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Differential regulation of Raf isozymes by growth versus differentiation inducing factors in PC12 pheochromocytoma cells

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Abstract PC12 pheochromocytoma cells possess four known MEK activators: A-, B-, c-Raf-1 and MEKK. In order to examine whether differentiation factors or growth factors have a Raf isozyme preference for activation of the mitogenic cytoplasmic Raf-MEK-MAPK protein kinase cascade, the activation kinetics of these enzymes in response to epidermal growth factor (EGF) and nerve growth factor (NGF) were compared. An initial activation of all three Raf kinases was noticed, but only A- and B-Raf showed sustained activation by NGF, which was not seen after EGF treatment. Furthermore, expression of oncogenic versions of all three Raf kinases as well as a potentially Raf-independent MEK activator, v-Mos, leads to activation of MAPK and to differentiation of PC12 cells. These data suggest a differential regulation of Raf kinases and that probably no alternative Raf substrates are involved in differentiation processes of PC12 cells.

Key words: Neuronal differentiation; Signalling pathway; A-Raf-1 kinase; B-Raf-1 kinase; c-Raf-1 kinase

1. Introduction

The function of Raf serine/threonine protein kinases is commonly associated with transmission of proliferation signals following growth factor receptor stimulation in mammalian cells. Signal transmission involves recruitment of a highly conserved protein kinase cascade consisting of the dual-specificity mitogen-activated protein kinase kinase (MEK) and its substrate, a serine/threonine specific mitogen-activated protein kinase (MAPK). Early work with an oncogenic version of Raf-1 (v-Raf) that was transduced as part of a retrovirus, 3611 MSV [1], demonstrated that constitutive expression of Raf-1 kinase also influenced differentiation processes (reviewed by Rapp et al. [2]). Specifically, upon infection of newborn mice an altered fate was observed in erythroid, B/myeloid and epithelial lineages [3] and upon infection of progenitor cells in culture erythroid, adipocyte or neuronal differentiation was induced [4-7]. When v-Raf was combined with another growth factor inducible cell cycle-progression regulator, v-myc, induction of differentiation of early progenitors of the B-lineage to mature lymphocytes was observed that was associated with a tendency to switch to the myeloid lineage [5,8]. When comparison was made with the activity of oncogenic forms of other Raf isozymes, A- and B-Raf, there was a suggestion of tissue specific transforming/differentiation effects [9]. The three Raf isozymes, A-, B- and c-Raf-1 differ in their tissue specific pattern of expression in that c-Raf-1 is present in all tissues, A-Raf adds to Raf-1 expression predominantly in urogenital tissues and B-Raf is primarily present in brain tissue [10]. It was therefore reasonable to expect that there may be functional differences between A-, B- and c-Raf-1s, although at least under conditions of overexpression they clearly overlapped in the ability to recruit the classic cytoplasmic cascade [11]. In fact, recent work on cAMP sensitivity of B-Raf versus c-Raf-1 in PC12 pheochromocytoma cells demonstrated differential regulation [12] and the use of chimeric oestrogen receptor Raf fusion proteins in NIH 3T3 and Rat1a fibroblast cell lines indicated differences in the specific activities towards the common substrate MEK and suggested differences in substrate choice [13]. One cell system in which all three Raf isozymes are expressed and which can be induced by growth factors to either differentiate or proliferate are PC12 pheochromocytoma cells [2,14,15]. In order to examine whether differentiation versus growth factor receptors would have an isozyme preference for activation of the classic cascade, we decided to determine the kinetics of activation of all three Raf isozymes by nerve growth factor (NGF) and epidermal growth factor (EGF), the effect of their oncogenic versions on activation of MAP kinase and on differentiation.

2. Materials and methods

2.1. Cell culture

PC12 cells were cultured as described by Troppmair et al. [6]. Titration of NGF or EGF showed that maximal activation of Raf is achieved with 25 ng/ml of growth factors. Therefore, all treatments with growth factors were performed using 50 ng/ml NGF or EGF (Gibco BRL). All experiments were performed in medium with serum. No starved cells were used. All retroviral infections were performed as described [6].

