Nerve Growth Factor-stimulated B-Raf Catalytic Activity Is Refractory to Inhibition by cAMP-dependent Protein Kinase*

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The cAMP-dependent protein kinase (PKA) exhibits both inhibitory and stimulatory effects upon growth factor signaling mediated by the mitogen-activated protein kinase signaling pathway. PKA has been demonstrated to inhibit Raf-1-mediated cellular proliferation. PKA can both prevent Ras-dependent Raf-1 activation and directly inhibit Raf-1 catalytic activity. In contrast to the inhibitory effect of PKA on Raf-1-dependent processes, PKA potentiates nerve growth factor-stimulated PC12 cell differentiation, a B-Raf mediated process. This potentiation, rather than inhibition, of PC12 cell differentiation is curious in light of the ability of PKA to inhibit Raf-1 catalytic activity. The kinase domains of Raf-1 and B-Raf are highly conserved, and it has been predicted that B-Raf catalytic activity would also be inhibited by PKA. In this study we examined the ability of PKA to regulate the kinase activity of the B-raf proto-oncogene. We report that nerve growth factor-stimulated B-Raf activity is not inhibited by PKA. By contrast, an N-terminally truncated, constitutively active form of B-Raf is inhibited by PKA both in vitro and in transfected PC12 cells. These results suggest that the N-terminal regulatory domain interferes with the ability of PKA to modulate B-Raf catalytic activity and provide an explanation for the observed resistance of B-Raf-dependent processes to PKA inhibition.

Members of the Raf family of serine/threonine protein kinases (Raf-1, B-Raf, and A-Raf) have been shown to be key regulators of growth factor signaling in diverse biological systems. Raf can directly phosphorylate and activate MEK (1–3), which in turn leads to the activation of mitogen-activated or extracellular signal-regulated protein kinase (MAPK) (4). In response to a variety of extracellular stimuli, the Raf/MEK/MAPK cascade mediates a signal relay from the plasma membrane to the nucleus, resulting in cell type-specific responses that include proliferation and differentiation (5–7). An early step in Raf-1 activation involves the binding of Ras and the recruitment of Raf-1 to the plasma membrane. However, subsequent events are then required to generate full Raf-1 activity (reviewed in Ref. 8).

Diverse extracellular signals are integrated within the cell to elicit a tailored cellular response to different environmental cues. Signal integration is achieved through cross-talk between intracellular signaling pathways. It has been demonstrated that inhibitory signal integration occurs between the cAMP-dependent protein kinase (PKA) pathway and the MAPK signaling pathway in a variety of cell types (9–18) through inhibition of Raf-1 activity. PKA has been shown to inhibit Raf-1 by two mechanisms: (i) inhibition of Ras binding (9) and (ii) direct inhibition of Raf-1 catalytic activity (17, 19, 20). Raf-1 signal transduction is also subject to negative regulation by the Ras-related small GTPase, Rap1. Elevation of intracellular cAMP levels and consequent PKA activation results in Rap1 GTP loading (21). The GTP-bound form of Rap1 can bind to the N-terminal regulatory domain of Raf-1 (22–24) and block Ras-dependent Raf-1 activation (25, 26). Thus, cAMP-dependent signaling can inhibit Raf-1 activity by several mechanisms including the direct phosphorylation of Raf-1 and the stimulation of the inhibitory Rap1 protein.

By contrast to Raf-1, less is known regarding the activation and regulation of B-Raf. B-Raf has been demonstrated to be required for early mouse development and is essential for vasculogenesis (27). B-Raf has been shown to be expressed at high levels in neural tissue (28) and has been most extensively characterized in the PC12 cell line. Raf-1 and B-Raf are closely related and share extensive homology between their catalytic domains (29). Raf-1 and B-Raf both phosphorylate and activate MEK1 and MEK2 the isoforms, albeit with different specific activities (30). In a manner similar to Raf-1 regulation, PKA can phosphorylate B-Raf and block Ras-dependent B-Raf activation (31). However, in contrast to the inhibitory effect of PKA on Raf-1-dependent proliferation, it has been demonstrated that PKA can potentiate B-Raf-dependent PC12 cell differentiation. This stimulatory effect of PKA appears to be indirect and mediated through Rap1 (26, 32, 33). Thus, Rap1 is inhibitory to Raf-1 but stimulatory to B-Raf.

