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Identification of a latent MAP kinase kinase kinase in PC12 cells as B-raf

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Abstract

A latent MAP kinase kinase kinase activity previously detected after chromatography of PC12 cell extracts on Mono Q [(1992) FEBS Lett. 314, 461–465] has been identified as B-Raf by immunoblotting and immunoprecipitation experiments and by its specific activation with B-Raf antibodies. B-Raf is phosphorylated after stimulation of PC12 cells with nerve growth factor or epidermal growth factor, but this is not accompanied by activation of the Mono Q-purified enzyme whether assayed in the absence or presence of GTP-Ras.

Key words: MAP kinase; PC12 cell; Raf; Nerve growth factor; Epidermal growth factor

1. Introduction

A protein kinase cascade has been dissected over the past few years which plays a critical role in mediating the actions of growth factors that signal via receptor protein tyrosine kinases. Cell stimulation by these growth factors leads within minutes to the activation of mitogen-activated protein (MAP) kinase, which translocates from the cytosol to the nucleus and regulates transcriptional events that culminate in either proliferation or differentiation [1].

The activation of MAP kinase by growth factors is thought to involve first, the formation of GTP-Ras, and then the activation of the protein kinase c-Raf. The latter activates MAP kinase kinase (MAPKK) by phosphorylating two serine residues [2], while MAPKK activates MAP kinase by phosphorylating a threonine and a tyrosine residue [3]. The phosphorylation sites in MAPKK and MAP kinase are situated in analogous regions of the kinase catalytic domain, between subdomains VII and VIII.

Rat phaeochromocytoma (PC12) cells undergo differentiation from immature chromaffin cells to a sympathetic neuron-like phenotype in response to nerve growth factor (NGF), and recent work strongly suggests that sustained activation of MAP kinase is not only required, but sufficient, to induce the differentiation of these cells [4,5]. NGF stimulation of PC12 cells activates the MAPKK1 isoform [2] which then activates the p42 and p44 isoforms of MAP kinase (p42/44^{mapk}) [6]. However, it is not clear which enzyme activates MAPKK1 in NGF-stimulated PC12 cells. Almost none of the MAPKKK activity in PC12 cell extracts is reported to be immunoprecipitated by c-Raf antibodies [7] and expression of a constitutively activated form of c-Raf in PC12 cells does not activate p42/44^{mapk} [8]. Two potential candidates for the NGF-stimulated MAPKKK are MEK kinase [9] and the protooncogene c-*mos* [10,11], both of which display MAPKKK activity in vitro. MEK kinase is a mammalian homologue of yeast protein kinases that lie in signalling pathways which coordinate the physiological changes that enable yeast cells to mate, and has been identified in a number of mammalian cells. In contrast c-Mos has only been detected in germ cells where it plays an important role in regulating meiosis.

We have previously detected a MAPKKK activity in PC12 cells after chromatography of cell lysates on Mono Q [12]. Unlike MAPKK1 and p42/44^{mapk}, this MAPKKK was inactive when the Mono Q fractions from NGF-stimulated PC12 cells were assayed immediately after chromatography, but became active after storage for several weeks at 4°C. Since these findings were made before the discovery that c-Raf, MEK kinase and c-Mos activate MAPKK in vitro, it became important to identity the latent MAPKKK activity in the Mono Q eluate. Here we identify this enzyme as B-Raf, an isoform of c-Raf.

2. Materials and methods

2.1. Materials

An affinity-purified antibody raised against the C-terminal sequence of B-Raf (ASPKTPIQAGGYGAFPVH) was a product of Santa Cruz Biotechnology Inc. and purchased from NBS Biologicals (North Mymms, Herts., UK). Antisera raised against C-terminal peptides from c-Raf (CTLTTSPRLPVF) and B-Raf were gifts from Dr. U. Rapp, NIH, Frederick, MD, USA, and antiserum raised against a C-terminal peptide of MEK kinase (DRPPSRELLKHPVFR) was a gift from Drs. C. Lange-Carter and G. Johnson, National Jewish Hospital, Denver, CO, USA. Sources of other materials are given elsewhere [12–14]. N-Ras from Triton-solubilised plasma membranes of the retinal cell line HER313A (where N-Ras comprises 5–10% of the plasma membrane protein) was purified on Mono Q and provided by Dr. D. Alessi in this Unit.

