Cell Type-Specific Regulation of B-Raf Kinase by cAMP and 14-3-3 Proteins

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SUMMARY

Cyclic AMP can either activate or inhibit the mitogen-activated protein kinase (MAPK) pathway in different cell types: MAPK activation has been observed in B-Raf-expressing cells and has been attributed to Rap1 activation with subsequent B-Raf activation, whereas MAPK inhibition has been observed in cells lacking B-Raf and has been attributed to cAMP-dependent protein kinase (A-kinase)-mediated phosphorylation and inhibition of Raf-1 kinase. We found that cAMP stimulated MAPK activity in CHO-K1 and PC12 cells, but inhibited MAPK activity in C6 and NB2A cells. In all four cell types, cAMP activated Rap1 and the 95 and 68 kDa isoforms of B-Raf were expressed. cAMP activation or inhibition of MAPK correlated with activation or inhibition of endogenous and transfected B-Raf kinase. Although all cell types expressed similar amounts of 14-3-3 proteins, approximately five-fold less 14-3-3 was associated with B-Raf in cells in which cAMP was inhibitory than in cells in which cAMP was stimulatory. We found that the cell type-specific inhibition of B-Raf could be completely prevented by overexpression of 14-3-3 isoforms, whereas expression of a dominant negative 14-3-3 mutant resulted in partial loss of B-Raf activity. Our data suggest that 14-3-3 bound to B-Raf protects the enzyme from A-kinase-mediated inhibition; the amount of 14-3-3 associated with B-Raf may explain the tissue-specific effects of cAMP on B-Raf kinase activity.
INTRODUCTION

The serine/threonine protein kinases of the Raf family (Raf-1, A-Raf and B-Raf) are key regulators of cell growth, differentiation and apoptosis in eukaryotic cells (1). They are activated by a large number of membrane receptors which stimulate Raf kinases indirectly, though small GTP-binding proteins of the Ras family (1-3). Activated Raf kinases phosphorylate and activate the dual-specificity kinases MEK-1 and -2 which in turn phosphorylate and activate the mitogen-activated protein kinases (MAPKs) Erk-1 and -2. Raf-1 is expressed ubiquitously, but A-Raf and B-Raf are differentially expressed with highest levels in urogenital tissues and brain, respectively (1). The Raf kinases differ in their response to upstream signals and their ability to activate the MAPK pathway (4-8). Raf-1 activation requires phosphorylation on Ser\textsuperscript{338} and Tyr\textsuperscript{340} subsequent to Ras\textsuperscript{GTP} binding; binding of Rap1\textsuperscript{GTP} to Raf-1 does not lead to activation (6,9-12). In B-Raf, the serine residue equivalent to Ser\textsuperscript{338} of Raf-1 is constitutively phosphorylated and the residue equivalent to Tyr\textsuperscript{340} of Raf-1 is an aspartate, leading to high basal activity of B-Raf compared to Raf-1; B-Raf can be activated fully by binding to Ras\textsuperscript{GTP} or Rap\textsuperscript{GTP} (4,6,12-14). Although both B-Raf and Raf-1 are expressed and activated by growth factors in neuronal cells, B-Raf seems to be the major MEK activator in these cells and possibly also in other cell types (4,7,15-18). Two major isoforms of B-Raf of 68 and 95 kDa differ by 115 amino acids at the N-terminus; alternative splicing of exons 8 and 10 yields additional isoforms which differ in their tissue distribution, basal MEK kinase activity and oncogenic properties (15,19).

In many cell types, including fibroblasts, glial cells, smooth muscle cells and epithelial tumor cells, MAPK signaling is inhibited by drugs and hormones which increase the intracellular cAMP concentration (20-27). This has been attributed to phosphorylation of two serine residues in Raf-1 by cAMP-dependent protein kinase (A-kinase): Ser\textsuperscript{43} phosphorylation near the Ras-binding domain of Raf-1 inhibits Ras\textsuperscript{GTP} binding and may effectively uncouple Raf-1 from Ras, and Ser\textsuperscript{621} phosphorylation in the catalytic domain can inhibit catalytic activity (28-31). However, a recent report suggests that A-kinase inhibits growth factor-induced Raf-1 activation.
in 293 human embryonal kidney cells independently of direct Raf-1 phosphorylation (32).

In other cell types, including neuronal cells, COS-7 kidney cells and S49 lymphoma cells, increased intracellular cAMP stimulates MAPK activity (27,33-38). In different strains of Chinese hamster ovary (CHO) cells, cAMP has been reported to either activate or inhibit MAPK activity (39-41). From studies of PC12 rat pheochromocytoma cells and NIH 3T3 fibroblasts, Vossler et al. (33) suggested that cAMP activates MAPK in cells expressing the 95 kDa isoform of B-Raf whereas cAMP inhibits MAPK in cells lacking this B-Raf isoform. In this model, cAMP activates Rap1 which then activates B-Raf; cAMP activation of Rap1 may occur through guanine nucleotide exchange factors (GEFs) which are regulated either directly by cAMP binding or indirectly by a mechanism requiring A-kinase phosphorylation (33,42-45). However, several reports showed that cAMP inhibits B-Raf activity in PC12 cells expressing both 95 and 68 kDa B-Raf with one report showing that cAMP inhibits B-Raf in serum-starved cells but not in cells maintained in serum-containing medium (18,34,35,46). Inhibition of B-Raf by cAMP was also described in unstimulated and phorbol ester-stimulated NIH3T3 cells and in chemoattractant-stimulated neutrophils; cAMP-mediated activation of a 68 kDa B-Raf isoform was observed in CHO cells and correlated with Rap1 activation (5,39,47). These results suggest that B-Raf regulation by cAMP may differ depending on growth conditions, expression of different B-Raf isoforms and presence of cell type-specific factors.

A-kinase phosphorylates B-Raf in vitro and in vivo, although the phosphorylation sites have not been mapped (46,48). B-Raf has no equivalent of Raf-1 Ser43, but Ser728 in B-Raf (numbers correspond to the 95 kDa isoform) corresponds to Ser621 of Raf-1 and the surrounding sequences are highly homologous suggesting that B-Raf Ser728 may also be a target for A-kinase phosphorylation (19,49). In co-transfection experiments, A-kinase activated full-length B-Raf but inhibited the isolated catalytic domain expressed in PC12 cells; when incubated with B-Raf in vitro, A-kinase had no effect on the activity of the full length enzyme but reduced the activity of the catalytic domain, suggesting that the N-terminal regulatory domain of B-Raf prevents A-kinase from inhibiting B-Raf catalytic activity (48).
The family of 14-3-3 proteins includes at least seven isoforms which are abundantly expressed in most tissues and bind as homo- or heterodimers to phosphoserine residues in the consensus sequence RSXpSXP (50-52). Raf-1 contains at least three 14-3-3 binding sites: one in the cysteine-rich domain between amino acids 136 and 187, a second surrounding Ser\(^{259}\), and a third surrounding Ser\(^{621}\) (50,53-55). Mutations in the two N-terminal sites which prevent 14-3-3 binding lead to Raf-1 activation (54,55); Ras\(^{\text{GTP}}\) and phosphatidylserine binding near these sites displaces bound 14-3-3 allowing full activation of Raf-1 via phosphorylation of Ser\(^{338}\) and Tyr\(^{340}\) and reassociation of 14-3-3 may be involved in returning Raf-1 to the inactive form (52,56-58). In contrast, binding of 14-3-3 to Ser\(^{621}\) of Raf-1 appears to be required for basal kinase activity, because: (i) mutation of Ser\(^{621}\) to any other residue destroys catalytic activity; (ii) removal of 14-3-3 from the catalytic domain of Raf-1 using specific detergents or competitive phospho-peptides completely abrogates kinase activity which is restored upon adding 14-3-3; and (iii) expression of a dominant negative 14-3-3 results in inhibition of the Raf-1 catalytic domain (53). These data have led to a model in which 14-3-3 binding to Raf-1 is necessary to keep the enzyme in an inactive, but activation-competent conformation (56,59,60). Some investigators have reported that over-expression of 14-3-3 potentiates Raf-1 activation while others have found no effect (53,54,56,60). All three 14-3-3 binding sites of Raf-1 are highly conserved in B-Raf and intracellularly, B-Raf appears to exists in a high molecular weight complex with 14-3-3 proteins, HSP90 and MEK-1 and -2 (8,49,50,61-63). Ser\(^{728}\) of B-Raf is a 14-3-3 binding site which appears to be necessary for B-Raf’s biological activity: a Ser\(^{728}\) to Ala substitution in the isolated B-Raf catalytic domain dramatically reduces the enzyme’s ability to induce Xenopus oocyte maturation or PC12 cell differentiation, although the mutant enzyme retains significant catalytic activity in vitro (64). B-Raf purified from Xenopus oocytes is synergistically activated by a combination of 14-3-3 and Ras\(^{\text{GTP}}\) (14).

