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Identification of a MAP kinase kinase kinase in phaeochromocytoma (PC12) cells

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A MAP kinase kinase kinase (MAPKKK) was identified in phaeochromocytoma (PC12) cells which reactivated homogeneous MAP kinase kinase (MAPKK) from rabbit skeletal muscle that had been inactivated by incubation with protein phosphatase 2A. Reactivation was accompanied by stoichiometric phosphorylation of MAPKK on a serine residue(s). Following stimulation of PC12 cells with nerve growth factor and chromatography of the extracts on Mono Q, MAP kinase and MAPKK were detected as active phosphorylated enzymes, whereas MAPKKK was inactive and only activated after prolonged storage at 4°C. The results suggest that the activation of MAPKKK by growth factors is likely to occur by a non-covalent mechanism.

MAP kinase; Nerve growth factor; PC12 cell; Protein phosphorylation; Signal transduction

1. INTRODUCTION

The initial steps in signal transduction by many growth factors involves their interaction with plasma membrane-bound receptors, followed by autophosphorylation of the receptors on tyrosine residues. Receptor autophosphorylation then leads within minutes to increases (and decreases) in the phosphorylation of many intracellular proteins on serine and threonine residues. Consequently, unravelling the molecular mechanisms by which receptor protein tyrosine kinases regulate cytoplasmic serine/threonine phosphorylation has become a major preoccupation of many laboratories.

Much attention is currently focussed on the activation of mitogen-activated protein (MAP) kinases, a family of protein serine/threonine kinases that are activated very rapidly in response to a variety of extracellular signals which cause cells to divide or to differentiate (reviewed in [1,2]). The activation of MAP kinase is triggered by phosphorylation of a threonine and a tyrosine residue [3,4], both catalysed by a single 'dual specificity' MAP kinase kinase (MAPKK) [5–10]. MAPKK and MAP kinase are activated with half times of 1–2 min and 3 min, respectively, following stimulation of rat phaeochromocytoma (PC12) cells with nerve growth factor (NGF), maximal activation occurring after 5 min (MAPKK) and 10–15 min (MAP kinase) [11]. MAPKK from PC12 cells [5] (and other tissues [6–8]) is inactivated in vitro by incubation with protein phosphatases that are specific for serine and threonine residues, but not by incubation with protein tyrosine phosphatases; nor can the deactivated MAPKK be reactivated by incubation with MgATP [5]. These observations imply that activation of MAPKK is likely to involve its phosphorylation on serine and/or threonine residues catalysed by a distinct MAP kinase kinase kinase (MAPKKK). In this paper we identify a MAPKKK activity in PC12 cells that activates MAPKK by phosphorylating it on serine residues. However, in contrast to MAPKK and MAP kinase which are detected as active phosphorylated species after Mono Q chromatography of extracts from NGF-stimulated cells, MAPKKK is detected as a latent, inactive enzyme. These results suggest that activation of this MAPKKK is is likely to occur by a non-covalent mechanism and not by a direct phosphorylation or dephosphorylation of this enzyme.

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2. MATERIALS AND METHODS

2.1. *Materials*

MAPKK ([7], S. Nakielny) and the catalytic subunit of protein phosphatase 2A ([12], D. Schelling) were purified to homogeneity from rabbit skeletal muscle in this laboratory by the investigators indicated in parentheses. Recombinant murine $p42^{mapk}$ (a generous gift from Miss S. Leevers and Dr. C.J. Marshall, Institute for Cancer Research, London) was expressed as a glutathione S-transferase fusion protein in *Escherichia coli*, purified by affinity chromatography on glutathione-Sepharose and cleaved with thrombin [13]. Okadaic acid was provided by Dr. Y.Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan) and the protein tyrosine phosphatase LAR by Dr. H. Saito (Dana Farber Cancer Institute, Boston, USA). Sources of other materials are given elsewhere [6,11].