The observation that NGF-stimulated PC12 cell neuronal differentiation is not inhibited by PKA (34, 35) is curious in light of the ability of PKA to inhibit Raf-1 catalytic activity. The high degree of homology between the kinase domains of Raf-1 and B-Raf has led to the prediction that B-Raf catalytic activity would also be inhibited by PKA (19). In this study we have directly tested the ability of PKA to regulate B-Raf catalytic activity. We report that even though the activity of the isolated C-terminal catalytic domain is negatively regulated by PKA, PKA did not inhibit the catalytic activity of NGF-stimulated, full-length B-Raf. Our data suggest that the N-terminal regulatory domain interferes with the ability of PKA to modulate B-Raf catalytic activity. This finding resolves the apparent
PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

discrepancy between the putative inhibitory effect of PKA upon B-Raf catalytic activity and the observed stimulatory effect of PKA on B-Raf-mediated PC12 cell differentiation.

EXPERIMENTAL PROCEDURES

**Plasmid Constructs, Polymerase Chain Reaction Mutagenesis, and RNA Preparation**—A plasmid encoding the full-length human B-raf (pSfL B-raf) was digested with StyI and Ndel to isolate the C-terminal catalytic domain (B-rafcat). The B-rafcat was subcloned into Small-digested pXen2, a Xenopus expression vector containing a glutathione S-transferase (GST) motif (20) to generate the GST-B-rafcat-GST. For -B-rafcat-GST fusion studies in PC12 cells, the same StyI/Ndel fragment was subcloned into EcoRV-digested Srα to generate Srα-B-rafcat. pSfL B-raf was digested with HindIII and Ndel to isolate the full-length B-raf and Klenow-treated and subcloned into EcoRV-digested Srα to generate Srα-B-raf.

Standard polymerase chain reaction-directed mutagenesis was employed to generate a lysine to methionine mutation at amino acid 482 (K482M) within pXen B-raf to generate the kinase-deficient B-rafcat K482M. The catalytic subunit of PKA in Srα (Srα-PKAc) (36) was utilized for expression of PKA in PC12 cells. The kinase-deficient C-terminal domain of Raf-1, rafcat K375M (pXen κNAF) has been described previously (37). For in vitro transcription, pXen plasmids were linearized with EcoRI and capped RNA synthesized with SP6 RNA polymerase and transcribed previously (20).

**Protein Expression in Xenopus Oocytes**—Defolliculated Xenopus oocytes were microinjected with in vitro transcribed RNA (10 ng of RNA per embryo) encoding GST or GST-Raf fusion proteins as indicated. Pools of 20 injected oocytes were lysed in Nonidet P-40 lysis buffer (10 mm Tris, pH 7.5, 137 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM NaPPi, 1% Nonidet P-40, 2 mm phenylmethylsulfonyl fluoride, 0.2 units of aprotinin (Sigma) /ml, and 25 μl leupeptin) 12 h after microinjection (37).

**PC12 Cell Culture and Transfections**—PC12 cells were grown in Dulbecco’s modified Eagle’s medium with 10% equine serum and 5% fetal calf serum. For transfections PC12 cells were plated on 35-mm polylysine-coated dishes at 50% confluency and transfected by the LipofectAMINE method (Life Technologies, Inc.). Transfected cells were then washed twice in lysis buffer and once in Tris-buffered saline (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM NaPPi, 1% Nonidet P-40, 2 mm phenylmethylsulfonyl fluoride, 0.2 units of aprotinin (Sigma) /ml, and 25 μl leupeptin) 12 h after microinjection (37).