We hypothesized that the variable results concerning cAMP regulation of B-Raf could be secondary to cell type-specific factors and, therefore, studied the effect of cAMP on B-Raf and
MAPK activity in CHO-K1, PC12, C6 glioma and NB2A neuroblastoma cells. In all four cell types, the 95 kDa isoform of B-Raf was expressed and membrane-permeable cAMP analogs increased the activation state of Rap1. However, the cAMP analogs stimulated B-Raf and MAPK activity in CHO-K1 and PC12 cells while inhibiting B-Raf and MAPK activity in C6 and NB2A cells. We found that inhibition of B-Raf by cAMP correlated with significantly lower amounts of enzyme-associated 14-3-3 and the cAMP-mediated inhibition was completely prevented by over-expression of 14-3-3; expression of a dominant negative 14-3-3 resulted in partial loss of B-Raf kinase activity. Our data suggest a model in which 14-3-3 protects B-Raf from A-kinase inhibition and may explain B-Raf’s varying response to cAMP in different cell types.
MATERIALS AND METHODS

Materials and DNA constructs

Rabbit polyclonal antibodies specific for Erk-1/2 (C16, SC93), for B-Raf kinase (C19, SC166), for Rap1 (121, SC65) and for 14-3-3 (K19, SC629) were from Santa Cruz Biotechnology, as was recombinant MEK-1. An anti-active MAPK antibody (V803A) was from Promega, an actin-specific antibody (A2066) was from Sigma, and a Raf-1 phospho-Ser621-specific antibody was from A. S. Shaw (53). The glutathione-S-transferase (GST)-tagged peptide encoding the Rap-binding domain of Ral-GDS (RBD peptide) was purified from bacteria transformed with the expression plasmid pGEX-RGF97 [provided by J. L. Bos (65)]. The membrane-permeable cAMP analogs 8-(4-chlorophenylthio)cAMP (8-pCPT-cAMP) and 8-bromo-cAMP (8-Br-cAMP) were from Biolog, and forskolin was from Calbiochem. Lipofectamine Plus™ and Lipofectamine 2000™ were from Life Technologies. An expression vector encoding the 95 kDa isoform of B-Raf but lacking exons 8b and 10 was from P. J. S. Stork [pcDNA3-BRaf (33)]. Either full-length B-Raf or the catalytic domain of B-Raf (amino acids 385 to 765) were fused in frame to GST in the vector pCMV-GST using BamHI (66). Wild type 14-3-3$,14-3-3J and the dominant negative mutant 14-3-3(R56,60A) were from A. S. Shaw (53). The reporter plasmid pGAL4-Luc was from M. Karin, and pElk-Gal4 was from G. L. Johnson (67).

Cell Lines and Transfections

CHO-K1 and PC12 cells were from the ATCC, and C6 and NB2A cells were provided by M. H. Ellisman and E. Koo, respectively. CHO-K1 and NB2A cells were grown in F-12K medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS); C6 and PC12 cells were maintained in Dulbecco’s modified Eagle Medium supplemented with 10% FBS or 5% FBS plus 5% horse serum, respectively. Cells were transfected using 9 µl of Lipofectamine Plus™ and 1.2 µg of DNA (for C6, CHO-K1 and PC12 cells) or 9 µl of Lipofectamine 2000™ and 1.2 µg of DNA (for NB2A cells) in 1 ml of OptiMEM™ medium according to the manufacturer’s protocol. After 5 h of transfection, cells were placed for 1 h in serum-containing
media and then some of the cells were transferred to media containing 0.1% FBS and 0.1%
bovine serum albumin (referred to as “low serum-containing medium”).

**Assessment of MAPK activation**

Cells were cultured for 48 h in either full (10%) serum-containing media or in low serum-
containing media prior to adding 250 µM 8-CPT-cAMP for the indicated time. Cells were lysed
in sodium dodecyl sulfate (SDS)- polyacrylamide electrophoresis (PAGE) sample buffer and
Western blots were generated as described previously (68) using an anti-active MAPK antibody
which specifically recognizes the dually phosphorylated, active form of Erk-1 and -2 (69). Equal
loading of protein was verified by reprobing the blot with an Erk-1 and -2-specific antibody. Western blots were developed using horse radish peroxidase-coupled secondary antibodies and enhanced chemiluminescence. In some experiments, MAPK activity was measured in Erk-1/2 immunoprecipitates using myelin basic protein as substrate (68).

**Measurement of Rap1-bound GTP and Total Rap1-bound Guanine Nucleotides**

Rap1•GTP was captured using RBD peptide (65) and GTP eluted from the isolated Rap was measured as described previously (70); total Rap1-bound guanine nucleotides were measured after converting Rap•GDP to Rap•GTP in half of the sample. This method, which has been described recently (71), correlated well with the method of Franke et al. (65) in which Rap1•GTP bound to RBD peptide is assessed by immunoblotting using a Rap1-specific antibody. Briefly, -3 x 10⁶ cells were extracted in a HEPES-based buffer containing 0.92 %
Triton X-114 and 0.08 % Triton X-45; to one half of the sample was added 20 mM MgSO₄ and
to the other half was added 10 µM GTP and 10 mM EDTA to fully exchange GDP for GTP (72).
After shaking 10 min at 4° C, extracts were warmed to 15° C for 1 min and centrifuged at room
temperature at 10,000 x g for 2 min to generate an aqueous and detergent phase. The detergent
phase, containing >95% of the Rap1, was diluted 10-fold with lysis buffer lacking detergent but
containing 20 mM MgSO₄ and the phase separation was repeated one more time. The diluted
detergent phase was then added to glutathione Sepharose beads pre-loaded with 80 µg of GST-
tagged RBD peptide and the mixture was shaken gently for 1 h at 4° C to allow quantitative
binding of Rap1•GTP to the RBD peptide (65,71). The beads were washed four times and then heated for 3 min at 100°C to elute the GTP bound to Rap1 which was measured using a coupled enzymatic assay (70). The affinity of the RBD peptide for Rap•GDP is undetectably low and we showed that the Ras•GTP present in cell lysates does not interfere with the Rap1 assay, probably because the affinity of RBD for Rap1•GTP is 100-fold higher than for Ras•GTP (71,73). In control experiments using purified Rap1, we found that the in vitro GTP exchange reaction was complete under the described conditions (71). The activation state of Rap1 was calculated as the amount of GTP bound to Rap1 determined in the sample which did not undergo exchange, divided by the total amount of nucleotide-bound Rap1 determined in the sample subjected to the exchange reaction.

**DNA Determination**

DNA was measured in the nuclear fraction by a standard fluorescence method using the fluorescent dye bisbenzimidazole (74)

**Immunoprecipitation and B-Raf Kinase Assay**

Cells were cultured in full serum-containing media or in low serum-containing media for 48 h before harvesting and were lysed in a HEPES-based buffer containing 1% Triton X-100, protease and phosphatase inhibitors. B-Raf was either immunoprecipitated using a B-Raf-specific antibody and Protein A agarose beads as described previously (68), or for cells transfected with GST-tagged B-Raf, the protein was isolated by incubation with glutathione Sepharose beads. After washing the beads, they were incubated with 300 ng of recombinant MEK-1 and 125 µM [γ-32PO4]ATP for 5 min at 30°C as described previously (68); control experiments indicated the assay was linear with respect to lysate input and time. Reaction products were subjected to SDS-PAGE, electroblotted onto polyvinylidene fluoride membranes and exposed to X-ray film. The amount of B-Raf present in the precipitates was determined by immunoblotting with a B-Raf-specific antibody. Control reactions were performed using precipitates obtained either with Protein A and control rabbit immunoglobulin or with glutathione Sepharose and mock-transfected cells.
**Determination of 14-3-3 Association with B-Raf**

