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Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase

(phorbol ester/epidermal growth factor/anti-phosphotyrosine antibody)

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ABSTRACT **pp42, a low-abundance 42-kDa protein, becomes transiently phosphorylated on tyrosine after stimulation of fibroblasts by a variety of mitogens, including epidermal growth factor, platelet-derived growth factor, phorbol 12-myristate 13-acetate, thrombin, and insulin-like growth factor II. The induction of pp42 phosphorylation on tyrosine by such diverse mitogenic agents suggests an important role for pp42 in the cascade of events necessary for cell transition from G_0 into the cell cycle. However, as with most proteins identified on the basis of their tyrosine phosphorylation, the function of pp42 in cellular regulation is unknown. In this manuscript we report evidence that suggests that pp42 is a serine/threonine-specific protein kinase. Stimulation of 3T3-L1 cells with insulin has been shown to activate a cytosolic serine/threonine kinase capable of phosphorylating microtubule-associated protein 2 (MAP-2) and ribosomal protein S6 kinase II. This cytosolic serine/threonine protein kinase, which itself is phosphorylated on tyrosine, has been termed "MAP kinase." We now report that pp42 phosphorylation and MAP kinase activation occur in fibroblasts in response to similar mitogens, that the two proteins comigrate on one- and two-dimensional polyacrylamide gels, and that the two proteins copurify chromatographically. The major peptides generated from purified MAP kinase by V8 protease digestion are present as a subset of the peptides in digests of pp42 excised from two-dimensional gels. Thus, the results suggest that MAP kinase is tyrosine-phosphorylated pp42.**

Many growth-factor receptors and oncogene products are tyrosine protein kinases. To understand the mechanisms of intracellular signaling used by these kinases, it will be important to identify and characterize their cellular substrate proteins. Although numerous tyrosine-phosphorylated proteins have been identified by gel electrophoresis in oncogenically transformed or growth factor-stimulated cells, in most cases the biological significance of these phosphorylations has not been determined. pp42 is among the most widely studied of these tyrosine-phosphorylated proteins; it was identified by gel electrophoresis in cells stimulated by any of a number of diverse mitogens [epidermal growth factor (EGF), platelet-derived growth factor, insulin-like growth factor II, thrombin, or phorbol 12-myristate 13-acetate (PMA; also called TPA)] (1–7) and also has been found in at least some oncogenically transformed cells (8–10). Because of the wide variety of agents that stimulate this phosphorylation, pp42 is believed to be involved in some unknown step in intracellular signaling that is shared by all of these mitogens.

Recently a novel serine/threonine protein kinase was identified in insulin-stimulated 3T3-L1 cells (11). This protein kinase has been shown to phosphorylate microtubule-associated protein 2 (MAP-2) as well as ribosomal protein S6 kinase II *in vitro* (12). The kinase was also found to migrate on sodium dodecyl sulfate (SDS)/polyacrylamide gels with a molecular mass of 40–42 kDa and to become phosphorylated on tyrosine during its insulin-stimulated activation (13)—properties similar to those of pp42. In this communication we compare in detail the properties of the serine/threonine kinase and pp42. We find that phosphorylation of pp42 and activation of the kinase occur in response to the same mitogens, that the two proteins comigrate on two-dimensional (2-D) polyacrylamide gels and have similar peptide maps, and that the two proteins copurify during sequential chromatography on anion-exchange, hydrophobic-interaction, and gel-filtration media. We propose that pp42 is a serine/threonine protein kinase, which we term "MAP kinase" based both on its substrate specificity and on the fact that it is a mitogen-activated protein kinase.

MATERIALS AND METHODS

Materials. EGF was purchased from Collaborative Research. V-8 protease was from ICN. DNase was purchased from Worthington, and RNase from Sigma.

Cell Culture. Swiss mouse 3T3 fibroblasts were grown to confluence on 100 mm dishes in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum.

