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Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins

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We have identified the beta (β) isoform of the 14-3-3 family of proteins as an activator of the Raf-1 protein kinase. 14-3-3 was isolated in a yeast two-hybrid screen for Raf-1 kinase domain binding proteins. Purified bovine brain 14-3-3 interacted specifically with both c-Raf-1 and the isolated Raf-1 kinase domain. Association was sensitive to the activation status of Raf-1; 14-3-3 bound to unactivated Raf-1, but not Raf-1 activated by protein kinase Cα or Ras and Lck. The significance of these interactions under physiological conditions was demonstrated by co-immunoprecipitation of Raf-1 and 14-3-3 from extracts of quiescent, but not mitogen-stimulated, NIH 3T3 cells. 14-3-3 was not a preferred Raf-1 substrate *in vitro* and did not significantly affect Raf-1 kinase activity in a purified system. However, in cell-free extracts 14-3-3 acted as a Ras-independent activator of both c-Raf-1 and the Raf-1 kinase domain. The same results were obtained *in vivo* using transfection assays; 14-3-3 enhanced both c-Raf-1- and Raf-1 kinase domain-stimulated expression of AP-1- and NF-κB-dependent reporter genes and accelerated Raf-1 kinase domain-triggered differentiation of PC12 cells. We conclude that 14-3-3 is a latent co-activator bound to unactivated Raf-1 in quiescent cells and mediates mitogen-triggered but Ras-independent regulatory effects aimed directly at the kinase domain.

Key words: 14-3-3 protein/kinase activator/mitogenic signaling/protein kinase/Raf-1

Introduction

Raf-1 is a cytoplasmic serine/threonine kinase whose activity plays a critical role in the control of proliferation and differentiation (reviewed by Li,P. *et al.*, 1991; Rapp, 1991; Heidecker *et al.*, 1992). Its importance as a signaling molecule is underscored by the fact that constitutively activated forms cause malignant transformation. Raf-1 protein is present in both quiescent and growing cells, but in quiescent cells possesses only a low level of intrinsic kinase activity. Raf-1 activity is induced by many signals that include not only growth factors, but also tumor promoting phorbol esters, such as PMA, inflamatory cytokines (TNF-α), differentiation signals (nerve growth factor; NGF), calcium mobilization, DNA damaging agents and oxygen radicals.

Work in many laboratories has delineated a signal transduction pathway from growth factor receptors to the nuclear immediate early response (Wasylyk *et al.*, 1989; Jamal and Ziff, 1990; Bruder *et al.*, 1992) that involves Ras, Raf-1 and the MAP kinase (MAPK) cascade (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Troppmair *et al.*, 1994). The Ras→Raf-1→Mek→MAPK pathway participates in the induction of AP-1 activity by activating c-*fos* transcription (Hill *et al.*, 1993; Marais *et al.*, 1993; Kortenjann *et al.*, 1994) and by phosphorylating c-Jun, which potentiates its transactivation activity (Smeal *et al.*, 1992). Raf-1 is also an important component of signal transduction pathways that lead to the activation of the NF-κB/Rel family of transcription factors. This is evidenced by the observation that dominant-defective mutants of Raf-1 interfere with serum-, PMA- and TNF-α-mediated induction of NF-κB activity (Finco and Baldwin, 1993).

In quiescent cells, Raf-1 is found phosphorylated on several serine residues. Mitogenic stimulation results in the phosphorylation of novel residues and causes an up to 15-fold activation of Raf-1 kinase activity (Kyriakis *et al.*, 1993; Morrison *et al.*, 1993). Both tyrosine phosphorylation mediated by Src family tyrosine kinases, such as Lck (Thompson *et al.*, 1991; Fabian *et al.*, 1993), and serine phosphorylation mediated by protein kinase Cα (PKCα) (Sozeri *et al.*, 1992; Kolch *et al.*, 1993; Carroll and May, 1994) have been shown to activate Raf-1 activity. In contrast, protein kinase A (PKA) phosphorylation of Raf-1 has been implicated in inhibition of kinase activity (Hafner *et al.*, 1994).

Both biochemical and genetic evidence has demonstrated direct interaction between Raf-1 and Ras (Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993). Raf-1 associates with the activated, GTP-bound form of Ras and competes with GTPase activating protein (GAP) for access to activated Ras (Moodie *et al.*, 1993). The molecular mechanism by which Ras activates Raf-1 has not yet been elucidated, but recent experiments suggest that Ras-mediated translocation of Raf-1 to the cytoplasmic membrane is a crucial step (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), perhaps to facilitate phosphorylation and concomitant activation by membrane-associated kinases.

In spite of intensive work over many years, the identity of Raf-1 substrates has until recently remained elusive. Work in many laboratories has now shown that Mek is a direct substrate of Raf-1 and that its phosphorylation

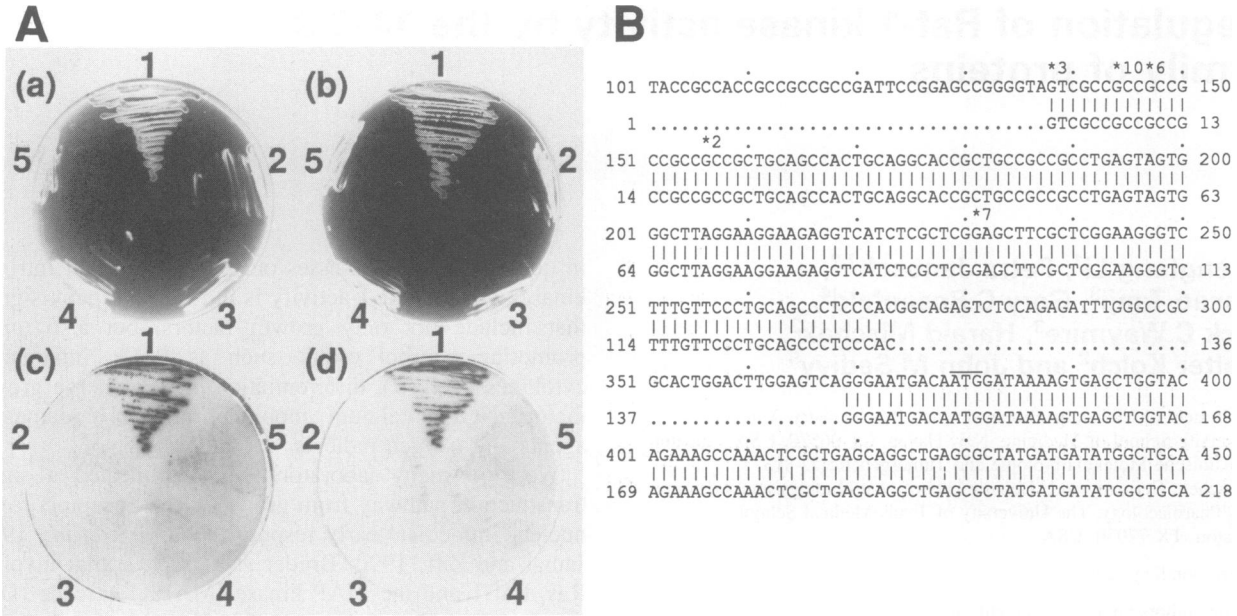


Fig. 1. (**A**) Biological activity of 14-3-3 clones in the two-hybrid system. The tester proteins, expressed as fusions with the GAL4 DNA binding domain, are: 1, wild-type Raf kinase domain (original 'bait'); 2, kinase-deficient kinase domain (K→W substitution at position 375); 3, Cdk2; 4, p53; 5, lamin. Two independent 14-3-3 clones are shown, pCORS3 (a and c) and pCORS7 (b and d), the longest and shortest inserts recovered respectively (B). (a and b) Growth on histidine-deficient medium. (c and d) β-Galactosidase activity. To assay β-galactosidase, cells were streaked on permissive medium (supplemented with histidine but lacking leucine and tryptophan to select for both plasmids), colonies were lifted onto nitrocellulose and activity was visualized *in situ* using X-gal. (**B**) Primary structure of 14-3-3 clones recovered in the two-hybrid screen. Upper line, the β isoform of the human 14-3-3 gene (GenBank X57346); lower line, our longest clone, pCORS3. The first nucleotides of each of the other cDNA clones recovered, pCORS10, pCORS6, pCORS2 and pCORS7 (in order of decreasing size), are identified with asterisks. In each case, immediately to the left of the cDNA sequence is an *Xho*I linker (not shown) used to clone the inserts into the pACT vector (Durfee *et al.*, 1993). All clones contained an identical 95 bp deletion in the region corresponding to the 5′ leader of the 14-3-3 mRNA. The ATG initiation codon of the 14-3-3 gene is indicated with a horizontal bar. All fusion proteins thus contain an N-terminal GAL4 activation domain, a variable length spacer derived from the 5′ leader of the 14-3-3 gene and the entire 14-3-3 protein coding region. In all clones the GAL4, spacer and 14-3-3 sequences comprise a single open reading frame. With the exception of the deletion, the sequence of all the clones was identical to the reported 14-3-3 sequence.

activates the MAPK pathway (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Wood *et al.*, 1992; Lange-Carter *et al.*, 1993; MacDonald *et al.*, 1993; Force *et al.*, 1994). The mechanism of NF-κB activation by Raf-1 involves phosphorylation of IκB in an inactive cytoplasmic IκB−NF-κB complex, which leads to the release of active NF-κB (Li,S. and Sedivy, 1993).

