Regulation of the MAP kinase cascade in PC12 cells: B-Raf activates MEK-1 (MAP kinase or ERK kinase) and is inhibited by cAMP

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Abstract In PC12 cells, cAMP stimulates the MAP kinase pathway by an unknown mechanism. Firstly, we examined the role of calcium ion mobilization and of protein kinase C in cAMP-stimulated MAP kinase activation. We show that cAMP stimulates p44[mapk] independently of these events. Secondly, we studied the role of B-Raf in this process. We observed that NGF, PMA and cAMP induce the phosphorylation of B-Raf as well as an upward shift in its electrophoretic mobility. We show that B-Raf is activated following NGF and PMA treatment of PC12 cells, and that it can phosphorylate and activate MEK-1. However, cAMP inhibits B-Raf autokinase activity as well as its ability to phosphorylate and activate MEK-1. This inhibition is likely to be due to a direct effect since we found that PKA phosphorylates B-Raf in vitro. Further, we show that B-Raf binds to p21[ras], but more important, this binding to p21[ras] is virtually abolished with B-Raf from PC12 cells treated with CPT-cAMP. Hence, these data indicate that the PKA-mediated phosphorylation of B-Raf hampers its interaction with p21[ras], which is responsible for the PKA-mediated decrease in B-Raf activity. Finally, our work suggests that in PC12 cells, cAMP stimulates MAP kinase through the activation of an unidentified MEK kinase and/or the inhibition of a MEK phosphatase.

Key words: cAMP; B-Raf; MAP kinase; MEK; p21[ras]; PC12 cell

1. Introduction

MAP kinases are key elements in the transduction of a large variety of extracellular stimuli (growth factors, hormones, neurotransmitters), which activate diverse signaling systems in the cell. Initial events in the various pathways include stimulation of tyrosine protein kinases, elevation of intracellular calcium, and activation of protein kinase C. Several signaling circuitries leading to MAP kinase activation converge on Ras, which leads to the sequential activation of Raf-1, MEKs and MAP kinases [1,2].

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Abbreviations: MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; CPT-cAMP, 8-(4-chlorophenylthio)-cyclic-AMP; NGF, nerve growth factor; MBP, myelin basic protein; PMA, phorbol 12-myristate 13-acetate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

We have previously reported that in the neuroendocrine PC12 cells cAMP activates MAP kinase and potentiates the stimulating effects of other agents [3]. The stimulatory effect of cAMP was observed also on MEK, but no further information is available on the mechanism used by cAMP in these cells. In contrast to PC12 cells, in smooth muscle cells [4], adipocytes [5] and some fibroblast cell lines [6,7], cAMP inhibits the MAP kinase cascade. In these cells it has been shown that cAMP-dependent protein kinase (PKA) phosphorylates Raf-1, rendering it less efficient in interacting with Ras [8–10]. The apparently cell-specific modulation of MAP kinase by cAMP is of considerable interest, since the biological action of cAMP in the different cell types is coherent with its action on the MAP kinase pathway. Indeed, in PC12 cells, cAMP stimulates the MAP kinase pathway and the key biological effect in these cells, i.e. neuronal differentiation [3]. In contrast, it decreases bioresponses in cells in which it inhibits the MAP kinase cascade [4–7].

To elucidate how cAMP may affect the MAP kinase route in PC12 cells, we firstly investigated the PKC- and calcium-dependence of this response. Secondly, we looked at B-Raf phosphorylation and activation, and its possible role as a kinase upstream of MEK. In contrast to the ubiquitous Raf-1, B-Raf is abundant in only a few tissues, such as neuronal cells and PC12 cells [11,12]. We show that exposure of PC12 cells to NGF, PMA and cAMP induces B-Raf phosphorylation. Furthermore, NGF treatment increases B-Raf autokinase activity but this is inhibited by cAMP. Importantly, in a reconstitution assay, B-Raf can phosphorylate and activate MEK-1. B-Raf activity is stimulated following NGF and PMA treatment of PC12 cells, but after cAMP co-treatment these activations returned to basal levels. Next, we show that PKA phosphorylates B-Raf in vitro. Moreover, B-Raf from untreated PC12 cells associates with p21[ras], but treatment of these cells with CPT-cAMP decreased the ability of B-Raf to associate with p21[ras]. It is therefore likely that PKA-mediated phosphorylation of B-Raf reduces its ability to interact with p21[ras], explaining the reduced activation of B-Raf in CPT-cAMP-treated PC12 cells.

Our results indicate that, while B-Raf can function as a MEK kinase, it does not appear to be actively implicated in the cAMP-induced stimulation of the MAP kinase pathway in PC12 cells.