2.2. *Chromatography of PC12 cell extracts on Mono Q*

PC12 cells were cultured [14], incubated with or without 50 ng/ml NGF and lysed in 20 mM Tris acetate pH 7.0 (20°C), 0.27 M sucrose,

1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (by vol.) Triton X-100, 1 mM benzamidine, 4 μg/ml leupeptin, 0.1% (by vol.) 2-mercaptoethanol (Buffer A). Extracts prepared from 10 dishes (6×10^7 cells) were applied to a 5×0.5 cm column of Mono Q equilibrated in 50 mM Tris-HCl pH 7.3 (20°C), 2 mM Na-EDTA, 2 mM Na-EGTA, 0.3 mM sodium orthovanadate, 5% (by vol.) glycerol, 0.03% (by weight) Brij 35, 0.1% (by vol) 2-mercaptoethanol, 1 mM benzamidine and 4 μg/ml leupeptin (Buffer B). After washing with 10 ml of equilibration buffer, the columns were developed with 40 ml linear salt gradients to 0.7 M NaCl in Buffer B. The flow rate was 1.0 ml/min and fractions of 1.0 ml were collected.

2.3. *Assay of protein kinases*

MAP kinase [5] and MAPKK [11] were assayed as described previously. One unit of MAP kinase was that amount of activity which catalysed the incorporation of 1.0 nmol of phosphate into myelin basic protein in one min. One unit of MAPKK was that amount of activity which produced 50% activation of recombinant MAP kinase in one min. [11].

MAPKKK was assayed routinely using inactive recombinant MAP kinase and inactive MAPKK from unstimulated PC12 cells. The inactive MAPKK eluted in the flowthrough fractions from Mono Q (section 2.2). 5 μl of inactive MAPKK in Buffer A was incubated with 5 μl of 4 μM recombinant inactive MAP kinase diluted in 50 mM Tris-HCl pH 7.5 (20°C), 0.1 mM Na-EGTA and 0.1% (by vol.) 2-mercaptoethanol (Buffer C) containing 0.04% (by weight) Brij 35 and 5 μl of MAPKKK (i.e. Mono Q eluate). After 3 min at 30°C the reaction was initiated with 5 μl of 40 mM magnesium acetate-0.8 mM unlabelled ATP. After a further 20 min at 30°C, a 0.01 ml aliquot was removed and added to 0.04 ml of ice cold Buffer C containing 1.0 mg/ml bovine serum albumin and 0.5 mM sodium orthovanadate. A 10 μl aliquot of this solution was then assayed for MAP kinase activity. Control incubations were carried in which inactive MAPKK was omitted and these values subtracted from those obtained in the presence of MAPKK. As shown in Fig. 1, the rate of production of active MAP kinase (monitored with myelin basic protein as substrate) increased with time as would be expected from increased formation of active MAPKK, and MAPKKK activity was completely dependent on the inclusion of both inactive MAPKK and MAPKKK in the assay. One unit of MAPKKK was defined as that amount which increased the activity of MAP kinase by 1.0 unit in the 20 min assay.

3. RESULTS

Extracts prepared from NGF-stimulated PC12 cells were chromatographed on Mono Q and assayed for MAPKK and MAP kinase activities. As reported previously (e.g. [5,11]), anion-exchange chromatography resolved two peaks of active MAPKK and two peaks of active MAP kinase activity, the latter corresponding to the $p42^{mapk}$ and $p44^{mapk}$ isoforms (Fig. 2A). In contrast, MAP kinase activity (Fig. 2B) and MAPKK (data not shown) were undetectable after Mono Q chromatography of extracts from unstimulated cells, as reported previously [5,14].

When assayed immediately after chromatography on Mono Q, no MAPKKK activity could be detected in any fraction from either NGF-stimulated or control cells (Fig. 2). However, upon storage at 4°C, a single peak of MAPKKK could be detected after about two weeks which eluted after $p44^{mapk}$ (Fig. 2A and B). Identical results were obtained whether PC 12 cells were stimulated with NGF for 2, 5, or 15 min prior to chromatography, or if NGF was omitted.

