# Regulation of the Raf-1 kinase domain by phosphorylation and 14-3-3 association

Michele T. YIP-SCHNEIDER*[1], Wenyan MIAO†[1], Amy LIN‡, Darlene S. BARNARD§, Guri TZIVION¶ and Mark S. MARSHALL§[2]

*Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A., †Millennium Pharmaceuticals Inc., Cambridge, MA 02142, U.S.A., ‡Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A., §Eli Lilly and Company, Lilly Corporate Center, Drop Code 1543, Indianapolis, IN 46285, U.S.A., and ¶Diabetes Research Laboratory, Department of Molecular Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, U.S.A.

The Raf-1 kinase domain is kept in an inactive state by the N-terminal regulatory domain. Activation of the kinase domain occurs following release from the N-terminal repression and possible catalytic upregulation. To distinguish the regulatory mechanisms that directly influence the catalytic activity of the enzyme from those which act through the inhibitory domain, the catalytic domain of Raf-1 (CR3) was expressed in COS-7 cells. The role of phosphorylation in the direct regulation of this domain was determined by substituting non-phosphorylatable amino acids for known serine and tyrosine phosphorylation sites. The intrinsic activity of each mutant protein was determined as well as stimulation by v-Src and phorbol esters. Both v-Src and phorbol esters were potent activators of CR3, requiring the serine 338/339 (p21-activated protein kinase, Pak) and tyrosine 340/341 (Src) phosphorylation sites for full stimulation of CR3. In contrast, loss of the serine 497/499 protein kinase C phosphorylation sites had little effect on CR3 activation by either v-Src or phorbol esters. Loss of serine 621, a 14-3-3 adaptor-protein-binding site, prevented activation of CR3 by v-Src or phorbol esters and partially decreased the high basal activity of the kinase fragment. When co-expressed in COS-7 cells, 14-3-3 associated strongly with full-length Raf-1, weakly with wild-type CR3 and not at all with the A621 and D621 CR3 mutants. The role of 14-3-3 in maintaining the activity of the catalytic domain of Raf-1 was investigated further by performing peptide-competition studies with wild-type CR3, wild-type CR3 and v-Src or constitutively active CR3 (CR3[YY340/341DD]). In each case, incubation of the proteins with a phosphoserine-621 Raf-1 peptide, which we show displaced Raf-1 and CR3[YY340/341DD] from 14-3-3, was found to substantially reduce catalytic activity. Taken together, our results support a model of Raf regulation in which the activity of the Raf-1 catalytic domain is directly upregulated by phosphorylation, following relief of inhibition by the N-terminal regulatory domain upon Ras-GTP binding. Moreover, the presence of serine 621 in the free catalytic fragment is required for full CR3 activation by stimulatory factors, and the continuous presence of 14-3-3 at this site is necessary for retaining activity once the kinase is activated.

Key words: CR3, Raf, serine 338.

## INTRODUCTION

The serine/threonine kinase Raf-1 is one of the primary effector proteins for membrane-bound Ras, responsible for relaying mitogenic signals from active Ras to the cell nucleus [1]. Upon binding to membrane-bound Ras-GTP, Raf-1 is recruited to the plasma membrane where activation occurs. Activated Raf-1 then transduces the signal through the MEK/ERK (mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase) cascade, thereby regulating the activity of numerous cytoplasmic and nuclear proteins involved in growth, cell division and differentiation (reviewed in [1,2]).