2.2. Chromatography of PC12 cell extracts on Mono Q and assay of protein kinases

Cells were cultured, stimulated [15], lysed [6], and the extracts chromatographed on Mono Q [12] as described previously. MAPKKK was

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assayed by its ability to reactivate MAPKK as in [13]. This assay, which employs bacterially expressed MAPKK1 and $p42^{mapk}$, is extremely sensitive and less than 0.5% conversion of MAPKK to its activated form can be quantitated accurately [2]. One unit of MAPKKK activity was that amount which increased the activity of MAP kinase by 1 U in 20 min assay [12,13]. Protein concentrations were determined by the method of Bradford [16].

2.3. Immunoprecipitation of Raf

Protein A–Sepharose (100 mg) was suspended in 1 ml of 50 mM Tris-HCl, pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.3 mM sodium orthovanadate, 5% (v/v) glycerol, 0.03% (w/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol (Buffer A) and a 0.04 ml aliquot incubated for 10 min at 4°C with Raf antibody (0.005 ml). An aliquot of activated MAPKKK from Mono Q (0.04 ml) was added to the Raf antibody/Protein A–Sepharose and the suspension incubated for 30 min at 4°C with occasional mixing. After centrifugation for 1 min at 13,000 × g, 0.065 ml of the supernatant was removed and the pellet washed once with 0.1 ml of buffer A, once with buffer A containing 0.5 M NaCl and twice with 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.04% (by mass) Brij 35, 0.1% (by vol.) 2-mercaptoethanol (buffer B). The pellet was resuspended in buffer B to a volume of 0.065 ml and aliquots of the resuspended pellet and supernatant were assayed for MAPKKK activity.

Raf was immunoprecipitated from PC12 cell extracts as described above except that the Protein A–Sepharose was suspended in lysis buffer (20 mM Tris-acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% (w/v) Triton X-100, 1 mM benzamidine, 4 μg/ml leupeptin, 0.1% (by vol.) 2-mercaptoethanol) and PC12 cell extract (0.1 mg, about 0.2 ml) replaced MAPKKK from Mono Q. The immunoprecipitates were washed with lysis buffer, then with lysis buffer containing 0.5 M NaCl and finally with buffer B containing the protein phosphatase inhibitor microcystin-LR (0.1 μM).

2.4. Immunoblotting with antibodies

Aliquots of PC12 cell extracts (20 μg) or Mono Q fractions (24 μl) were denatured in SDS and subjected to SDS-PAGE on 7.5% (B-Raf) or 10% (c-Raf or MEK kinase) gels [17]. The proteins were transferred to nitrocellulose, immunoblotted and detected as described previously [13].

3. Results

3.1. Identification of the latent MAPKKK activity as B-Raf

PC12 cells were chromatographed on Mono Q and, as reported previously [12], MAPKKK activity could not be detected in any of the column fractions if they were assayed immediately. However, immunoblotting with B-Raf antibodies revealed a protein of the expected mass (95 kDa [18]) which co-eluted with the peak of MAPKKK activity that appears when the fractions are assayed several weeks later (Fig. 1). Furthermore, most of the MAPKKK activity that appeared on storage was precipitated by B-Raf antibodies (Fig. 2), and two different B-Raf antibodies raised against a C-terminal peptide

