 h, cells were washed with phosphate-free Dulbecco's modified Eagle's medium supplemented with dialyzed 10% fetal bovine serum and radiolabeled for 4 h in the same medium with 1.5 mCi of \(^{32}\)P orthophosphate in a total volume of 3.5 ml. To induce moderate stress, cells were stressed with 0.4 M sorbitol for 30 min after 3.5 h of labeling. For induction of apoptosis, 2 mM H\(_2\)O\(_2\) was added at the beginning of the labeling. After 4 h, the cells were collected and stored at \(-80^\circ\)C.

Cells from one dish were thawed in 0.5 ml of lysis buffer containing 50 mM HEPES, pH 7.5, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1% Nonidet P-40, phosphatase inhibitors (20 mM NaF, 5 mM Na\(_3\)P\(_2\)O\(_7\), 2 mM Na\(_2\)VO\(_4\), 10 mM okadaic acid), and protease inhibitors (20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml pepstatin, 0.5 mM phenylmethanesulfonfyl fluoride). After 25 min on ice, the cell lysate was centrifuged at 16,000 × g for 15 min at 4 ºC. GST-Mnk1 was purified using 50 μl of glutathione-Sepharose 4B at 4 ºC for 1 h and analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Phosphorylation of Mnk1 by Pak2—To determine whether Mnk1 was a substrate for Pak2, GST-Mnk1 was examined using Pak2 activated by cleavage with caspase 3 or by binding of Cdc42(GTP\(_{\gamma}\)S). GST-Mnk1 was incubated with \([\gamma^{32}\P\)ATP in the presence and absence of Pak2 and analyzed by SDS-PAGE; GST-Mnk1 was readily phosphorylated by caspase-cleaved Pak2 but was not a substrate for Pak2 activated by Cdc42 (Fig. 1A). The GST fusion protein of Mnk1 migrated at a molecular mass of 72 kDa, while Mnk1 migrated at 49 kDa (Fig. 1B, left panel). Mnk1 and GST-Mnk1 were readily phosphorylated by Pak2 (Fig. 1B, right panel). No phosphorylation was observed in the absence of Pak2.

Phosphorylation of Mnk1 was quantified using increasing concentrations of Pak2 activated by caspase 3 (Fig. 1C). Maximal phosphorylation was obtained at an enzyme to substrate molar ratio of 1:6, yielding about 1.5 mol of \(^{32}\)P incorporated per mol of GST-Mnk1 following incubation for 1 h. When the molar ratio was reduced to 1:15, the amount of \(^{32}\)P incorporated was reduced to 1.0 mol/mol. Analysis of the double reciprocal Lineweaver-Burk plot (data not shown) yielded a \(K_m\) value for GST-Mnk1 of 0.60 μM and a \(V_{max}\) value of 14.9 pmol/min/μg of Pak2.

Identification of the Sites in Mnk1 Phosphorylated by Pak2—Tryptic phosphopeptides from Mnk1 phosphorylated by Pak2 were separated by two-dimensional phosphopeptide mapping and visualized by autoradiography. Three major phosphopeptides were observed with GST-Mnk1 (Fig. 2A). Mnk1 alone yielded two major phosphopeptides corresponding to peptides 1 and 2 of GST-Mnk1 (Fig. 2B), indicating that phosphopeptide 3 originated from GST or the GST-linker region. Phosphoamino-
acid analysis showed peptide 1 contained phosphoserine, peptide 2 contained phosphothreonine (Fig. 2C), and peptide 3 contained both phosphoaminoacids (data not shown).

Phosphopeptide 1, purified further by reverse phase HPLC, yielded a major peak containing 87% of the radioactivity, which was collected, concentrated, and subjected to manual Edman degradation and automated amino acid sequencing (Fig. 3A). Microsequence analysis of peptide 1 yielded the sequence ATDXLPG, corresponding to amino acids 24–30 of mouse Mnk. Parallel manual Edman degradation released $^{32}$P exclusively at cycle 4. Thus $X$ was the phosphoserine at position 27 (Ser27) in mouse Mnk1.

Phosphopeptide 2 could not be purified further by HPLC, as it was not retained on the C-18 column (data not shown). Edman degradation of peptide 2 released $^{32}$P exclusively at cycle 3 (Fig. 3B). Given the specificity of trypsin, there was only one possible threonine that could yield release of $^{32}$P at cycle number 3, Thr$^{22}$ in the sequence RKTR, that corresponded to amino acids 20–23 of mouse Mnk. The assignment of Thr$^{22}$ as a phosphorylation site for Pak2 was consistent with CNBr cleavage data, which yielded a single phosphopeptide corresponding to the N terminus of Mnk1 starting from amino acid 2 (data not shown). Additional support for the identification came from analysis of human Mnk1, which contained a glycine (Gly$^{24}$) at the position corresponding to Thr$^{22}$. With human Mnk1 phosphorylated by Pak2, only phosphopeptide 1 was detected by two-dimensional phosphopeptide mapping (data not shown). Thus mouse Mnk1 was phosphorylated by Pak2 on Thr$^{22}$ and Ser$^{27}$. The phosphorylation sites were located in an extremely basic region important for binding to eIF4G (15).