To identify novel substrates, we have used the yeast two-hybrid system (Chien *et al.*, 1991) in a systematic search for proteins that interact with the Raf-1 kinase domain. The major candidate to emerge from this screen is the 14-3-3 protein (reviewed by Aitken *et al.*, 1992). 14-3-3 was initially identified as an abundant brain protein (Moore and Perez, 1967) and was shown to be a mixture of 7–8 isoforms (Ichimura *et al.*, 1988; Isobe *et al.*, 1991). 14-3-3 belongs to a growing gene family of at least 16 members (Fu *et al.*, 1993). 14-3-3 is expressed in most tissues (Isobe *et al.*, 1991; Leffers *et al.*, 1993) and shows remarkable evolutionary conservation extending to lower eukaryotes and plants (Hirsch *et al.*, 1992; Martens *et al.*, 1992; Ford *et al.*, 1994). Such conservation of structure implies a fundamental importance in cellular physiology.

The first known function of 14-3-3 was activation of enzymes involved in neurotransmitter synthesis (tyrosine and tryptophan hydroxylases; Ichimura *et al.*, 1987). Other activities have since been described: inhibition (Toker *et al.*, 1992) or activation of PKC (Isobe *et al.*, 1992; Tanji *et al.*, 1994); activation of catecholamine exocytosis (Morgan and Burgoyne, 1992) and Ca^{2+}-activated phospholipase A activity (Zupan *et al.*, 1992). 14-3-3 proteins may participate in signal transduction pathways regulating cellular proliferation: (i) some 14-3-3-related proteins are differentially expressed in normal and malignant cells (Prasad *et al.*, 1992; Leffers *et al.*, 1993); (ii) 14-3-3 associates with the polyomavirus middle T antigen in transformed cells (Pallas *et al.*, 1994); (iii) yeast 14-3-3 functions in DNA damage-induced cell cycle control (Ford *et al.*, 1994).

In summary, Raf-1 has been implicated as a key regulatory molecule that integrates upstream signals from tyrosine kinase, PKC and Ras proteins and transmits them into the MAPK and NF-κB pathways. Several lines of evidence indicate that additional Raf-1 substrates remain to be discovered. For example, activated Raf-1 causes the constitutive activation of the Mek/MAPK pathway in NIH 3T3 cells but not Rat-1a cells, yet it can readily transform both cell lines (Gallego *et al.*, 1992; Gupta *et al.*, 1992). Results presented in this report show that 14-3-3 is unlikely to be a major substrate of Raf-1, but functions instead as an important regulator of Raf-1 kinase activity.

Results

Screening for proteins that interact with the kinase domain of Raf-1

The yeast two-hybrid system (Chien *et al.*, 1991) was used to identify proteins which interact specifically with

the kinase domain of Raf-1. The histidine selection system (Durfee *et al.*, 1993), which utilizes two distinct GAL4-dependent reporter genes, *HIS3* (a histidine biosynthetic enzyme) and *lacZ* (β-galactosidase), was employed. The 'bait' was the Raf-1 kinase domain fused to the GAL4 DNA binding domain (GBD−Raf; Li,S. and Sedivy, 1993). Approximately 500 000 primary transformants of a human T cell cDNA library expressed as fusion products with the GAL4 activation domain (Durfee *et al.*, 1993) were screened.

Several hundred clones showed significant histidine prototrophy and of those 10 were strongly β-galactosidase-positive. For nine of these clones histidine prototrophy and β-galactosidase activity depended on the presence of both plasmids. As a further control, segregants containing the cDNA library plasmids only were mated with cells expressing several tester GAL4 (DNA binding domain) fusion proteins: (i) the original Raf-1 bait; (ii) an analogous construct expressing a kinase-deficient Raf-1 protein (Li,S. and Sedivy, 1993); (iii) Cdk2; (iv) p53; and (v) lamin. Histidine prototrophy and β-galactosidase activity were recovered only in combination with the original GBD−Raf-encoding plasmid (Figure 1A).

The cDNA library plasmids were transferred into *Escherichia coli* and subjected to DNA sequencing. The results showed that four distinct genes were recovered; one was recovered five times, another two times and the remaining two as single isolates. A search of the GenBank database revealed that the gene recovered five times encoded the β isoform of the 14-3-3 protein (Isobe *et al.*, 1991). DNA sequencing showed that the recombinant junctions between the 14-3-3 cDNA and the GAL4 vector were distinct in all the recovered clones (Figure 1B).

14-3-3 activates Raf-1 in mammalian cells

The effect of 14-3-3 expression on Raf-1 activity in mammalian cells was investigated in transfection assays using NF-κB- and AP-1-dependent reporter genes as indicators of Raf-1 activity. 14-3-3 cDNA was subcloned into a mammalian expression vector and co-transfected into NIH 3T3 cells, in various combinations, with reporter- and Raf-1-expressing plasmids. After electroporation, cells were incubated in 10% serum for 24 h, serum starved (0.2% serum) for 24 h, stimulated with 10% serum for 20 h and harvested for chloramphenicol acetyltransferase (CAT) assays. 14-3-3 did not stimulate in the absence of co-transfected Raf-1 plasmids (Figure 2A, bars 1 and 2). c-Raf-1 alone did not stimulate NF-κB reporter activity (Figure 2A, bar 3), whereas c-Raf-1 plus 14-3-3 stimulated it ~3- to 4-fold (Figure 2A, bar 4). Raf-BXB (the isolated kinase domain) by itself stimulated ~2- to 3-fold (Figure 2A, bar 6) and inclusion of 14-3-3 raised the stimulation to ~14-fold (Figure 2A, bar 7). Assays performed with an AP-1 reporter instead of the NF-κB reporter produced analogous results: both c-Raf-1- and Raf-BXB-mediated stimulation of AP-1 activity was enhanced by 14-3-3 and the effect was more pronounced with Raf-BXB (not shown). In a separate series of experiments, the transfections were repeated using cells under exponential growth conditions during the entire assay (Figure 2B). The strong effect of 14-3-3 on the isolated Raf kinase domain was again apparent; Raf-BXB plus 14-3-3 elicited ~8-fold more AP-1 activity than Raf-BXB alone (Figure 2B, bars

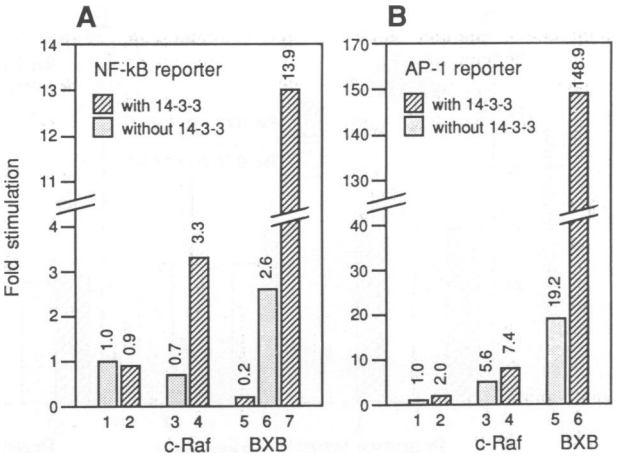


Fig. 2. Stimulation of Raf-1-mediated activation of NF-κB and AP-1 by 14-3-3. NIH 3T3 cells growing exponentially in 10% serum were harvested, electroporated with the indicated plasmids and assayed for CAT activity. (**A**) Stimulation of NF-κB activity during the $G_0 \rightarrow S$ transition. After electroporation, cells were incubated in 10% serum for 24 h, serum starved (0.2% serum) for 24 h, stimulated with 10% serum for 20 h and harvested for CAT assays. Bar 1, NF-κB reporter alone; bar 2, NF-κB reporter with 14-3-3; bar 3, NF-κB reporter with c-Raf-1; bar 4, NF-κB reporter with c-Raf-1 plus 14-3-3; bar 5, NF-κB reporter containing a mutated NF-κB-binding site with Raf-BXB; bar 6, NF-κB reporter with Raf-BXB; bar 7, NF-κB reporter with Raf-BXB plus 14-3-3. (**B**) Stimulation of AP-1 activity in exponentially growing cells. After electroporation, cells were incubated continuously in 10% serum for 48 h. Bar 1, AP-1 reporter alone; bar 2, AP-1 reporter with 14-3-3; bar 3, AP-1 reporter with c-Raf-1; bar 4, AP-1 reporter with c-Raf-1 plus 14-3-3; bar 5, AP-1 reporter with Raf-BXB; bar 6, AP-1 reporter with Raf- BXB plus 14-3-3. All electroporations contained a total of 50 μg of plasmid DNA. The reporter in (A) was pMHC-NF-κB CAT (10 μg per electroporation), except in bar 5, where pMHC-NF-κB*mut* CAT was used. In (B) the reporter was pB4X. Assays with 14-3-3 contained 20 μg of pcDNA3/14-3-3 plasmid DNA; assays without 14-3-3 contained 20 μg of empty pcDNA3 vector. The full-length Raf (c-Raf) plasmid was pMNC-Raf and the isolated Raf kinase domain (BXB) plasmid was pRSV-Raf-BXB. Both were included (where indicated) at 20 μg per assay. Activities are expressed as fold activation calculated with respect to the activity elicited by the empty pcDNA3 vector (column 1). Values from a single representative experiment are shown. The experiments were performed twice with consistent results.

