pp54 Microtubule-associated Protein-2 Kinase Requires Both Tyrosine and Serine/Threonine Phosphorylation for Activity*

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John M. Kyriakias‡‡, David L. Brautigan‖, Thomas S. Ingber‡‡†, and Joseph Avruch‡‡†

From the ‡Diabetes Unit and Medical Services, Massachusetts General Hospital and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114, the †Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, and the ‡Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011.

pp54 microtubule-associated protein-2 (MAP-2) kinase, a recently discovered protein serine/threonine kinase (Kyriakias, J., and Avruch, J. (1990) J. Biol. Chem. 265, 17355-17363), is shown to contain immunoreactive phosphotyrosine residues. Treatment with recombinant rat brain protein tyrosine phosphatase-1 deactivates pp54 MAP-2 kinase, concomitant with the removal of phosphorytosine residues. Protein (serine/threonine) phosphatase-1 also deactivates pp54 MAP-2 kinase in a specific fashion. pp54 MAP-2 kinase joins pp42 MAP-2 kinase and cdc2/matruration-promoting factor as one of only three serine/threonine protein kinases known to be regulated by phosphorylation at both tyrosine and, independently, at serine/threonine residues. In view of these shared regulatory properties, a role for pp54 MAP-2 kinase in the control of cell division is likely.

A number of Ser/Thr protein kinases are activated by mitogens which bind to receptor tyrosine kinases. These include protein kinase C (1), the 60- and 70-kDa S6 protein kinases (2, 3), casein kinase-2 (4), the c-ras protooncogene product (5), and a 42-kDa microtubule-associated protein-2 (MAP-2) kinase (6). The mechanisms which underlie mitogen regulation of these kinases are diverse and largely indirect, e.g. involving intermediate catalysts, such as the phospholipase C-catalyzed accumulation of diacylglycerol (the proximate activator of kinase C), initiated by the PDGF receptor (7). The S6 kinases (2, 3) and probably c-ras (8, 9) are activated by serine/threonine kinases situated upstream in the signal transduction cascade. An example of such an upstream protein kinase appears to be pp54 MAP-2 kinase, which can phosphorylate and partially reactivate the phosphatase-inactivated 85-kDa S6 kinase II purified from Xenopus eggs (10). Although other candidate physiologic substrates have not yet been identified, a significant role for pp42 MAP-2 kinase in the regulation of cell growth seems likely inasmuch as its primary sequence shows considerable homology to two cell cycle control genes from Saccharomyces cerevisiae, FUS3 and KSS1 (11). Moreover, consistent with an upstream location, pp42 MAP-2 kinase is phosphorylated at both Tyr and Thr residues in response to mitogens and phorbol esters (6) and requires both types of phosphorylation for activation (12). This observation suggests that pp42 MAP-2 kinase couples tyrosine kinases to intracellular mitogen-activated Ser/Thr kinases. The only other Ser/Thr protein kinase known to be regulated by phosphorylation of both Ser/Thr and tyrosine residues is the 34-kDa catalytic subunit of cdc2/MFP; this Tyr phosphorylation is inhibitory (13).

pp54 MAP-2 kinase is a recently discovered 54-kDa Ser/Thr protein kinase present in rat liver which is activated 10-fold after cycloheximide administration, in vivo (14). The enzyme is distinct from pp42 MAP-2 kinase based on size, chromatographic properties, and substrate specificity (14). Notably, pp54 MAP-2 kinase does not phosphorylate or reactivate the 85-kDa Xenopus S6 kinase II (14). Nevertheless, pp42 and pp54 MAP-2 kinases do exhibit an overlapping, relatively narrow substrate specificity (MAP-2, myelin basic protein, and histone H1), and, like pp42 MAP-2 kinase (10), pp54 MAP-2 kinase can be completely and specifically inactivated by protein Ser/Thr phosphatase-2A (14).

We now show that pp54 MAP-2 kinase exhibits a further similarity with pp42 MAP-2 kinase (15). Treatment of pp54 MAP-2 kinase with recombinant rat brain protein (rbbP) tyrosine phosphatase-1 specifically and completely inactivates the kinase through selective dephosphorylation of tyrosine residues. Moreover, protein phosphatase-1, which is highly specific for serine/threonine residues, also deactivates pp54 MAP-2 kinase in a selective manner. These results indicate that pp54 MAP-2 kinase, like pp42 MAP-2 kinase, requires both serine/threonine and tyrosine phosphorylation for activity; together with cdc2/MFP, which is respectively regulated by tyrosine phosphorylation, these enzymes exemplify an emerging group of Ser/Thr protein kinases which function in the control of cell growth and require phosphorylation (or dephosphorylation) at both Ser/Thr and Tyr residues concomitantly to express their protein kinase activity.