Cells were transfected with pcDNA3-BRaf or pCMV-GST-BRaf and after 48 h of culture in full serum-containing media, cells were lysed and B-Raf was immunoprecipitated or isolated on glutathione Sepharose beads as described above. Beads were washed four times in lysis buffer and eluted in SDS-PAGE sample buffer. Proteins eluted from the beads and 5% of the input cell lysates were analyzed by SDS-PAGE/Western blotting. For untagged B-Raf immunoprecipitates, blots were first developed using an antibody which recognizes all major 14-3-3 isoforms and then re-probed with a B-Raf-specific antibody. For GST-tagged B-Raf, blots were simultaneously developed with 14-3-3- and B-Raf-specific antibodies.
RESULTS

Cell Type-Specific Regulation of the MAPK Pathway by 8-pCPT-cAMP

Vossler et al. (33) were the first to explain cAMP activation of the MAPK pathway by a model in which cAMP activation of Rap1 can lead to B-Raf activation and subsequent MAPK activation in cells expressing the 95 kDa B-Raf isoform; results in support of this model have been published subsequently (27). However, other results suggest negative regulation of B-Raf by cAMP in different cell types (5, 18, 34, 35, 46, 47). We, therefore, decided to examine regulation of the MAPK pathway in four different cell lines which express the 95 kDa B-Raf isoform (Fig. 3, described below). Since Erhardt et al. (34) found that regulation of B-Raf by cAMP in PC12 cells was different under high or low serum concentrations in the culture medium, we examined the effect of 8-pCPT-cAMP on MAPK activity under both conditions. Fig. 1 shows MAPK phosphorylation in the different cell lines at various times after adding 250 µM 8-pCPT-cAMP: the blots were developed with an antibody specific for the dually phosphorylated, active form of MAPK (upper half of each panel) with equal protein loading demonstrated by reprobing the blots with an antibody which recognizes Erk-1 and -2 irrespective of their phosphorylation state (lower half of each panel). In C6 rat glioma cells and NB2A murine neuroblastoma cells, MAPK phosphorylation was significantly inhibited within 5 min of adding 8-pCPT-cAMP; the inhibition was somewhat more pronounced in low serum-containing media compared to full serum-containing media and lasted for at least 60 min. On the other hand, in CHO-K1 Chinese hamster ovary cells and PC12 cells, MAPK phosphorylation was stimulated within 5 min of adding 8-pCPT-cAMP. The stimulation was similar in media containing low or high serum concentrations and lasted for 10-20 min in CHO-K1 cells and for at least 20-60 min in PC12 cells; these results are similar to the cAMP-mediated MAPK stimulation in CHO and PC12 cells reported by others (33, 34, 39, 41). Assays in which Erk-1 and -2 were immunoprecipitated from cells and MAPK activity was determined using myelin basic protein as substrate confirmed the results shown in Fig. 1 (data not shown). The effect of 8-pCPT-cAMP was maximal at 250 µM and 1 mM 8-Br-cAMP or 20 µM...
forskolin yielded similar results (data not shown).

**Effect of 8-pCPT-cAMP on Rap1 Activation**

MAPK activation by cAMP in PC12 cells is mediated by Rap1, but Rap1 activation by cAMP appears to be cell type-specific (75,76). Since differential activation of Rap1 by cAMP could explain differences in MAPK regulation by cAMP, we measured the effect of 8-CPT-cAMP on Rap1 activation in the four cell types under study using a novel, quantitative enzymatic method (71). Fig. 2 shows that treating C6, CHO-K1 or NB2A cells with 250 µM 8-pCPT-cAMP for 5 min increased the amount of GTP bound to Rap1 by 2- to 3-fold (open and filled bars represent data obtained in the absence and presence of cAMP, respectively). All three cell types contained similar amounts of total nucleotide-bound Rap1 per µg of cellular DNA (Fig. 2, striped bars), indicating similar absolute amounts of Rap1•GTP when the cells were treated with cAMP. These results are in agreement with recent reports of Rap1 activation by cAMP in CHO10001 cells and C6 cells, which used semiquantitative Western blot analysis of RBD-bound Rap1•GTP (27,39,42). In PC12 cells, the activation state of Rap1 increased from 8.6 ± 1.5% in untreated cells to 18.6 ± 2% in cAMP-treated cells, confirming previous reports that 8-pCPT-cAMP increased the amount of GTP bound to transfected His-tagged Rap1 by about two-fold in 32PO4-labeled PC12 cells (33,77). Thus, while Rap1 activation by cAMP correlated with MAPK activation in CHO-K1 and PC12 cells, a similar degree of Rap1 activation in C6 and NB2A cells did not result in MAPK activation.

**B-Raf Expression**

Multiple, alternatively spliced B-Raf isoforms have been described which may vary in their ability to activate MEK and, therefore, MAPK (15,19,49,78). We compared endogenous B-Raf expression in the different cell types by Western blotting (Fig. 3, upper panel). Although the amounts of the 95 and 68 kDa B-Raf isoforms differed to some extent between the different cell lines, the 95 kDa isoform was present in all four cell lines with NB2A and PC12 cells expressing similar levels. Duplicate blots were probed with an antibody to actin (Fig. 3, middle panel) and with an antibody which recognizes all major isoforms of 14-3-3 (Fig. 3, lower panel); these
blots demonstrated equal protein loading and comparable levels of 14-3-3 in all lanes, respectively. Treating cells for 15 min with 8-pCPT-cAMP did not affect B-Raf or 14-3-3 levels (Fig. 3). Apparently, not all C6 cells express B-Raf (27), and in different strains of CHO and PC12 cells varying amounts of the 68 kDa or 95 kDa B-Raf isoform are present (33,35,39,79).

Cell Type-Specific Regulation of B-Raf by 8-pCPT-cAMP

We examined the effect of 8-pCPT-cAMP on B-Raf activity measured in B-Raf immunoprecipitates using recombinant MEK-1 and [(-32PO4)]ATP. In C6 and NB2A cells, B-Raf activity was inhibited by cAMP, whereas in CHO-K1 and PC12 cells, B-Raf activity was stimulated by cAMP; the effects of 8-pCPT-cAMP were noticeable at 5 min and maximal by 10 min (Fig. 4, upper panels; the lower panels show comparable amounts of the 95 kDa B-Raf isoform in the immunoprecipitates of cAMP-treated and untreated cells, the 68 kDa B-Raf isoform was obscured by the immunoglobulin heavy chain). The effect of cAMP on B-Raf activity was similar in cells cultured in either 0.1% or 10% serum-containing media (data not shown). Thus, cAMP’s effects on endogenous B-Raf activity reflected its effects on MAPK activity in all four cell types. Our results also indicate that Rap1 activation is not sufficient to activate B-Raf in C6 and NB2A cells. Similarly, Rap1 activation is not associated with B-Raf activation in phorbol ester-stimulated Rat-1 cells and bombesin-treated NIH3T3 cells (75,80).

Effect of 8-pCPT-cAMP on the MAPK Pathway in Cells Transfected With the 95 kDa B-Raf Isoform

The cell type-specific differences in cAMP regulation of B-Raf and MAPKs could be due to the presence of different B-Raf isoforms. To eliminate this variable, we measured the effect of cAMP on the MAPK pathway in cells transfected with the 95 kDa B-Raf isoform which is positively regulated by cAMP in PC12 cells (33). Using the transcription factor Elk-1 as a physiological target of Erk-1 and -2, we measured activation of a Gal4-dependent reporter gene by a chimeric Elk-Gal4 construct (33,67). In the absence of exogenous B-Raf, 8-pCPT-cAMP inhibited Elk-Gal4 activity by >50 % in C6 and NB2A cells, and cAMP increased Elk-Gal4
activity 2.9-fold in CHO-K1 cells (Fig. 5, open and filled bars represent data in the absence and presence of cAMP, respectively). These results reflect the effects of cAMP on endogenous Erk activators and are similar to the short-term effects of cAMP shown in Fig. 1. When cells were co-transfected with the 95 kDa B-Raf isoform, Elk-Gal4 activity increased approximately 3-fold in C6 and NB2A cells and 7-fold in CHO-K1 cells (only relatively small amounts of B-Raf were transfected to avoid artifacts that could occur with extremely high B-Raf expression).

Treating cells with 8-pCPT-cAMP inhibited Elk-Gal4 activity by >50% in B-Raf-transfected C6 cells and by >80% in NB2A cells, while cAMP increased Elk-Gal4 activity 3.4-fold in B-Raf-transfected CHO-K1 cells. We obtained similar results in PC12 cells as shown for CHO-K1 cells, confirming previous work with PC12 cells (33). Thus, regulation of the MAPK pathway by cAMP was similar in cells transfected with the 95 kDa B-Raf isoform as in cells expressing endogenous B-Raf, and the effect of cAMP on the 95 kDa B-Raf isoform was cell type-specific.