Mammalian Raf isoforms are highly conserved within three distinct regions designated conserved regions (CRs) 1, 2 and 3. CR1 and CR2 are located within the N-terminal 300 amino acids of Raf-1 and constitute a negative regulatory domain; CR3 constitutes the C-terminal kinase domain. The N-terminal regulatory domain of Raf-1 maintains the kinase domain in an inactive state and promotes activation of the kinase through specific interactions with Ras at the plasma membrane. When expressed without the N-terminus, the catalytic CR3 domain possesses a constitutive level of activity, sufficient to transform fibroblasts [3]. The inactive conformation of Raf-1 is maintained by association with the 14-3-3 family of adaptor proteins, which have been found to assemble as dimers and bind to specific phosphorylated serines on many different signalling proteins within the cell [4–11]. While the 14-3-3 dimer has been shown to bridge Raf-1 with other signalling proteins, the 14-3-3 dimer also binds to at least two distinct sites within Raf-1 itself, specifically phosphoserines 259 in CR2 and 621 in CR3 [12,13]. Upon association of Raf-1 with Ras-GTP at the plasma membrane, the 14-3-3 dimer is displaced from the 259 site, followed by rapid catalytic activation of Raf-1. Three different models have been proposed to explain the role of 14-3-3 in Raf-1 regulation. One model proposes that upon displacement from phosphoserine 259, 14-3-3 rebinds to a third, unidentified binding site within CR3 [14]. The new bridge formed between this third site and phosphoserine 621 is hypothesized to constrain the catalytic domain in an active conformation. A second, similar model, suggests that 14-3-3 protects phosphorylated serine 621 from phosphatases, and therefore stabilizes a kinase-competent conformation of Raf [15]. The third model predicts that the 14-3-3–Raf interaction is required for Ras to localize Raf-1 to the plasma membrane for activation by protein kinases [16]. Once at the plasma membrane, the 14-3-3 dimer is proposed to release completely from active Raf-1. Reassociation with 14-3-3 then occurs during inactivation and subsequent translocation of Raf-1 back to the cytosol.

Abbreviations used: CR, conserved region of Raf-1; Pak, p21-activated protein kinase; PKC, protein kinase C; MEK/ERK, mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase; GST, glutathione S-transferase; MBP, myelin basic protein.

[1] These authors contributed equally to this paper.

[2] To whom correspondence should be addressed (e-mail Marshall_Mark@Lilly.com).

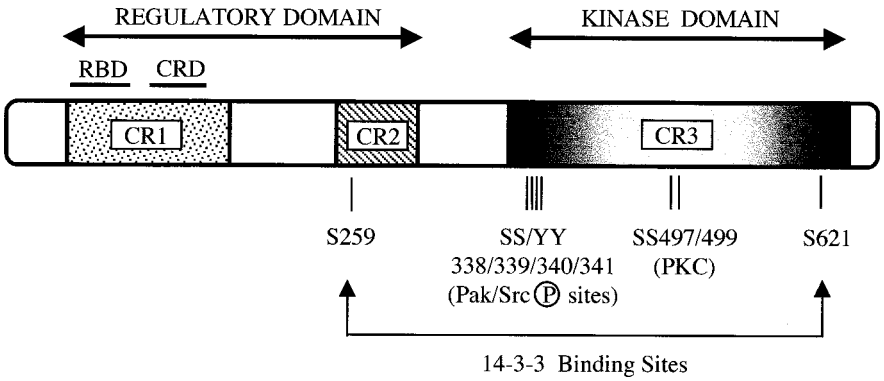


**Figure 1 The structural features and phosphorylation sites within Raf-1**

Phosphorylation sites located within the human Raf-1 kinase domain (CR3) as indicated were mutated by site-directed mutagenesis. RBD, Ras-binding domain; CRD, cysteine-rich domain.

As illustrated by the three models described above, the precise role of 14-3-3 in Raf regulation is unclear. This confusion also applies to the role of CR3 phosphorylation in the regulation of Raf. Distinct bodies of evidence exist that both dispute and support a role for catalytic regulation by direct CR3 phosphorylation (reviewed in [17]). While it has been established that phosphorylation of CR3 is important for Raf-1 regulation, whether these phosphorylations directly influence the catalytic function of Raf-1 or act in concert with Ras to relieve the N-terminal inhibition remains to be clarified. A growing number of distinct phosphorylated residues within CR3 have been identified and characterized. We have recently reported that p21-activated protein kinase (Pak)-family protein kinases phosphorylate Raf-1 serine 338 directly [18]. Src-family kinases have been shown previously to phosphorylate tyrosines 340 and 341 [19]. Phosphorylations of serines 338, 339, 497 and 499 and of tyrosines 340 and 341 have all been proposed to be necessary for Raf-1 activation by diverse stimuli (Figure 1). Phosphorylation of the serines and tyrosines at positions 338–341 has been shown to be induced by both oncogene expression and growth-factor stimulation [12,19–21]. Furthermore, replacement of serines 338 and 339 or tyrosines 340 and 341 with non-phosphorylatable amino acids dramatically impairs the activation of Raf-1 by Ras, Src, epidermal growth factor and phorbol esters [17,22]. Conversely, substitution of serines 338 and 339 with negatively charged amino acids restores regulation by both Ras and Src, presumably by mimicking the phosphorylated serines. Acidic amino acid substitution of tyrosines 340 and 341 is even more striking and results in the constitutive activation of Raf-1, suggesting a hierarchy of function for phosphorylation of the serine/tyrosine patch at 338–341.