2.2. Antisera

Rabbit polyclonal antisera to MAPK, and MEKK were from Santa Cruz Biotechnology. Antibodies against c-Raf-1 and B-Raf were directed to the isozyme specific C-terminal 13 amino acids. Anti A-Raf serum was generated with a 30 kDa peptide of the kinase domain. No cross reaction of the antibodies with the different Raf isozymes was observed in immonoprecipitation or immunoblotting [16,17].

2.3. Expression and purification of recombinant substrates

GST-MEK and the mutant enzyme K97M MEK were purified as described [18,19] except that for K97M MEK an additional Mono Q purification step was performed and that the eluate was dialysed against buffer containing 25 mM HEPES, pH 7.5, 25 mM Na-glycerophosphate, 1.5 mM EGTA, 5% glycerol and 1 mM DTT. The bacterially expressed ERK2His6 (K52R) protein was purified as described [20].

2.4. Immunoprecipitations and kinase assays

PC12 cells were rinsed twice with phosphate-buffered saline, scraped into chilled buffer A (50 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 10 mM Na-pyrophosphate, 25 mM Na-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 0.1% 2-mercaptoethanol, 1 mM Na-vanadate, 25 mM NaF, 0.01% leupeptin, 0.01% aprotinin, 2 mM pepstatin) and centrifuged at 1000×*g* for

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5 min at 4°C. 400 μg of protein per sample were rotated at 4°C for 2 h with 25 μl protein A agarose beads and anti-Raf sera. The immune complexes were washed twice with buffer A and once with buffer B (25 mM HEPES pH 7.5, 25 mM Na-glycerophosphate, 1.5 mM EGTA, 5% glycerol, 1 mM DTT) and pellets were resuspended in 30 μl buffer B supplemented with 10 mM $MgCl_2$, 100 μM ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mM, Amersham) and with 4 μg K97M MEK and incubated at 30°C for 15 min. Reactions were stopped by the addition of 4×Laemmli sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. Quantification of the amount of substrate phosphorylation was performed by a BAS 2000 II Fuji phosphoimager. The quality of immunoprecipitations was checked by immunoblot analysis using horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Amersham). To minimise experimental variability, the enzymatic activity of all Raf kinases as well as MEK and MAPK was quantified simultaneously in the same PC12 cell extract. The kinase activation values of stimulated cells were normalised to unstimulated cells, the activity of which was taken as unity. For the coupled Raf-MEK-MAPK kinase assay the Raf immune complexes were incubated for 10 min at 30°C as described above but without radiolabeled ATP and with 2.0 μg GST-MEK. Afterwards 2.5 μg of the kinase inactive mutant of ERK2 and 10 μCi [γ-^{32}P]ATP was added and the mixture was incubated for additional 15 min at 30°C. The kinase activity of MEK and MAPK enzymes was estimated in the same manner as Raf kinases. As substrates 2.5 μg of ERK2His6 and 2.5 μg of myelin basic protein (MBP, UBI), respectively, were used.

3. Results

3.1. NGF induces sustained activation of A-Raf and B-Raf in PC12 cells

The enzymatic activation of all three Raf kinases as well as MEKK after addition of NGF or EGF to PC12 cell culture is presented in Fig. 1. The levels of Raf proteins did not change during the time course of stimulation as judged from immunoblot experiments (data not shown). Both growth factors induced rapid activation of c-Raf-1 with a maximum after 1–2.5 min of stimulation. No difference between differentiation (NGF) and proliferation (EGF) inducing signals was noticed. The 4–5-fold activation of c-Raf-1 declined after 30 min of growth factor stimulation to only 1.5-fold basal level and was indistinguishable from unstimulated cells after 3 h (Fig. 1A).