5 and 6). Under these assay conditions, 14-3-3 had only a slight, if any, stimulatory effect on c-Raf-1 (Figure 2B, bars 3 and 4).

The biological effects of 14-3-3 on Raf-1 activity in mammalian cells were confirmed and extended in experiments using PC12 cells. 14-3-3 cDNA and/or Raf-BXB expression constructs were co-electroporated with a pRSV*lacZ* indicator plasmid into PC12 cells. At various times after electroporation cells were histochemically stained for β-galactosidase activity (to identify cells that had productively taken up DNA) and the extent of differentiation was scored by measuring the length of neurite outgrowths by microscopic observation (Figure 3). As reported previously (Wood *et al.*, 1993), the active Raf-1 kinase domain (Raf-BXB) accelerated differentiation induced by NGF and was capable of triggering differentiation even in the absence of NGF. Both effects were significantly stimulated by the inclusion of the 14-3-3 expression vector.

14-3-3 protein binds Raf-1 in vitro

Glutathione S-transferase (GST)-tagged Raf-1 proteins, either full-length or the isolated kinase domain (Li,S. and

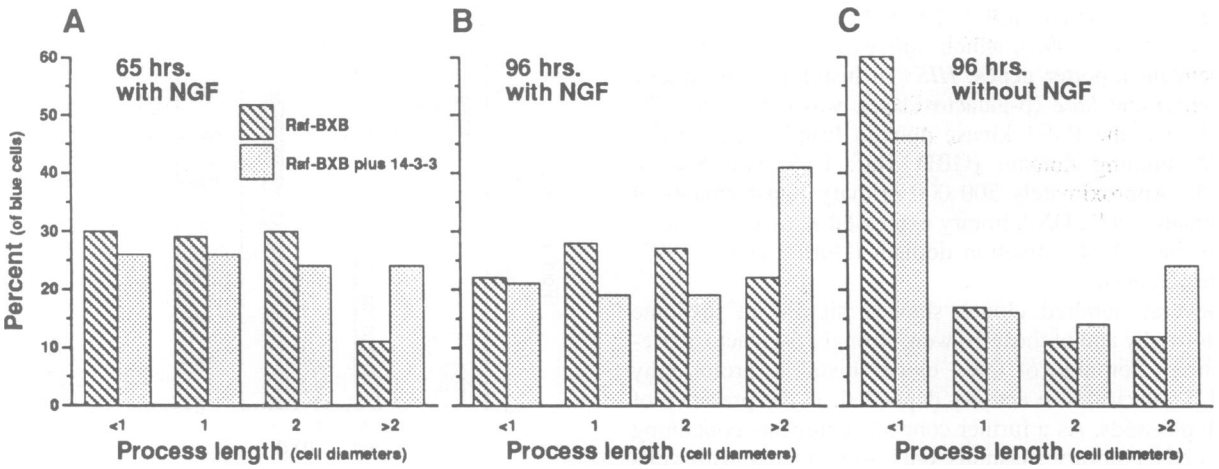


Fig. 3. Effect of Raf-1 kinase and 14-3-3 on differentiation of PC12 cells. PC12 cells were cultured on plastic in Dulbecco's modified Eagle's medium supplemented with 10% donor horse serum and 5% fetal bovine serum at 37°C in an atmosphere of 10% CO_2. Cells were electroporated as described (Myers *et al.*, 1994) with a total of 50 μg of plasmid DNA; 10 μg of pRSV*lacZ*, 20 μg of pRSV-Raf-BXB and 20 μg of pcDNA3/14-3-3 or empty pcDNA3 vector. After electroporation, cells were plated on collagen-coated dishes. Where indicated, NGF (50 ng/ml) was added 12 h after electroporation. At the indicated times (after electroporation) cells were fixed, permeabilized and stained with X-Gal as described (Herzing and Meyn, 1993). Cells staining blue were scored for neurite outgrowth (length of processes in cell diameters) by microscopic observation (<1% of total cells surviving electroporation were stained blue). (**A**) Sixty five hours after electroporation in the presence of NGF. (**B**) Ninety six hours after electroporation in the presence of NGF. (**C**) Ninety six hours after electroporation in the absence of NGF. In control experiments, 70–80% of cells induced with NGF for 6 days had processes of >2 cell diameters, whereas 90–95% of cells in the absence of NGF had processes of <1 cell diameter. Electroporation of pRSV*lacZ* with or without pcDNA3/14-3-3 had no effect. Approximately 90% of white cells in (C) had processes of <1 cell diameter. This shows that Raf-BXB induces differentiation in the absence of NGF. In (B) (3.5 days in the presence of NGF) ~10 and 50% of white cells had processes of >2 and <1 cell diameters respectively. This shows that Raf-BXB significantly accelerates differentiation induced by NGF.

Sedivy, 1993), were expressed in *E.coli* and absorbed to glutathione–agarose beads. Immobilized Raf was incubated with purified bovine 14-3-3 protein (Tanji *et al.*, 1994) and retention on the beads was analyzed by SDS–PAGE and Western blotting (Figure 4A,a). The results showed that 14-3-3 bound strongly to full-length c-Raf-1; binding to the kinase domain was weaker but clearly detectable. Consistent with the results of the two-hybrid experiments, binding to a kinase-defective kinase domain was substantially decreased. At least two isoforms of 14-3-3 interacted with Raf-1 (the upper band contains the ε isoform, while the lower band is a mixture of the other isoforms). In a separate experiment, immobilized Raf-1 was incubated with a crude extract of HeLa cells (Figure 4A,b). Since interaction could also be demonstrated in a complex mixture of proteins, the observed binding is unlikely to be an artefact of adventitious sticking between two highly purified proteins.

To further delineate the region of Raf-1 that binds to 14-3-3, constructs bearing various fragments of Raf-1 were employed. The source of 14-3-3 protein was a crude extract of mouse brain (Figure 4B). The N-terminal portion of Raf-1 lacking the zinc finger sequence in CR1 (GNH) did not bind 14-3-3, while a longer segment containing the entire CR1 region and extending into the variable region between CR1 and CR2 (GNS) bound 14-3-3 strongly. A segment encompassing the entire CR1 and CR2 domains (GRS) also bound 14-3-3 well. A segment containing only the CR3 domain (GNX) bound 14-3-3 weakly, but at a clearly detectable level. Discrete binding was also detected to the hinge region between CR2 and CR3 (not shown). These results show that 14-3-3 binds to both the regulatory and kinase domains of c-Raf-1, but not to the region of the regulatory domain that interacts with Ras. Identical results were obtained in a separate experiment using purified recombinant 14-3-3 zeta (ζ) expressed in *E.coli* instead of the bovine brain preparation. The robustness of the interaction with the kinase domain observed in the two-hybrid system can be explained by the known sensitivity of that assay.

14-3-3 protein associates preferentially with unactivated c-Raf-1 in vivo

Raf-1 immunoprecipitates prepared from either quiescent or serum-stimulated NIH 3T3 cells were assayed for Raf-1 activity in a linked kinase assay which contained purified kinase-active Mek and kinase-defective MAPK as a Mek substrate. The kinase reactions were displayed by SDS–PAGE, blotted and autoradiographed to visualize Mek/MAPK phosphorylation. The same blots were then subjected to Western analysis using a 14-3-3 antiserum. Significant amounts of 14-3-3 protein were detected in Raf-1 immunoprecipitates from quiescent cells, while this association was greatly decreased after 3 min and completely lost after 15 min of serum stimulation (Figure 5). The co-immunoprecipitation experiments show that 14-3-3 and Raf-1 interact at physiological protein concentrations and the sensitivity of complex formation to mitogenic stimulation indicates that the interactions are biologically relevant.

The parallel kinase assays clearly showed the onset of Raf-1 activation after 3 min and significant activation after 15 min. This argues that the 14-3-3 association was not due to non-specific contamination of Raf-1 immunoprecipitates with the abundant 14-3-3 protein. 14-3-3 and Raf-1 association was also clearly detected when immunoprecipitations were performed with 14-3-3 antiserum and subsequently blotted with cRaf-VI antiserum.