The activation of MAPKKK as a function of time is shown in Fig. 3. MAPKKK could barely be detected after storage at 4°C for 5 days, but became easily detectable after 10 days and was still rising after several weeks. The rate of activation was similar in fractions obtained from NGF-stimulated or control cells (Fig. 3). Activation was not prevented by addition of the protein serine/threonine phosphatase inhibitor okadaic acid (0.1 μM) or the protein tyrosine phosphatase inhibitor sodium orthovanadate (0.1 mM) to the column fractions, but was prevented by freezing the fractions at −70°C. MAPKKK that had been activated by storage at 4°C for several weeks could not be inactivated by incubation with protein phosphatase 2A (30 mU/ml, see [12] for definition of units) or the protein tyrosine phosphatase LAR (0.1 mg/ml) under conditions where MAP kinase activity was decreased by 90% [5]. MAPKKK that had been activated by storage, eluted from Mono Q at the same position as the inactive enzyme following rechromatography on the anion-exchange column (data not shown).

MAPKKK was assayed in Figs. 2 and 3 using an inactive form of MAPKK from PC12 cells which elutes

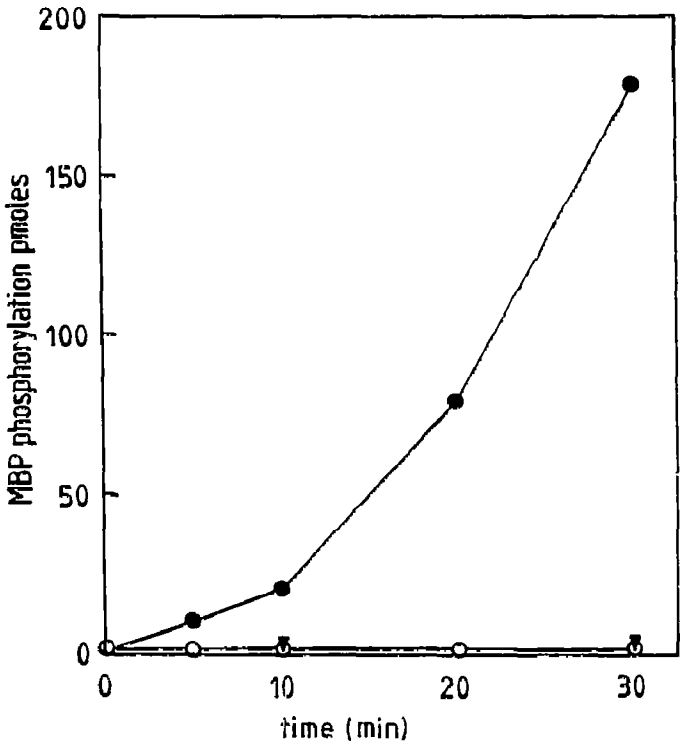

Fig. 1. Assay of MAP kinase kinase kinase activity. MAPKKK from Mono Q (Fig. 2) was incubated with inactive MAPKK, inactive MAP kinase and MgATP as described in section 2.3. The closed circles show the production of active MAP kinase as measured by the phosphorylation of myelin basic protein (MBP), while the closed triangles and open circles show control incubations in which either MAPKKK or inactive MAPKK were omitted from the assays, respectively.