The activation kinetics of B- and A-Raf (Fig. 1C,D) were different from those of c-Raf-1. Initial induction was less rapid and the maximally achieved stimulation was not higher than 3-fold. Besides differences in levels, the pattern of activation differed remarkably between the Raf isozymes. Stimulation of A- and B-Raf kinase activity was biphasic with an early peak after 5 min being followed by a second peak after 60–90 min. Biphasic kinetics were noted for exposure to both NGF and EGF, but were more pronounced, especially in the case of A-Raf, after NGF stimulation. It is interesting that similar kinetics with peaks after 5 and 60 min were described in [21] for p21 Ras activation after NGF stimulation of PC12 cells and it was expected that the activity of B-Raf would follow most closely that of Ras. Surprisingly, it was the stimulation of A-Raf that best paralleled Ras activation (Fig. 1D and [21]). As Raf kinases are not the only MEK activators in PC12 cells, we also examined other enzymes. Both NGF and EGF induced biphasic activation of MEKK, however, in this case there was no significant difference in the level of induction. If anything, levels after EGF treatment were higher than after NGF treatment (Fig. 1B). These data suggest that A- and B-Raf but not c-Raf-1 or MEKK are involved in differentiation induction by NGF. In order to show that the phosphorylation of MEK by Raf kinases is an activating phosphorylation, we performed the coupled MEK-MAPK kinase assay. Immune complexes of all Raf kinases and of MEKK were able to induce MAPK phosphorylation after NGF or EGF stimulation through activation of GST-MEK (data not shown).

The pattern of MEK activation differs from that of c-Raf-1, as the initial peak is 4-fold higher with either EGF or NGF and the activity remains elevated for more than 90 min after NGF treatment (data not shown). The differential ability of NGF but not EGF to induce sustained activity was also reflected at the level of MEK and MAPK (data not shown and [21-23]). Thus, the rapid and high activation of MEK and subsequently of MAPK by NGF and EGF correlated with the rapid and high activation of c-Raf-1, and the prolonged activity of MEK and MAPK in response to NGF compared to EGF correlated with a sustained activity of A-Raf and B-Raf.

3.2. Effect of oncogenic forms of A-, B-, and c-Raf-1 on differentiation and MAP kinase activation in PC12 cells

We [6] and others [24] have previously shown for oncogenic forms of c-Raf-1 that they induce differentiation of PC12 cells. As our kinetic data on activation of endogenous A- and B-Raf suggest that these are more likely mediators of differentiation, we examined whether oncogenic versions of A- and B-Raf were also able to induce neuronal differentiation of PC12 cells. Comparison was made with NGF and another retroviral oncogene v-mos, which encodes a serine/threonine kinase that has been reported to directly phosphorylate and activate MEK in vitro [25,26]. All four constitutively active MEK activators induced neurite outgrowth (Fig. 2). However, there were subtle differences in morphology of differentiating cells. Whereas NGF treated cells formed numerous and very branched neurites, the retrovirally introduced kinases induced formation of only 2–3 low-branched extensions. After 3 days all infected cells had neuronal extensions longer than 3–5 cell body diameters, while NGF induced neurits achieved these only on day 5 or 6. Fig. 3 shows that only infected cells differentiate, since only cells with neurites were stained with anti-c-Raf-1 antibodies on day 5 after infection with the constitutively active form of c-Raf-1.

As Raf kinases might have multiple substrates and constitutively active c-Raf-1 was previously thought to be unable to induce MAPK activity in PC12 cells, we determined the level of MAPK activity in all of the infected cells and compared it to EGF induction. Fig. 4 shows that MAPK is activated in PC12 cells expressing any of the three constitutively activated Raf kinases. v-Mos, an alternative MEK activator, was also active in this differentiation assay. The lower level of activity observed relative to the EGF control may be partly explained by the small fraction of exogenous Raf expressing cells (20–30%). Kinetic analysis of MAPK activation after retroviral infection shows that MAPK activation is only observed after 36 h (Fig. 4A), the earliest time at which neurite outgrowth was detected (data not shown).