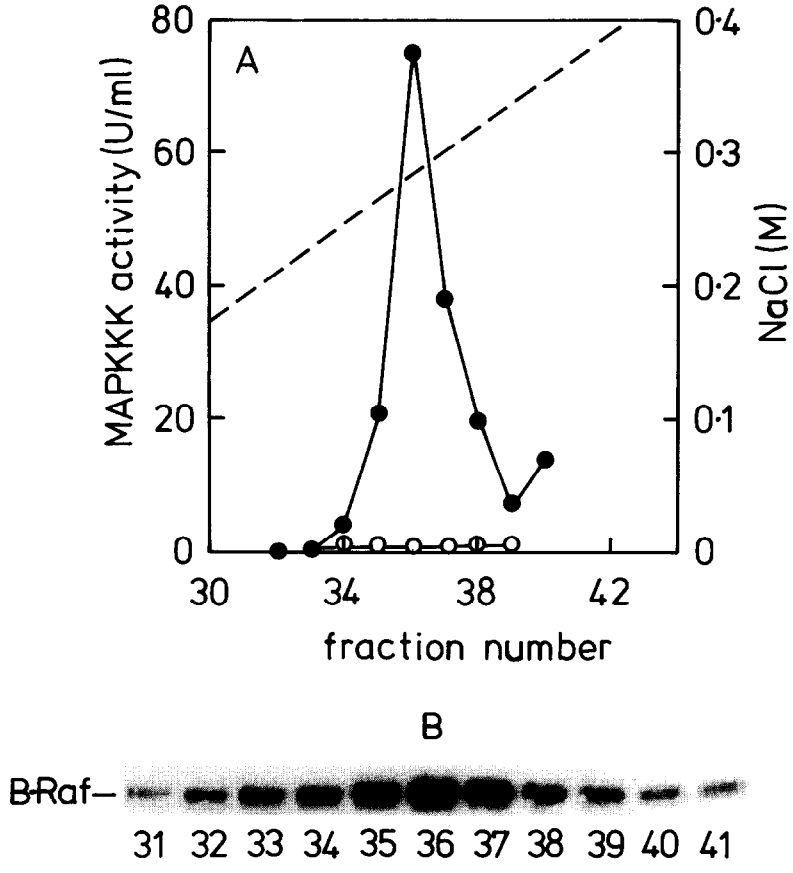

Fig. 1. The latent MAPKKK in PC12 cells co-elutes on Mono Q with B-Raf. Cell lysates were chromatographed on Mono Q and (A) assayed for MAPKKK activity immediately (○), or after storing the fractions for 4 weeks at 4°C (●). The broken line shows the salt gradient. (B) 24 μl of the freshly isolated fraction were subjected to SDS-PAGE and immunoblotted with B-Raf antibodies.

of B-Raf activated the latent MAPKKK activity when added to the freshly isolated Mono Q fractions (Fig. 3). This activation did not occur if the antibodies were first preincubated with peptide immunogen (Fig. 3), indicating that activation results from a specific interaction with the B-Raf antibody. In contrast, no MAPKKK activity was immunoprecipitated when the B-Raf antibody was replaced by either buffer or c-Raf antibodies (Fig. 2), although under identical conditions the c-Raf antibody immunoprecipitated most of the MAPKKK activity associated with the solubilised plasma membranes of Ras-transformed retinal cells [13]. Immunoblotting of the Mono Q fractions with c-Raf antibodies revealed a protein with the mass of c-Raf (70 kDa) which eluted as a very broad peak overlapping with B-Raf. However, the fractions containing the highest levels of c-Raf eluted later (at fractions 38 and 39) than those containing the latent MAPKKK activity (data not shown). Immunoblotting of the same fractions with MEK kinase antibodies showed no immunoreactive band of the expected mass (78 kDa [9]).

3.2. *Lack of correlation between phosphorylation and activation of Raf*

It has been reported that the phosphorylation of B-Raf increases in response to NGF or EGF, resulting in a small decrease in its electrophoretic mobility, which is maximal 5–15 min after growth factor stimulation [18,19]. Similar results were obtained in the present work, where the decrease in electrophoretic mobility of the 95 kDa band in PC12 cell lysates was detectable 3 min after stimulation with NGF, was maximal after 4 min and sustained for at least 30 min. After stimulation with EGF the mobility shift was detectable after 2 min, maximal after 3 min and sustained for at least 1 h (Fig. 4A). Thus the decrease in mobility of B-Raf does not correlate with the activity of MAPKK-1, which is maximally activated after stimulation with EGF for 2 min and then rapidly inactivated [14]. The decreased electrophoretic mobility of B-Raf was retained after chromatography on Mono Q (Fig. 4B), but no MAPKKK activity was detectable in the Mono Q fractions immediately after chromatography, again indicating that phosphorylation per se is insufficient for activation. The inclusion of GTP (0.1 mM) and N-Ras in the assays also failed to activate Mono Q-purified B-Raf from NGF-stimulated or unstimulated PC12 cells (not shown).