Serum Starvation, Cell Lysis, and Radiolabeling. Confluent cells ($\approx 10^7$ cells per dish) were washed several times with Krebs–Ringer bicarbonate (KRB) Hepes buffer (14), incubated for 2 hr at 37°C in the same buffer, and then stimulated with EGF (400 ng/ml) for 10 min before lysis. Samples were prepared by the method of Kazlauskas and Cooper (15) for 2-D analysis of pp42A. Briefly, cells were lysed with 1 ml of lysis buffer (0.3% SDS/0.065 M dithiothreitol/0.001 M EDTA/0.02 Tris, pH 8.0) preheated to 100°C. The cells were scraped from the dish, and 0.1 ml of DNase/RNase solution (1 mg of DNase and 500 μg of RNase per ml of 0.5 M Tris, pH 7.0/0.05 M $MgCl_2$) was added. Lysates were passed several times through a 22-gauge needle and then lyophilized. Samples were reconstituted with 1 ml of Garrels sample buffer (16), and 70 μl of this material was used for each 2-D polyacrylamide gel. Lysates for immunoblots were prepared by adding 1 ml of hot SDS electrophoresis sample buffer (0.05 M Tris, pH 6.8/1% SDS/10% (vol/vol) glycerol/0.005% bromphenol blue/1% 2-mercaptoethanol) per dish (17); 70 μl of this lysate per lane was used for SDS gel electrophoresis. When it was necessary to use labeled cell lysate, 1 mCi (1 Ci = 37 GBq) of $^{32}PO_4^{3-}$ (New England Nuclear) per ml was added directly to the KRB Hepes buffer at the start of the 2 hr starvation.

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Abbreviations: MAP, microtubule-associated protein; PMA, phorbol 12-myristate 13-acetate; 2-D, two-dimensional; EGF, epidermal growth factor.

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Anti-Phosphotyrosine Antibodies and Immunoblotting. Rabbit anti-phosphotyrosine antiserum was generated by a modification of the procedure described by Kamps and Sefton (18) followed by fractionation on a phosphotyrosine-coupled Affi-Gel column. Specificity of these antibodies for phosphotyrosine was confirmed by immunoblotting total cellular protein extract from EGF- or PMA-treated cells [bound to nitrocellulose (S&S, BA 83)] in the presence or absence of 0.04 M phosphotyrosine, phosphoserine, or phosphothreonine. Only phosphotyrosine significantly prevented antibody binding (data not shown). Lysates were electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS followed by electroblotting onto nitrocellulose. Filters were blocked overnight in 3% bovine serum albumin/0.15 M NaCl/50 mM Tris, pH 7.4. The filters were incubated with affinity-purified anti-phosphotyrosine antibody (5 μg/ml) in blocking solution supplemented with 0.5% Nonidet P-40 and 0.1% Tween-20 and then incubated at room temperature with shaking for 1.5 hr. Filters were washed in detergent-supplemented blocking solution, followed by incubation with ^{125}I-labeled protein A (1 μCi/ml;Amersham) for 2 hr, washing, and autoradiography (19, 20).

Purification of MAP Kinase. MAP kinase was partially purified by DEAE-cellulose and phenyl-Superose chromatography and was assayed by the procedures of Ray and Sturgill (14). Aliquots from each purification step were precipitated with deoxycholate/trichloroacetic acid as described by Bensadoun and Weinstein (21) and reconstituted with hot SDS sample buffer for electrophoresis and anti-phosphotyrosine antibody immunoblotting.

V8 Protease Peptide Maps. V8 protease (1.2 μg) was used to digest ^{32}P-labeled proteins excised directly from dried gels by the method of Cleveland *et al.* (22). Peptides were resolved on a 15% polyacrylamide/SDS gel.

2-D Electrophoresis and Alkali Treatment. 2-D gel electrophoresis followed the methods of O'Farrell (23) and Garrels (16) for comparison of pp42 and MAP kinase. Phenyl-Superose-purified ^{32}P-labeled MAP kinase (^{32}P-MAP kinase; 60 μl) was precipitated with deoxycholate/trichloroacetic acid and then reconstituted with 70 μl of ^{32}P-labeled or unlabeled EGF-stimulated 3T3 cell lysate before 2-D gel electrophoresis. All 2-D gel samples were loaded on isoelectric focusing gels containing a mixture of pH 3.5–10, 5–7, and 6–8 Ampholines (LKB); the resolving gel for the second dimension contained 10% acrylamide. Proteins were electroblotted onto Immobilon (Millipore) and then alkali-treated with 1 M KOH at 56°C for 2 hr (10, 24).