4. Discussion

We show here that the differentiation inducing NGF receptor differs from the proliferation inducing EGF receptor in its

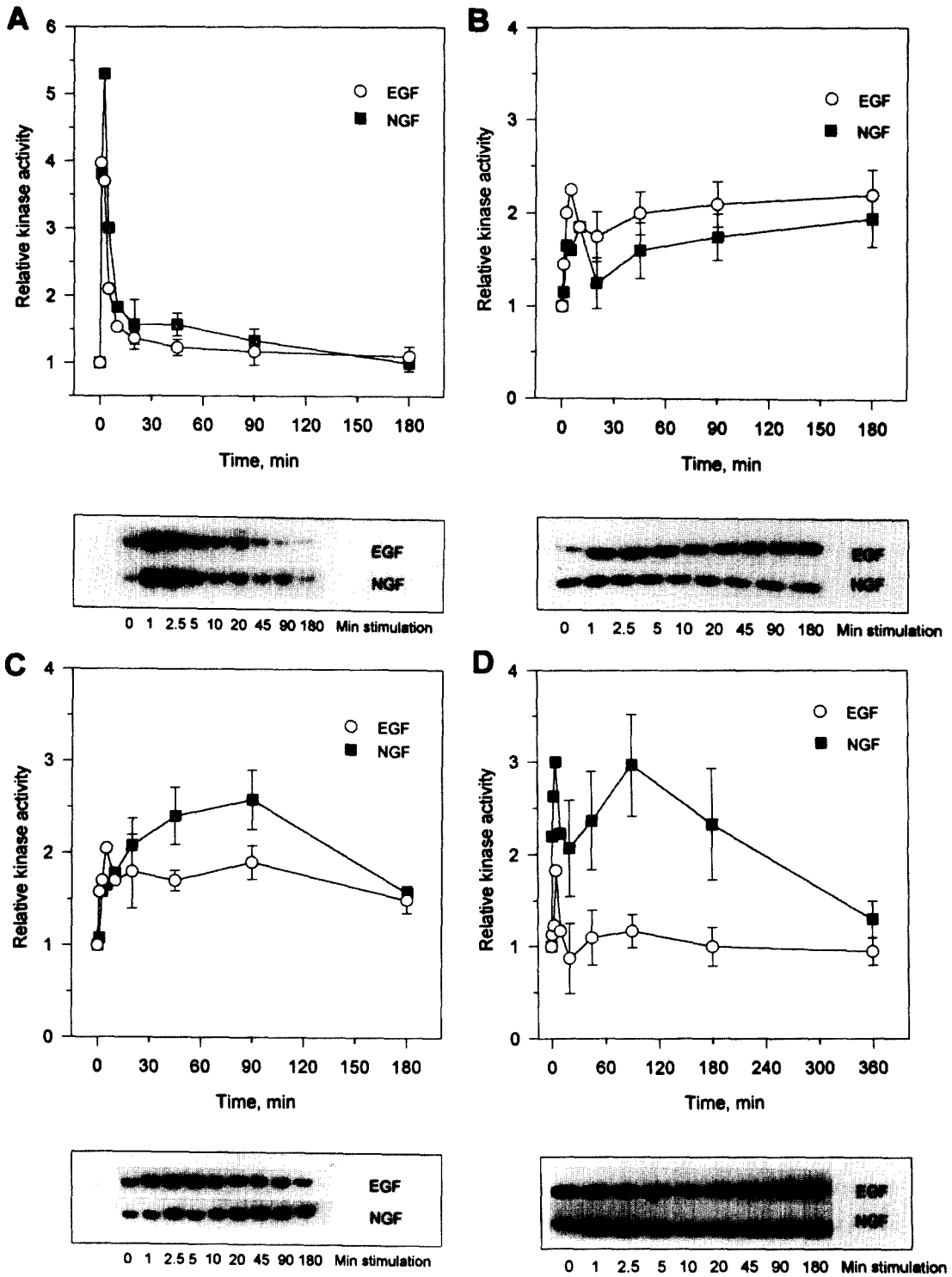

Fig. 1. Kinetics of c-Raf-1 (A), MEKK (B), B-Raf (C) and A-Raf (D) activation following NGF or EGF stimulation. 5×10^7 PC12 cells were treated with 50 ng/ml NGF or 50 ng/ml EGF for the times indicated. The appropriate kinases were immunoprecipitated from the cell lysates and in vitro kinase assays were performed with the kinase inactive K97M MEK as a substrate as described in section 2. The activity of untreated cells was taken as equal to unity and the relative values are presented. The results presented are the means of three separate experiments (error bars represent standard error of the mean). The relative basal MEK phosphorylating activities of immunoprecipitated emzymes in unstimulated cells were 1:5:1:0.6 for A-, B-, c-Raf-1 and MEKK, respectively. The specificity of the immunoassays was tested by competition with the corresponding peptides to which the antibodies were raised. In vitro kinase conditions were the same for all three Raf kinases as well as MEKK, following established protocol [13,33]. A temporal pattern of MEK phosphorylation of one representative experiment is shown under each time-course curves. The amounts of immunoprecipitated Raf kinases and MEKK throughout the time course were controlled by immunoblotting analysis. No time-dependent changes in amounts of immunoprecipitated proteins were observed.