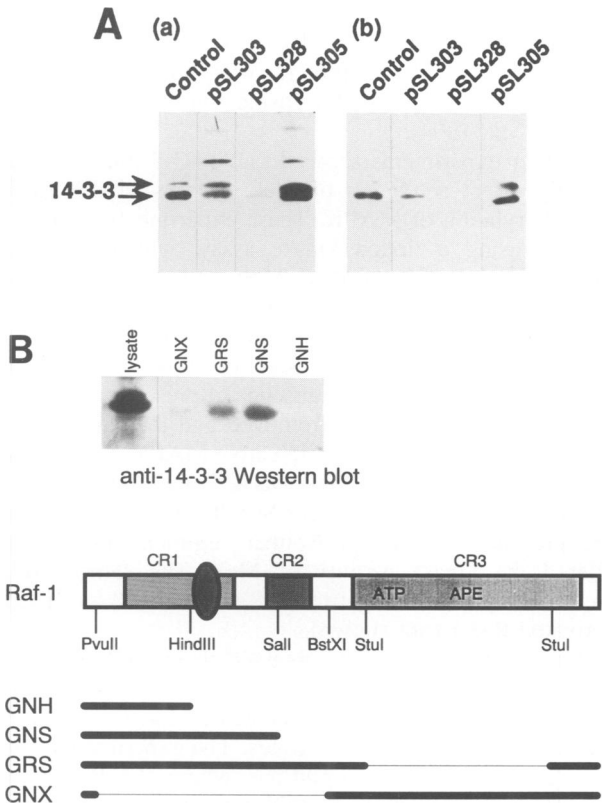


Fig. 4. (**A**) Binding of 14-3-3 to Raf-1 *in vitro*. GST−Raf proteins encoded by the indicated plasmids (pSL305, full-length Raf-1; pSL303, Raf kinase domain; pSL328, kinase-deficient kinase domain) were immobilized on glutathione−agarose beads. Equal amounts of purified Raf-1 proteins (~200 ng per assay) were incubated with (a) 150 ng of purified bovine brain 14-3-3 protein or (b) 100 µl (800 µg) of cytoplasmic HeLa cell extract. The beads were washed five times with PBS (containing 1 mM each of EDTA, DTT and PMSF, 1% Triton X-100, 10 µg/ml aprotinin and 100 µg/ml leupeptin) and analyzed for bound 14-3-3 protein by Western blotting. Equal input of Raf-1 protein per assay was controlled on parallel silver stained gels. The control lane contains 20 ng of purified 14-3-3 protein. 14-3-3 protein did not bind to glutathione−agarose beads or to immobilized GST (not shown). The experiment was performed twice with consistent results. (**B**) Mapping the region of Raf-1 which interacts with 14-3-3. The indicated deletion constructs were expressed as GST fusion proteins in *E.coli*. The regions of Raf-1 encoded by the constructs are indicated with bold lines; deletions are shown as thin lines. The zinc finger motif in CR1 is indicated as a filled oval. Equal amounts of purified Raf-1 proteins (~100 ng per assay) immobilized on glutathione−Sepharose beads were incubated with 200 µl of mouse brain lysate for 1 h at 4°C (one brain was washed in ice-cold PBS, snap-frozen in liquid nitrogen, dounce homogenized in TBST, sonicated and insoluble material was removed by centrifugation at 15 000 *g* for 30 min), the beads were washed four times with TBST and analyzed for bound 14-3-3 protein by Western blotting. Equal input of Raf-1 protein was controlled on parallel Coomassie brilliant blue stained gels. The control lane contains 10 µl of the brain lysate. The experiment was performed twice with consistent results.

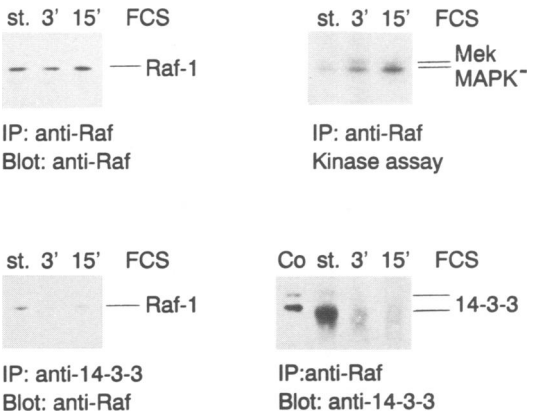


Fig. 5. Co-immunoprecipitation of Raf-1 and 14-3-3 from quiescent and stimulated cells. NIH 3T3 cells were serum starved for 24 h and stimulated with 20% serum for either 3 or 15 min, as indicated. Lysates were prepared, immunoprecipitated with cRaf-VI antiserum and assayed for Raf kinase activity (Materials and methods). The kinase assays included 100 ng each of purified kinase-active Mek and kinase-deficient MAPK as substrates. Kinase reactions were separated on a 12.5% SDS−polyacrylamide gel and blotted. The same blot was sequentially autoradiographed (top right panel), stained with 14-3-3 antiserum (bottom right panel) and finally stained with cRaf-VI antiserum to control for input of Raf-1 protein (top left panel). The mobility shift of Raf-1 associated with its activation is not apparent because of the high percentage of acrylamide used (top left panel); activation (~3-fold) is, however, demonstrated by the direct kinase assay (top right panel). In a separate experiment, the antibodies were used in reverse order; immunoprecipitation was with 14-3-3 antiserum followed by blotting with cRaf-VI antiserum (bottom left panel). Equal amounts of 14-3-3 protein were precipitated in each lane (not shown). This experiment was subject to some variability because the abundance of 14-3-3 in NIH 3T3 cells makes its quantitative immunoprecipitation difficult (we estimated that 14-3-3 was some 100-fold more abundant than Raf-1 in the extracts). St, stationary phase (quiescent) cells; Co, control lane loaded with purified 14-3-3 protein; FCS, fetal calf serum. Numbers above the lanes indicate the duration (in min) of serum stimulation. The experiment was performed three times with consistent results.

Furthermore, TNF-α, which results in a very rapid and transient activation of Raf-1 and MAPK in NIH 3T3 cells (T.Seitz, W.Kolch and H.Mischak, unpublished observations), caused a parallel dissociation and reassociation of Raf-1 and 14-3-3; binding was decreased at 1, 2, 5 and 10 min after TNF-α treatment and was re-established at the 15 and 20 min time points (not shown).

The preferential association of 14-3-3 with unactivated Raf was corroborated in binding experiments using GST−Raf (full-length) expressed in the baculovirus−Sf9 system either alone or activated by co-expression with PKCα or Ras plus Lck (Hafner *et al.*, 1994). GST-tagged Raf proteins were purified from extracts of insect cells by glutathione−Sepharose affinity chromatography and tested for interaction with bovine brain 14-3-3 in a fashion analogous to that employed with *E.coli*-expressed proteins. 14-3-3 bound to unactivated Raf-1, but not to Raf-1 activated by either PKCα or Ras/Lck (not shown). Kinase assays of the same preparations showed that Raf-1 activity was stimulated 4- to 5-fold and 60- to 70-fold by co-expression with PKCα and Ras/Lck, respectively. Longer exposures revealed a small amount of binding to PKCα-activated Raf-1. This residual binding may be indicative of the fact that PKCα activates Raf-1 weakly relative to Ras/Lck. Consistent with previous results, the kinase-defective Raf-301 mutant protein (expressed by itself in insect cells) did not bind 14-3-3 to an extent detectable by immunoblotting. The experiments were performed on three occasions with consistent results. In addition, endogenous 14-3-3 protein present in crude extracts of mouse brain behaved identically to the bovine brain preparation.

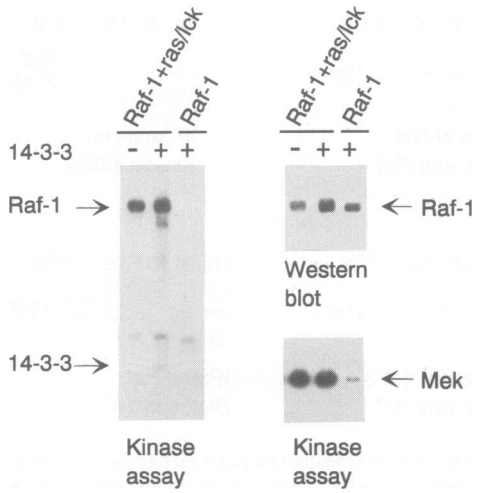


Fig. 6. Phosphorylation of 14-3-3 by Raf-1. Raf-1 protein, either unactivated or activated by co-expression with Ras/Lck (as indicated), was purified from insect cells. Aliquots of 5–10 ng of immobilized Raf-1 were used to phosphorylate 250 ng purified bovine brain 14-3-3 or 100 ng purified kinase-defective Mek as control, using standard kinase assay conditions (Materials and methods). The presence of 14-3-3 in assay reactions is indicated above the lanes. The reactions shown in the right panel included Mek. Kinase reactions were separated by SDS−PAGE and blotted. Blots were autoradiographed and subsequently stained with cRaf-VI antiserum to control for input of Raf-1 protein. The experiment was performed three times with consistent results. We, as well as others (Freed *et al.*, 1994), have observed a 30 kDa insect cell protein, immunologically related to 14-3-3, that interacts with Raf proteins expressed in the baculovirus−Sf9 system. Two lines of evidence indicate that insect cell 14-3-3 is not present in significant amounts in our Sf9-expressed Raf preparations: first, the GST−Raf proteins are expressed to a level of ~5% of total cellular protein, which is well in excess of endogenous 14-3-3 protein; second, the purity of the GST−Raf preparations was estimated to be at least 90% by Coomassie blue staining.