The electrophoretic mobility of c-Raf is also decreased when PC12 cells are stimulated with NGF or EGF [20]. In the present study, the mobility shift was apparent after stimulation for 5 min with NGF, was maximal after 15 min and sustained for at least 30 min. In cells stimulated with EGF the mobility shift was only apparent after 15 min, and sustained for at least 1 h (data not shown). Thus phosphorylation of c-Raf in response to EGF does not

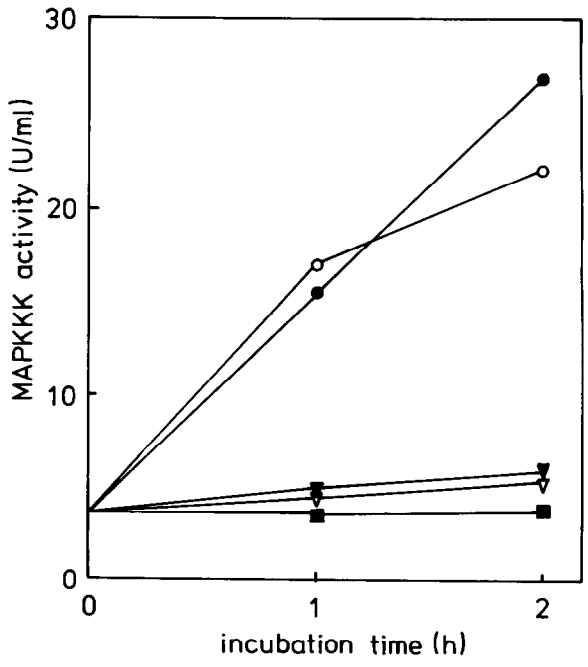

Fig. 3. Activation of B-Raf by B-Raf antibodies. An aliquot of fraction 36 from chromatography on Mono Q containing very little MAPKKK activity (Fig. 1, ○) was incubated at 4°C with two different B-Raf antibodies raised against a C-terminal peptide of B-Raf in the absence (open and closed circles) or presence (open and closed triangles) of peptide immunogen (10 μg/ml). The closed squares show an incubation in which antibody and peptide immunogen were omitted. Each incubation was assayed for MAPKKK activity at the times indicated.

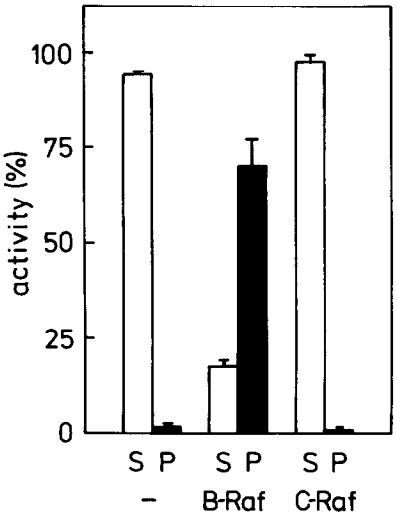

Fig. 2. The MAPKKK activity from PC12 cells is immunoprecipitated with B-Raf antibodies. Fraction 36 from chromatography on Mono Q (Fig. 1) was stored for 4 weeks at 4°C to activate the latent MAPKKK activity and then incubated with Protein A–Sepharose alone (−), B-Raf antibodies coupled to Protein A–Sepharose (B-Raf) or c-Raf antibodies coupled to Protein A–Sepharose (C-Raf). After centrifugation of the Protein A–Sepharose, MAPKKK activity was measured in the immunoprecipitate (P, filled bars) and supernatant (S, open bars). Activities are expressed as a percentage of the values measured in the fraction before addition of antibody. The results are expressed as S.E.M. for three separate experiments.