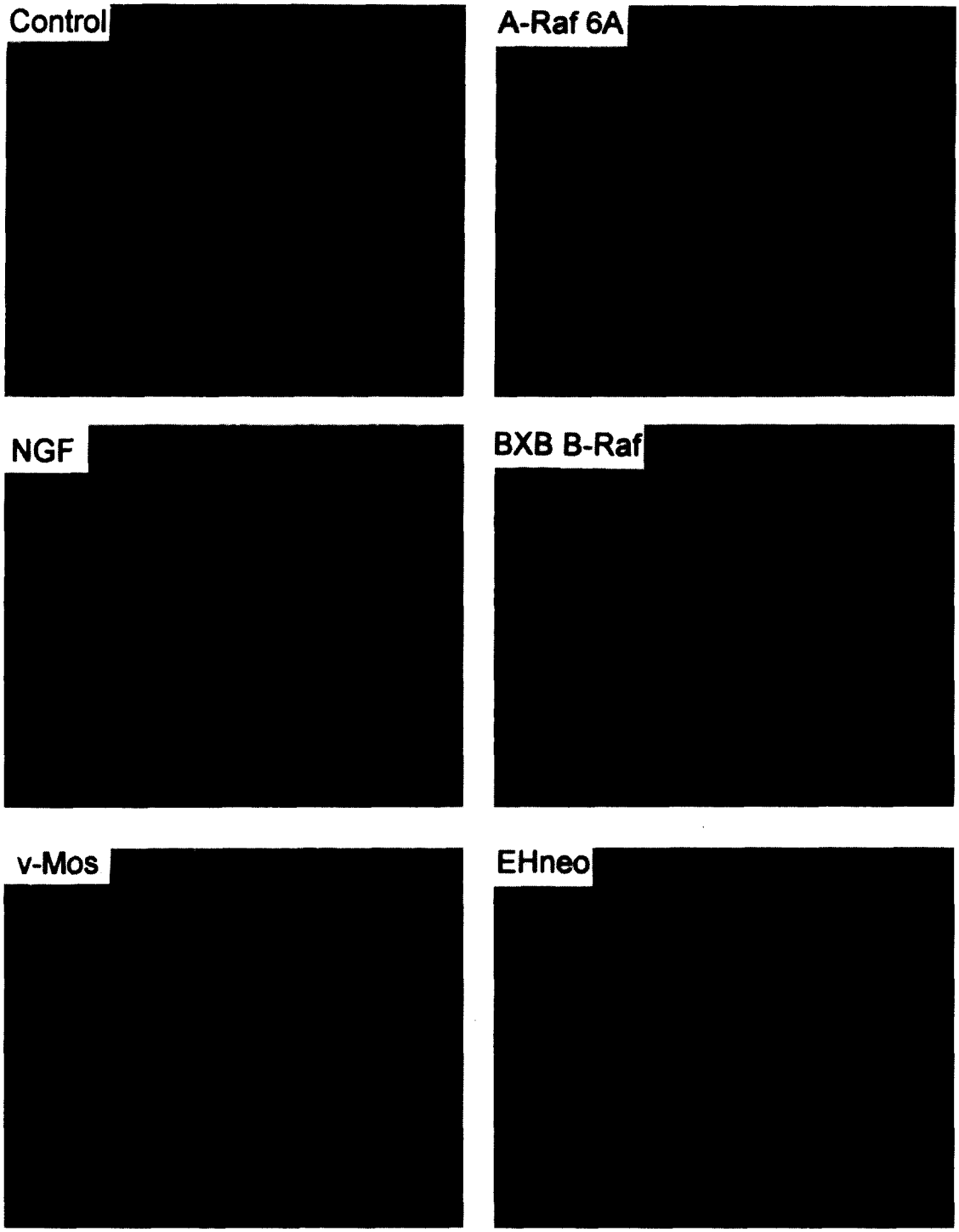

Fig. 2. Neurite formation in PC12 cells following infection with retroviruses carrying constitutively active forms of raf and mos DNAs. PC12 cells were infected with 2×10^6 cfu/ml of retroviruses containing cDNA encoding constitutively active mutants of A-Raf (A-Raf 6A), B-Raf (BXB B-Raf), c-Raf-1 (EHneo), Mos (v-Mos). Uninfected and NGF stimulated cells were used as controls. On day 6 after treatment the cells were fixed and photographed (×60).

ability to induce sustained activation of A- and B-Raf isozymes. We further show that all three constitutively active forms of Raf kinases are able to induce neuronal differentiation of PC12 cells and to activate MAPK. Finally, we illustrate by use of a Raf independent MEK activator, v-mos that Raf is not required for induction of differentiation consistent with earlier reports on the activity of oncogenic MEK in PC12 cells [27,28].

Perhaps the most striking finding was the differential response of the three Raf isozymes to growth factor treatment. Clearly, all three enzymes, which overlap in their mode of activation in that they all contain a Ras binding domain, become rapidly activated upon treatment of cells with either EGF or NGF. However, after NGF treatment only A- and B-Raf showed sustained activation reflecting the sustained activation of Ras under these conditions [21], whereas