**RESULTS**

The B-Raf and Raf-1 proteins are highly homologous within their catalytic domains (81% amino acid identity). Because PKA can directly phosphorylate the C-terminal domain and inhibit the catalytic activity of Raf-1 (17, 19, 20), we wished to determine whether the catalytic activity of B-Raf was subject to similar regulation by PKA.

We first tested whether the catalytic domain of B-Raf was a substrate for phosphorylation by PKA. The catalytic domain of B-Raf (amino acids 385–765) was fused to an N-terminal glutathione S-transferase moiety (designated B-Rafcat-GST). A point mutation was then introduced within the ATP binding site of B-Raf to render the enzyme catalytically inactive and prevent autophosphorylation (designated B-Rafcat K482M-GST). The B-Rafcat K482M-GST protein was expressed in Xenopus oocytes and partially purified by glutathione-Sepharose affinity chromatography. Fig. 1 demonstrates that the B-Raf catalytic domain (B-Rafcat K482M-GST), like the Raf-1 catalytic domain (Rafcat K375M), is a substrate for PKA. No phosphorylation of B-Rafcat K482M-GST was observed in the absence of PKA (data not shown). The B-Rafcat K482M-GST protein migrates as a doublet under these assay conditions. The GST moiety when expressed alone was not phosphorylated by PKA (GST, Fig. 1), demonstrating that PKA phosphorylates site(s) within the Raf-1 and B-Raf catalytic domains.

We next determined whether the activity of the B-Raf catalytic domain could be inhibited by PKA phosphorylation. The B-Rafcat-GST protein was expressed in Xenopus oocytes and partially purified by glutathione-Sepharose affinity chromatography. B-Rafcat-GST was incubated in the absence or presence of PKA, as described for Fig. 1, but with nonradioactively labeled ATP. The glutathione-Sepharose B-Rafcat-GST complex was then washed extensively, and the phosphotransferase activity of the B-Rafcat-GST was determined using a kinase-
PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

Fig. 2. B-Raf catalytic activity is inhibited by PKA phosphorylation in vitro. A, the isolated catalytic domain of B-Raf was expressed in Xenopus oocytes as a GST fusion protein (B-Rafcat-GST). The glutathione-purified protein was incubated in the absence (−PKA) or presence (+PKA) of PKA in vitro, and the subsequent activity of B-Rafcat-GST was measured using kinase-negative MEK (KN-MEK). A GST immunoblot analysis of the same samples indicates that an equal amount of B-Rafcat-GST fusion protein was present in each assay. B, a histogram summarizing the inhibitory effect of PKA on B-Raf activity from three independent experiments. The error bar indicates the standard error of the mean.

PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

Fig. 3. The catalytic activity of B-Rafcat is inhibited by co-transfection of PKAcat in PC12 cells. A, a plasmid encoding B-Rafcat-GST was expressed in PC12 cells in the absence (−PKA) or presence (+PKA) of co-transfected PKAcat. MEK kinase activity of the glutathione-Sepharose-purified proteins was determined as described in Fig. 2. An anti-B-Raf immunoblot analysis of the sample used for the MEK kinase assay indicates that an equal amount of B-Rafcat-GST fusion protein was present in each reaction. B, a histogram summarizing the inhibitory effect of PKAcat on B-Rafcat activity in transfected PC12 cells from four independent experiments. The error bar indicates the standard deviation of the mean.

negative MEK substrate in the presence of radioactively labeled ATP. Preincubation with PKA significantly reduced B-Rafcat activity (Fig. 2, 44.7% inhibition ± 6.5 S.E., n = 3).