Phosphorylation of Mnk1 by Pak2 and Analysis of Mnk1 Activity—To determine whether phosphorylation of Mnk1 by Pak2 and Erk2 were mutually exclusive, Mnk1 was phosphorylated with Erk2 or Pak2 under conditions where approximately 1 mol of $^{32}$P was incorporated per mol of Mnk1. The extent of phosphorylation of Mnk1 by Pak2 and Erk2 was similar at 90 min with about 25% more $^{32}$P incorporated into Mnk1 by Pak2, as compared with Erk2 (Fig. 4). Sequential addition of Pak2 following removal of Erk2, or Erk2 following removal of Pak2, showed that phosphorylation was additive and independent of the order of phosphorylation.

To analyze the effects of phosphorylation on Mnk1, a recombinant form of eIF4E (NΔ27–4E) was used as substrate. NΔ27–4E was incubated with nonphosphorylated Mnk1 or Mnk1 phosphorylated by Pak2 and Erk2 alone or in combination as described above and analyzed by SDS-PAGE; the radiolabel was detected by phosphorimaging (Fig. 5, upper panels). Mnk1 activated by Erk2 phosphorylated eIF4E, while Mnk1 phosphorylated by Pak2 had no activity, as indicated by a lack of phosphorylation of eIF4E. Sequential phosphorylation of Mnk1 showed that Pak2 did not appreciably alter the activity of Mnk1 toward eIF4E, as compared with Erk2 alone, regardless of the order of phosphorylation (Fig. 5, lower panel).

It has been shown that eIF4E phosphorylation is regulated when bound to eIF4G (21, 25); therefore the effects of phosphorylation by Pak2 on the activity of Mnk1 toward eIF4E as part of eIF4F were analyzed. As with NΔ27–4E, nonphosphorylated Mnk1 and Mnk1 phosphorylated by Pak2 did not phosphoryl-
ate eIF4E (Fig. 6, left panel). Similar to the data shown in Fig. 5, sequential phosphorylation of Mnk1 did not alter the activity of Mnk1 toward eIF4E. The 4G subunit of eIF4F was also phosphorylated by active Mnk1, while nonphosphorylated Mnk1 and Mnk1 phosphorylated by Pak2 showed no activity toward eIF4G (Fig. 6, right panel). The total amount of phosphate incorporated into eIF4G was similar to that of eIF4E. In contrast to the results obtained with eIF4E, a 50% reduction in the phosphorylation of eIF4G was observed when Mnk1 was phosphorylated by both Erk2 and Pak2. The reduction in phosphorylation of eIF4G was observed regardless of the order of phosphorylation. These data indicated that phosphorylation of active Mnk1 by Pak2 did not affect phosphorylation of eIF4E or the 4E subunit of eIF4F but inhibited phosphorylation of the 4G subunit of eIF4F.

Binding of Mnk1 to eIF4G—The N-terminal 23 amino acids of Mnk1 have been determined to be essential for binding of Mnk1 to eIF4G (15, 25), while the binding site on Mnk1 for eIF4G has been localized to the C-terminal 200 amino acids (25). Recombinant peptides of eIF4G fused to an NH2-terminal tag containing the S-peptide of RNase A (40) were utilized to examine the effects of phosphorylation on binding of Mnk1 to eIF4G. Nonphosphorylated GST-Mnk1 (no phosphorylation by Erk2 or Pak2) was incubated with two S-tagged eIF4G peptides; S-4G-(877–1078) was outside the Mnk1 binding region, while S-4G-(1317–1560) contained the Mnk1 binding region (Fig. 7A). S-4G-(1317–1560) did bind to Mnk1, whereas S-4G-(1078–1560) did not bind to Mnk1 (Fig. 7B). When Mnk1 was phosphorylated by Pak2, there was a significant decrease (47%) in binding of Mnk1 to the peptide. Phosphorylation of the S-4G-(1078–1560) peptide alone by Pak2 had no effect on Mnk1 binding. When both Mnk1 and the

FIG. 3. Manual and automated sequencing of Mnk1 phosphopeptides. A, phosphopeptide 1 was subjected to Edman degradation and microsequence analysis as described under “Experimental Procedures.” The sequence identified by automated microsequencing and the corresponding Mnk1 sequence are shown. B, the sequence shown for phosphopeptide 2 by Edman degradation is the only one possible, as described under “Results.”