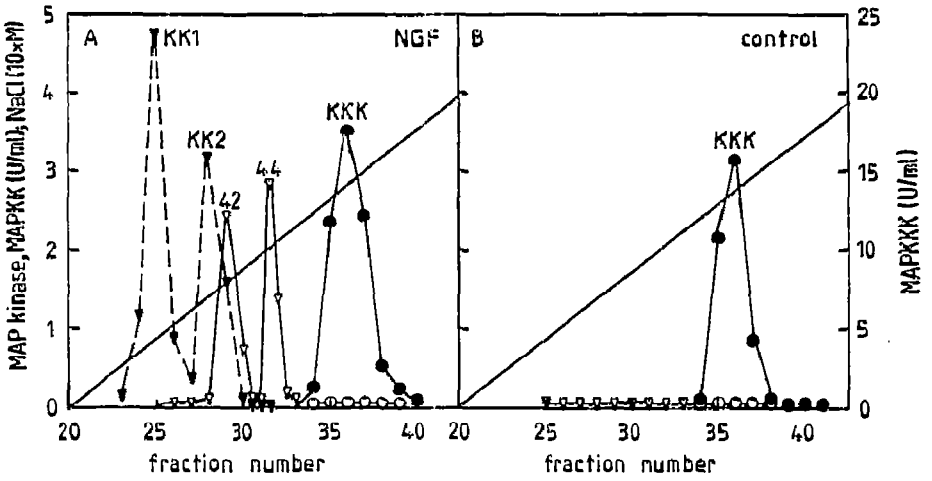

Fig. 2. Detection of MAP kinase kinase kinase after chromatography of PC12 cell extracts on Mono Q. Extracts were chromatographed on Mono Q as described in section 2.2. and assayed immediately after chromatography for MAP kinase (▽—▽), MAPKK (▼---▼) and MAP kinase kinase kinase (KKK, ○—○). The column was subsequently reassayed for MAPKKK 12 days later (●—●). Similar results were obtained in about 15 different experiments. Although in this particular experiment only fractions 34–40 were assayed for MAPKKK, in other experiments all the column fractions were assayed, but no MAPKKK was detected anywhere, except between fractions 34 and 40 after prolonged storage at 4°C. A. Results obtained using extracts prepared from PC12 cells that had been stimulated for 5 min with 50ng/ml NGF. B. Results from unstimulated (control) cells. Chromatography on Mono Q resolves two isoforms of MAP kinase, termed $p42^{mapk}$ (42) and $p44^{mapk}$ (44) and two different forms of MAPKK (KK1 and KK2). KK2 may be a more highly phosphorylated form of KK1 [11].

in the flowthrough fractions from Mono Q (section 2.2). However, MAPKKK also activated a homogeneous

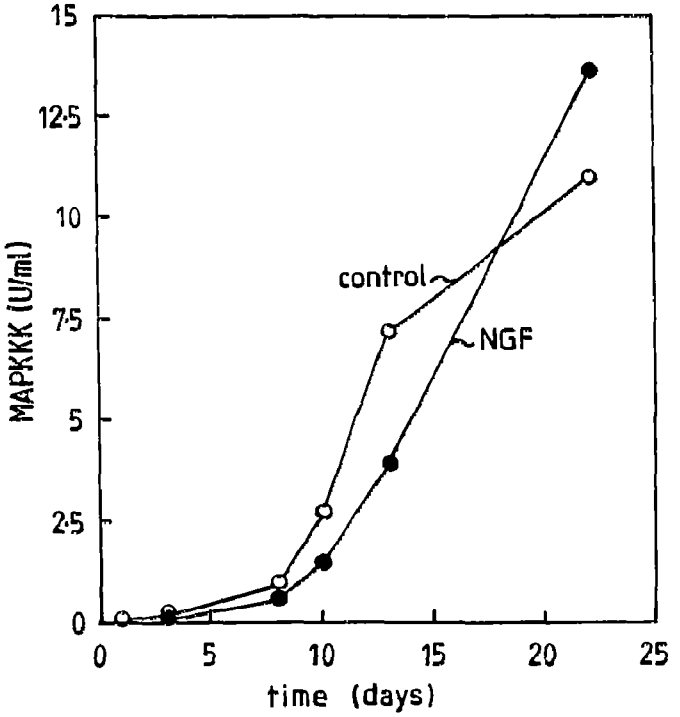

Fig. 3. Time dependent activation of MAP kinase kinase kinase. Fraction 36/37 from the Mono Q column (Fig. 2) was assayed for MAPKKK activity immediately (day 1) and at various times up to 22 days. The closed circles show the results with MAPKKK from NGF-stimulated cells and the open circles results from unstimulated (control) cells.

preparation of MAPKK from skeletal muscle [7] which had been inactivated by preincubation with protein phosphatase 2A (Fig. 4). Reactivation was accompanied by the phosphorylation of MAPKK (Fig. 5A) to 0.8 mol per mol 44 kDa subunit after 1 h (the protein concentration being estimated by the method of Bradford [15]). Nearly all the phosphate was attached to a serine residue(s) (Fig. 5B), and the traces of phosphothreonine and phosphotyrosine can be accounted for by the slow autophosphorylation of these residues catalysed by activated MAPKK [7]. MAPKK that had not been preincubated with protein phosphatase 2A was activated up to 2-fold (Fig. 4) and phosphorylated (Fig. 5) by MAPKKK, indicating that the purified muscle enzyme is not isolated in a fully phosphorylated state. No phosphorylation or activation of MAPKK occurred upon incubation with MgATP and either MAPKKK that had been stored at −70°C to prevent activation or the inactive MAPKKK in freshly isolated Mono Q fractions (data not shown).