RESULTS

Comparison of pp42 and MAP Kinase by 2-D Gel Electrophoresis. Since pp42 was best characterized on 2-D polyacrylamide gels, we used this system to compare the relative mobility of ^{32}P-labeled pp42 (^{32}P-pp42) to that of ^{32}P-MAP kinase. pp42 on 2-D gels resolves into two phosphoforms—pp42A and pp42B—with pp42B the more basic of the two (7). Kazlauskas and Cooper (15) have observed differences in the relative abundance of pp42A and pp42B, depending on the conditions used to prepare lysates for 2-D electrophoresis; pp42A is the principal phosphoform detected with the lysis method used here. Alkali-resistant pp42A, with an apparent molecular mass of 42 kDa and a pI of 6.8, was observed in EGF-stimulated 3T3 fibroblasts (Fig. 1*A*, arrow) but not in the unstimulated control cells (Fig. 1*B*). ^{32}P-MAP kinase, partially purified by DEAE-cellulose and phenyl-Superose chromatography, was also subjected to 2-D analysis. As with pp42A, phosphorylation of MAP kinase was stable in alkali, and the protein has a similar molecular mass and pI (Fig. 1*C*). When the autoradiographs shown in Fig. 1 *A* and *C* were superimposed, MAP kinase was found to comigrate exactly with pp42A. To confirm this finding, we mixed a precipitated aliquot of ^{32}P-MAP kinase with ^{32}P-labeled cell lysate from EGF-treated cells. The mixture was subjected to 2-D gel analysis, and, indeed, MAP kinase exactly comigrated with pp42A (Fig. 1*D*). We conclude from the 2-D gel comigration study that pp42A and MAP kinase display identical apparent molecular masses and isoelectric points.

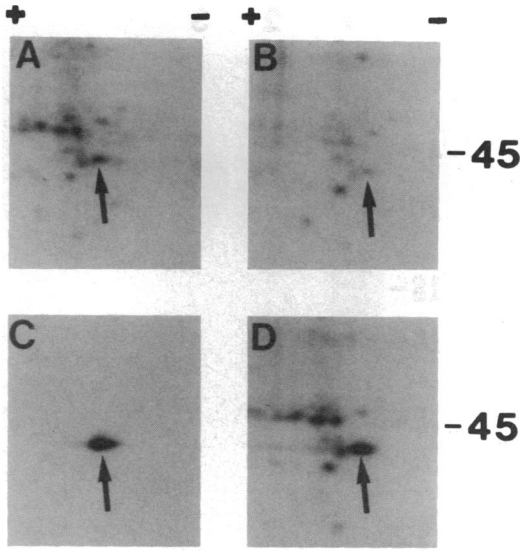

FIG. 1. Comigration of pp42A and MAP kinase on 2-D polyacrylamide gels. Total lysates from ^{32}P-labeled Swiss 3T3 fibroblasts were prepared by the procedure of Kazlauskas and Cooper (15), separated on 2-D polyacrylamide gels, electroblotted onto Immobilon, and alkali-treated as described. *A–D* show only the portion of the 2-D autoradiographs that include the proteins with an apparent pI of 6.8 and a molecular mass of 42 kDa; the migration position of pp42A is indicated by arrows. (*A*) Lysate from EGF-treated cells. (*B*) Lysate from untreated cells. ^{32}P-MAP kinase was purified from EGF-stimulated cells by DEAE-cellulose and phenyl-Superose chromatography as described by Ray and Sturgill (14). Purified MAP kinase was precipitated with deoxycholate/trichloroacetic acid, reconstituted with unlabeled (*C*) or ^{32}P-labeled (*D*) lysates from EGF-stimulated cells, and processed as above. A molecular mass of 45 kDa is indicated.

pp42A and MAP Kinase V8 Protease Peptide Map Comparisons. Further evidence relating ^{32}P-pp42A and ^{32}P-MAP kinase was obtained when we compared their V8 protease phosphopeptide maps. A single pp42A spot, comparable to that in Fig. 1*A*, was excised from a 2-D gel and subjected to partial digestion with staphylococcal protease V8 (Fig. 2). The major band in the digested pp42A (29 kDa; Fig. 2, lane 1) corresponds to the major band seen in the digested MAP kinase (Fig. 2, lanes 2 and 3), consistent with the identity of the two proteins. In addition, the digested pp42 contains several lower molecular mass bands, some of which have not been seen in the purified MAP kinase, even when its digestion was more extensive. We suspect that the pp42 spot as excised may contain phosphoproteins in addition to MAP kinase. Consistent with this suggestion, we found that tryptic phosphopeptide maps of MAP kinase yielded a single peptide, which corresponded to one of three major peptides generated by trypsin treatment of pp42 (ref. 2; data not shown).