2. Materials and methods

2.1. Materials

Antibodies to p44[mapk] [13], MEK-1 [3] were obtained as previously described. Antibodies to B-Raf were generated against a peptide corresponding to exon 11 of the Rmil protein [14], the avian homologue of

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the B-Raf gene product, which also recognize murine B-Raf protein (Barnier et al., unpublished results). The PKA catalytic subunit, PKI, and MBP were from Sigma. Activated CH Sepharose 4B was from Pharmacia.

2.2. Cell culture

PC12 cells were cultured in RPMI 1640 supplemented with 10% horse serum and 5% fetal calf serum. Prior to stimulation, cells were starved overnight in RPMI 1640 containing 0.25% fetal calf serum and 0.2% bovine serum albumin.

2.3. $p44^{mapk}$ assay

$p44^{mapk}$ activity was measured in an immune complex kinase assay as described [3]. Briefly, cells were solubilized in stop buffer (50 mM HEPES, pH 7.5, 10 mM EDTA, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 2 mM sodium orthovanadate, 100 mM NaF, 1% Triton X-100 (v/v), 100 U/ml aprotinin, 20 mM leupeptin, and 0.18 mg/ml $PhMeSO_2F$). $p44^{mapk}$ was immunopurified from cell extracts with antibody preabsorbed to protein A-Sepharose, and its kinase activity measured by incubation with [γ-^{32}P]ATP and myelin basic protein as a substrate. Radioactivity incorporated in myelin basic protein was quantitated by spotting an aliquot of the kinase reaction mixture onto phosphocellulose paper, followed by washing, and determination of the radioactivity associated with the papers.

2.4. ^{32}P labeling

PC12 cells were incubated in serum- and phosphate-free RPMI, containing 0.5 mCi/ml [^{32}P]orthophosphate for 3.5 h. Thereafter, cells were stimulated, solubilized in stop buffer and cell extracts were immunoprecipitated with antibody preadsorbed to protein A-Sepharose. Immunopurified proteins were subjected to SDS-PAGE analysis under reducing conditions followed by autoradiography.

2.5. B-Raf autokinase assay

After starvation, PC12 cells were treated with the indicated compounds and lysed in stop buffer. Cell extracts were immunopurified using an antibody to B-Raf preadsorbed to protein A-Sepharose. Immunopurified proteins were then incubated in kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 40 mM pNPP) and autokinase activity was initiated by addition of 1 μM [γ-^{32}P]ATP (7000 Ci/mmol). After 10 min at room temperature the reaction was stopped by addition of Laemmli buffer and proteins were subjected to SDS-PAGE analysis on a 7.5% resolving gel.

2.6. B-Raf activity towards MEK-1

B-Raf was immunopurified as described above and incubated in the presence or absence of immunopurified MEK-1 in kinase buffer supplemented with 1 μM [γ-^{32}P]ATP (7000 Ci/mmol). After 10 min at room temperature, the kinase reaction was stopped by addition of Laemmli buffer and proteins were subjected to SDS-PAGE analysis on a 10% resolving gel. MEK-1 was immunopurified from CHO cells transfected with an expression vector coding for human MEK-1.

2.7. MEK-1 activation mediated by B-Raf

After the appropriate treatment, PC12 cells were lysed in stop buffer and B-Raf was immunopurified as described above. B-Raf was then incubated with or without immunopurified MEK-1 in kinase buffer enriched with 15 μM ATP. After 10 min, recombinant $p44^{mapk}$ was added or not for 10 min, then 1 μM [γ-^{32}P]ATP (7000 Ci/mmol) and 150 μg/ml myelin basic protein were added for 10 min. Proteins were subjected to SDS-PAGE analysis on a 12.5% resolving gel and autoradiographed.

2.8. PKA-mediated B-Raf phosphorylation

B-Raf was immunoprecipitated from PC12 cells, thereafter incubated in kinase buffer supplemented with 50 μM ATP for 15 min. Pellets were washed and incubated in HEPES 30 mM, pH 7.4, 30 mM NaCl, Triton X-100 0.1% (v/v), 100 mM NaF and 0.1 μM okadaic acid, and treated or not with PKA catalytic subunit (10 U) and PKI (10 ng); thereafter a mixture containing 8 mM $MgCl_2$, 4 mM $MnCl_2$, and 15 μM [γ-^{32}P]ATP (100 Ci/mmol) was added. After 10 min at room temperature the reaction was stopped by the addition of Laemmli buffer, and proteins were subjected to SDS-PAGE analysis on a 7.5% resolving gel.