14-3-3 protein is a poor Raf-1 substrate and does not affect Raf-1 activity in a purified in vitro system

Immobilized c-Raf-1 expressed in insect cells was used to phosphorylate purified bovine brain 14-3-3 and kinase-negative Mek, as a control. The kinase reactions were displayed by SDS−PAGE and blotted. The blots were autoradiographed and subsequently stained with Raf-1 antiserum to control for Raf-1 protein input (Figure 6). The lower band of the 14-3-3 preparation was very weakly phosphorylated by Ras/Lck-activated Raf-1 (on a molar basis, 14-3-3 phosphorylation was some 750-fold less efficient than Mek phosphorylation). Inclusion of both Mek (100 ng) and 14-3-3 (500 ng) in a kinase assay resulted in Mek phosphorylation only, indicating that Mek completely competed out 14-3-3. PKCα-activated Raf-1 did not phosphorylate 14-3-3 at a detectable level (not shown).

The ability of 14-3-3 to modulate Raf-1 activity was investigated in kinase assays containing kinase-defective Mek as substrate and increasing amounts of purified 14-3-3 (Figure 7A). Both Mek phosphorylation and the Raf-1 input were quantitated on the same blot, the former by PhosphorImager analysis and the latter by laser scanning of enhanced chemiluminescence signals following staining with Raf-1 antiserum (not shown). The results indicate that 14-3-3 does not significantly modulate Raf kinase activity in this assay. The same result was obtained whether GST−Raf was expressed alone or activated by co-expression with either PKCα or Ras/Lck. The same preparation of 14-3-3 protein was fully active when assayed for activation of tyrosine hydroxylase or PKC (Tanji *et al.*, 1994).

Further experiments showed that 14-3-3 did not significantly affect activation of Mek towards MAPK or its phosphorylation of MAPK. These experiments were performed using a linked kinase assay, which contained immobilized Raf-1, purified Mek and kinase-defective MAPK as a Mek substrate. The influence of 14-3-3 on individual steps of this phosphorylation cascade was examined (Figure 7B). First, Ras/Lck-activated Raf-1 was used to activate Mek in the absence or presence of 14-3-3, the GST−Raf beads were removed and the supernatants were incubated with kinase-defective MAPK as substrate. In a second experiment, Mek was activated in the absence of 14-3-3 and then assayed for MAPK phosphorylation in the presence of 14-3-3. Neither regimen significantly altered the kinase activity of Mek. The same results were obtained with unactivated Raf-1, as well as PKCα-activated Raf-1 (not shown).

Analogous results were obtained when IκB was used instead of Mek as a Raf-1 substrate (not shown). This shows that 14-3-3 does not modulate Raf-1 activity towards either of its two known substrates. The experiments were also performed under conditions where the Raf-1 and 14-3-3 concentrations were held constant (50 and 250 ng respectively) and the substrate was titered from 100 to 0.1 ng. This experiment was designed to investigate whether 14-3-3 can facilitate the interaction of Raf with its substrates as the substrate concentration becomes limiting. In addition, the experiments were also performed under conditions where substrate and 14-3-3 concentrations were held constant (Mek, 50 ng, IκB, 100 ng, 14-3-3, 250 ng) and Raf was titered from 50 to 0.5 ng. This setting more closely reflects the situation in the cell, where c-Raf-1 is less abundant than both 14-3-3 and its substrates. In both experiments, the Raf proteins tested were: GST−Raf (full-length), GST−Raf activated with PKCα, GST−Raf activated with Ras/Lck, GST−GNX (isolated kinase domain, Figure 4B) and GST−GNX activated with PKCα. Under all conditions of assay, 14-3-3 did not activate Raf-1 kinase activity. Furthermore, the same results were obtained whether bovine brain of recombinant 14-3-3 proteins were used.

14-3-3 activates Raf-1 in a cell-free system

Triton-disrupted mammalian cell extracts described by Dent *et al.* (1993) are capable of recapitulating the activation of the MAPK pathway by Ras *in vitro*. This cell-free system was employed to investigate the effect of 14-3-3 on Raf-1 activity in the presence of a complex mixture of signal transducing proteins. Purified bovine brain 14-3-3 protein (Tanji *et al.*, 1994), activated Ras, or both, were incubated in the cell extracts, endogenous Raf-1 was removed by immunoprecipitation and its activation status was determined in a linked kinase assay using Mek and kinase-defective MAPK as substrates (Figure 8A). Addition of bovine brain 14-3-3 caused activation of Raf-1 in a dose-dependent manner. The maximal activation was about 3-fold and was reproducible in three separate experiments. The addition of activated Ras stimulated

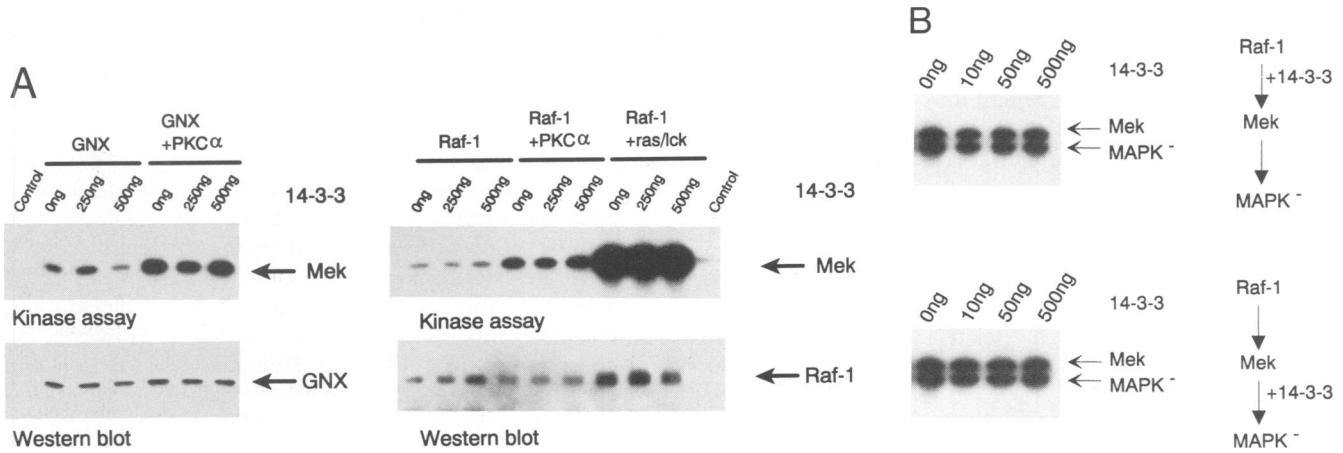


Fig. 7. (**A**) Effect of 14-3-3 on Raf kinase activity *in vitro*. The indicated Raf-1 proteins were expressed in insect cells (Raf-1, full-length Raf; GNX, isolated kinase domain; see Figure 4B). Raf-1 proteins were activated, as indicated, by co-expression with PKCα or Ras/Lck. Aliquots of 5–10 ng of immobilized Raf-1 proteins were used to phosphorylate 100 ng purified kinase-defective Mek in the presence of increasing amounts of purified bovine brain 14-3-3, as indicated, using standard kinase assay conditions (Materials and methods). The kinase reaction shown in the lane marked Control was performed in the absence of Raf-1 protein. Kinase reactions were separated by SDS−PAGE and blotted. Blots were autoradiographed (top panel) and subsequently stained with cRaf-VI antiserum to control for input of Raf-1 protein (bottom panel). The experiment was performed three times with consistent results. (**B**) Effect of 14-3-3 on Mek activation and activity *in vitro*. The effect of 14-3-3 on the Raf-1→Mek→MAPK phosphorylation cascade (see diagram on right) was investigated in a linked assay system. Aliquots of 5–10 ng of immobilized, Ras/Lck-activated Raf-1 were incubated in RK buffer with 100 ng kinase-active Mek in the presence of 30 μM ATP for 30 min at 25°C. The reactions were incubated either in the absence (bottom panel) or presence (top panel) of the indicated amounts of 14-3-3. Raf-1 beads were sedimented and 15 μl of the supernatant was removed and added to 10 μl of a mix containing 5 μCi [γ-^{32}P]ATP and 100 ng kinase-defective MAPK. The reactions were incubated either in the absence (top panel) or presence (bottom panel) of the indicated amounts of 14-3-3 for 30 min at 25°C. Kinase reactions were separated by SDS−PAGE, blotted and autoradiographed. The blots were subsequently stained with cRaf-VI antiserum to ensure equal input of Raf-1 protein (not shown). The experiment was performed three times with consistent results.

Raf-1 to a similar extent, which was expected from previous work (Dent *et al.*, 1993) and confirms that the extracts were fully active. Interestingly, the stimulatory effects of Ras and 14-3-3 were additive, suggesting that 14-3-3 regulates Raf-1 independently of Ras.

To assay the activation of the isolated Raf-1 kinase domain, baculovirus-expressed GST-tagged Raf proteins were added to the Triton-disrupted cell lysates, either alone or in conjunction with 14-3-3 and activated Ras (Figure 8B). Following incubation to allow activation, GST−Raf proteins were recovered with glutathione–Sepharose beads and assayed using kinase-defective Mek as substrate. In addition to 14-3-3 purified from brain (Tanji *et al.*, 1994), the effect of recombinant, *E.coli*-expressed 14-3-3 ζ was also assayed. The results showed that: (i) exogenously added full-length Raf (GST−Raf) was regulated by activated Ras and 14-3-3 in an analogous fashion to that of endogenous Raf-1; (ii) both 14-3-3 preparations activated GST−Raf in a dose-dependent manner and (iii) the effect of 14-3-3 was additive to the activation elicited by GTP-loaded Ras.