Phosphorylation of serines 497 and 499 within the activation loop of CR3 has been proposed to be responsible for Raf-1 activation by protein kinase C (PKC) [23,24]. Phosphorylation of the Raf-1 activation loop would be a direct means of increasing kinase activity, analogous to phosphorylation of the ERK activation loop by MEK. In recent studies, the loss of both of these residues was observed to have little effect on Raf-1 activation by phorbol esters. In fact, phosphorylation of serine 499 repressed the activity of Raf-1 [17,25,26]. The primary effect of phorbol ester treatment was to promote an increase in Ras-GTP which then resulted in Raf-1 activation via the same mechanisms as utilized by epidermal growth factor.

We have recently demonstrated in a mammalian cell-culture model that full-length Raf-1 kinase is regulated by a common set of events, irrespective of the activating stimulus [17]. Among these controlling events is phosphorylation of the catalytic domain on residues 338–341. However, since that study involved only full-length Raf-1, the activating role of these phosphorylations could not be determined. Similarly, although we found that phorbol esters stimulated Raf-1 primarily via Ras-GTP, regulatory phosphorylation of the kinase-activation loop could not be ruled out completely due to the presence of the N-terminal regulatory domain. In the present study, we used a defined mammalian transient expression system to investigate the role of phosphorylation and 14-3-3 association in the direct regulation of the Raf-1 catalytic domain (CR3), in the absence of the intrinsic inhibition by the regulatory domain. We report that multiple phosphorylation sites within the catalytic domain of Raf-1 are required for the direct regulation of Raf-1 catalytic activity, exclusively through effects on the catalytic domain rather than disrupting interactions between the catalytic domain and the N-terminal regulatory domain. Furthermore, we confirm that association with 14-3-3 is required to maintain the activated state of the Raf-1 CR3 catalytic domain in the absence of the regulatory N-terminus of the protein.

## MATERIALS AND METHODS

### PCR cloning of the Raf-1 kinase domain (CR3)

Nucleotide sequences encoding residues 307–648 (CR3) of human c-Raf-1 were PCR-amplified using the 5′ primer 5′-GGTGGA-ATTCAGCCGAAAACCCCCGTG-3′ and the 3′ primer 5′-GCCCCTCGAGCTAGAAGACAGGCAGCCTCG-3′. The amplified CR3 fragment was digested with *Eco*RI/*Xho*I and cloned into the vector pAlter-1 (Promega) to generate pAlter-CR3, which was used for the mutagenesis of CR3. The DNA sequence of the cloned CR3 fragment was confirmed by sequencing (ThermoSequenase kit, Amersham).

### Mutagenesis and plasmid construction

CR3 mutants were generated by site-directed mutagenesis of pAlter-CR3 using the appropriate oligonucleotides to introduce the desired base changes (Altered sites™ *in vitro* mutagenesis system, Promega). The specific mutations were confirmed by DNA sequencing.

The mammalian expression vector pECE [27] was modified for subcloning and attachment of a Myc-epitope tag to the N-terminus of CR3. The mutated CR3 fragments generated above were then subcloned into the modified pECE vector as *Eco*RI/*Hin*dIII inserts. The resulting pECE-CR3 plasmids were used to transiently express Myc-tagged CR3 proteins in COS-7 cells.