2.9. Association of B-Raf with $p21^{Ha\text{-}ras}$

Recombinant $p21^{Ha\text{-}ras}$ was immobilized on Sepharose beads. Thereafter, $p21^{Ha\text{-}ras}$ was complexed with GDP, or with the non-hydrolysable GTP analogue, GTPγS, by incubating the $p21^{Ha\text{-}ras}$ beads with GDP (100 μM) or GTPγS (100 μM) for 30 min at 37°C in 50 mM HEPES, pH 7.5, 2 mM EDTA. Control experiments were performed using heat-denaturated $p21^{Ha\text{-}ras}$. The beads (corresponding to approximately 300 μg of $p21^{Ha\text{-}ras}$) were incubated for 3 h at 4°C with 3 mg of extracts from untreated PC12 cells or PC12 cells treated for 15 min with 1 mM CPT-cAMP. Cell extracts were obtained as described above except that the stop buffer lacked EDTA. After 4 washes in stop buffer supplemented with 0.1% SDS, the proteins associated with $p21^{Ha\text{-}ras}$ were eluted in Laemmli buffer and subjected to Western blot analysis using B-Raf as a probe.

3. Results and discussion

In contrast to observations made in several other cell types (smooth muscle cells [4], adipocytes [5] and fibroblasts [6,7]), we have previously reported that, in PC12 cells, cAMP activates MAP kinase ($p42^{mapk}$ and $p44^{mapk}$) and MEK-1 [3]. While the inhibitory effect of cAMP has been shown to be induced through PKA-mediated Raf-1 phosphorylation [9,10,15], nothing is known about the mechanism underlying the stimulatory action of cAMP on MAP kinase. cAMP is known to exert most of its biological effects by means of PKA, but it has also been shown to modulate directly the Glut 4 transporter [16] and some ion channels [15,17,18]. Thus it is possible that in PC12 cells the stimulatory effect of cAMP on MAP kinase could result from a cross-talk with other second messengers, in particular calcium ions or PKC, which are known to stimulate MAP kinase activity in several cell types [19,20]. To investigate whether cAMP might feed into the MAP kinase cascade by mobilizing extracellular calcium or by activating protein kinase C, we tested the effects of chelating extracellular calcium and of down-regulation of PKC by overnight incubation of PC12 cells with PMA (Fig. 1). Neither EGTA nor down-regulation

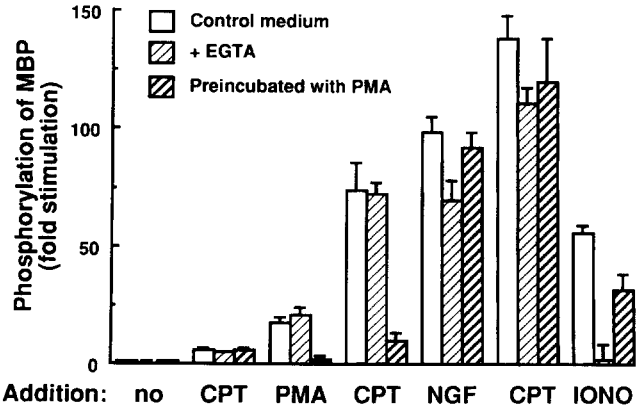

Fig. 1. Effects of EGTA and of preincubation with PMA on $p44^{mapk}$ activation by cAMP in PC12 cells. Cells were treated with buffer (open bars), incubated with 5 mM EGTA (hatched bars), or incubated overnight with 1 μM PMA (solid bars). One hour before the stimulation PMA was removed by two washes. Cells were then exposed for 15 min to 1 mM CPT-cAMP, 1 μM PMA or 50 nM NGF or for 10 min to 1 μM ionomycin and thereafter solubilized. $p44^{mapk}$ was immunoprecipitated and its kinase acitivity was measured as described in section 2. $p44^{mapk}$ activity is expressed as fold-stimulation above the level seen in untreated cells. Data represent the mean±S.D. of triplicate wells from an experiment performed twice with similar results.