**Association of 14-3-3 with B-Raf Kinase**

B-Raf and Raf-1 are tightly associated with proteins of the 14-3-3 family and 14-3-3 proteins appear to be necessary to keep Raf-1 in an activation-competent conformation (56, 59, 61). Mutation of Ser728 in the B-Raf catalytic domain prevents 14-3-3 binding and interferes with B-Raf biological functions in intact cells (64), and B-Raf activity is synergistically enhanced by adding 14-3-3 and RasCGTP in vitro (14). Since 14-3-3 binding to B-Raf could alter the regulation of B-Raf by cAMP, we examined the amount of 14-3-3 associated with B-Raf in C6, CHO-K1, NB2A and PC12 cells. All four cell types expressed comparable amounts of total 14-3-3 proteins (Fig. 3, lower panel).

Immunoprecipitation of endogenous B-Raf from the different cell types resulted in co-immunoprecipitation of small amounts of 14-3-3 which were difficult to quantitate (Fig. 6 A, C6 and CHO-K1 cells transfected with empty vector are shown in lane 1); we, therefore, expressed the 95 kDa B-Raf isoform to produce higher, but comparable B-Raf levels in the different cell types. For both C6 and CHO-K1 cells, immunoprecipitates from cells expressing
transfected B-Raf contained increased amounts of 14-3-3 compared to immunoprecipitates from control cells (Fig. 6 A, for both cell types compare lanes 2 and 3, cells transfected with B-Raf vector to lane 1, cells transfected with empty vector). When the amount of 14-3-3 protein present in the immunoprecipitates was compared to the amount of 14-3-3 present in the cell lysates, it became clear that more 14-3-3 was associated with B-Raf in CHO-K1 cells than in C6 cells (Fig. 6 A, lanes 4-6 show 5% of the cell lysates used for B-Raf immunoprecipitation).

To confirm these results, we transfected C6, CHO-K1, NB2A and PC12 cells with increasing amounts of an expression vector encoding GST-tagged full-length B-Raf and isolated the enzyme on glutathione Sepharose beads. Proteins bound to the washed beads were analyzed by Western blots developed simultaneously with B-Raf- and 14-3-3-specific antibodies (Fig. 6 B). We chose cell lysates which contained comparable amounts of GST-tagged B-Raf for the pull-down assay, and found significantly more 14-3-3 associated with B-Raf in CHO-K1 and PC12 cells compared to C6 and NB2A cells (Fig. 6 B). The differences in the amounts of 14-3-3 associated with B-Raf were not due to differences in B-Raf expression in successfully transfected cells, because transfection efficiencies were similar for C6 and PC12 cells and for CHO-K1 and NB2A cells, respectively (data not shown). Results of three independent experiments, in which the signal intensities for B-Raf and 14-3-3 were quantitated by laser densitometry, indicate that about 5-fold more 14-3-3 protein was associated with B-Raf in cells in which cAMP stimulated B-Raf activity compared to cells in which cAMP inhibited B-Raf activity, although the amount of total 14-3-3 protein expressed was similar in all cell types studied (Fig. 6 C and Fig. 3).

Effect of 14-3-3 on the Regulation of B-Raf Activity by 8-pCPT-cAMP
To measure the effects of 8-pCPT-cAMP and 14-3-3 on the activity of the 95 kDa B-Raf isoform directly, we transfected C6 and CHO-K1 cells with a GST-tagged full-length B-Raf construct, isolated B-Raf on glutathione Sepharose beads and incubated the washed beads with MEK-1 and [(\textsuperscript{32}P)\textsubscript{4}]ATP. Similar to the results shown for endogenous B-Raf in Fig. 4, activity of the transfected B-Raf was inhibited 75% by cAMP in C6 cells, whereas it was increased 2.3-fold by cAMP in CHO-K1 cells (Fig. 7 A shows a typical experiment and Fig. 7 B summarizes the results of three independent experiments). Co-transfection of expression vectors encoding either 14-3-3\$ or 14-3-3\$J with GST-tagged B-Raf increased B-Raf activity in both C6 and CHO-K1 cells and completely prevented the inhibitory effect of 8-pCPT-cAMP in C6 cells (Fig. 7 A and B, the effects of 14-3-3 J are shown only for C6 cells). In contrast, transfecting CHO-K1 cells with a dominant negative form of 14-3-3 [14-3-3mut (R56,60A)], which shows impaired Raf-1 binding and forms heterodimers with endogenous wild type 14-3-3 (53), significantly decreased B-Raf activity (Fig. 7 A and B). When MEK phosphorylation was normalized to the amount of B-Raf present, no significant cAMP stimulation of B-Raf could be demonstrated in C6 cells transfected with wild type 14-3-3 and in CHO-K1 cells transfected with 14-3-3mut (R56,60A) (Fig. 7 B). For comparison, over-expression of wild type 14-3-3 increases the activity of Raf-1 in HeLa, NIH3T3 and COS cells, whereas expression of the dominant negative 14-3-3 mutant inhibits Raf-1 in 293 cells (53,56,60).

In PC12 cells, A-kinase inhibits the activity of the B-Raf catalytic domain whereas it stimulates the activity of full-length B-Raf (48). When we transfected the B-Raf catalytic domain into C6 and CHO-K1 cells, we found that 8-pCPT-cAMP inhibited its activity in both cell types, although inhibition was more pronounced in C6 cells than in CHO-K1 cells; co-transfection of a 14-3-3\$ expression vector prevented the inhibition in both cell types (Fig. 8). Over-expression of 14-3-3 increased the amount of B-Raf catalytic domain in several independent experiments, suggesting that 14-3-3 may stabilize the protein. We found that co-transfection of the catalytic subunit of A-kinase inhibited the catalytic domain of B-Raf in both C6 and CHO-K1 cells and inhibited full-length B-Raf in C6 cells but not in CHO-K1 cells.
similar to the results shown for 8-pCPT-cAMP (data not shown). Thus, the cell type-specific activation of B-Raf by cAMP/A-kinase requires B-Raf’s N-terminal regulatory domain which mediates Ras/Rap binding, while the isolated catalytic domain is subject to inhibition by cAMP/A-kinase in all cell types examined. However, over-expression of 14-3-3 can protect both full-length and isolated catalytic domain of B-Raf from cAMP-mediated inhibition.

Effect of cAMP and 14-3-3 on B-Raf Ser^{728} Phosphorylation
Ser²² in the catalytic domain of B-Raf is required for 14-3-3 binding and represents a potential A-kinase phosphorylation site; Ser²² in B-Raf is analogous to Ser⁶²¹ in Raf-1 which binds 14-3-3 and can be phosphorylated by A-kinase (28,30,49,64). Since the sequences surrounding these two sites are nearly identical, we used an anti-Raf-1 phospho-Ser⁶²¹-specific antibody to examine the effect of cAMP on B-Raf Ser²² phosphorylation (53). Very low amounts of Ser²² phosphorylation were detectable on GST-tagged B-Raf isolated from untreated C6 and CHO-K1 cells and treating cells with 8-pCPT-cAMP increased the signal modestly in both cell types (Fig. 9, upper panels). Over-expression of 14-3-3$ significantly increased the amount of Ser²² phosphorylation in both cell types with cAMP treatment inducing a further modest increase in phosphorylation (Fig. 9). Western blots demonstrated that similar amounts of B-Raf were present in the absence and presence of cAMP and that co-transfection of the 14-3-3 vector increased the amount of 14-3-3 associated with B-Raf (Fig. 9, lower panels). When Figs. 7 and 9 are compared, the amount of Ser²² phosphorylation did not correlate with B-Raf activity. B-Raf activity may be differentially influenced by Ser²² phosphorylation in the presence and absence of bound 14-3-3; the modest degree of cAMP-induced Ser²² phosphorylation suggests that Ser²² may not be the major target for A-Kinase inhibition of B-Raf.
DISCUSSION

The model developed by Vossler et al. (33) describing cAMP activation of MAPKs via activation of Rap1 and B-Raf in PC12 cells has been difficult to reconcile with reports by other workers of cAMP-mediated inhibition of B-Raf activity in PC12 cells (18,34,35) and in other cell types (5,47); the differences in cAMP response have been attributed to variable expression of the 95 and 68 kDa B-Raf isoforms (33). However, transfection of the 95 kDa B-Raf isoform into B-Raf-deficient cells resulted in MAPK stimulation by cAMP in NIH3T3 but not in Rat-1 fibroblasts (33,34); transfection of this B-Raf isoform into B-Raf-deficient C6 cells increased basal MAPK activity, which was inhibited by the A-kinase inhibitor HA120, but cAMP activation was not demonstrated (27). On the other hand, Rap1-dependent activation of a 68 kDa B-Raf isoform by cAMP has been described in CHO cells but not in Rat-1 fibroblasts (39,81). These results from different laboratories suggest that differential expression of the 95 and 68 kDa B-Raf isoforms may not be sufficient to explain cAMP activation or inhibition of B-Raf in different cells. We hypothesized that B-Raf regulation may be modulated by cell type-specific factors and found that: (i) cAMP activation of Rap1 was not sufficient to activate B-Raf in all cells; (ii) cAMP activation or inhibition of the 95 kDa B-Raf isoform correlated with high or low amounts of 14-3-3 associated with the enzyme; and (iii) over-expression of 14-3-3 protected both full-length and the catalytic domain of B-Raf from cAMP-mediated inhibition.