### COS-7 cell transfections

COS-7 cells were transfected with the pECE-CR3 plasmids by electroporation using a Bio-Rad Gene Pulser as described previously [28]. For PMA stimulation, the cells were starved overnight (Dulbecco's modified Eagle's medium with 0.5% bovine calf serum) 24 h after transfection. The cells were then stimulated with 50 nM PMA for 5 min prior to harvesting.

### Immunoprecipitation and coupled Raf assay

Cells were lysed in buffer A (20 mM Tris/HCl, pH 8.0/2 mM EDTA/50 mM $\beta$-glycerophosphate/1 mM $Na_3VO_4$/1% Triton X-100/10% glycerol/1 mM PMSF/1 $\mu$g/ml pepstatin/1 $\mu$g/ml leupeptin/2.2 $\mu$g/ml aprotinin) [29] followed by passage through a 25-gauge needle. Cell lysates were clarified by centrifugation at 10000 ***g*** for 10 min at 4 °C. To immunoprecipitate Myc-tagged CR3 proteins, the clarified COS-7 cell lysates were incubated with anti-Myc 9E10 antibody [30] (a gift from Robert Deschenes, University of Iowa, Iowa City, Iowa, U.S.A.) prebound to GammaBind Plus Sepharose (Pharmacia) at 4 °C for 2 h. Complexes were washed with lysis buffer and then used in the coupled Raf kinase assay described previously [17]. Briefly, the complexes were incubated with recombinant glutathione S-transferase (GST)-MEK and GST-ERK; activation of the MEK/ERK cascade was assayed by the addition of myelin basic protein (MBP) as the substrate. The extent of MBP phosphorylation was quantified by scintillation counting of filters spotted with an aliquot of the reaction mixture. Raf kinase activity was normalized to the amount of CR3 present in each immunoprecipitate as determined by Western-blot analysis.

### Western blotting

Proteins were resolved by SDS/PAGE (10% gels, NOVEX) and transferred to Immobilon-P membrane (Millipore). Western blotting to detect Raf-1/CR3 was performed with anti-Myc 9E10 as the primary antibody and anti-mouse Ig-horseradish peroxidase (Amersham) as the secondary antibody. Alternatively, other primary antibodies were employed as indicated. Proteins of interest were visualized by ECL® (Amersham) and quantified by densitometry (Imaging Densitometer GS-670, Bio-Rad).

### 14-3-3/CR3 interaction in COS-7 model system

For 14-3-3/CR3 co-immunoprecipitation experiments, Myc-tagged full-length Raf-1 or CR3 was transiently expressed in COS-7 cells together with GST-tagged 14-3-3 [14]. Following transfection (72 h), cells were harvested for immunoprecipitation as described above. Lysates were incubated with glutathione-Sepharose 4B with mixing for 2 h. The complexes were collected and analysed by Western blotting with anti-GST and anti-Myc 9E10 antibodies (Santa Cruz Biotechnology) to detect GST-tagged 14-3-3 and Myc-tagged full-length Raf-1/CR3 proteins respectively. For peptide competition studies, lysates were incubated with the S621 Raf-1 phosphopeptide (Raf-1 residues 613–627, LPKINRSApSEPSLHR, phosphorylated on serine 621) or the control peptide as detailed below prior to mixing with glutathione-Sepharose beads to purify the complexes for analysis.

For the peptide competition experiments with Myc-tagged Raf-1/CR3, COS-7 cells were transiently transfected with expression plasmids for either Myc-tagged full-length Raf-1 or CR3 proteins as described above. After transfection (3 days), cells were harvested and lysed in buffer A. Lysates were incubated with the S621 Raf-1 phosphopeptide or the unphosphorylated control peptide (final concentrations, 0.5 mM) for 1 h at 4 °C, followed by the addition of anti-Myc 9E10 antibody prebound to GammaBind Sepharose beads. The resulting complexes were assayed for kinase activity as described above.