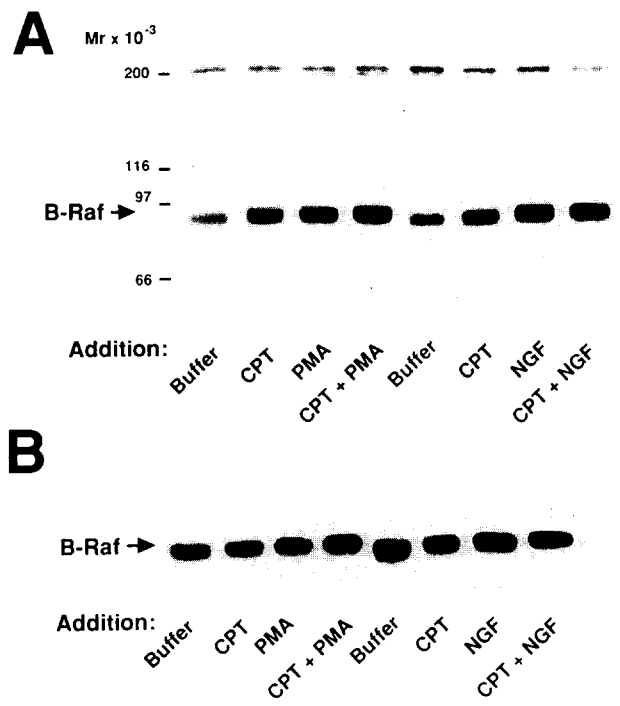

Fig. 2. B-Raf phosphorylation in PC12 cells. (A) In vivo ^{32}P labeling. ^{32}P-Loaded PC12 cells were incubated for 15 min with 1 mM CPT-cAMP, 1 μM PMA, CPT-cAMP plus PMA, 0.1 μM NGF and CPT-cAMP plus NGF and solubilized. B-Raf was then immunopurified and subjected to SDS-PAGE analysis. This autoradiograph is representative of an experiment performed twice. (B) B-Raf Western blot analysis. PC12 were incubated for 15 min with 1 mM CPT-cAMP, 1 μM PMA, 0.1 μM NGF, and CPT-cAMP together with PMA or NGF, and solubilized. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane which was probed with an antibody to B-Raf. The membrane was incubated with ^{125}I-labelled protein A and autoradiographed. This autoradiograph is representative of an experiment performed three times with comparable results.

of PKC inhibited MAP kinase activation by CPT-cAMP, a membrane-permeant cAMP analogue. Chelation of intracellular calcium by BAPTA was also without effect (results not shown). Furthermore, no rise in intracellular calcium was observed with a calcium probe in CPT-cAMP-treated PC12 cells. As reported by others [21], the NGF-induced MAP kinase activation was inhibited by 30% upon calcium chelation, but was unaffected by PKC down-regulation. In PC12 cells exposed to NGF and CPT-cAMP, CPT-cAMP increased NGF-stimulated MAP kinase activity by approximately 40% [3]. As found in cells treated with NGF only, this activation is inhibited by 20% after calcium chelation, and not significantly modified after PKC down-regulation. Confirming the efficiency of PKC down-regulation, we found that PMA pretreatment totally abolished MAP kinase activation by PMA. PKC down-regulation was also shown to partially inhibit the effect of ionomycin, a calcium ionophore. Confirming efficient chelation of extracellular calcium, EGTA abolished completely MAP kinase activation by ionomycin. These findings indicate that cAMP activates MAP kinase independently of calcium mobilization and of stimulation of a PMA-sensitive PKC.

We then tried to determine at which step, upstream of MAP kinase, the stimulatory effect of cAMP is initiated. Firstly, we determined whether PKA was able to phosphorylate MEK. To do this, we performed an in vitro kinase assay using recombinant GST-MEK-1 or GST-MEK-2, and purified PKA catalytic subunit. In these experiments, we were unable to detect PKA-mediated phosphorylation of either of the MEK proteins (data not shown). However, since MEK-1 and MEK-2 do not contain canonical consensus PKA phosphorylation sites, these results are not surprising. We then looked at a potential upstream activator of MEK. While Raf-1 has been shown to activate MEK, a similar function of B-Raf has not been clearly documented. In contrast to the ubiquitously expressed Raf-1, B-Raf is abundant in only a few tissues, including neuronal tissue and PC12 cells [11,12]. It could therefore confer cell type specificity with respect to the regulation of the MAP kinase cascade. PC12 cells were loaded with [^{32}P]orthophosphate, treated with appropriate compounds and B-Raf was immunoprecipitated with a specific antibody. As observed in Fig. 2A, CPT-cAMP increased the phosphorylation of B-Raf (2-fold above the level observed in untreated cells). PMA also induced B-Raf phosphorylation (2.5-fold), which is slightly increased (3-fold) after a co-treatment with CPT-cAMP. NGF alone increased B-Raf phosphorylation (3-fold), and this phosphorylation is not modified after a CPT-cAMP co-treatment. The phosphorylation was linked to an upward shift in electrophoretic mobility as observed by Western blot analysis of B-Raf (Fig. 2B). We then looked to see whether the PKA-mediated phosphorylation of B-Raf modified its autokinase activity, and whether B-Raf could phosphorylate MEK. To do this, PC12 cells were treated for 10 or 60 min with CPT-cAMP, NGF or both and then lysed. B-Raf was immunoprecipitated from the cell extracts and its autokinase activity was measured as described in section 2. As shown in Fig. 3, NGF augmented the autokinase activity of