We next wished to determine whether the inhibitory effects of PKA on B-Raf catalytic activity observed in vitro could be reproduced following transfection of mammalian tissue culture cells. For these experiments we utilized PC12 cells, which had previously been utilized to demonstrate cross-talk between the PKA and B-Raf signaling pathways (26, 31, 39, 40). PC12 cells were transfected with B-Rafcat-GST in the presence or absence of a co-transfected plasmid encoding the catalytic subunit of PKA (PKAcat). Transfection of this PKAcat construct results in a high and persistent level of PKA activity in PC12 cells as measured with a CAMP response element-CAT reporter plasmid (data not shown). B-Rafcat-GST was partially purified by glutathione-Sepharose affinity chromatography from PC12 cell lysates prepared 24 h after transfection. We observed that co-transfection with PKAcat inhibited the activity of B-Rafcat-GST in PC12 cells (Fig. 3A, +PKA). As in the in vitro study (Fig. 2), we did not observe complete inhibition of B-Rafcat activity by PKA (55.5% inhibition ± 4.4 S.E.M., n = 4). Immunoblot analysis of the same lysates revealed that similar amounts of B-Raf protein were present in each sample (Fig. 3B), indicating that expression of PKAcat did not lead to translational inhibition of the co-transfected B-Rafcat-GST. Thus, the difference in catalytic activity reflects changes in the specific activity of the B-Rafcat-GST protein following PKA co-transfection. It would appear that PKA co-transfection results in a mobility shift of a portion of the B-Rafcat-GST protein (Fig. 3B).

Given the inhibitory effect of PKA on the isolated B-Raf catalytic domain in transfected PC12 cells, we wished to determine whether PKA exerted an inhibitory influence on the activity of the full-length B-Raf enzyme present in PC12 cells. To directly assess the effects of PKA, we immunoprecipitated active B-Raf from NGF-stimulated PC12 cells. The B-Raf immune complexes were incubated in the presence or absence of PKA in vitro and subsequently assayed for B-Raf activity. PKA treatment did not result in any inhibition of B-Raf activity (Fig. 4A). By contrast, PKA treatment reduced PKC-activated Raf-1 activity (Fig. 4B, 38.2% inhibition ± 4.1 S.E., n = 3) as previously reported (17, 19). We conclude that following NGF stimulation, the catalytic activity of B-Raf is not subject to inhibition by PKA. Moreover, rather than exerting an inhibitory influence on catalytic activity, PKA actually stimulated B-Raf activation. PC12 cells were transfected with full-length B-Raf-GST in the presence or absence of co-transfected PKAcat. Immunoblot analysis of the glutathione-Sepharose affinity-purified lysates indicated that similar amounts of full-length B-Raf-GST were expressed in the absence or presence of co-transfected PKAcat (Fig. 5A). In contrast to the inhibitory effect of PKAcat upon the activity of the transfected B-Raf catalytic domain (Fig. 3), PKA co-transfection stimulated the activity of full-length B-Raf-GST approximately 3-fold (Fig. 5B). This activation of B-Raf-GST by PKAcat is in agreement with a published report that demonstrated that elevated levels of cAMP in PC12 cells led to activation of B-Raf (26).

DISCUSSION

It has previously been shown that PKA can negatively regulate Raf-1 activity by two distinct mechanisms. First, PKA can prevent Raf-1 activation by phosphorylating the Raf-1 N-terminal regulatory domain and blocking Ras interactions. Second, PKA can phosphorylate the Raf-1 C-terminal catalytic domain and inhibit Raf-1 catalytic activity. The association of Ras with B-Raf is also inhibited by PKA (31), and PKA blocks Ras-dependent B-Raf activation. Similarly, based on sequence homology, it has been predicted that B-Raf catalytic activity would also be subject to inhibition by PKA (19). However, recent evidence has suggested that rather than play an obligate inhibitory function in the regulation of B-Raf, PKA can actually stimulate B-Raf activity in certain PC12 cell lines via the small G-protein, Rap1 (26, 33). To address these apparently contradictory roles for PKA in the control of B-Raf, we have investigated whether B-Raf catalytic activity is inhibited by PKA. We report here that contrary to the effects on Raf-1, PKA does not
PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