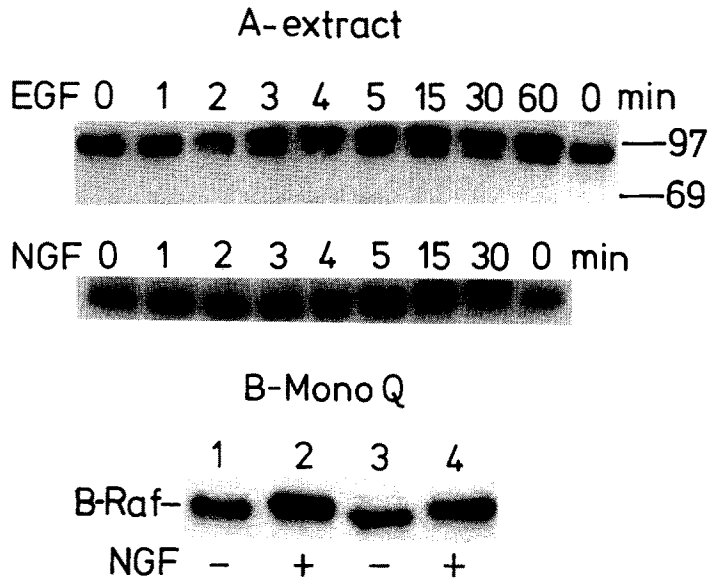

Fig. 4. Phosphorylation of B-Raf in PC12 cells in response to EGF and NGF. (A) PC12 cells were stimulated with EGF (100 ng/ml) or NGF (50 ng/ml) for the times indicated, lysed and 20 μg of extract subjected to SDS-PAGE and immunoblotted with B-Raf antibodies. The stronger immunoreactivity of the more highly phosphorylated (lower mobility) forms of B-Raf was observed in most experiments. The arrows denote the positions of the marker proteins phosphorylase (97 kDa) and bovine serum albumin (69 kDa). (B) PC12 cell extracts were chromatographed on Mono Q and fraction 36 from Fig. 1 was immunoblotted with B-Raf antibodies. Lanes 1 and 3, fractions from unstimulated PC12 cells; lanes 2 and 4, fractions from PC12 cells stimulated with NGF for 15 min.

correlate with either the rate of activation or inactivation of MAPKK1. No MAPKKK activity was found when the c-Raf-containing fractions from Mono Q were assayed immediately after chromatography. Furthermore, no MAPKKK activity was detected when c-Raf was immunoprecipitated from the lysates of NGF-stimulated PC12 cells at any time point (data not shown). In contrast, the same c-Raf antibody immunoprecipitated most of the MAPKKK activity from the solubilised membranes of Ras-transformed retinal cells under the same conditions [13].

4. Discussion

The immunoblotting and immunoprecipitation experiments (Figs. 1 and 2) and specific activation of the latent MAPKKK activity by the B-Raf antibody (Fig. 3) demonstrate that this enzyme is not MEK kinase, c-Mos or c-Raf, but an isoform of c-Raf termed B-Raf. B-Raf has a restricted tissue distribution and is found only in foetal brain and testis [20a], although its presence in PC12 cells has been noted before [18,19]. The C-terminal catalytic domain of B-Raf is 79% identical to c-Raf. The N-terminal regulatory domain is less similar, but contains the two conserved regions (CR1 and CR2) present in c-Raf [18,21], suggesting an analogous activation mechanism. Why B-Raf becomes activated when the Mono Q fractions are stored for several weeks at 4°C is unclear. N-Terminal truncation of c-Raf is known to generate a constitutively activated enzyme [22,23], and in several experiments we have observed the partial conversion of B-Raf during storage from the native 95 kDa form to a 63 kDa species by immunoblotting with the B-Raf antibodies which are raised against a C-terminal peptide (section 2.1). However, we have been unable to prevent activation with any proteinase inhibitors, and in other experiments a similar activation of B-Raf occurred during storage without significant formation of the 63 kDa species, suggesting that limited proteolysis may not be the mechanism of activation. A further possibility, consistent with effects of the antibody and of prolonged storage, is that activation results from the aggregation of B-Raf.