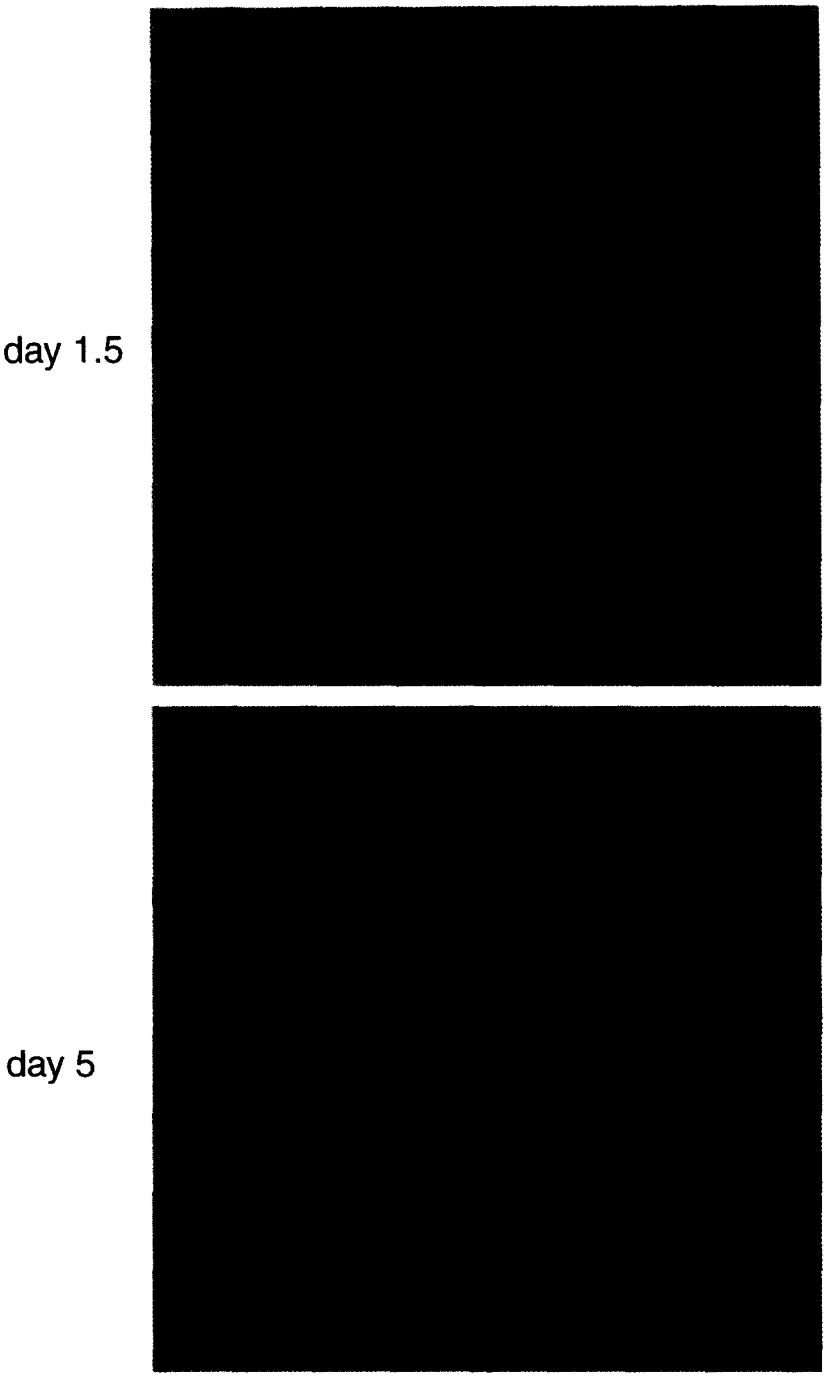

Fig. 3. Overexpression of v-Raf protein induces neurite formation. After 36 h or 5 days of infection with constitutively active c-Raf-1 kinase (EHneo) PC12 cells were fixed at −20°C for 5 min with methanol and Raf protein was visualised using the polyclonal rabbit anti-sp-63 antibodies and the peroxidase ABC staining kit from Vector Laboratories.

c-Raf-1 which demonstrated the highest degree of initial activation was rapidly inactivated. Negative regulation of Raf has been observed previously both at the level of activation and with active Raf. Phosphorylation of c-Raf-1 on Ser-43 of the regulatory domain trough PKA inhibits the Ras dependent activation of Raf-1 kinase leading to the downregulation of the classic cascade and to inhibition of growth factor induced cell proliferation of Rat1a and NIH 3T3 cells [12,29,30]. Inactivation of constitutively active c-Raf-1 was observed in Jurkat T-cells [31]. Downregulation of the MEK phosphorylating ability of a truncation activated form of Raf-1 called BXB, which essentially consists of the kinase domain of c-Raf-1 was complete and reversible. As the oncogenic forms of all three Raf isozymes were active in the PC12 differentiation assay, yet only kinase active c-Raf-1 was subject to downregulation, it may be more likely that in these cells negative regulation targets the full-length molecule. Alternatively, it may be that like in Jurkat cells the kinase domain is modified so as to render it inactive, but that the capacity of this process is limited and becomes overwhelmed by the overexpressed v-Raf. In this case we would expect the specific activity of v-Raf from PC12 cells to be lower than that in NIH 3T3 cells.