The isolated Raf kinase domain, GNX, was also activated by 14-3-3 in a dose-dependent fashion to an extent comparable to that of full-length Raf. The addition of activated Ras, however, did not further increase the activity of GNX, demonstrating that activation by 14-3-3 is not dependent on Ras. That Mek phosphorylation in these assays was mediated by GST−Raf and not by a contaminating kinase was shown in controls with the kinase-defective GST−Raf-301 mutant protein; Mek was not phosphorylated to a significant extent and no response to the addition of 14-3-3 and/or Ras was observed.

In summary, the cell-free extract experiments showed that: (i) 14-3-3 activated both full-length Raf and the isolated Raf kinase domain; (ii) Ras and 14-3-3 activated full-length Raf in an additive manner; and (iii) addition of Ras had no effect on the isolated kinase domain, indicating that 14-3-3 activates Raf-1 in a Ras-independent fashion.

Discussion

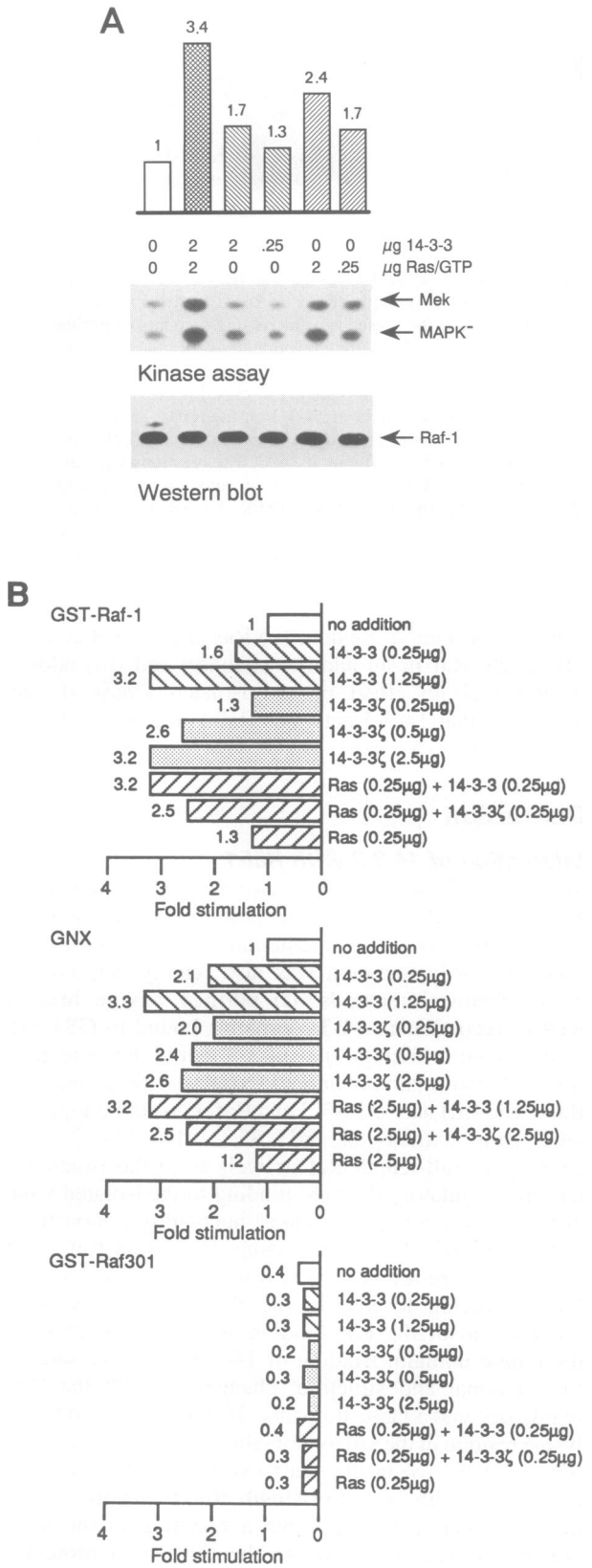
Interaction of 14-3-3 with Raf-1

We identified 14-3-3 β in a yeast two-hybrid screen as a Raf-1 kinase domain binding protein. In the context of this genetic assay the interaction was both robust and specific. The association was also readily demonstrable by biochemical methods: (i) purified bovine brain as well as recombinant 14-3-3 proteins bound to GST−Raf recombinant proteins; (ii) 14-3-3 could be effectively selected from mammalian cell extracts with immobilized Raf-1 and (iii) native 14-3-3 and Raf-1 co-immunoprecipitated from extracts of NIH 3T3 cells. 14-3-3 bound strongly to full-length Raf as well as to the isolated N-terminal regulatory domain; binding to the isolated kinase domain was significantly weaker, but readily demonstrable.

Mapping of the 14-3-3 binding domain on Raf-1 indicates that there are at least two interaction sites; a strong binding region located in the Raf-1 regulatory region downstream of the Ras binding site and another site in the kinase domain. Binding of 14-3-3 was very sensitive to functional and structural changes in both the Raf-1 regulatory and kinase domains. Mutation of a conserved lysine residue in the kinase domain greatly reduced binding (observed *in vivo* in the yeast two-hybrid system as well as *in vitro* with both full-length Raf-1 and the isolated kinase domain). The fact that a mutation in the kinase domain abrogated binding to the full-length protein, in

spite of the strong association mapped to the regulatory domain, indicates that the interaction with the kinase domain is highly specific and functionally significant.

Association with full-length Raf-1 was also profoundly influenced by its activation status. 14-3-3 bound to unactivated Raf-1, but binding to Raf-1 activated by co-expression with either PKCα or Ras plus Lck was significantly reduced. These observations suggest that in the context of the full-length Raf molecule, 14-3-3 binding is a co-operative process which is regulated by conformational changes in Raf-1. It has been suggested that in the inactive state the Raf-1 regulatory domain is folded over the kinase domain. Activation is envisioned to be accompanied by a conformational change that unfolds the molecule and thereby allows the kinase domain efficient access to substrates (Heidecker *et al.*, 1992; Stokoe *et al.*, 1994). The verification of this model has to await elucidation of the three-dimensional structure of Raf-1. However, our observations that 14-3-3 preferentially associates with unactivated Raf-1 *in vitro* and that a 14-3-3–Raf-1 complex can be co-immunoprecipitated from extracts of quiescent but not activated NIH 3T3 cells are consistent with the conformational activation model.



Effect of 14-3-3 on Raf-1 activity

In vitro assays using purified proteins showed no significant effect of 14-3-3 on Raf-1 kinase activity. The assays were performed systematically to ensure that small effects would not escape detection: (i) unactivated, PKCα- or Ras/Lck-activated full-length Raf, as well as the isolated kinase domain, were used; (ii) direct Mek phosphorylation, as well as Mek activation towards MAPK, were examined; (iii) activity towards IκB, the second Raf-1 substrate, was examined; (iv) both bovine brain and recombinant *E.coli*-expressed 14-3-3 preparations were tested; (v) 14-3-3 was titrated to high concentrations and Raf as well as substrates were singly titrated to low concentrations; (vi) results were quantitated by PhosphorImager and input Raf-1 protein was controlled by immunoblotting of the kinase

Fig. 8. (**A**) Effect of 14-3-3 on endogenous Raf kinase activity in cell-free extracts. Triton-disrupted NIH 3T3 cell lysates (~10^6 cells in 25 μl) were incubated with the indicated amounts of bovine brain 14-3-3 and activated Ras for 15 min at 32°C. Reactions were terminated by addition of 1 ml ice-cold TBST, centrifuged at 13 000 *g* and the supernatants were immunoprecipitated with 1.5 μl cRaf-VI antiserum. Immunoprecipitates were assayed with the linked system employing kinase-active Mek and kinase-deficient MAPK (Figure 7B). The combined Mek/MAPK signals were quantitated with a PhosphorImager (top panel; numbers above bars indicate the fold activation). The middle panel shows the corresponding autoradiograph. The blots were also stained with cRaf-VI antiserum to ensure equal input of Raf-1 protein (bottom panel). The experiment was performed three times with consistent results. (**B**) Activation of GST-tagged Raf-1 proteins by Ras and 14-3-3 in cell-free extracts. The indicated Raf-1 proteins were expressed in the baculovirus–Sf9 system. Aliquots of 20–40 ng of GST-tagged Raf and the indicated amounts of Ras and/or 14-3-3 proteins were added to 50 μl cell lysates and incubated for 10 min at 37°C. Reactions were terminated as above, GST-tagged Raf proteins were recovered by absorption to glutathione–Sepharose beads and assayed using kinase-negative Mek as substrate. Results were quantitated with a PhosphorImager and are expressed as fold activation with respect to the untreated control. GNX is the isolated Raf-1 kinase domain (Figure 4B). GST–Raf-301 is full-length Raf containing the kinase-negative K375W mutation (Heidecker *et al.*, 1990). Note that in the co-activation assays of GNX with Ras and 14-3-3, the amounts of protein used were greater than in the corresponding assays with GST–Raf and GST–Raf-301. This was done to show that even high amounts of Ras do not activate the isolated kinase domain and that activation elicited by 14-3-3 is not affected by Ras. The experiments were performed three times with consistent results (with the exception of the GST–Raf-301 assays, which were done once); a representative data set is presented in the figure.

reactions and (vii) all assays were performed on multiple separate occasions. The only effect was an occasional slight inhibition of Raf-1 activity, which was judged not significant because any differences were always <2-fold in magnitude, did not titrate with increasing 14-3-3 concentrations and were not reproducible. The same preparation of bovine brain 14-3-3 was active in tyrosine hydroxylase assays.