Copurification of MAP Kinase and a 42-kDa Phosphoprotein. EGF stimulated the appearance of a 42-kDa phosphotyrosine-containing protein in Swiss 3T3-L1 cell lysates immunoblotted with anti-phosphotyrosine antibodies (Fig. 3*B*, arrow). This band was also found in cells stimulated with insulin and PMA (data not shown). The chromatographic properties of this tyrosine-phosphorylated 42-kDa protein were compared with those of MAP kinase by using anti-phosphotyrosine antibodies in conjunction with the MAP kinase assay (14). MAP kinase activity copurified with the

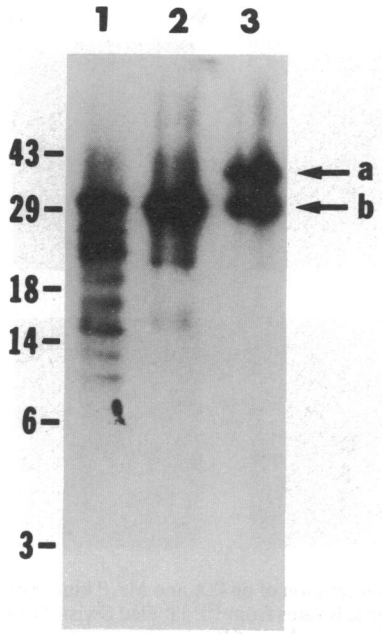

FIG. 2. Staphylococcal V8 protease phosphopeptide map comparisons of partial pp42A and MAP kinase digests. ^{32}P-pp42A was excised from a dried 2-D gel and partially digested with V8 protease (lane 1) as described by Cleveland *et al.* (22). ^{32}P-MAP kinase was purified by DEAE-cellulose, phenyl-Superose chromatography, and SDS gel electrophoresis and then subjected to partial V8 digestion (lane 2) exactly as for pp42A. MAP kinase in control lane 3 (i.e., no protease) is partially digested by contaminating traces of V8 protease from the adjacent lane. Arrows: a, undigested MAP kinase; b, the major comigrating 29-kDa phosphopeptide present in both pp42A and MAP kinase digests. Molecular masses are shown in kDa.

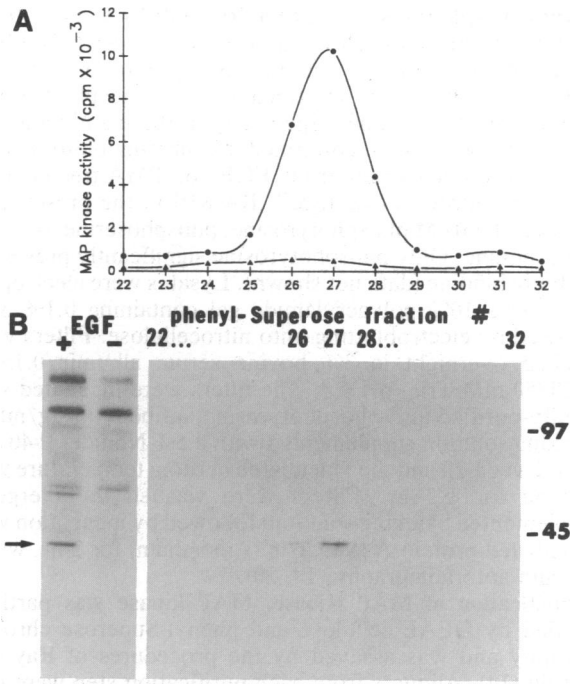

FIG. 3. Copurification of MAP kinase with a 42-kDa phosphotyrosine protein from EGF-stimulated Swiss 3T3 fibroblasts. EGF-treated or untreated 3T3 fibroblast homogenates were subjected to sequential DEAE-cellulose and phenyl-Superose chromatography. (*A*) Elution of EGF-stimulated (●) or unstimulated (▲) MAP kinase activity from phenyl-Superose. (*B Left*) Anti-phosphotyrosine immunoblotting of total proteins from EGF-treated cells (lane +) or untreated cells (lane −). Cells were lysed with hot SDS electrophoresis sample buffer. (*B Right*) Anti-phosphotyrosine immunoblotting of the same phenyl-Superose fractions shown in *A*, obtained from EGF-treated cells. Molecular mass is shown in kDa.