## RESULTS

### Intrinsic kinase activity of Raf-1 kinase domain phosphorylation mutants

Seven residues within the Raf-1 kinase domain (CR3) reported to be inducibly phosphorylated and important for activation were altered using site-directed mutagenesis (Figure 1). Serines were substituted with alanine, and tyrosines were changed to phenylalanine to eliminate each phosphorylation site. Serines 338 and 339, tyrosines 340 and 341, and serines 497 and 499 were each substituted in pairs to control for potentially redundant phosphorylation of adjacent residues. Tyrosines 340 and 341 were also changed to aspartic acid residues to mimic the negative charge of phosphorylated amino acids. Serine 621, the C-terminal 14-3-3-binding site, was also replaced with either alanine or aspartic acid. These amino acid substitutions were each introduced into CR3, which was then transiently expressed as an isolated kinase domain (residues 307–648) in COS-7 cells.

To determine whether the phosphorylation of each specific site affected the basal, constitutive activity of the free CR3 catalytic fragment, the intrinsic kinase activity of each phosphorylation mutant was measured in serum-deprived cells. Myc-tagged wild-type and mutant CR3 proteins were transiently expressed in COS-7 cells and immunoprecipitated with an anti-Myc-epitope antibody. The intrinsic kinase acitivity of the immunoprecipitated CR3 proteins was determined and expressed relative to v-Src-stimulated wild-type CR3 activity (Figure 2, white bars). As reported previously, independent expression of the CR3 fragment resulted in constitutive activation of the kinase. While alanine substitution of serines 497 and 499 had no effect on the intrinsic activity, amino acid substitution of serines 338/339, tyrosines 340/341 and serine 621 decreased the level of constitutive kinase activity by approx. 40–60%. Interestingly, in no case did the alteration of these individual sites result in a complete loss of basal kinase activity. When aspartic acid residues were used to mimic phosphorylated tyrosines 340 and 341, the basal constitutive activity of this mutant increased more than 30-fold above that of wild-type CR3 fragment (Figure 2B).

### v-Src stimulation of CR3 kinase activity

To determine whether any of the phosphorylation sites are required for stimulation of the intrinsic CR3 kinase activity by Src, wild-type and mutant CR3 proteins were co-expressed with v-Src in COS-7 cells and assayed for activity (Figure 2, black bars). As predicted by the high intrinsic activity of the con-