14-3-3 was a poor substrate for Raf-1 (at least 750-fold less efficient than Mek), which makes the biological significance of the phosphorylation questionable. Since native brain 14-3-3 protein was used, however, it is possible that an isoform of 14-3-3 not abundant in the preparation may be a good Raf-1 substrate. Although 14-3-3 has consensus phosphorylation sites for several protein kinases, work to date has shown only low levels of phosphorylation either *in vivo* or *in vitro* (Toker *et al.*, 1992; M.Tanji and J.C.Waymire, unpublished observations). The attractive hypothesis that the activity of 14-3-3 proteins may be regulated by phosphorylation thus needs further investigation.

14-3-3 was found to activate Raf-1 in a cell-free system. The extracts were prepared by partially disrupting quiescent NIH 3T3 cells with Triton X-100, a procedure that was developed to study the regulation of the MAPK pathway (Dent *et al.*, 1993). Both activated Ras, used as a positive control, and 14-3-3 activated endogenous Raf-1 ~3-fold. The significance of this result is indicated by four lines of evidence. First, the magnitude of the activation compares well with data in the original report describing the development of the cell-free system (Dent *et al.*, 1993). Second, the activation was reproducible in multiple independent experiments. Third, the activation effect titered reproducibly with increasing amounts of input 14-3-3. Fourth, the magnitude of Raf-1 activation falls in the same range as that elicited *in vivo* by serum stimulation of NIH 3T3 cells (3- to 5-fold as measured by immunoprecipitation kinase assays; see Figure 5). We thus concur with Dent *et al.* (1993) that the Triton-disrupted cell extracts represent a faithful *in vitro* recapitulation of physiological conditions.

The simplest explanation for our findings is that the Triton-disrupted cell lysates provide a cofactor essential for 14-3-3-mediated activation of Raf-1 which is missing from *in vitro* assay systems composed of purified components. This situation is reminiscent of the activation of Raf-1 by Ras, which is not simply due to binding, but requires Ras-mediated membrane translocation of Raf-1, where Raf-1 is exposed to an activator (Dent and Sturgill, 1994; Leevers *et al.*, 1994; Stokoe *et al.*, 1994).

The validity of the activation observed in cell-free extracts was corroborated by co-immunoprecipitation experiments, which showed that 14-3-3 and Raf-1 interact physically at physiological protein concentrations. The sensitivity of complex formation to mitogenic stimulation indicates that the interactions are biologically relevant. Most importantly, transfection assays demonstrated that 14-3-3 can activate Raf-1 kinase activity *in vivo* in mammalian cells. First, co-transfection of a 14-3-3 expression construct markedly enhanced the activation of both AP-1- and NF-κB-dependent reporters by c-Raf-1, as well as by the isolated kinase domain, in NIH 3T3 cells. Second, expression of 14-3-3 significantly accelerated differentiation of PC12 cells mediated by the isolated Raf-1 kinase domain.

During the preparation of this manuscript, two papers demonstrating the interaction of Raf-1 with 14-3-3 were published (Freed *et al.*, 1994; Irie *et al.*, 1994), and the reported data are in good agreement with ours. The data we present here extend our understanding of the Raf-1–14-3-3 relationship in three important new areas: (i) we present detailed studies of the activation process; (ii) we show that 14-3-3 regulates the Raf-1 kinase domain directly and independently of Ras and (iii), most importantly, we show that 14-3-3 proteins can elicit a physiologically significant activation of Raf-1 in mammalian cells. One discrepancy between our data and that of Freed *et al.* (1994) and Irie *et al.* (1994) is that they reported a small (2- to 3-fold) *in vitro* activation of Raf-1 by 14-3-3. Since they only assayed mammalian Raf proteins immunoprecipitated from yeast cells, the reason for the discrepancy could be due to differences in experimental systems.

Mechanism of Raf-1 activation by 14-3-3

It is becoming increasingly evident that Raf-1 can be activated by several pathways, that activation involves multiple steps and that both the regulatory and kinase domains are targets for regulation. For example, PKCα and PKA have been shown to phosphorylate activating and inhibitory sites respectively on the kinase domain (Hafner *et al.*, 1994). This regulation is independent of membrane recruitment, since it affects v-Raf, which does not interact with Ras. Reversion of the malignant phenotype of v-Raf-transformed cells by cAMP agonists demonstrates the biological significance of regulatory effects directed at the kinase domain (Hafner *et al.*, 1994).

The first step of Raf-1 activation occurs by extracellular signal-triggered translocation to the cytoplasmic membrane, mediated by the activation of Ras, which functions as a docking protein (Hallberg *et al.*, 1994). As demonstrated by constitutively targeting Raf-1 to the cytoplasmic membrane, membrane recruitment itself causes sufficient activation to elicit transformation (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). The ability of mitogens to further activate membrane-tethered Raf-1 implies the existence of secondary mitogen-dependent activation events. The isolated Raf kinase domain does not bind to Ras and lacks membrane-targeting signals. The fact that it still responds to stimulation by 14-3-3 indicates that at least some mitogen-triggered activating steps can occur independently of membrane localization. Raf-1 activation can thus be conceptually subdivided into five stages: (i) recruitment to the cell membrane; (ii) activation at the membrane; (iii) release from the membrane; (iv) further modulation in the cytoplasm and (v) facilitation of interactions with substrates. Existence of step 2 is implied by the membrane localization of kinases, such as Lck or PKCα, known to activate Raf-1. Steps 2 and 4 may occur concurrently and/or independently if cytoplasmic effectors such as PKA or 14-3-3 can efficiently access membrane-bound Raf-1.

The highly specific interaction and strong activation of the isolated kinase domain with 14-3-3 suggest that 14-3-3 does not act in the Ras-dependent membrane recruitment step. The conclusion that Ras and 14-3-3 act at different steps in the Raf-1 activation pathway is supported by their additive effects in a cell-free system.

Furthermore, Ras and 14-3-3 bind Raf-1 independently *in vitro*, do not compete for binding and their combined presence does not activate Raf-1 (W.Kolch, unpublished observations). That simple binding of 14-3-3 to Raf-1 is not sufficient to cause activation is evident from *in vitro* experiments and is also suggested by the observation that Raf-1 and 14-3-3 are complexed in quiescent cells.

We propose that 14-3-3 interacts strongly with the Raf-1 activation domain, perhaps directly monitoring the activation status, but that its activation is aimed directly at the kinase domain. This would explain the observation that *in vivo* the isolated Raf-1 kinase domain responds more strongly than c-Raf-1 to 14-3-3 activation. We envision that due to the large physiological excess of 14-3-3, c-Raf-1 could be close to saturation *in vivo*. In contrast, the more weakly interacting isolated kinase domain would be largely unoccupied and thus could respond robustly to an increase in 14-3-3 concentration.

The picture of 14-3-3 that emerges is that of a latent co-activator bound to unactivated Raf-1 in quiescent cells. Once Raf-1 is fully activated, 14-3-3 apparently becomes dispensable and is released. Our data do not support the possibility that 14-3-3 facilitates the interaction between the Raf-1 kinase domain and its substrates. Although it is conceivable that 14-3-3 has multiple roles in the Raf-1 activation pathway, the simplest hypothesis that explains the observed effects towards both c-Raf-1 and the isolated kinase domain is that 14-3-3 mediates regulatory effects aimed at the kinase domain. The precise step in the Raf-1 activation pathway mediated by 14-3-3 is currently under investigation.

Materials and methods

Yeast two-hybrid screen

The Raf-1 kinase domain was fused to the GAL4 DNA binding domain in vector pGBD9 (Chien *et al.*, 1991) to produce pGBD-Raf, which was used as the 'bait' in the screen. Yeast genetic techniques and media composition were as described (Durfee *et al.*, 1993). Histidine-selective medium contained 25 mM 3-aminotriazole (Sigma). The human cDNA library in vector pACT (Durfee *et al.*, 1993) was provided by Stephen Elledge (Baylor College of Medicine) and screened in strain Y190 (Harper *et al.*, 1993). *In situ* colony assay for β-galactosidase activity was performed by lifting colonies onto nitrocellulose and permeabilization by submersion in liquid nitrogen as described (Vojtek *et al.*, 1993).