42-kDa phosphoprotein during sequential chromatography with DEAE-cellulose and phenyl-Superose. EGF-stimulated MAP kinase was eluted from phenyl-Superose as a single peak in three fractions at ≈0.1 M NaCl and 37% ethylene glycol (Fig. 3*A*). Aliquots of these and surrounding fractions were immunoblotted with anti-phosphotyrosine antibodies. Only a single 42-kDa phosphoprotein, whose apparent concentration corresponded exactly with MAP kinase activity, was detected in these fractions by anti-phosphotyrosine antibodies (Fig. 3 *A* vs. *B*). Phenyl-Superose fractions from control fibroblasts not treated with EGF showed little, if any, active MAP kinase, and no 42-kDa band was observed. In further experiments, MAP kinase purified through the phenyl-Superose step was applied to a Superose 12 gel filtration column; MAP kinase activity and the 42-kDa tyrosine-phosphorylated protein (as detected by anti-phosphotyrosine antibodies) were eluted in the same fractions (data not shown). This result is identical to that obtained previously when using ^{32}P-labeling for phosphoprotein detection (14).

In order to characterize the 42-kDa phosphoprotein/MAP kinase relationship in greater detail, all flow-throughs, washes, and fractions from the MAP kinase purification were subjected to analysis by immunoblotting with anti-phosphotyrosine antibodies. Samples were normalized on the basis of volume (relative to the homogenate volume), and the proteins were precipitated with deoxycholate/trichloroacetic acid. Where protein concentrations were very low, larger volumes (up to 1.2 ml, representing 60–70% of a given sample) were precipitated with deoxycholate/trichloroacetic acid and analyzed in a single SDS gel lane to be certain that lack of the 42-kDa protein was not an artifact that could be attributed to low protein concentrations. The 42-kDa phosphotyrosine protein was only detected in those fractions containing MAP kinase activity (data not shown), with the following exception. When 1.2 ml of the phenyl-Superose wash (i.e., a pool of fractions 1–6) was precipitated and then immunoblotted with anti-phosphotyrosine antibodies, a faint band that appeared to comigrate with pp42A/MAP kinase was detected. This band was present at very low concentrations and may represent unbound pp42A or possibly the other phosphorylated form of pp42—namely, pp42B (see *Discussion*).

Map kinase copurified on DEAE-cellulose, phenyl-Superose, and Superose 12 with a 42-kDa phosphoprotein detected by anti-phosphotyrosine antibodies. This copurification and the fact that the protein could not be detected elsewhere support the suggestion that the 42-kDa tyrosine-phosphorylated protein is MAP kinase. This is further supported by previous phosphoamino acid analyses, which showed that MAP kinase was the only mitogen-stimulated, tyrosine-phosphorylated protein present in the phenyl-Superose eluate (14).

DISCUSSION

pp42 is a principal target protein for mitogen-stimulated tyrosine protein kinases in quiescent cells (1–7). Although numerous tyrosine kinase substrates have been identified in growth-factor-stimulated and oncogenically transformed cells (8–10), little is known about the functional significance of these phosphorylations. Several reported observations led us to suspect that pp42 might be related to MAP kinase, a recently identified mitogen-activated serine/threonine kinase. For example, both proteins become tyrosine-phosphorylated after agonist stimulation, and both have molecular masses of 42 kDa (1–7, 13, 14). Moreover, maximal tyrosine phosphorylation of pp42 occurs within 5 min of stimulation and slowly decays, with a half-life of 1–2 hr (4, 5); the activation of MAP kinase follows a similar time course (11).

In addition, pp42 is found almost exclusively in the cytosol (ref. 2; our unpublished results) as is mitogen-stimulated MAP kinase activity (14). Therefore, we compared in detail the relatedness of these two proteins, utilizing the criteria by which each is best characterized: 2-D polyacrylamide gel electrophoresis for pp42, and purification by DEAE-cellulose, phenyl-Superose, and Superose 12 chromatography for MAP kinase.