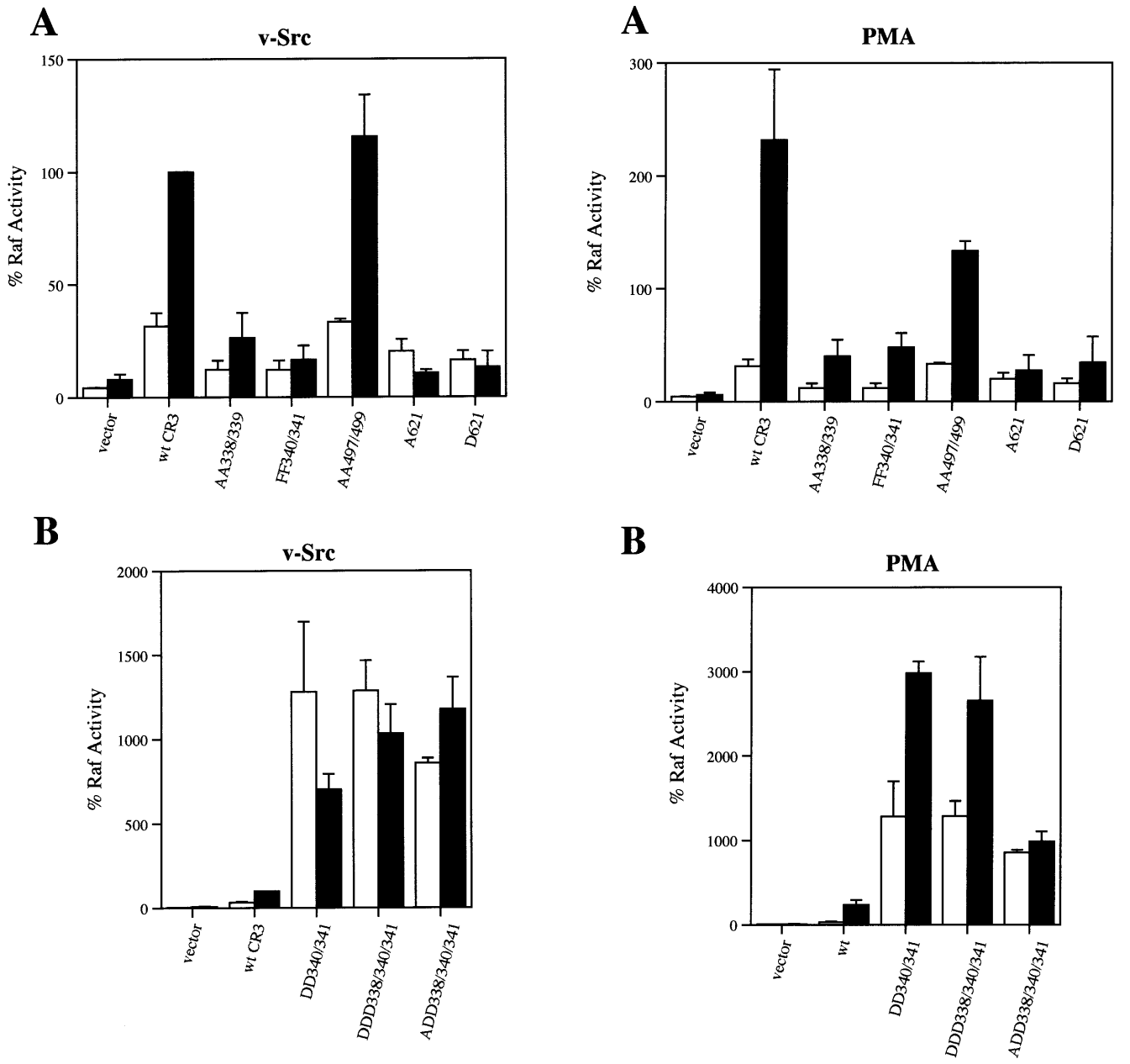


**Figure 2 Intrinsic and oncogenic v-Src-induced CR3 kinase activity**

COS-7 cells were transiently transfected with Myc-tagged wild-type (wt) CR3, mutant CR3 or co-transfected with v-Src expression plasmids. Myc-tagged CR3 proteins were immunoprecipitated with anti-Myc 9E10 antibody and Raf kinase activity was measured using a coupled MEK/ERK/MBP assay. The kinase activities were normalized to the amount of CR3 in the immunoprecipitates and expressed relative to v-Src-induced wild-type CR3 kinase activity (set to 100%). (**A**) The kinase activity profiles of the lower-activity CR3 proteins in the absence (white bars) or presence (black bars) of v-Src. (**B**) Kinase activities of the super-active CR3 proteins in the absence (white bars, note the difference in scale) or presence (black bars) of v-Src.




**Figure 3 PMA-stimulated CR3 kinase activity**

COS-7 cells were transiently transfected with expression plasmids for Myc-tagged wild-type CR3 (wt) or mutant CR3. Following treatment of the cells with PMA (50 nM), the CR3 proteins were immunoprecipitated and assayed for Raf kinase activity. The kinase activities were normalized to the amount of CR3 proteins in the immunoprecipitates and standardized to v-Src-induced wild-type CR3 kinase activity (set to 100%). (**A**) The kinase activity profiles of the lower-activity CR3 proteins, unstimulated (white bars) or stimulated (black bars) with PMA. (**B**) The kinase activity profiles of the super-active CR3 proteins, unstimulated (white bars, note the difference in scale) or stimulated (black bars) with PMA.