Protein expression and purification

pSL303 contains the kinase domain of murine c-*raf-1* from the *Bgl*I site inserted into pGEX3X (Smith and Johnson, 1988). pSL328 is the same construct with Lys375 of Raf-1 changed to Trp (the mutation is analogous to Raf-301; Heidecker *et al.*, 1990). Both mutations eliminate Raf kinase activity. pSL303 and pSL328 have previously been referred to as pGST-Raf and pGST-Raf-K375W (Li,S. and Sedivy, 1993). pSL305 contains full-length murine c-*raf-1* cDNA (*Dra*I fragment) inserted into pGEX1X. To construct GST−Raf baculoviruses, the GST portion (including the polyglycine linker and thrombin cleavage site) of pGEX-KG (Guan and Dixon, 1991) was inserted into the baculovirus vector pAcC5 upstream of the polylinker to create pGST/AcC5 (Hafner *et al.*, 1994). An *Nco*I site was introduced at the ATG start codon of human c-*raf-1*, which allowed in-frame insertion into the *Nco*I site in the polylinker of pGST/AcC5. An in-frame deletion between the *Pvu*II and *Bst*XI sites (Figure 3B) was introduced to remove the regulatory domain. This construct was designated GNX and is identical to the BXB construct (Heidecker *et al.*, 1990). Analogous constructs were made in pGEX-KG for expression in *E.coli*. Derivatives containing various portions of c-*Raf-1* were also made (Figure 3B); GNH contains the *Nco*I−*Hin*dIII fragment, GNS contains the *Nco*I−*Sal*I fragment and GRS contains an internal in-frame *Stu*I−*Stu*I deletion that removes most of the kinase domain. In all cases the Raf-1 proteins were tagged with GST at their N-terminus. GST fusion proteins were purified from *E.coli* cultures in the presence of 1% Triton X-100 as described (Guan and Dixon, 1991). Aliquots of washed glutathione−Sepharose beads were analyzed by SDS−PAGE and purity of the immobilized Raf-1 preparations was estimated to be 20–60% by Coomassie brilliant blue or silver staining of gels. The gels were also used to ensure equal Raf-1 input in subsequent experimental procedures. GST fusion proteins expressed in the baculovirus−Sf9 cell system were purified in the presence of 1% Triton X-100 as described (Hafner *et al.*, 1994). GST−Raf (full-length) was expressed either by itself (referred to as unactivated Raf-1) or by co-infection with either PKCα- or Ras plus Lck-expressing baculovirus constructs (the Ras construct contained the activating Gly12→Val mutation). GST fused to the N-terminus of Raf-1 does not affect the activity of the kinase domain or regulation of the full-length protein by PKCα or Ras/Lck (Hafner *et al.*, 1994). The purification of 14-3-3 protein to homogeneity from bovine brain has been described elsewhere (Tanji *et al.*, 1994). The preparation contains a mixture of several 14-3-3 isoforms and can be resolved into three closely migrating bands of 32, 30 and 29 kDa by SDS−PAGE. In the experiments presented here, the 30 and 29 kDa bands migrated as a single broad band. Purified recombinant kinase-active Mek, kinase-defective Mek and kinase-defective MAPK (Gardner *et al.*, 1993) were kindly provided by Manuela Baccarini (University of Vienna). Purified recombinant 14-3-3 ζ protein expressed in *E.coli* was kindly provided by Haian Hu (Emory University).

Raf-1 kinase assays

GST−Raf proteins immobilized on glutathione−Sepharose beads were washed three times with TBST [20 mM Tris−HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, supplemented with protease inhibitors, 1 mM phenylmethylsulfonylfluoride (PMSF) and 1 μg/ml leupeptin, and phosphatase inhibitors, 10 mM β-glycerophosphate, 5 mM NaF, 10 mM Na pyrophosphate and 1 mM orthovanadate], once with Raf kinase (RK) buffer [20 mM Tris−HCl, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)] and resuspended in RK buffer. Aliquots of 5–10 ng of immobilized Raf-1 protein were used in each kinase reaction. Kinase reactions (25 μl) included 20 μM ATP and 5 μCi [γ-^{32}P]ATP; other components are indicated in Figure legends. Incubation was for 30 min at 25°C; reactions were terminated by addition of Laemli sample buffer and boiling. Samples were separated by SDS−PAGE and blotted onto membranes. Following autoradiography, membranes were processed for Western analysis using the indicated antibodies. Mek phosphorylation by Raf-1 co-expressed with activators is not due to contamination with PKCα or Lck, since neither could be detected in the Raf-1 preparations by immunoblotting. In addition, neither PKCα nor Lck can phosphorylate Mek to a detectable extent *in vitro* and inclusion of a specific PKC inhibitor (GF109203X) in the assays had no effect on observed Raf-1 activity (Hafner *et al.*, 1994).

Immunoprecipitations and Western blotting

Antiserum against whole 14-3-3 protein prepared in goat (Tanji *et al.*, 1994) and antiserum against a C-terminal peptide of Raf-1 (cRaf-VI) prepared in rabbits (Hafner *et al.*, 1994) have been described. For each immunoprecipitation, a confluent 15 cm diameter dish of NIH 3T3 cells was rinsed with ice-cold phosphate-buffered saline (PBS) and the cells were lysed in 2 ml of TBST. One half of the lysate was precipitated with 1.5 μl of cRaf-VI antiserum and 5 μl of protein A−agarose (Boehringer) for 1 h at 4°C. Immunoprecipitates were washed five times with TBST, once with RK buffer, resuspended in RK buffer and assayed for Raf-1 activity as indicated above. For Western analysis, SDS gels were electroblotted onto Immobilon-P membranes (Millipore). Membranes were blocked with TBSM (20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 5% non-fat dry milk) and incubated with a 1:500 or 1:2000 dilution of 14-3-3 or cRaf-VI antiserum respectively in TBSM. Blots incubated with cRaf-VI antiserum were washed (4–5 times, 5 min each wash) with TBS−Tween (20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 1% Tween-20); blots incubated with 14-3-3 antiserum were washed (8–10 times, 5 min each wash) with TBS−Tween containing 0.2% SDS. Incubation with appropriate secondary antibody coupled to horseradish peroxidase was in TBS−Tween. Blots were washed 5–6 times with TBS−Tween, rinsed with water and developed with the enhanced chemiluminescence system (Amersham).

Triton-disrupted cell lysates

Triton-disrupted cell lysates were prepared from quiescent NIH 3T3 cells as described by Dent *et al.* (1993). Approximately 1×10^6 cells in a total volume of 25 or 50 μl were used per assay. The lysate was left untreated or else incubated with different amounts of 14-3-3 protein

and/or purified Ha-ras protein (kindly provided by Alan Wolfman) loaded with the non-hydrolyzable GTP analog GMP-PNP (Boehringer). After 15 min incubation at 32°C, 1 ml of TBST supplemented with protease and phosphatase inhibitors was added to the lysate. After 10 min on ice, the sample was centrifuged for 2 min at 13 000 *g*. The pellet contained <10% Raf-1 protein and no detectable Raf-1-specific kinase activity. Immunoprecipitation with cRaf-VI antiserum and assay in the linked Mek→MAPK assay system was as described above. Controls showed that the majority of Raf protein and Raf kinase activity were recovered by immunoprecipitation. Each assay contained 100 ng purified kinase-active Mek and 100 ng of kinase-negative MAPK. Exogenously added GST−Raf proteins were assayed using 100 ng of purified kinase-defective Mek. Kinase reactions contained 20 μM ATP and 5 μCi [γ-^{32}P]ATP. After incubation for 30 min at 25°C, reactions were stopped by addition of Laemli sample buffer. Samples were displayed by SDS−PAGE, blotted and autoradiographed. Blots were subsequently stained with cRaf-VI antiserum.

CAT assays

The 14-3-3 cDNA was subcloned from the pACT yeast vector into the mammalian cDNA expression vector pcDNA3 (InVitrogen). The other plasmids used in CAT assays (Figure 2) were: the empty pcDNA3 vector; pMNC-Raf (full-length Raf; Kolch *et al.*, 1991); pRSV-Raf-BXB (Raf kinase domain; Heidecker *et al.*, 1990); pB4X (AP-1-dependent reporter; Bruder *et al.*, 1992); pMHC-NF-κB CAT and pMHC-NF-κB*mut* CAT (NF-κB-dependent reporter and the analogous construct containing mutant NF-κB sites; Baldwin *et al.*, 1991). NIH 3T3 cells were electroporated with the indicated plasmid DNAs and equal amounts of protein were assayed for CAT activity as described (Li,S. and Sedivy, 1993). In each electroporation experiment, the requisite number of dishes were harvested (two per electroporated sample) and the harvested cells were pooled, centrifuged and washed in transfection buffer. Pooled cells were divided into the requisite number of aliquots, which were electroporated in rapid succession and immediately replated in pre-warmed medium. Using this procedure, electroporation efficiencies are essentially identical among different samples in a single experiment.

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Note added in proof

The preferential association of 14-3-3 with unactivated Raf-1 has been corroborated in experiments where 14-3-3β was expressed in *E.coli* as a GST fusion protein, immobilized on glutathione-sepharose beads and incubated with extracts of Sf-9 cells which had been infected with untagged Raf-1-expressing baculovirus. Activation of Raf-1 with PKCα significantly decreased (but did not eliminate) binding to GST-14-3-3. Raf-1 activated by Ras/Lck did not bind to GST-14-3-3 at a level detectable by immunoblotting.