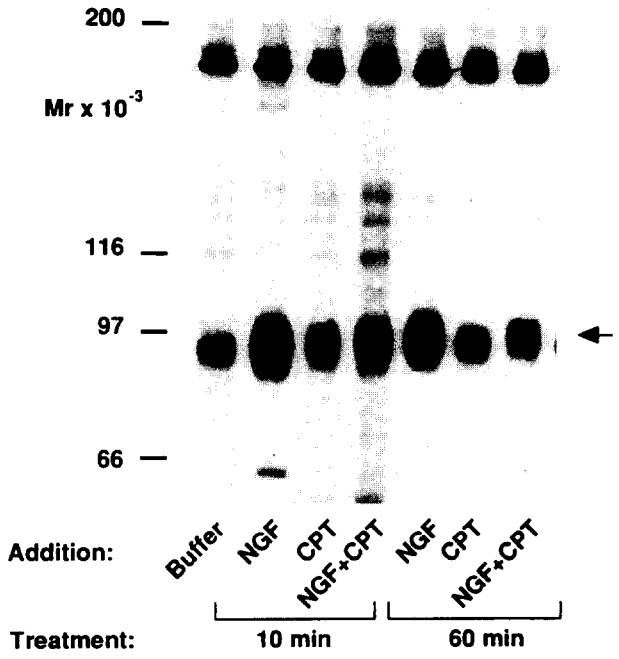

Fig. 3. B-Raf autokinase assay. PC12 cells were incubated for 10 or 60 min with 0.1 μM NGF, 1 mM CPT-cAMP, or NGF with CPT-cAMP and solubilized. B-Raf was then immunopurified, submitted to an autokinase assay, as described in section 2, analyzed by SDS-PAGE on a 7.5% resolving gel and autoradiographed. This autoradiograph is representative of an experiment performed twice with comparable results.

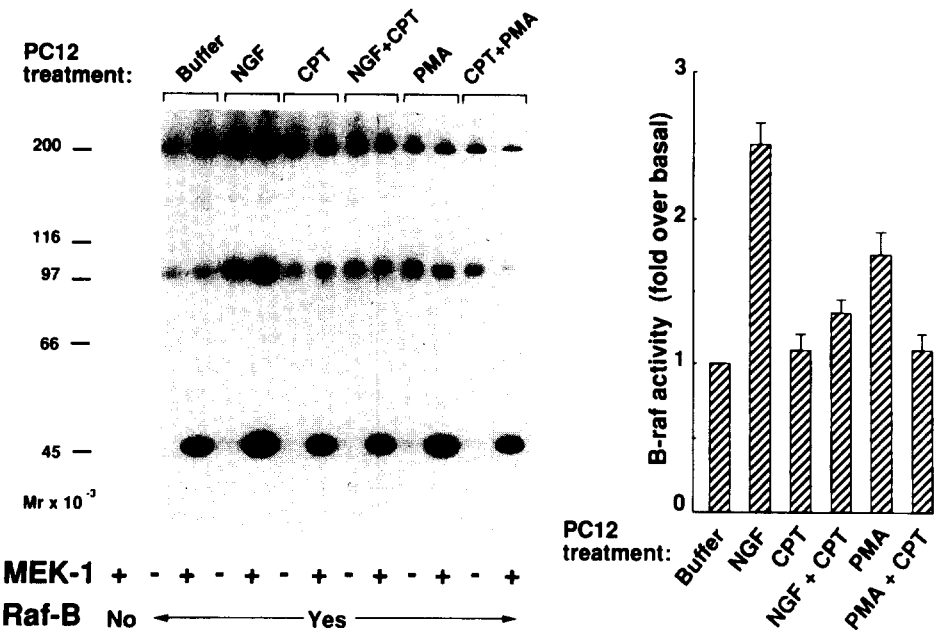

Fig. 4. MEK-1 phosphorylation by B-Raf. PC12 cells were incubated for 15 min with 0.1 μM NGF, 1 mM CPT-cAMP, CPT-cAMP with NGF, 1 μM PMA and PMA with CPT-cAMP. B-Raf was immunoprecipitated from these cells and incubated in presence or in absence of immunopurified MEK-1 as described in section 2. The results have been quantified by scanning densitometry, and are expressed as means ± S.E.M. (n = 3–4) of B-Raf activity above basal (left panel). This autoradiogaph is representative of 3–4 experiments which gave comparable results.