Our finding that cAMP activation of Rap1 was not sufficient to activate B-Raf in C6 and NB2A cells is in agreement with previous observations that Rap1 activation in phorbol ester-stimulated Rat-1 cells and bombesin-stimulated NIH3T3 cells does not lead to B-Raf activation (75,80). Activation of B-Raf by Rap1 may require tissue-specific co-activators or there may be mechanisms that interfere with Rap1 activation of B-Raf. While purified Rap1·GTP activates purified B-Raf in vitro in the absence of added co-factors, 14-3-3 proteins present in B-Raf preparations may be important for this effect of Rap1, similar to 14-3-3 proteins synergistically enhancing B-Raf activation by Ras·GTP (13,14,60,61). Thus, the amount of 14-3-3 associated
with B-Raf in intact cells could potentially influence the ability of Rap1 to activate B-Raf; however, overexpression of 14-3-3 in C6 cells was not sufficient for cAMP to activate B-Raf. Cell type-specific differences in the subcellular localization of Rap1 and B-Raf could account for the cell type-specific ability of Rap1 to activate B-Raf. More work is necessary to determine the factor(s) required for B-Raf activation by Rap1•GTP in vivo.

The cAMP/A-kinase-mediated inhibition of B-Raf may involve direct phosphorylation, because B-Raf is phosphorylated by A-kinase in vitro and in vivo with A-kinase phosphorylation of the catalytic domain of B-Raf inhibiting its activity both in vitro and in vivo (46,48). The modest increase in Ser^{728} phosphorylation in cAMP-treated cells did not correlate with B-Raf activity; over-expression of 14-3-3 led to increased Ser^{728} phosphorylation of B-Raf and increased kinase activity. Similarly, overexpression of 14-3-3 in HeLa cells leads to increased Ser^{621} phosphorylation of Raf-1, which is associated with increased kinase activity, but A-kinase phosphorylation of Ser^{621} has been reported to inhibit Raf-1 activity (28,30,53). A-kinase is probably not the major kinase phosphorylating Ser^{621} of Raf-1 in vivo and the effect of Ser^{621} phosphorylation on Raf-1 activity remains controversial (28,32,82). The amount 14-3-3 bound to phospho-Ser^{621}/ Ser^{728} may determine Raf-1/B-Raf kinase activity rather than the amount of Ser^{621}/ Ser^{728} phosphorylation per se. This way, A-kinase phosphorylation of B-Raf Ser^{728} may be inhibitory in C6 cells with low amounts of 14-3-3 bound to B-Raf while cells with high amounts of 14-3-3 bound to B-Raf (CHO cells or C6 cells over-expressing 14-3-3) were protected from the inhibitory effect. However, Ser^{728} may not be the major target for A-kinase phosphorylation and other potential A-kinase phosphorylation sites include Ser^{429} and Ser^{446}, both located at the N-terminus of the catalytic domain (19,49). Alternatively, it is possible that A-kinase phosphorylation of other proteins could alter their interaction with B-Raf. Although most 14-3-3 isoforms contain a potential A-kinase phosphorylation site, they are not A-kinase substrates (83), and we found that the amount of 14-3-3 associated with B-Raf was not detectably altered in cAMP-treated cells (Fig. 8).
Binding of 14-3-3 to target proteins, which often is dependent on serine phosphorylation, may result in activation, inhibition, translocation or association of the target protein with other proteins (50,51,84). For example, phosphorylation of the pro-apoptotic protein BAD by Akt-1/protein kinase B promotes 14-3-3 binding which prevents BAD’s heterodimerization with the anti-apoptotic proteins BCL-2 and BCL-X\(_L\), and 14-3-3 binding to the phosphorylated form of the mitotic inducer Cdc25 leads to cytoplasmic retention and nuclear export (85,86). 14-3-3 proteins may also function as adaptor proteins, which mediate the association of different proteins, as in the example of Raf-1 and BCR (52,87). Through the use of dominant negative forms of 14-3-3 it has been demonstrated that inhibition of apoptosis is an important function of 14-3-3 proteins; this effect of 14-3-3 proteins is mediated partially by the regulation of MAPK pathways (88). Several investigators have demonstrated that 14-3-3 association with Raf-1 is required to maintain basal Raf-1 catalytic activity and to stabilize Raf-1 in an “activation-competent conformation”; mutation of the 14-3-3 binding site in the catalytic domain of Raf-1 (Ser\(^{621}\)) leads to a virtually inactive enzyme (53,56,57,59). Mutation of the analogous site in B-Raf (Ser\(^{728}\)) leads to only 50–60% loss of \textit{in vitro} kinase activity, but results in almost complete loss of biological activity \textit{in vivo}, suggesting that 14-3-3 binding at this site is required to couple B-Raf to its downstream effectors (64). Our results suggest a model in which 14-3-3 bound to B-Raf prevents the inhibitory effects of A-kinase phosphorylation and add a new aspect to the growing list of 14-3-3 functions. Since the catalytic domain of B-Raf is inhibited by cAMP, even in cells in which full-length B-Raf is stimulated, it appears that higher amounts of 14-3-3 are required to protect the isolated catalytic domain from cAMP-mediated inhibition compared to the full-length enzyme. Binding of 14-3-3 to N-terminal sites in B-Raf may contribute to keeping B-Raf in an active conformation and it may be necessary, albeit not sufficient, to allow positive interaction of Rap1 with the N-terminus.

The amount of 14-3-3 associated with B-Raf appears to be regulated by cell type-specific factors. Similar amounts of total 14-3-3 were present in the four cell lines we studied, but significantly less 14-3-3 was associated with B-Raf in cells in which cAMP inhibited B-Raf
compared to cells in which cAMP simulated B-Raf. Only a small amount of total cellular 14-3-3 was associated with B-Raf even in PC-12 or CHO-K1 cells in which B-Raf was over-expressed. Cell type-specific differences in the subcellular localization of B-Raf and 14-3-3 could potentially limit association of the two proteins (6,9,51). Numerous other 14-3-3 target proteins, including the protein kinase BCR and the death agonist BAD, may compete with B-Raf for 14-3-3 binding (52,85). Recently, the product of the early response gene BRF1 has been demonstrated to interact tightly with 14-3-3$ and J and to interfere with the binding of 14-3-3 to Raf-1 (89). Thus, B-Raf association with 14-3-3 proteins may vary between different cell types due to the expression of proteins which enhance or diminish 14-3-3 binding to B-Raf or due to expression of different 14-3-3 isoforms which vary in their affinities for specific target proteins (51,89). More work is clearly necessary to examine the factors which influence 14-3-3 association with B-Raf and the mechanism(s) by which 14-3-3 proteins modulate B-Raf functions.
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REFERENCES