The fact that purified MAP kinase comigrates with pp42A on 2-D polyacrylamide gels provides strong evidence for the relatedness of these two proteins. The fact that the two have similar phosphopeptide maps strengthens this suggestion. Thus, we propose that MAP kinase is one of the two principal pp42 phosphoforms—namely, pp42A. We cannot as yet account for pp42B. Perhaps it is retained by the phenyl-Superose column; however, this explanation seems unlikely given the strigent conditions used to elute proteins off the column. Alternatively, pp42B may be the low-abundance 42-kDa band detected in the phenyl-Superose wash by anti-phosphotyrosine antibody immunoblotting. Finally, differences in the apparent relative abundance of pp42A and pp42B have been observed, depending on the exact procedures used for sample preparation and 2-D gel analysis. Kazlauskas and Cooper (15) have suggested this possibility to explain why pp42A is preferentially seen in their 2-D gels. In any event, we will not be able to identify pp42B with confidence and determine its relationship to MAP kinase until immunological reagents are available that specifically recognize pp42 and/or MAP kinase.

MAP kinase can be isolated rapidly by sequential chromatography on DEAE-cellulose and phenyl-Superose fast protein liquid chromatography. Utilizing this procedure as another criterion for comparison, we have determined that MAP kinase activity copurified with a 42-kDa tyrosine-phosphorylated protein. Furthermore, this phosphoprotein represents most, if not all, of the mitogen-stimulated tyrosine phosphorylation at 42 kDa as detected by our anti-phosphotyrosine antibodies. These results, along with the 2-D gel analyses, suggest that MAP kinase is (or at least is a component of) the tyrosine-phosphorylated pp42.

In addition to the copurification data described above, we also have found that MAP kinase activity and pp42 tyrosine phosphorylation are coordinately increased in response to agonists (data not shown). MAP kinase originally was described in insulin-stimulated 3T3-L1 cells, but in this paper we show that it also is activated in EGF-stimulated fibroblasts. Moreover, activators of protein kinase C, such as PMA, have been shown to stimulate pp42 phosphorylation (3, 4), and we have found that PMA can also stimulate MAP kinase activity (data not shown). The MAP kinase activity stimulated by PMA is chromatographically indistinguishable from that seen after EGF or insulin stimulation. Finally, chronic PMA treatment, which depletes cellular protein kinase C (25), reduces the extent to which pp42 becomes phosphorylated on tyrosine when subsequently treated with heterologous mitogens such as EGF (15, 26), and we have obtained preliminary results that chronic PMA treatment also reduces the ability of EGF to stimulate MAP kinase activity. These results indicate that a similar requirement for protein kinase C exists for both tyrosine phosphorylation of pp42 and activation of MAP kinase. The nature of this requirement is a subject for future investigation.

In the past, studies of pp42 have been extremely difficult because of its low abundance, requiring the use of high $^{32}PO_4^{3-}$ levels to visualize it on 2-D polyacrylamide gels. In addition, pp42 could be identified only under denaturing conditions, and no enzymatic assay was available for its activity, making it difficult to design purification strategies. Using anti-phosphotyrosine antibodies, pp42/MAP kinase can be identified easily by immunoblotting one-dimensional gels, thereby eliminating the need for high $^{32}PO_4^{3-}$ and 2-D polyacrylamide gels. In addition, pp42 can be efficiently monitored under differing experimental conditions by the MAP kinase assay. Both the anti-phosphotyrosine antibodies and the MAP kinase assay should be invaluable for future pp42/MAP kinase characterization and purification. Purification of MAP kinase to homogeneity and cloning its gene will be necessary to define unambiguously its cellular function as well as its precise relationship to pp42.

Evidence is accumulating that a network of protein kinases may participate in the regulation of the cell cycle. Of potential relevance to this, MAP kinase has been shown to phosphorylate and reactivate *Xenopus* S6 kinase II which had been deactivated by phosphatase 1 or 2A (12). Thus, we hypothesize that pp42/MAP kinase is one of the kinases in this interlocking network. We also suggest that "MAP kinase" be retained as the name of the enzyme. The acronym was originally taken from "microtubule-associated protein," one of the preferred *in vitro* substrates. MAP kinase may now also stand for "mitogen-activated protein" kinase and is consistent with the relatively promiscuous activation of the enzyme by diverse mitogens.

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