B-Raf, and this increase was inhibited by a CPT-cAMP co-treatment. Interestingly, these results show that, as observed for Raf-1 [22], the upward B-Raf shift in electrophoretic mobility can not be linked to a change in its kinase activity. To determine whether the autokinase activity of B-Raf correlates with its ability to activate MEK-1, PC12 cells were treated with CPT-cAMP, PMA and NGF, and then the kinase activity of B-Raf towards MEK-1 was measured as described in section 2. As illustrated in Fig. 4, NGF stimulated the B-Raf-mediated phosphorylation of MEK-1 by 2.5 ± 0.2-fold over basal. PMA was also able to activate B-Raf, albeit with lower efficiency than NGF (1.7 ± 0.1-fold over basal). CPT-cAMP alone had no effect on B-Raf activity (1.1 ± 0.1-fold over basal). Strikingly, we found that CPT-cAMP inhibits B-Raf activation induced either by NGF (from 2.5 ± 0.2 to 1.35 ± 0.1), or by PMA (from 1.7 ± 0.1 to 1.1 ± 0.1). Western blot experiments performed after B-Raf immunoprecipitation from cells treated with these compounds revealed that the amount of B-Raf immunoprecip-

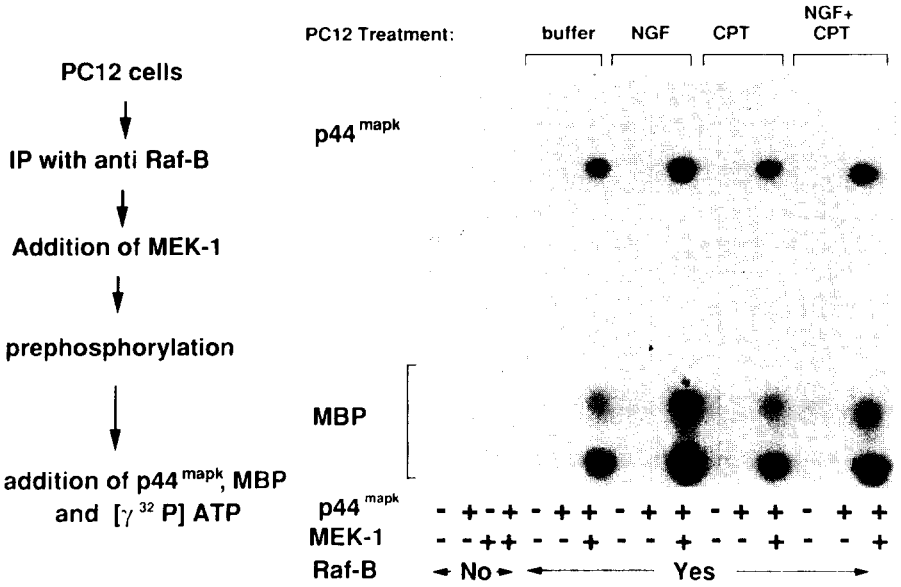

Fig. 5. MEK-1 activation mediated by B-Raf. PC12 cells were incubated for 15 min with 0.1 μM NGF, 1 mM CPT-cAMP and CPT-cAMP with NGF. B-Raf was immunopurified from these cells and incubated or not with immunopurified MEK-1 in the presence of unlabeled ATP for 10 min. Recombinant p44^{mapk} was then added for 10 min followed by a 10 min incubation with [γ-^{32}P]ATP and myelin basic protein. Proteins were then subjected to SDS-PAGE analysis on a 12.5% resolving gel and autoradiographed.