EXPERIMENTAL PROCEDURES

Immunoprecipitation of pp54 MAP-2 Kinase with Anti-phosphotyrosine Antibodies—pp54 MAP-2 kinase was purified and, where indicated, autophosphorylated in the presence of [γ-32P]ATP as described (14). A high affinity rabbit polyclonal anti-phosphotyrosine antisera (Rat73D-24) was employed (16). For immunoprecipitations, normal or anti-phosphotyrosine antisera were bound to protein A-Sepharose in the presence or absence of 2 mM phosphotyrosine or phosphoserine (20 ml of serum/100 ml of settled bed volume of beads in 20 ml Hepes, pH 7.4, 2 mM EDTA, 2 mM EGTA) by incubating...
for 2 h at 4 °C. The beads were washed 3 times in incubation buffer containing 150 mM NaCl and resuspended in buffer B (20 mM Hepes, pH 7.2, 2 mM EGTA, 5 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100) plus or minus 2 mM phosphotyrosine or 2 mM phosphoserine. Purified, autophosphorylated pp54 MAP-2 kinase, in buffer B, with or without phosphoamino acids, was then added and the mixtures incubated for 4 h at 4 °C. The beads were pelleted by centrifugation and the supernatants removed. The beads were then washed three times in buffer B containing 150 mM NaCl, either plus or minus phosphoamino acids. The immune complexes and the immunosupernatants were then either assayed for MAP-2 kinase as described (14) or brought to 1% SDS, 250 mM sucrose, heated to 100 °C, and subjected to SDS-PAGE. For immunoblotting studies, purified pp54 MAP-2 kinase was transferred to nitrocellulose after SDS-PAGE. The transfers were probed with anti-phosphotyrosine antibodies (1:500 dilution) either plus or minus 2 mM phosphotyrosine and counterstained with 125I-protein A.

Treatment of pp54 MAP-2 Kinase with Recombinant Rat Brain rrbP Tyrosine Phosphatase-1—rrbP tyrosine phosphatase-1 is a truncated, recombinant form of rat brain protein tyrosine phosphatase-1, produced by replacing the Lys-323 codon with a stop codon and expressing the construct in Escherichia coli behind the bacteriophage T7 promoter (17). The recombinant enzyme retained the entire catalytic domain and was purified to homogeneity by a two-step procedure that employed chromatography on DEAE-cellulose followed by affinity chromatography on thiophosphorylated reduced carboxymethylated-lysozyme-Sepharose. Details of the purification and characterization of the enzyme will be published elsewhere. Purified pp54 MAP-2 kinase (0.2 unit, where 1 unit will transfer 1 pmol of phosphate/min to MAP-2 (14)) was incubated in assay buffer (50 mM Tris-HCl, pH 7.0, 250 mM EDTA, 0.1% β-mercaptoethanol, 0.01% Brij-35, 0.5 mg/ml crystallized, lyophilized bovine serum albumin) with rrbP tyrosine phosphatase-1 at the indicated concentrations (1 unit of rrbP tyrosine phosphatase-1 will remove 1 nmol of phosphotyrosine per minute at 30 °C) either in the presence or absence of 1 mM Na₃VO₄. The phosphatase reaction was stopped by the addition of NaF (20 mM), and aliquots were taken for assay of MAP-2 kinase activity and/or anti-phosphotyrosine immunoblot (14). The preparation of phosphatase-1 employed in these studies was virtually devoid of phosphotyrosine phosphatase activity inasmuch as the dephosphorylation of 32P-Tyr-reduced carboxymethylated lysozyme was catalyzed at rate equivalent to 1/150,000 that observed for 32P-Ser phosphorylase a (data not shown).