1. Hagemann, C. and Rapp, U. R. (1999) *Exp.Cell Res.* **253**, 34-46
2. Daum, G., Eisenmann-Tappe, I., Fries, H.-W., Troppmair, J., and Rapp, U. R. (1994) *TIBS* **19**, 474-480
3. Morrison, D. K. and Culter, R. E., Jr. (1997) *Curr.Opin.Cell Biol.* **9**, 174-179
4. Jaiswal, R. K., Moodie, S. A., Wolfman, A., and Landreth, G. E. (1994) *Mol.& Cell.Biol.* **14**, 6944-6953
5. Reuter, C. W. M., Catling, A. D., Jelinek, T., and Weber, M. J. (1995) *J.Biol.Chem* **270**, 7644-7655
6. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. (1997) *J.Biol.Chem.* **272**, 4378-4383
7. Pritchard, C. A., Samuels, M. L., Bosch, E., and McMahon, M. (1995) *Mol.& Cell.Biol.* **15**, 6430-6431
8. Moodie, S. A., Paris, M. J., Kolch, W., and Wolfman, A. (1994) *Mol.& Cell.Biol.* **14**, 7153-7162
9. Jelinek, T., Dent, P., Sturgill, T. W., and Weber, M. J. (1996) *Mol.& Cell.Biol.* **16**, 1027-1034
10. Hu, C.-D., Kariya, K., Kotan, G., Shirouzu, M., Yokoyama, S., and Kataoka, T. (1997) *J.Biol.Chem.* **272**, 11702-11705
11. Zhang, X.-F., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) *Nature* **364**, 308-313
12. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais,
13. Ohtsuka, T., Shimizu, K., Yamamori, B., Kuroda, S., and Takai, Y. (1996) *J.Biol.Chem.*

14. Shimizu, K., Kuroda, S., Yamamori, B., Matsuda, S., Kaibuch, K., Yamauchi, T., Isobe, T., Irie, K., Matsumoto, K., and Takai, Y. (1994) *J.Biol.Chem.* **269**, 22917-22920

15. Papin, C., Denouel-Galy, A., Laugier, D., Calothy, G., and Eychene, A. (1998) *J.Biol.Chem.* **273**, 24939-24947

16. Catling, A. D., Reuter, C. W. M., Cox, M. E., Parsons, S. J., and Weber, M. J. (1994) *J.Biol.Chem.* **269**, 30014-30021

17. Eychene, A., Dusanter-Fourt, I., Barnier, J. V., Papin, C., Charon, M., Gisselbrecht, S., and Calothy, G. (1995) *Oncogene* **10**, 1159-1165

18. Lange-Carter, C. A. and Johnson, G. L. (1994) *Science* **265**, 1458-1461

19. Stephens, R. M., Sithanandam, G., Copeland, T. D., Kaplan, D. R., Rapp, U. R., and Morrison, D. K. (1992) *Mol. & Cell.Biol.* **12**, 3733-3742

20. Hordijk, P. L., Verlaan, I., Jalink, K., vanCorven, E. J., and Moolenaar, W. H. (1994) *J.Biol.Chem.* **269**, 3534-3538

21. Cook, S. J. and McCormick, F. (1993) *Science* **262**, 1069-1071

22. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993) *Science* **262**, 1065-1068

23. Graves, L. M., Bornfeldt, K. E., Raines, E. W., Potts, B. C., Macdonald, S. G., Ross, R., and Krebs, E. G. (1993) *Proc.Natl.Acad.Sci.USA* **90**, 10300-10304

24. Chen, J. and Iyengar, R. (1994) *Science* **263**, 1278-1281

25. Matousovic, K., Grande, J. P., Chini, C. C. S., Chini, E. N., and Dousa, T. P. (1995)
26. Chen, J., Bander, J. A., Santore, T. A., Chen, Y., Ram, P. T., Smit, M. J., and Iyengar, R. (1998) *Pharmacology* **95**, 2648-2652

27. Dugan, L. L., Kim, J. S., Zhang, Y., Bart, R. D., Sun, Y., Holtzman, D. M., and Gutmann, D. H. (1999) *J.Biol.Chem.* **274**, 25842-25848

28. Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A., and Kolch, W. (1996) *Mol. & Cell.Biol.* **16**, 5409-5418

29. Chuang, E., Barnard, D., Hettich, L., Zhang, X.-F., Avruch, J., and Marshall, M. S. (1994) *Mol. & Cell.Biol.* **14**, 5318-5325

30. Häfner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Fippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994) *Mol. & Cell.Biol.* **14**, 6696-6703

31. Kikuchi, A. and Williams, L. T. (1996) *J.Biol.Chem.* **271**, 588-594

32. Sidovar, M. F., Kozlowski, P., Lee, J. W., Collins, M. A., He, Y., and Graves, L. M. (2000) *J.Biol.Chem.*, in press.

33. Vossler, M. R., Yao, H., York, R. D., Pan, M.-G., Rim, C. S., and Stork, P. J. S. (1997) *Cell* **89**, 73-82

34. Erhardt, P., Troppmair, J., Rapp, U. R., and Cooper, G. M. (1995) *Mol. & Cell.Biol.* **15**, 5524-5530

35. Vaillancourt, R. R., Gardner, A. M., and Johnson, G. L. (1994) *Mol.Cell.Biol.* **14**, 6522-6530

36. Englaro, W., Rezzonico, R., Durand-Clement, M., Lallemand, D., Ortonne, J.-P., and Ballotti, R. (1995) *J.Biol.Chem.* **270**, 24315-24320

37. Wan, Y. and Huang, X.-Y. (1998) *J.Biol.Chem.* **273**, 14533-14537
38. Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) *J.Biol.Chem.* **269**, 7851-7854

39. Seidel, M. G., Klinger, M., Freissmuth, M., and Höller, C. (1999) *J.Biol.Chem.* **274**, 25833-25841

40. Sevetson, B. R., Kong, X., and Lawrence, J. C. Jr. (1993) *Proc.Natl.Acad.Sci.USA* **90**, 10305-10309

41. Verheijen, M. H. G. and Defize, L. H. K. (1997) *J.Biol.Chem.* **272**, 3423-3429

42. De Rooij, J., Wittinghofer, A., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., and Bos, J. L. (1998) *Nature* **396**, 474-477

43. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) *Science* **282**, 2275-2279

44. Altschuler, D. L., Peterson, S. N., Ostrowski, M. C., and Lapetina, E. G. (1995) *J.Biol.Chem.* **270**, 10373-10376

45. Hata, Y., Kaibuchi, K., Kawamura, S., Hiroyoshi, M., Shirataki, H., and Takai, Y. (1991) *J.Biol.Chem.* **266**, 6571-6577

46. Peraldi, P., Frödin, M., Barnier, J. V., Calleja, V., Scimeca, J.-C., Filloux, C., Calothy, G., and van Obberghen, E. (1995) *FEBS.Lett.* **357**, 290-296

47. Buhl, A. M., Avdi, N., Worthen, G. S., and Johnson, G. L. (1994) *Proc.Natl.Acad.Sci.USA* **91**, 9190-9194

48. MacNicol, M. C. and MacNicol, A. M. (1999) *J.Biol.Chem.* **274**, 13193-13197

49. Sithanandam, G., Kolch, W., Duh, F.-M., and Rapp, U. R. (1990) *Oncogene* **5**, 1775-1780

50. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84**, 889-897

51. Aitken, A., Jones, D., Soneji, Y., and Howell, S. (1995) *Biochem.Soc.Transct.* **23**, 605-611
52. Burbelo, P. D. and Hall, A. (1995) Curr. Biol. 5, 95-96

53. Thorson, J. A., Yu, L. W. K., Hsu, A. L., Shih, N.-Y., Graves, P. R., Tanner, J. W., Allen, P. M., Piwnica-Worms, H., and Shaw, A. S. (1998) Mol. & Cell. Biol. 18, 5229-5238

54. Michaud, N. R., Fabian, J. R., Mathes, K. D., and Morrison, D. K. (1995) Mol. & Cell. Biol. 15, 3390-3397

55. Clark, G. J., Drugan, J. K., Rossman, K. L., Carpenter, J. W., Rogers-Graham, K., Fu, H., Der, C. J., and Campbell, S. L. (1997) J. Biol. Chem. 272, 20990-20993

56. Hancock, J. F., Roy, S., McPherson, R. A., Appoloni, A., Yan, J., Lane, A., and Clyde-Smith, J. (1998) Mol. & Cell. Biol. 18, 3947-3955

57. McPherson, R. A., Harding, A., Roy, S., Lane, A., and Hancock, J. F. (1999) Oncogene 18, 3862-3869

58. Rommel, C., Radziwill, G., Moelling, K., Noeldeke, J., Heinicke, T., Jones, D., and Aitken, A. (1996) Oncogene 12, 609-619

59. Tzivion, G., Luo, Z., and Avruch, J. (1998) Nature 394, 88-92

60. Li, S., Janosch, P., Tanji, M., Rosenfeld, G. C., Waymire, J. C., Mischak, H., Kolch, W., and Sedivy, J. M. (1995) EMBO J. 14, 685-696