4. DISCUSSION

In contrast to MAPKK and MAP kinase, MAPKKK is not detected as an active enzyme after Mono Q chromatography of extracts from NGF-stimulated PC12 cells. Instead, it is found in a latent inactive form, activity only being generated after prolonged storage of the fractions at 4°C. One possible explanation for activation is limited proteolysis catalysed by a proteinase present as a contaminant in these fractions. This proteinase may either destroy an inhibitory domain on

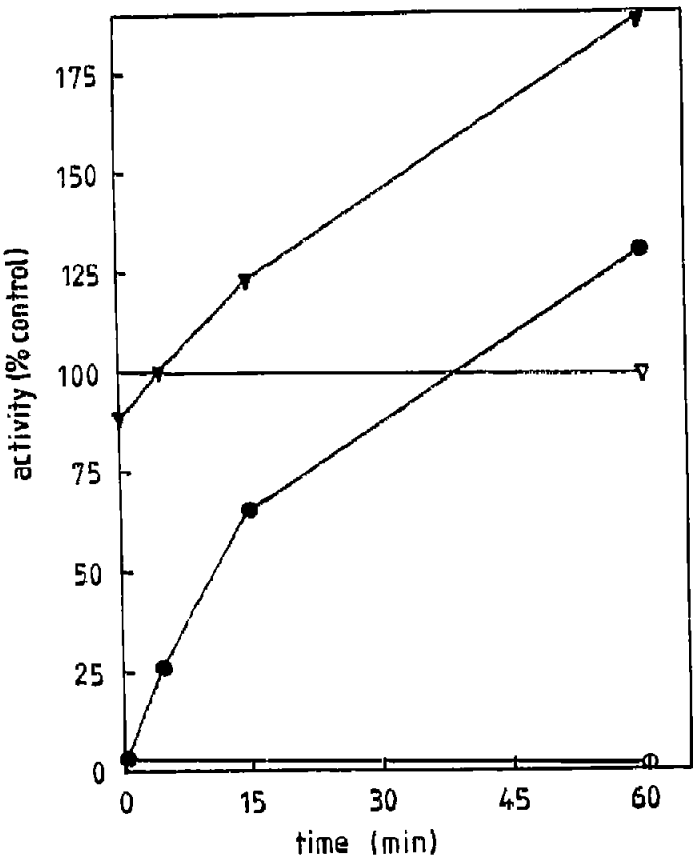

Fig. 4. Activation of MAP kinase kinase from rabbit skeletal muscle by MAP kinase kinase kinase. A homogeneous preparation of MAPKK from rabbit skeletal muscle (2 μl, 75 μg/ml) in 25 mM Tris-HCl pH 7.4 (20°C), 0.2 M NaCl, 1.0 mM Na-EDTA, 0.5 mM Na-EGTA, 5% (v/v) glycerol, 0.02% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol was inactivated by incubation for 30 min at 30°C with 2 μl of 20 mU/ml protein phosphatase 2A (see ref. 12 for definition of units) in 50 mM Tris-HCl pH 7.0 (20°C), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, then 2 μl of 10 μM okadaic acid was added to inactivate the phosphatase. A 2 μl aliquot of the inactivated MAPKK was mixed with 3 μl of Buffer B, 2 μl of active MAPKKK (i.e. fraction 36 in Fig. 2) and warmed at 30°C for 3 min. Activation of MAPKK was then initiated with 3 μl of 33 mM magnesium acetate-0.67 mM unlabelled ATP. At different times aliquots were diluted and assayed for MAPKK activity (●—●). The open circles show a control experiment in which MAPKKK was omitted The closed and open triangles show experiments in the presence and absence of MAPKKK, respectively, in which active MAPKK (i.e. not preincubated with protein phosphatase 2A) was used instead of inactivated MAPKK. The value of 100% corresponds to the activity of MAPKK that has not been inactivated by protein phosphatase 2A.