The observation that antibodies raised against a C-terminal peptide of B-Raf activated its MAPKKK activity was surprising, because c-Raf is not activated by antibodies raised against its C-terminus. Activation of B-Raf was blocked by the peptide immunogen (Fig. 3), indicating that it results from a specific interaction between the antibody and B-Raf, and not from proteolysis catalysed by a contaminating proteinase. These antibodies should therefore be useful for detecting latent forms of B-Raf in cell extracts. However, the present study suggests that they should not be used to study the activation of B-Raf in vivo, despite the fact that they have been employed for this purpose previously [18,19]. When B-Raf was immunoprecipitated from PC12 cell extracts there was a high basal level of MAPKKK activity even in unstimulated cells, as expected from the activating

effect of the antibodies. This basal activity increased nearly 2-fold after stimulation of PC12 cells for 3–4 min with NGF or EGF, and remained at this elevated value for at least 30 min (S. Traverse, unpublished results). However, this activation may be artefactual if a post-translational modification, such as phosphorylation, potentiated activation by the antibody.

In many mammalian cells the activation of c-Raf, MAPKK and MAP kinase is potently inhibited by agonists that elevate the intracellular concentration of cyclic AMP or by cell permeant analogues of cyclic AMP [24–28]. Wu et al. [27] reported that the inhibition of c-Raf was accompanied by increased in vivo phosphorylation of Ser-43, the residue phosphorylated by cyclic AMP-dependent protein kinase in vitro. They also found that phosphorylation of Ser-43 reduced the affinity of c-Raf for GTP-Ras [27], suggesting that Ser-43 phosphorylation may underlie the inhibition of c-Raf activation by cyclic AMP. Our attempts to trigger differentiation by microinjecting the activating B-Raf antibodies into PC12 cells have so far been unsuccessful (S. Traverse and A. Prescott, unpublished experiments). It is therefore unclear whether B-Raf or another as yet unidentified Ras-dependent protein kinase activates MAPKK1 in NGF- or EGF-stimulated PC12 cells [7]. However, if B-Raf was the relevant activity, the lack of Ser-43 in this isoform could explain why the activation of MAP kinase is not inhibited by cyclic AMP in PC12 cells [29].

Our finding that c-Raf is not activated by NGF in PC12 cells is in agreement with data reported in [7], but disagrees with the work of Ohmichi et al. [30]. These investigators did not measure c-Raf activity using the physiological substrate MAPKK, but by the phosphorylation of c-Raf which occurred when the immunoprecipitates were incubated with MgATP [30]. It is therefore possible that phosphorylation was not catalysed by c-Raf itself, but by another NGF-stimulated protein kinase present in the immunoprecipitates as a trace contaminant. Because c-Raf and MAPKK are substrates for other kinases such as the p42 and p44 isoforms of MAP kinase, it is essential to measure c-Raf by its ability to activate MAPKK and hazardous to measure activation by the phosphorylation of MAPKK [7], c-Raf [30] or other proteins, as noted previously [2].

The molecular mechanism by which growth factors activate either c-Raf or B-Raf is unknown. Although GTP-Ras is known to interact with the N-terminal domain of c-Raf [31–34], and c-Raf is translocated to the plasma membrane and activated in Ras-transformed retinal cells [13], neither we [13] nor others [34] have been able to detect any direct activation of c-Raf or B-Raf by GTP-Ras in vitro. Furthermore, although B-Raf and c-Raf are phosphorylated in PC12 cells in response to NGF or EGF, phosphorylation does not lead to, or correlate with, activation. Similarly c-Raf associated with the plasma membranes of Ras-transformed retinal cells [14] is not inactivated by incubation with high concentrations of a protein phosphatase 'cocktail', and c-Raf in insect Sf9 cells (which had been activated by co-expression with oncogenic *Ras*) is not deactivated by the same phosphatase treatment, although all covalently bound phosphate is removed (D. Alessi and P. Cohen, unpublished). Thus neither allosteric activation by GTP-Ras nor phosphorylation appear to account for the activation of Raf by growth factors, and the role of GTP-Ras may be to recruit Raf to the plasma membrane where it is activated by an as yet unidentified mechanism.

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