RESULTS AND DISCUSSION

A high affinity polyclonal anti-phosphotyrosine antiserum immunoprecipitates purified pp54 MAP-2 kinase (Fig. 1A) in a specific manner and in an active form (Fig. 1A, left), indicating that the kinase contains immunoreactive phosphotyrosine. Preadsorption of the serum with phosphotyrosine completely prevents immunoprecipitation, and the active kinase remains in the supernatant (as with normal serum), whereas preadsorption with phosphoserine does not prevent immunoprecipitation. The purified kinase polypeptide, 32P-labeled by autophosphorylation (Fig. 1B) by anti-phosphotyrosine antibodies in a reaction blocked by phosphotyrosine. Finally, the anti-phosphotyrosine antibodies immunoblot a 54-kDa polypeptide corresponding to purified pp54 MAP-2 kinase (Fig. 1C), a reaction that is prevented by phosphotyrosine.

The importance of these immunoreactive phosphotyrosine residues in the regulation of pp54 MAP-2 kinase activity was explored using a highly purified recombinant, truncated rrbP tyrosine phosphatase-1 (17). Treatment with this Tyr-specific phosphatase gave, in a concentration-dependent fashion, essentially complete deactivation of pp54 MAP-2 kinase activity (Fig. 2A). This rrbP tyrosine phosphatase-1-mediated kinase deactivation was prevented by inclusion of Na₃VO₄ during the phosphatase incubation.
Regulation of pp54 MAP-2 Kinase

Fig. 2. Inactivation of pp54 MAP-2 kinase with rrbP tyrosine phosphatase-1. A, pp54 MAP-2 kinase (0.2 unit) was treated with various concentrations of rrbP tyrosine phosphatase-1 either in the presence (closed symbols) or absence (open symbols) of 1 mM vanadate. Each point is the mean of duplicate samples which varied 10% or less. Two experiments were performed yielding essentially identical results. rrbPTP-1, recombinant rat brain protein tyrosine phosphatase-1. B, pp54 MAP-2 kinase (0.6 unit) was autophosphorylated with [γ-32P]ATP (14) and treated with vehicle (open bars), rrbP tyrosine phosphatase-1 (600 units/ml) (filled bars), or rrbP tyrosine phosphatase-1 (600 units/ml) plus 1 mM Na3V04 (hatched bars). Aliquots were removed for MAP-2 kinase assay (Activity) (14). Additional aliquots were subjected directly to SDS-PAGE (32-P pp54 polypeptide: Total) or to immunoprecipitation with anti-phosphotyrosine antibodies, followed by SDS-PAGE (32-P pp54 polypeptide: anti P-tyr I.P.). Estimates of MAP-2 kinase activity as well as 32P incorporated into MAP-2 were quantitated by liquid scintillation counting of the cognate 32P-labeled polypeptide, excised after autoradiography. Means for duplicate samples are shown. Two experiments were performed yielding essentially the same results. C, the presence with various concentrations of rrbP tyrosine phosphatase-1 either in the pp54 MAP-2 kinase polypeptide were quantitated by liquid scintillation counting of the cognate 32P-labeled polypeptide, excised after autoradiography. Means for duplicate samples are shown. Two experiments were performed yielding essentially the same results. B, effect of protein phosphatase-1 on pp54 MAP-2 kinase phosphotyrosine immunoreactivity. Approximately 0.5 unit of pp54 MAP-2 kinase was treated with vehicle (open bar), 75 units/ml protein phosphatase-1 (filled bar), or 75 units/ml protein phosphatase-1 + 10 mM NaF (hatched bar) for 60 min at 30°C. Reactions were stopped with 10 mM NaF, and fractions assayed (left) or subjected to immunoblotting with anti-phosphotyrosine (Anti P-Tyr) antibodies as described (right). and Ser residues, at sites separate from those which regulate activity, inasmuch as autophosphorylation has no effect on pp54 MAP-2 kinase activity (14). As such, 32P incorporated via autophosphorylation can serve as a useful marker for confirming the specificity of the rrbP tyrosine phosphatase-1 treatment for phosphotyrosine and the absence of significant proteolysis. rrbP tyrosine phosphatase-1 treatment failed to remove 32P which was incorporated during autophosphorylation (Fig. 2B, right, and 2C). However, concomitant with deactivation of kinase activity (Fig. 2B, left), protein phosphatase-1B treatment of pp54 MAP-2 kinase resulted in a substantial loss of phosphotyrosine immunoreactivity, as indicated by the decreased ability of the anti-phosphotyrosine antibodies to precipitate the rrbP tyrosine phosphatase-1-treated pp54 polypeptide (Fig. 2B, center, and 2C). Thus, rrbP tyrosine phosphatase 1 treatment deactivates pp54 MAP-2 kinase with an accompanying loss of phosphotyrosine and without apparent Ser/Thr dephosphorylation (Fig. 2, B and C). These findings show that the immunoreactive phosphotyrosine residues detected in pp54 MAP-2 kinase (Fig. 1) include residues critical to kinase activity.