61. Yamamori, B., Kuroda, S., Shimizu, K., Fukui, K., Ohtsuka, T., and Takai, Y. (1995) J. Biol. Chem. 270, 11723-11726

62. Jaiswal, R. K., Weissinger, E., Kolch, W., and Landreth, G. E. (1996) J. Biol. Chem. 271, 23626-23629

63. Papin, C., Eychene, A., Brunet, A., Pages, G., Pouyssegur, J., Calothy, G., and Barnier, J. V. (1995) Oncogene 10, 1647-1651

64. MacNicol, M. C., Muslin, A. J., and MacNicol, A. M. (2000) J. Biol. Chem. 275, 3803-3809
65. Franke, B., Akkerman, J.-W., and Bos, J. L. (1997) *EMBO J.* **16**, 252-259

66. Tsai, R. Y. L. and Reed, R. R. (1997) *BioTechniques* **23**, 794-800

67. Johnson, N. L., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Gleavy, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I., and Johnson, G. L. (1996) *J.Biol.Chem.* **271**, 3229-3237

68. Suhasini, M., Li, H., Lohmann, S. M., Boss, G. R., and Pilz, R. B. (1998) *Mol. & Cell.Biol.* **18**, 6983-6994

69. Payne, D. M., Rossomando, A. J., Martino, 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

70. Sharma, P. M., Egawa, K., Huang, Y., Martin, J. L., Huvar, I., Boss, G. R., and Olefsky, J. M. (1998) *J.Biol.Chem.* **273**, 18528-18537

71. von Lintig, F. C., Pilz, R. B., and Boss, G. R. (2000) *Oncogene* **19**, 4029-4034

72. Quilliam, L. A., Der, C. J., Clark, R., O’Rourke, E. C., Zhang, K., McCormick, F., and Bokoch, G. M. (1990) *Mol.Cell.Biol.* **10**, 2901-2908

73. Herrmann, C., Horn, G., Spaargaren, M., and Wittinghofer, A. (1996) *J.Biol.Chem.* **271**, 6794-6800

74. Brunk, C., Jones, K., and James, T. (1979) *Anal.Biochem.* **92**, 497-500

75. Zwartkruis, F. J. T., Wolthuis, R. M. F., Nabben, N. M. J. M., Franke, B., and Bos, J. L. (1998) *EMBO J.* **17**, 5905-5912

76. Bos, J. L., Franke, B., M’Rabet, L., Reedquist, K., and Zwartkruis, F. (1997) *FEBS.Lett.* **410**, 59-62

77. Yao, H., York, R. D., Misra-Press, A., Carr, D. W., and Stork, P. J. S. (1998) *J.Biol.Chem.* **273**, 8240-8247

78. Barnier, J. V., Papin, C., Eychene, A., Lecoq, O., and Calothy, G. (1995) *J.Biol.Chem.* **270**
79. Sugawara, F., Ninomiya, H., Okamoto, Y., Miwa, S., Mazda, O., Katsura, Y., and Masaki, T. (1996) *Mol. Pharmacol.* **49**, 447-457

80. Posern, G., Weber, C. K., Rapp, U. R., and Feller, S. M. (1998) *J. Biol. Chem.* **273**, 24297-24300

81. Faure, M. and Bourne, H. R. (1995) *Mol. Biol. Cell* **6**, 1025-1035

82. Sprenkle, A. B., Davies, S. P., Carling, D, Hardie, D. G., and Sturgill, T. W. (1997) *FEBS Letters* **403**, 254-258

83. Toker, A., Sellers, L. A., Amess, B., Patel, Y., Harris, A., and Aitken, A. (1992) *Eur. J. Biochem.* **206**, 453-461

84. Aitken, A. (1995) *TIBS* **20**, 95-97

85. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) *Cell* **87**, 619-628

86. Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999) *Nature* **397**, 172-175

87. Braselmann, S. and McCormick, F. (1995) *EMBO J.* **14**, 4839-4848

88. Xing, H., Zhang, S., Weinheimer, C., Kovacs, A., and Muslin, A. J. (2000) *EMBO J.* **19**, 349-358

89. Bustin, S. A. and McKay, I. A. (1999) *DNA Cell Biol.* **18**, 653-661
FOOTNOTES

The abbreviations used are: A-kinase, cAMP-dependent protein kinase; 8-Br-cAMP, 8-bromo-cAMP; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GEF, guanine nucleotide exchange factor; GST, glutathione-S-transferase; MAPK, mitogen-activated protein kinase; 8-pCPT-cAMP, 8-(4-chlorophenylthio)cAMP; RBD, Rap-binding domain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis.
FIGURE LEGENDS

Fig. 1: Cell type-specific regulation of MAPK by 8-pCPT-cAMP. C6, CHO-K1, NB2A and PC12 cells were cultured either in low serum or full serum-containing media for 48 h prior to adding 250 µM 8-pCPT-cAMP for the indicated times (0’ to 60’) as described in Materials and Methods. Western blots were probed with an antibody specific for active Erk-1 and -2 dually phosphorylated on Thr and Tyr (P-MAPK) (69). To demonstrate equal loading, blots were re-probed with an antibody recognizing ErK-1 and -2 irrespective of their phosphorylation state (MAPK).

Fig. 2: Effect of 8-pCPT-cAMP on Rap1 Activation. C6, CHO-K1 and NB2A cells were cultured in serum-free medium for 48 h and either left untreated (open bars) or were treated with 250 µM 8-pCPT-cAMP for 5 min (filled bars). The activation state of Rap1 was determined as described in Materials and Methods and is (Rap1•GTP/total Rap1-bound guanine nucleotides) x100 (left panel). Total guanine nucleotides bound to Rap1 are shown in the right panel for cAMP-treated cells (striped bars) and are expressed as fmoles of guanine nucleotides per µg of cellular DNA; similar results were obtained for untreated cells (not shown). The data are the mean ± S.D. of at least three independent experiments performed in duplicate.

Fig. 3: B-Raf expression. Logarithmically growing C6, CHO-K1, NB2A and PC12 cells were left untreated (-) or were treated for 15 min with 250 µM 8-pCPT-cAMP (+). Cells were lysed and equal amounts of protein were subjected to Western blotting with a B-Raf-specific antibody which recognizes both the 95 kDa and 68 kDa isoforms (upper panel). Duplicate blots were probed with an antibody which recognizes the conserved C-terminus of actin (middle panel) and with an antibody which recognizes all major isoforms of 14-3-3 (lower panel). Note that at least two different 14-3-3 isoforms were resolved on this 10% acrylamide gel.
Fig. 4: Cell type-specific regulation of B-Raf by 8-pCPT-cAMP.  C6, CHO-K1, NB2A and PC12 cells were cultured in full serum-containing media and treated with 250 µM 8-pCPT-cAMP for the indicated times (0’ to 20’). Cell lysates were prepared from one (CHO-K1, NB2A and PC12) or two (C6) plates and subjected to immunoprecipitation using a B-Raf-specific antibody that recognizes both the 95 kDa and 68 kDa isoforms. Immunoprecipitates were incubated in the presence of MEK-1 and [(-32PO4]ATP for 5 min, subjected to SDS-PAGE/electroblotting and autoradiography to assess MEK phosphorylation as described in Materials and Methods (P-MEK, upper half of the panels). The blots were later probed with a B-Raf-specific antibody to determine the amount of B-Raf present in the immunoprecipitates (lower half of the panels; only the 95 kDa B-Raf isoform is shown because the 68 kDa isoform was obscured by the immunoglobulin heavy chain). No signal was observed on the autoradiographs when control rabbit immunoglobulin was substituted for the B-Raf-specific antibody indicating that MEK auto-phosphorylation was not detectable under the conditions used (not shown).