stitutively active YY340/341DD CR3 mutant, v-Src stimulated wild-type CR3 kinase activity more than 3-fold. Phenylalanine substitution of tyrosines 340 and 341 rendered CR3 unresponsive to v-Src, although some intrinsic activity was detectable. The extremely high constitutive activity of the YY340/341DD and the triple mutants was not increased further by v-Src (Figure 2B). These results confirmed that the phosphorylation of tyrosines 340 and 341 regulates the catalytic domain of Raf-1 directly, not by affecting intramolecular interactions between the regulatory N-terminus of Raf-1 and its catalytic domain. The SS338/339AA mutant had greatly reduced Src-stimulated activity, below the constitutive activity measured for wild-type CR3. Nevertheless, v-Src stimulated the lower intrinsic activity of the SS338/339AA mutant 2-fold, suggesting a possible co-operative effect between the phosphoserines and phosphotyrosines at this site. Elimination

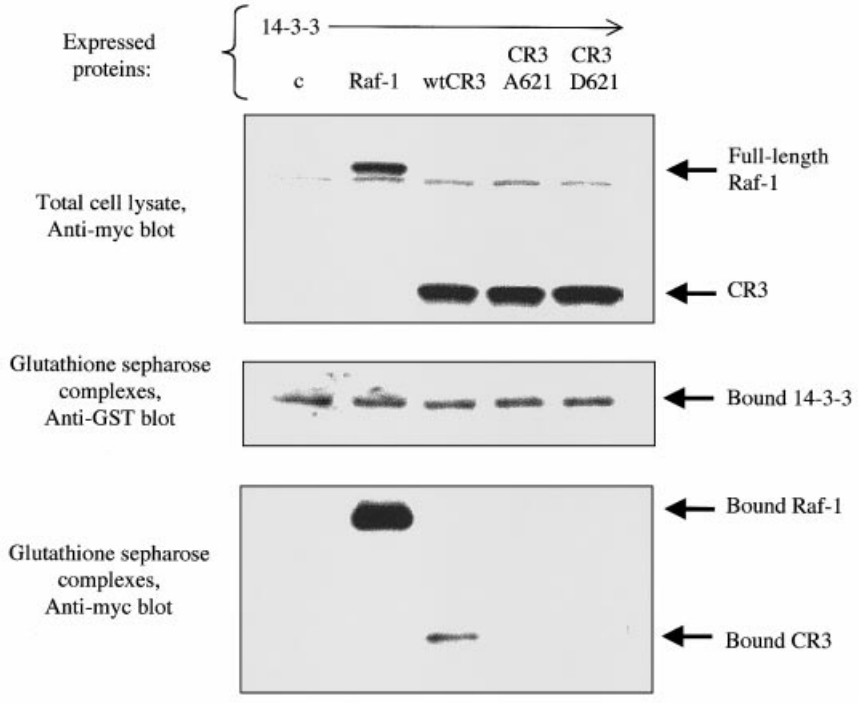


**Figure 4** ***In vivo*** **complex formation between CR3 and 14-3-3 proteins**

COS-7 cells were transiently co-transfected with plasmids expressing GST-tagged 14-3-3ζ and either Myc-tagged full-length Raf-1 or wild-type/mutant CR3 proteins as indicated (c = control, vector alone transfected). Following purification of the complexes by glutathione-Sepharose, full-length Myc-Raf-1, Myc-CR3 or GST-14-3-3 proteins present in the complexes as well as total Myc-tagged Raf/CR3 in the cell lysates were detected by Western blotting analysis using the appropriate primary antibodies as indicated.

of the serine 621 site completely blocked stimulation of the basal kinase activity of this mutant by v-Src. In contrast, loss of the PKC phosphorylation sites in the SS497/499AA mutant had no effect upon the ability of v-Src to fully induce the activity of CR3.

### Phorbol ester stimulation of CR3 activity

PMA is a potent tumour-promoting phorbol ester that activates PKC. PKC has been proposed to activate Raf-1 through direct phosphorylation of serines 497 or 499 in the activation loop [23,24]. We and others have reported that phorbol esters can stimulate full-length Raf-1 by increasing the amount of intracellular Ras-GTP [17,31,32]. By testing CR3 alone, it should be possible to distingush between phorbol ester activation of Raf-1 activity mediated through either Ras binding or direct phosphorylation of the catalytic domain. As shown in Figure 3, treatment of COS-7 cells expressing CR3 with PMA for 5 min resulted in a significant stimulation of Raf activity (7-fold). PMA also stimulated the SS497/499AA mutant, although less robustly. This result further argues against the model that PKC activates Raf-1 exclusively through phosphorylation of serines 497 or 499 in the activation loop.