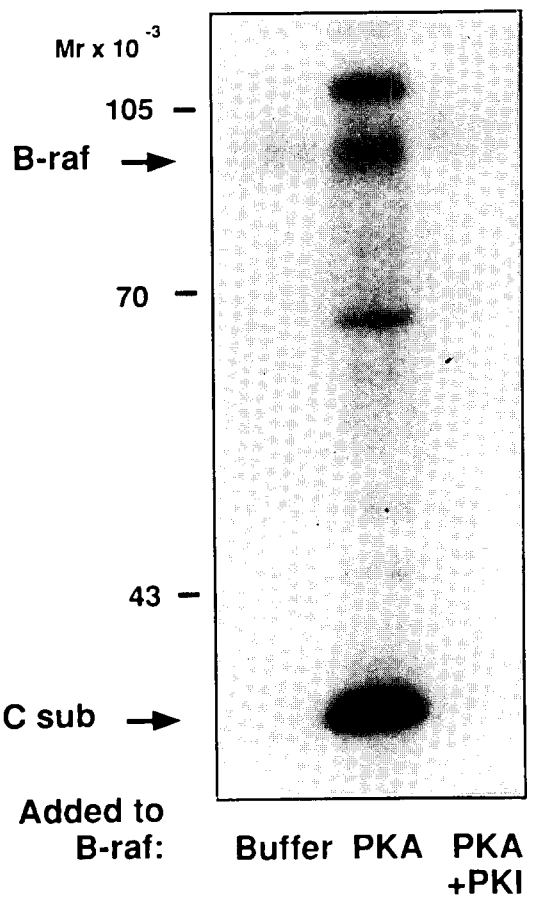

Fig. 6. PKA-mediated B-Raf phosphorylation. B-Raf was immunopurified from PC12 cells, thereafter incubated in the presence of ATP and then treated with buffer alone, PKA or PKA together with PKI for 10 min, as described in section 2. Proteins were then subjected to SDS-PAGE analysis on a 7.5% resolving gel and autoradiographed. This autoradiogram is representative of an experiment performed twice with comparable results.

itated was comparable under each condition, which excludes the possibillity that the changes in B-Raf activity could be due to variations in the B-Raf quantity present in the experiments (data not shown). The results in Fig. 5 demonstrate that the NGF-induced B-Raf phosphorylation is activating since it enables MEK-1 to phosphorylate and stimulate $p44^{mapk}$. Indeed, B-Raf from NGF-treated cells induced a 2.3-fold activation of MEK-1 and of $p44^{mapk}$. While CPT-cAMP alone had no effect, it inhibited the NGF-induced activation to the level observed in basal conditions. These results show that, similar to Raf-1, B-Raf can function as an activator of MEK-1. In a very recent study Traverse and Cohen reported that B-Raf activity can be measured only after B-Raf aggregation obtained after a 4 week-long storage of the protein at 4°C [23]. Here we show that freshly isolated B-Raf is endowed with enzymatic activity toward MEK-1.

It should be noted that the activity of B-Raf obtained from untreated cells is elevated (Figs. 4 and 5). This may be related to the finding that Raf-1 is activated through phosphorylation of Tyr-340 and Tyr-341 after activation of the PDGF receptor, and in $p60^{v\text{-}src}$-transformed fibroblasts [24]. Moreover, mutation of these amino acids to negatively charged residues (Asp) has been shown to activate the catalytic activity of Raf-1 [24]. The sequence alignment of the catalytic domain for the Raf proteins revealed that all Raf-1 and A-Raf proteins have tyrosine residues at positions analogous to Tyr-340 and Tyr-341, while B-Raf has negatively charged amino acids at these positions. This difference in the amino acid sequence could render B-Raf 'constitutively active' as compared to Raf-1, and could explain, at least in part, the high basal activity of B-Raf. It could be envisioned that to restrain the 'unstimulated' MEK and MAPK, the high 'basal' activity of B-Raf toward MEK-1 must be negatively regulated by phosphatases at the level of MEK and/or MAP kinase, as we previously suggested [25].

cAMP inhibits B-Raf activation as illustrated by the repressive action of CPT-cAMP on the NGF response (Figs. 4 and 5). It has recently been reported that PKA phosphorylates Raf-1 on Ser-43 [8,10], and that this phosphorylation results in a profound inhibition of Ras binding which is necessary for Raf-1 activation [8–10]. Moreover, in their study, Chuang et al. demonstrated that this Ser-43 is located in a 50-amino acid PKA regulatory segment which is found only in Raf-1, and not in A-Raf or B-Raf [10]. In contrast, B-Raf possesses two potential phosphorylation sites for PKA in its C-terminus, upstream of the kinase domain (Ser-429 in a RKSS sequence, and Ser-446 in a RRDS sequence). To determine whether B-Raf is indeed a direct substrate of PKA, we performed an in vitro phosphorylation experiment. Thus, B-Raf was immunopurified from PC12 cells, then incubated in the presence or absence of purified PKA catalytic subunit without or with the protein kinase inhibitor, PKI. As observed in Fig. 6, PKA was able to phosphorylate B-Raf, and this phosphorylation appears to be specific since it was inhibited by PKI. Next, we tried to determine whether B-Raf associates with Ras, and whether this association was modified after CPT-cAMP treatment of PC12 cells. A reconstitution experiment was performed as described in