FIG. 4. Sequential Phosphorylation of Mnk1 by Erk2 and Pak2. GST-Mnk1 was phosphorylated by Erk2, by Pak2, or by sequential addition of Erk2 and Pak2, as described under “Experimental Procedures.” The beads were washed between incubations to remove the first protein kinase. Following SDS-PAGE, the radiolabel in Mnk1 was visualized and quantified by phosphorimaging. Phosphorylation of Mnk1 by Erk2 was set at 100%.

FIG. 5. Phosphorylation of eIF4E (N27–4E) by differentially phosphorylated Mnk1. The activity of Mnk1 following phosphorylation by Erk2, Pak2, or both protein kinases, as indicated in the legend to Fig. 4, was analyzed with recombinant eIF4E (N27–4E). Following SDS-PAGE, eIF4E was stained with Coomassie blue (Stain) and the radiolabel was visualized and quantified (Image). Phosphorylation of 4E by Mnk1 phosphorylated by Erk2 was set at 100%.

Phosphorylation of Mnk1 by Pak2
peptide were phosphorylated by Pak2 the decrease in binding was similar to that observed when only Mnk1 was phosphorylated by Pak2 (data not shown).

Phosphorylation of Mnk1 in 293T Cells—293T cells expressing GST-Mnk1, Pak2, and Cdc42 were incubated with $^{32}$P, and GST-Mnk1 was purified on glutathione-Sepharose and analyzed by SDS-PAGE. The sites of phosphorylation were examined by tryptic phosphopeptide mapping and phosphorimaging and compared with GST-Mnk1 phosphorylated in vitro by purified Pak2. With kinase-inactive Pak2 T402A and dominant-negative Cdc42N17, two major phosphopeptides were observed; one migrated at the position of Ser27, and the other phosphopeptide was not observed in vitro (Fig. 8A). With wild type Pak2 and constitutively activated Cdc42L61 alone, and following subjection to hyperosmolarity (conditions where Pak2 was activated), similar data were obtained (Fig. 8, B and C). However, when cells were subjected to treatment with H$_2$O$_2$, which induced apoptosis and activated caspase 3, a phosphopeptide that migrated toward the anode was identified as Thr22 (Fig. 8, D–F). Thus, under conditions of apoptosis, Thr22 was shown to be a site of phosphorylation on Mnk1. This coincides with data obtained in vitro wherein caspase-cleaved Pak2 phosphorylated Mnk1, while Cdc42-activated Pak2 did not.

**DISCUSSION**

In general, there is a positive correlation between the growth status of the cell, the phosphorylation state of eIF4F, and the rate of translation in dividing cells (reviewed in Refs. 6, 8, and 9). In contrast, less experimentation has been conducted on the effects of phosphorylation on protein synthesis in stressed, nondividing cells and during early apoptosis. Overall phosphorylation is significantly decreased under conditions of stress. Serum deprivation reduces translation and phosphorylation of eIF4B, ribosomal protein S6, and elongation factor 1 (41–43). Incubation of cells at elevated temperatures (43–44°C) for 20 min causes an inhibition of translation with concomitant dephosphorylation of eIF4B and eIF4E (44–46) and increased binding of 4E-BP1 to eIF4E (47). During early apoptosis a number of factors involved in translation are cleaved by caspase 3 (48–50).

In the studies herein, phosphorylation of Mnk by Pak2, a protein kinase involved in both stress and apoptosis, and the effects of phosphorylation on Mnk function were examined. A schematic representation of Mnk1 is shown in Fig. 9, in which

**FIG. 6. Phosphorylation of eIF4F by differenntially phosphorylated Mnk1.** Phosphorylation of eIF4F by Mnk1 phosphorylated by Erk2, Pak2, or both protein kinases was analyzed. Following SDS-PAGE, eIF4F was stained with Coomassie blue and the $^{32}$P was visualized and quantified by phosphorimaging. Left panel, Coomassie stain (Stain) and phosphorimage (Image) of the radiolabeled 4E of eIF4F. Right panel, the phosphorylated 4G subunit of eIF4F. Quantification of phosphorylation is shown graphically in the lower panel. Incorporation of $^{32}$P into eIF4E and eIF4G by Mnk1 phosphorylated by Erk2 was set at 100%.