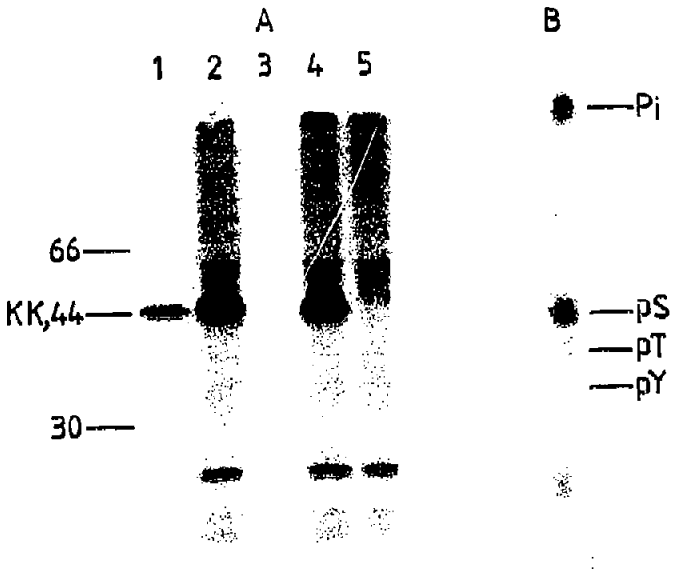

Fig. 5. Phosphorylation of MAP kinase kinase from rabbit skeletal muscle by MAP kinase kinase kinase. A. Homogeneous skeletal muscle MAPKK was activated by MAPKKK as described in the legend to Fig. 4, except that [γ^{32}P]ATP (10^6 cpm/nmol) replaced unlabelled ATP. After incubation of MAPKK with MAPKKK and MgATP for 60 min at 30°C, the samples were denatured in SDS, subjected to electrophoresis on 10% SDS-polyacrylamide gels [22] and autoradiographed. Migration is from top to bottom and the arrows denote the positions of, bovine serum albumin (66 kDa), MAPKK (44 kDa, KK) and carbonic anhydrase (30 kDa). Lane 1, autophosphorylation of active MAPKK in the absence of MAPKKK; lane 2, phosphorylation of active MAPKK in the presence of active MAPKKK; lanes 3 and 4, same as lanes 1 and 2 except that MAPKK was first inactivated by preincubation with protein phosphatase 2A; lane 5, same as lanes 2 and 4, except that MAPKK was omitted. B. MAPKK from lane 4 in A was eluted from the gel, precipitated with trichloroacetic acid and the precipitated protein washed with water as described previously [6]. The sample was then hydrolysed for 1.5 h at 110°C in 6 N HCl, electrophoresed on thin layer cellulose to resolve phosphorylated amino acids and autoradiographed [6] The positions of phosphotyrosine (pY), phosphothreonine (pT) and phosphoserine (pS) and inorganic phosphate (Pi) are marked. The other ^{32}P-labelled spot is a phosphopeptide resulting from incomplete hydrolysis.

MAPKKK or an associated inhibitory subunit. However, attempts to mimic activation by incubation with trypsin or chymotrypsin have so far been unsuccessful, while incubation at ambient temperature for up to 16 h failed to cause significant activation of the freshly isolated Mono Q fractions.

The failure to detect any other enzyme in the Mono Q fractions capable of activating MAPKK, suggests that the MAPKKK we have identified may be responsible for activating MAPKK after NGF stimulation. However, in this case, its mechanism of activation cannot involve phosphorylation or dephosphorylation per se, otherwise MAPKKK (like MAP kinase and MAPKK) should have been detected as an active enzyme in the Mono Q eluate from NGF-stimulated cells. Our results therefore imply that MAPKKK is activated by a non-covalent mechanism. Two potential candidates for the physiological activator of MAPKKK are the GTP-liganded form of p21ras and phosphatidylinositol 3,4,5-trisphosphate both of which are formed very rapidly in many cells in response to growth factors whose receptors are protein tyrosine kinases. In PC12 cells NGF causes partial conversion of p21ras to its active GTP-liganded state within 2 min [16], while trans-

fection of PC12 cells with the dominant-negative (Asn17) mutant of p21ras blocks the ability of NGF to activate MAP kinase [17]. Similarly, NGF stimulation of PC12 cells activates phosphatidyl inositol 3-kinase increasing the intracellular level of phosphatidyl inositol 3,4,5-trisphosphate over 10-fold within a minute [18].