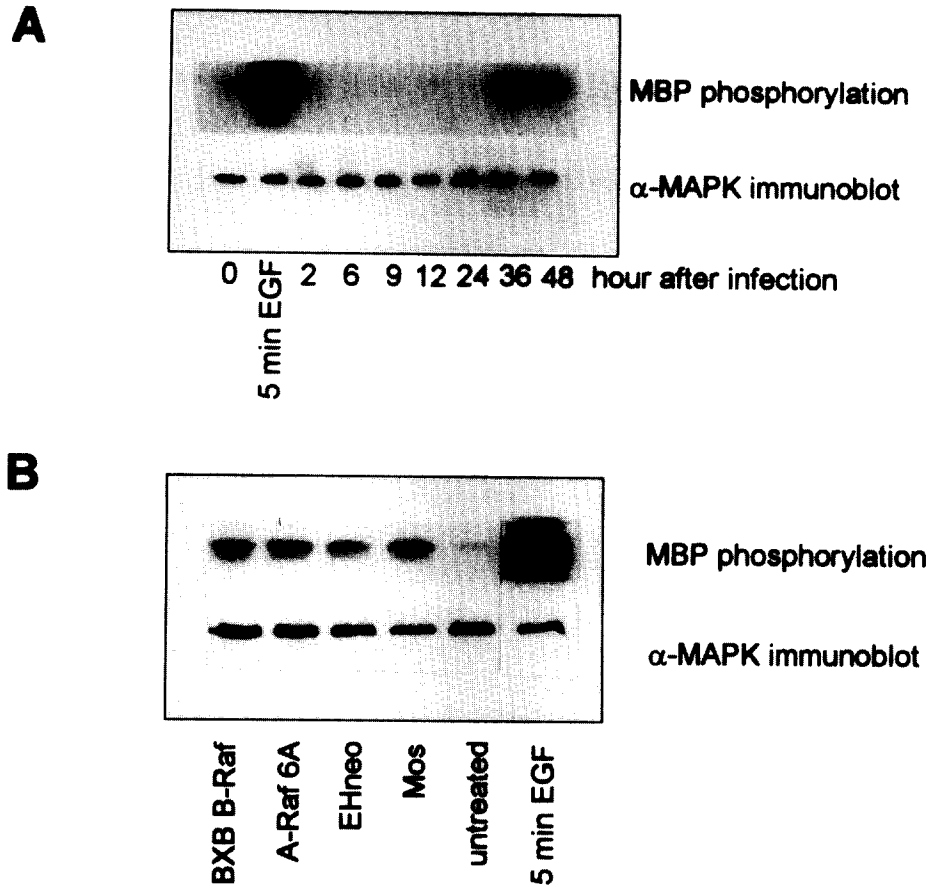

Fig. 4. Overexpression of oncogenic forms of Raf and Mos kinases in PC12 cells induce MAPK activation. PC12 cells were infected with retroviruses containing constitutively active form of A-Raf (A-Raf 6A), B-Raf (BXB B-Raf), c-Raf-1 (EHneo) and Mos (v-mos) as described in Fig. 3. MAPK was immunoprecipitated from cell lysates after different times of infection with EHneo (A) or after 36 h of infection with the corresponding retroviruses (B) and phosphorylation of MBP by MAPK immune complexes was analysed. 5 min stimulation of uninfected cells with 50 ng/ml EGF was used as a positive control. The immunoblot was performed using the ECL detection kit as described in section 2.

Our demonstration of MAPK activity in BXB Raf-1 expressing PC12 cells is in conflict with an earlier report by Wood et al. [32] were induction of BXB Raf-1 by dexamethasone from an MTV-LTR based vector led to neurite outgrowth apparently in the absence of MAPK activation. These data are often used to argue for the existence of alternative Raf substrates. A more trivial explanation may be the low level of MAPK activity in BXB Raf-1 PC12 cells relative to that induced by EGF treatment (Fig. 4). Whichever way this may be, we consider it unlikely that Raf specific differentiation substrates come into play, because a Raf independent activator of MEK, v-mos like constitutively activated MEK [27] is a very potent differentiation inducer. This is the first report of differential regulation of components of the highly conserved Ras-Raf-MEK-MAPK signalling cascade by NGF and EGF. These findings now raise important questions about the underlying mechanisms. Factors important for the reactivation of c-Raf-1 have to be to identified as well as the basis for resistance to downregulation of A- and B-Raf. The relevant experiments are now in progress.

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