**FIG. 7. Binding of Mnk1 to eIF4G.** A, schematic representation of human eIF4GI and the eIF4G peptides. PABP, poly(A)-binding protein. B, phosphorylated and nonphosphorylated GST-Mnk1 (1 μg) were incubated with the peptides indicated; the protein complexes were isolated on S-protein-agarose, separated by SDS-PAGE, and transferred to nitrocellulose. Ponceau S staining (Stain) and Western blotting of GST-Mnk1 by anti-GST antibody (Blot) are shown. +, phosphorylated protein; P, phosphorylated protein.

peptide were phosphorylated by Pak2 the decrease in binding was similar to that observed when only Mnk1 was phosphorylated by Pak2 (data not shown).
The identified sites phosphorylated by Pak2 are depicted in relation to those of Erk/p38. The two sites phosphorylated by Pak2, Thr22 and Ser27, are in a region of Mnk1 that binds the C terminus of 4G (15). Thr22 is adjacent to a basic cluster of amino acids (14KRRKKRKT22), which fit the recognition/phosphorylation sequence previously identified for substrates of Pak2 (KRXST) (51). This region is a nuclear localization sequence, and Mnk1 has been identified in the nucleus (52).

The second phosphorylation site does not fit the consensus site, but basic residues upstream can provide an alternative recognition sequence for phosphorylation at Ser27. An examination of the sequences of human and *Xenopus* Mnk1, mouse Mnk2, and human Mnk2a and 2b reveals that the serine at position 27 in the mouse Mnk1 homologue is conserved in all these species. This could indicate the importance of this serine and its phosphorylation state in regulating binding to eIF4G. In contrast, the threonine at position 22 is found only in the mouse and *Xenopus* homologues of Mnk1. Similar sequences in mouse Mnk2 (17RKKKRC22RATD34SF) and human Mnk2a and -b (28KKKRRG34RATDSL) contain the equivalent of Ser27 in human Mnk1, while the Thr22 site contains either glycine or cysteine.

In 293T cells, phosphorylation of Mnk1 on Ser27 is observed under conditions where Pak2 is inactive. Activation of Pak2 by Cdc42 in response to sorbitol leads to an overall enhancement of phosphorylation of Mnk1 at several sites, including Ser27, but Thr22 is not phosphorylated. In contrast, induction of apoptosis leads to phosphorylation of both Ser27 and Thr22 (Fig. 8). The ratio of phosphorylation of these sites in vivo is similar to that seen in vitro with caspase-cleaved Pak2. Since Mnk1 is

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In serum-stimulated cells, both eIF4E and eIF4G are phosphorylated (17, 23). Three serine residues in eIF4G are identified as serum-stimulated sites, 1108, 1148, and 1192 (23), while phosphorylation at two other sites is repressed by serum. Mnk1 is phosphorylated and activated by Erk under conditions of growth, and by p38 under conditions of stress, and appears to be the major protein kinase responsible for phosphorylation of eIF4E (13–15). The protein kinases responsible for phosphorylation of eIF4G in vivo have not been identified; however, regulation of phosphorylation of the serum-stimulated serine residues in eIF4G is responsive to phosphatidylinositol 3-kinase and FRAP/mTOR signaling, as shown with the protein kinase inhibitors wortmannin, LY294002 and rapamycin (23).

Phosphorylation of eIF4E by Mnk1 occurs when Mnk1 is associated with eIF4F; however, Mnk1 can phosphorylate eIF4E alone, although less efficiently (Ref. 25; data not shown). In vivo, there is a decrease in phosphorylation of eIF4E when eIF4E mutants lacking the Mnk1 binding region are used (25). Scheper et al. (24) showed that activation of Mnk1 by PMA (via stimulation of Erk, as shown in 293 cells using inhibitors), results in decreased binding of Mnk1 and to a lesser extent Mnk2 to eIF4F. Five phosphorylation sites on Mnk2 were identified in vivo in response to PMA, serines 27, 384, 387, 399, and Thr403. Ser387 and Thr403 are adjacent to a proline and would be phosphorylated by a proline-directed kinase, while Ser27 could be phosphorylated by Pak2.

A schematic for the regulation of phosphorylation of eIF4F by Mnk through Erk/p38 or through Pak2 is presented in Fig. 10. The left side of the figure shows the activation of Mnk1 by Erk/p38 and binding of Mnk to the 4G subunit of eIF4F, with the subsequent phosphorylation of eIF4E and eIF4G. Upon activation of the Pak2 (right side) there would be an increase in the phosphorylation of Ser27 in Mnk2 and Ser27 and Thr422 in Mnk1, with a concomitant decrease in the association of Mnk1 with eIF4G. This would be a rapid and effective mechanism to alter translation in conjunction with the cleavage of eIF4G by caspase 3.

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Fig. 10. Model for the regulation of eIF4F by phosphorylation of Mnk1.
The model shows Erk/p38-mediated phosphorylation of eIF4F by Mnk1 (left) and Pak2-mediated phosphorylation of Mnk1 (right). A detailed description of the model is presented under “Discussion.”

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