Transfection of several mammalian cells with the viral oncogene v-raf, a protein serine/threonine kinase, or N-terminally truncated (active) forms of the cellular homologue c-raf, cause constitutive activation of MAP kinase in the absence of growth factor stimulation [19–21], while transfection of COS-1 cells with c-raf itself, potentiates the activation of MAP kinase by phorbol esters and epidermal growth factor [21]. Furthermore, recombinant v-raf expressed in *Escherichia coli* [20], raf immunoprecipitated from cells that overexpress N-terminally truncated c-raf [19], or c-raf immunoprecipitated from phorbol ester-stimulated cells overexpressing full length c-raf [21], activate MAPKK in vitro. The purification of MAPKKK to homogeneity and peptide sequencing will be needed to determine whether it is related to c-raf or other known protein kinases. Nevertheless, the detection of a MAPKKK in an untransfected mammalian cell for the first time will facilitate both its characterisation and the elucidation of its activation mechanism.

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REFERENCES

[1] Sturgill, T.W. and Wu, J. (1991) Biochim. Biophys. Acta 1092, 350–357.
[2] Cobb, M.H., Boulton, T.G. and Robbins, D.S. (1991) Cell Regulation 2, 965–978.
[3] Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Nature 343, 651–653.
[4] Payne, D.M., Rossomando, A.J., Martin, P., Erickson, A.K., Her, J.H., Shabanowitz, J., Hunt, D.F., Weber, M.J. and Sturgill, T.W. (1991) EMBO J. 10, 885–892.
[5] Gomez, N. and Cohen, P. (1991) Nature 353, 170–173.
[6] Nakielny, S., Cohen, P., Wu, J. and Sturgill, T.W. (1992) EMBO J. 11, 2123–2129.
[7] Nakielny, S., Campbell, D.G. and Cohen, P. (1992) FEBS Lett. 308, 183–189.
[8] Matsuda, S., Kosako, H., Takenaka, K., Moriyama, K. Sakai, H., Akiyama, T., Gotoh, Y. and Nishida, E. (1992) EMBO J. 11, 973–982.
[9] Seger, R., Ahn, N.G., Posada, J., Munar, E.S., Jensen, A.M., Cooper, J.A., Cobb, M.H. and Krebs, E.G. (1992) J. Biol. Chem. 267, 14373–14381.
[10] Crews, C.M. and Erikson, R.L. (1992) Proc. Natl. Acad. Sci. USA 89, 8205–8209.
[11] Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Biochem. J. 288, 351–355.
[12] Cohen, P., Alemany, S., Hemmings, B.A., Strålfors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, 390–408
[13] Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C. and Cohen, P. (1992) EMBO J. 11, 3985–3994
[14] Gomez, N, Tonks, N.K., Morrison, C., Harmar, T. and Cohen, P. (1990) FEBS Lett. 271, 119–122
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
[16] Nakafuku, M., Satoh, J. and Kaziro, Y. (1992) J. Biol. Chem. 267, 19448–19454.
[17] Thomas, S.M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J.S. (1992) Cell 68, 1031–1040.
[18] Carter, A.N. and Downes, C.P. (1992) J. Biol. Chem. 267, 14563–14567
[19] Kyriakis, J.M., App, H., Zhang, X-f., Banerjee, P., Brautigan, D. L., Rapp, U.R. and Avruch, J. (1992) Nature 358, 417–421.
[20] Dent, P., Haser, W., Haystead, T.A.J., Vincent, L.A., Roberts, T.M. and Sturgill, T.W. (1992) Science 257, 1404–1407.
[21] Howe, L.R., Leevers, S.J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C.J. (1992) Cell 71, 335–342.
[22] Laemmli, U.K. (1970) Nature 227, 680–685