The susceptibility of pp54 MAP-2 kinase to deactivation shown is a representative autoradiograph of pp54 MAP-2 kinase, autophosphorylated, treated with protein phosphatase-1B and immunoprecipitated, from the experiment performed in B. PTP-1, protein tyrosine phosphatase-1.
by protein phosphatase-2A observed previously (14) was interpreted to reflect hydrolysis of phosphoserine and/or phosphothreonine residues critical to kinase activity. Protein phosphatase-2A, however, can catalyze tyrosine dephosphorylation under certain conditions (19). In view of the effects of Tyr dephosphorylation on pp54 MAP-2 kinase activity, it appeared necessary to reexamine the regulatory role of phosphoserine/phosphothreonine. This was accomplished using protein phosphatase-1 which is highly specific for these two phosphoamino acids (see "Experimental Procedures"). Protein phosphatase-1 deactivated pp54 MAP-2 kinase extensively in a concentration-dependent fashion; deactivation was prevented by inclusion of either okadaic acid, protein phosphatase inhibitor-2 (Fig. 3A), or NaF (Fig. 3B). Protein phosphatase-1 treatment did not diminish the pp54 MAP-2 kinase phosphotyrosine content as judged by immunoblot (Fig. 3B) confirming that the deactivation by protein phosphatase-1 is due to selective dephosphorylation of phosphoserine and/or phosphothreonine residues on the kinase. Thus, selective hydrolysis of either phosphotyrosine or phosphoserine/phosphothreonine residues leads to inactivation of pp54 MAP-2 kinase.

Two well characterized protein Ser/Thr kinases, pp42 MAP kinase and cdc2/MPF, have previously been shown to be regulated by phosphorylation at both Ser/Thr as well as Tyr residues (the regulation of c-raf by Tyr (5) phosphorylation in vivo is presently unclear). pp42 MAP-2 kinase is activated in response to a variety of mitogens, whereas cdc2/MPF controls progression through the cell cycle, and in fission yeast is required for transit both from GI to S and from Gz to M. Based on its similar regulatory properties and especially its regulation by tyrosine phosphorylation, a role for pp54 MAP-2 kinase in growth control is inferred. The identity of the upstream regulators of cdc2, pp42 MAP-2 kinase, and pp54 MAP-2 kinase is of considerable interest. Mitogens like PDGF and epidermal growth factor, which act through receptor kinases, and through receptor protein kinases absolutely specific for tyrosine residues, tyrosine-specific kinases), followed by an intramolecular autophosphorylation of the MAP-2 kinase, by phosphorylation of enzyme tyrosine residues (either directly or through recruited cellular tyrosine-specific kinases), followed by an intramolecular autophosphorylation of the MAP-2 kinase on Ser/Thr residues, which transforms the MAP-2 kinase to a conformation active on protein substrates. Alternatively, epidermal growth factor and PDGF activate multiple Ser/Thr kinases, such as kinase C, casein kinase-II, c-ras, etc., one of which might catalyze the phosphorylation of the Ser/Thr residues crucial to the activation of pp42 and pp54 MAP-2 kinase. An entirely novel mechanism, however, is suggested by the recent finding that the protein product of the wee1 gene, a protein kinase which is a negative regulator of cdc2 kinase in fission yeast, is able to catalyze its autophosphorylation on both tyrosine and serine residues (20), as well as the phosphorylation of the model peptide substrate, angiotensin II, on tyrosine residues (20). Thus, the wee1+ gene product is apparently capable of phosphorylating both Ser/Thr and Tyr residues. Such dual specificity has also been attributed to three protein kinases, Clk (21), Spk1 (22), and STY (23), whose activity was characterized after cloning and expression of the enzyme in prokaryotic hosts. Thus, the upstream regulator of the pp54 and pp42 MAP kinases may include protein kinases with such dual specificity. In recent experiments, we have detected an enzyme activity in soluble extracts of HeLa cells, capable of reactivating the phosphotyrosine phosphatase-deactivated pp54 MAP-2 kinase. Further studies of the molecular structure, proximate upstream activators, and cellular targets of the pp54 MAP-2 kinase are being actively pursued.

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REFERENCES