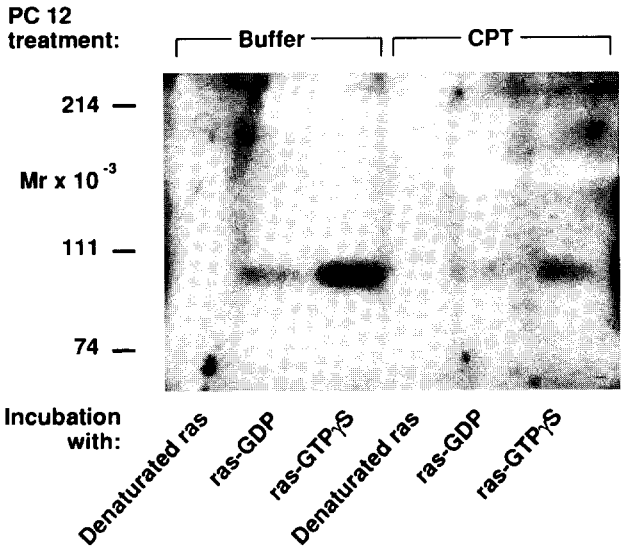

Fig. 7. B-Raf association with $p21^{Ha\text{-}ras}$. PC12 cells were treated with buffer or CPT-cAMP for 15 min then solubilized. Cell extracts were incubated in the presence of Sepharose beads associated with heat-denaturated $p21^{Ha\text{-}ras}$ (denaturated Ras), or with $p21^{Ha\text{-}ras}$ loaded with GDP (Ras-GDP), or GTPγS (Ras-GTPγS). After 3 h at 4°C, beads were washed and the presence of B-Raf associated with $p21^{Ha\text{-}ras}$ was determined as described in section 2. This autoradiogram is representative of an experiment performed 4 times with comparable results.

section 2, and the results are shown in Fig. 7. It was found that B-Raf associates with Ras-GTPγS, but not with heat-denaturated Ras and only very weakly with Ras-GDP. Note that similar studies with Raf-1 have previously shown that, whereas Raf-1 associates preferentialy with Ras-GTPγS, it associates also, albeit with much lower affinity, with Ras-GDP [26,27]. Interestingly, when cells were treated with CPT-cAMP, the ability of B-Raf to bind to Ras was dramatically decreased. However, the activity of B-Raf obtained from untreated or NGF-stimulated cells was not modified upon its in vitro phosphorylation by PKA. These data suggest that the association of B-Raf with Ras is essential for B-Raf activation and that its interruption might be sufficient to explain the inhibition of PKA on B-Raf functioning. In addition, it is likely that the mechanism of B-Raf inhibition after CPT-cAMP treatment of PC12 cells is the same as that previously observed for Raf-1. However, while we favour the idea that this effect is due to a direct phosphorylation of B-Raf by PKA, we can not exclude the action of another kinase which could be previously activated by PKA. In summary, from our data taken together we draw the key conclusion that B-Raf appears to be subjected to the same regulatory mechanisms as Raf-1, concerning both its activation by Ras and its inhibition by PKA phosphorylation. The latter point is particularly intriguing since the PKA phosphorylation sites are not found in the same domains in both Raf proteins.

The stimulation of MAP kinase by cAMP seen in PC12 cells implies that there exist mechanisms which are obviously able to overcome the reduced B-Raf function in PKA-treated cells. To explain the stimulatory action of cAMP on MAP kinase in PC12 cells, it could be hypothesized that cAMP, either through PKA or directly, interferes with negative modulators of MEK, such as phosphatases, or activates MEK kinase(s) distinct from the Raf kinases, such as MEKK identified by Lange-Carter and colleagues [28]. Indeed, PKA has been shown to regulate the Ser/Thr phosphatase PP1G [29], tyrosine phosphatase activities [30] and the tyrosine phosphatase PTP-PEST [31]. However, whereas the phosphorylation sites modulating MEK activity have been determined [32], no data on phosphatase(s) regulating MEK are available. An emerging hypothesis suggests that Raf-1 is not the major or sole MEK activator involved in growth factor activation of cells. This is supported by observations made in Swiss 3T3 cells [33] and adipocytes [34,35] where MAP kinase stimulation can be dissociated from Raf-1 activation. Further, in Rat 1a cells, expression of constitutively activated v-ras and v-raf did not result in a marked change in MEK activity [36]. Finally, transfection of PC12 cells with activated Raf-1 did not induce significant increases in MAP kinase activity [37]. As a whole, these data indicate that in PC12 cells, MAP kinase activation is probably not mediated solely by Raf kinases. This could explain why NGF and cAMP have antagonistic effects on B-Raf activation, while they act in synergy to stimulate MEK-1. Clearly, additional MEK activator(s) remain to be identified in PC12 cells. In this context, enzymes of the MEK kinase family certainly offer a promising track for future research. Indeed, at least five isoforms have been reported and some, but not all, appear to be activated in response to growth factors [1]. It is possible that one of these MEK kinases is specific for PC12 cells and that it is activated by cAMP, thus explaining why the cAMP stimulatory action on MAP kinase appears to be restricted to PC12 cells.

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