Fig. 5: Effect of 8-pCPT-cAMP on the MAPK pathway in cells transfected with the 95 kDa B-Raf isoform.  C6, NB2A and CHO-K1 cells were transfected with 1 ng of expression vector encoding the chimeric transcription factor Elk-Gal4, 50 ng of the reporter pGAL4-Luc, 50 ng of pRSV-$Gal (internal control) and either 10 ng of expression vector encoding full-length B-Raf or empty vector as described in Materials and Methods. Cells were cultured in low serum-containing media for 24 h after transfection and were either left untreated (open bars) or were treated with 250 µM 8-pCPT-cAMP (filled bars). The cells were harvested 8 h later and luciferase and $-galactosidase activities were measured as described in Materials and Methods. For each cell type, the ratio of luciferase to $-galactosidase activity in untreated cells transfected with Elk-Gal4 alone was assigned the value of one. The data represent the mean ± S.D. of at least three independent experiments performed in duplicate.
**Fig. 6: Association of 14-3-3 with B-Raf kinase.** Panel A: To achieve comparable B-Raf expression, C6 cells were transfected with 0.6 or 1.2 µg of a B-Raf expression vector and CHO-K1 cells were transfected with 0.2 or 0.4 µg of the B-Raf vector (lanes 2 & 5 and lanes 3 & 6, respectively). Total DNA was adjusted to 1.2 µg with empty vector. In lanes 1 & 4 are shown cells transfected with empty vector alone. After 48 h in full serum-containing media, cell lysates were subjected to immunoprecipitation with a B-Raf-specific antibody. Lanes 1-3 show the washed immunoprecipitates and lanes 4-6 show 5% of the cell lysate input. Western blots were probed first with an antibody recognizing all major 14-3-3 isoforms (lower blot) and then reprobed with a B-Raf-specific antibody (upper blot). Panel B: C6, CHO-K1, NB2A and PC12 cells were transfected with 0.1µg to 0.8 µg of an expression vector encoding GST-tagged full-length B-Raf and 48 h later, cell lysates were incubated with glutathione-agarose beads. Proteins bound to the washed beads were analyzed by Western blotting using antibodies specific for B-Raf and 14-3-3 simultaneously. C6 and PC12 cells were transfected with 0.4 µg (lanes 1 and 7) or 0.8 µg (lanes 2 and 8) and CHO-K1 and NB2A cells were transfected with 0.1 µg (lanes 3 and 5) or 0.2 µg (lanes 4 and 6) of pCMV-GST-BRaf. In lanes 9 and 10 are shown mock-transfected CHO-K1 cells and CHO-K1 cells transfected with 0.3 µg of pCMV-GST-BRaf, respectively. Panel C: Experiments were performed as described in panel B; autoradiographs in the linear range of exposure were analyzed by laser densitometry and the ratio of the 14-3-3 signal intensity to the B-Raf signal intensity was calculated. The data represent the mean ± S.D. of three independent experiments.

**Fig. 7: Effect of 14-3-3 on regulation of B-Raf activity by 8-pCPT-cAMP.** Panel A: C6 cells and CHO-K1 cells were transfected with 50 ng of an expression vector encoding full-length GST-tagged B-Raf; cells were co-transfected with 100 ng of expression vectors encoding 14-3-3$, 14-3-3J, a dominant negative mutant form of 14-3-3 [14-3-3mut (R56,60A)], or empty pcDNA3 expression vector (first two lanes) as indicated. The total amount of transfected DNA was adjusted to 1.2 µg by adding pGem3Z. After 48 h of culture in full serum-containing
medium, cells were treated for 15 min with 250 µM 8-pCPT-cAMP as indicated. Cell lysates were prepared and GST-tagged B-Raf was isolated on glutathione agarose beads; the beads were incubated with recombinant MEK-1 and [γ-32P]ATP for 5 min and subjected to SDS-PAGE/electroblotting/autoradiography to assess MEK phosphorylation as described in Materials and Methods (upper half of panel). The blots were probed with a B-Raf-specific antibody to quantitate the amount of B-Raf on the beads (lower half of panel). Control experiments demonstrated expression of the wild type and mutant 14-3-3 forms by Western blotting of cell lysates (not shown). Panel B: Experiments were performed as described in panel A; autoradiographs in the linear range of exposure were scanned by laser densitometry and the amount of MEK phosphorylation was normalized to the amount of B-Raf. The amount of MEK phosphorylation in untreated cells without transfected 14-3-3 was assigned a value of one. The data represent the mean ± S.D. of three independent experiments.

Fig. 8: Effect of 14-3-3 on the catalytic domain of B-Raf. C6 cells (left panels) and CHO-K1 cells (right panels) were transfected with 50 ng of an expression vector encoding the GST-tagged catalytic domain of B-Raf; cells were co-transfected with 100 ng of 14-3-3 vector (lanes 5-7) or empty pcDNA3 vector (lanes 2-4) as indicated. Cells were treated with 8-pCPT-cAMP for the indicated time, the GST-tagged catalytic domain of B-Raf was isolated and MEK phosphorylation was determined as described in Fig. 7. In the lanes labeled “C” (lane 1), cells were transfected with pCMV-GST empty vector instead of the B-Raf catalytic domain vector and cell lysates were processed in parallel with the samples shown in the other lanes. Blots probed with a B-Raf-specific antibody are shown in the lower half of the panel. Similar results were obtained in one other experiment.

Fig. 9: Effect of 8-pCPT-cAMP and 14-3-3 on B-Raf Ser728 phosphorylation. C6 cells (left panels) and CHO-K1 cells (right panels) were transfected with an expression vector encoding full-length GST-tagged B-Raf and co-transfected with either empty pcDNA3 vector (lanes 1
and 2) or an expression vector encoding 14-3-3$ (lanes 3 and 4) as described in Materials and Methods. Cells were cultured in low serum-containing media and treated for 15 min with 250 µM 8-pCPT-cAMP as indicated. GST-tagged B-Raf was isolated on glutathione agarose beads and the beads were subjected to SDS-PAGE/electroblotting as described in Methods. The blot was probed first with an antibody specific for phospho-Ser$^{621}$ of Raf-1, with Ser$^{621}$ of Raf-1 corresponding to Ser$^{728}$ in the catalytic domain of B-Raf [upper panel, designated Ser$^{728}$-P] (19,49,53). The blot was re-probed with a B-Raf antibody which does not distinguish phospho- and dephospho-forms, and an 14-3-3 antibody (lower panel). Similar results were obtained in two other experiments.
Fig. 3

|       | C6 | CHO | NB2A | PC12 | cAMP |
|-------|----|-----|------|------|------|
| -     | -  | -   | -    | -    | -    |
| +     | +  | +   | +    | +    | +    |

- 95 kDa - B-Raf
- 68 kDa -
- 42 kDa - actin
- 30 kDa - 14-3-3
Fig. 4

|       | C6     | CHO   |
|-------|--------|-------|
| 0'    |        |       |
| 5'    | ![P-MEK](image) | ![P-MEK](image) |
| 10'   | ![B-Raf](image)  | ![B-Raf](image) |
| 20'   | ![P-MEK](image)  | ![P-MEK](image) |

|       | NB2A   | PC12  |
|-------|--------|-------|
| 0'    |        |       |
| 5'    | ![P-MEK](image) | ![P-MEK](image) |
| 10'   | ![B-Raf](image)  | ![B-Raf](image) |
| 20'   | ![P-MEK](image)  | ![P-MEK](image) |
Fig. 5

Bar charts showing relative luciferase activity in different cell lines:

- **C6**
  - Empty Vector: 1
  - B-Raf: 3

- **NB2A**
  - Empty Vector: 1
  - B-Raf: 3

- **CHO**
  - Empty Vector: 0
  - B-Raf: 30
Fig. 7

A

C6

+14-3-3β  +14-3-3γ  cAMP

- -  +  -  +  -  +

P-MEK

B-Raf

CHO

+14-3-3mut

+14-3-3β (R56,60A)

cAMP

- -  +  -  +  -  +

B

MEK Phosphorylation

C6

Control  +14-3-3β  +14-3-3γ

CHO

Control  +14-3-3β  +14-3-3mut (R56,60A)
Fig. 8

**C6**

|     | C   | 0'  | 10' | 20' | 0'  | 10' | 20' | cAMP |
|-----|-----|-----|-----|-----|-----|-----|-----|------|
| + 14-3-3β |     |     |     |     |     |     |     |      |

- **P-MEK**
- **B-Raf**

**CHO**

|     | C   | 0'  | 10' | 20' | 0'  | 10' | 20' | cAMP |
|-----|-----|-----|-----|-----|-----|-----|-----|------|
| + 14-3-3β |     |     |     |     |     |     |     |      |

- **P-MEK**
- **B-Raf**
Fig. 9

C6

+14-3-3β

-  +  -  +  cAMP

-  +  -  +

Ser^{728}\text{-P}

B-Raf

-14-3-3

CHO

+14-3-3β

-  +  -  +  cAMP

-  +  -  +

Ser^{728}\text{-P}

B-Raf

-14-3-3