 activated B-Raf was immunoprecipitated from NGF-stimulated PC12 cells and incubated in the absence (−PKA) or presence (+PKA) of PKA in vitro. B-Raf was isolated from immature Xenopus oocytes, activated with purified PKC, and subsequently treated in the presence or absence of PKA. MEK kinase activity of the B-Raf and Raf-1 proteins was measured as described in the legend to Fig. 2. In this experiment, B-Raf was activated by NGF to levels 4-fold higher than the activity of B-Raf in unstimulated PC12 cells. Raf-1 was activated by PKC 12.9-fold higher than the level of Raf-1 in immature Xenopus oocytes. KN-MEK, kinase-negative MEK. A, B-Raf immunoblot analysis of glutathione-Sepharose affinity-purified proteins, further studies will be required to determine why PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

Our findings indicating that the catalytic activity of full-length B-Raf is not subject to inhibition by PKA are compatible with several recent observations concerning PKA and B-Raf cross-talk. It has been shown that elevated intracellular cAMP levels had differential effects on Raf-1 and B-Raf when the cells were maintained in serum-containing media (39). This would be consistent with cAMP-induced PKA inhibiting active Raf-1 but having little effect on active, full-length B-Raf. PKA has also been demonstrated to stimulate MAPK in certain cell types (26, 43, 44). This stimulation is mediated indirectly by PKA through the Rap1-dependent activation of B-Raf (26, 33). Unlike the Ras-dependent activation of B-Raf, Rap1-dependent activation is refractory to PKA.

These regulatory differences between Raf isoforms may tailor the cellular response following extracellular ligand stimulation. For example, although simultaneous cAMP elevation generally results in a block to growth factor-stimulated signaling, in PC12 cells (which express B-Raf) the combination results in the potentiation of growth factor-stimulated differentiation. The mechanistic basis of the isoform-specific resistance to inhibition by PKA has not hitherto been fully understood. In addition to Ras, Rap1-dependent B-Raf activation was necessary for both the phenotypic extension of neurites and the induction of differentiation-specific gene expression in PC12 cells (33). Moreover, the Rap1-dependent activation of differentiation was dependent upon endogenous PKA activity (32). Our findings would suggest that although Raf-1 activity is susceptible to PKA-mediated inhibition, Rap1-stimulated B-Raf catalytic activity is refractory to the inhibitory action of PKA. Thus, the differential regulation of Raf isoforms by PKA would allow certain cell types to interpret PKA signaling in a stimulatory rather than an inhibitory manner on MAPK-dependent processes. Indeed, we observe activation rather than inhibition of full-length B-Raf in response to PKA co-transfection of PC12 cells (Fig. 5). Taken together, the primary means of negatively regulating B-Raf activity via PKA may be through regulation of Ras-dependent B-Raf activation rather than through inhibition of catalytic activity. Indeed, elevated cAMP and PKA have been shown to block Ras binding to B-Raf (31). B-Raf activation by Rap1, on the other hand, is stimulated by PKA (26, 33). Because Ras and Rap1 have highly related effector domains and have been shown to bind to the same target proteins, further studies will be required to determine why Rap1 binding is refractory to PKA phosphorylation of B-Raf.

The results of our study are most compatible with the hypothesis that following NGF-stimulated activation of B-Raf, the N-terminal regulatory domain serves to protect the catalytic domain from the inhibitory action of PKA. The idea that communication between the N- and C-terminal domains of Raf proteins contributes to the overall regulation of activity is not
PKA Does Not Inhibit NGF-Stimulated B-Raf Catalytic Activity

without precedent (45). Further, the 14-3-3 family of proteins has been shown to interact with both the N-terminal and C-terminal domains of Raf-1, and it has been proposed that these interactions may be dynamic in nature resulting in the stabilization of active or inactive Raf-1 conformations (46–48). Curiously, the N-terminal regulatory domain does not protect the catalytic domain of Raf-1 from inhibition by PKA (17, 19, 41). Thus, amino acid differences in the catalytic domain of Raf-1 from inhibition by PKA (17, 19, 41).

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