The SS338/339AA, YY340/341FF, S621A and S621D CR3 mutants were also examined for inducibility by PMA (Figure 3, black bars). The PMA-induced kinase activities of these mutants were significantly lower than that of wild-type CR3, although some stimulation was measured above their intrinsic activities. The S621A and S621D mutants were the least responsive to PMA stimulation. The superactive CR3 mutants, YY340/341DD and SYY338/340/341DDD, were further activated upon PMA stimulation (Figure 3B). However, the SYY338/340/341ADD mutant lost its ability to respond to PMA stimulation. Taken together, these results confirm that phosphorylation of serines 338/339, tyrosines 340/341 and serine 621 is required for full Raf catalytic activation in response to a variety of stimuli.

### 14-3-3 and activation of the CR3 catalytic kinase domain

As discussed in the Introduction, several models exist to explain the role of 14-3-3 in Raf-1 regulation. One model proposes that 14-3-3 maintains Raf in an inactive conformation that is competent to associate with Ras localized in the plasma membrane. According to this hypothesis, 14-3-3 dissociates from Raf-1 following kinase activation and is not necessary for maintaining activity. This model predicts that the isolated CR3 kinase domain should function independently of 14-3-3. A second model proposes that in addition to maintaining Raf-1 in an inactive conformation, 14-3-3 plays an essential role during and following activation by maintaining the Raf-1 catalytic domain in an active conformation. This model predicts that an independent CR3 domain would require 14-3-3 for activity.

To address the question of whether CR3 requires 14-3-3 association for activity, we followed three experimental approaches. The first approach involved evaluating the activity of a CR3 mutant which lacked the serine 621 binding site for 14-3-3 (Figures 2 and 3). Alanine substitution at serine 621 partially reduced the basal constitutive activity of the CR3 fragment and completely blocked further stimulation of the kinase by v-Src or phorbol ester, suggesting that 14-3-3 association is critical for CR3 activation by v-Src or phorbol esters.

The second approach involved comparing the association between 14-3-3 and the different CR3 proteins relative to full-

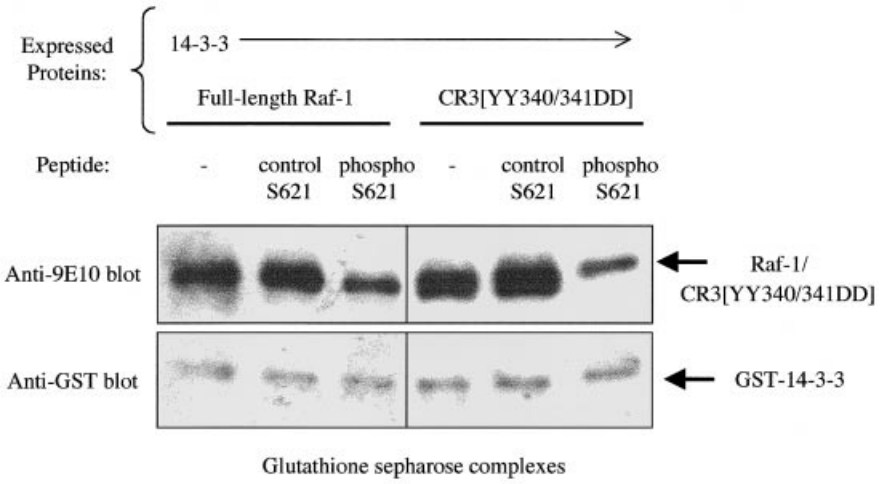


**Figure 5 Displacement of Raf-1 and CR3[YY340/341DD] from 14-3-3 by the phosphoserine 621 Raf-1 peptide**

Cell lysates prepared from COS-7 cells transiently expressing GST-tagged 14-3-3ζ and either Myc-tagged Raf-1 or CR3[YY340/341DD] were incubated for 1 h with 0.5 mM S621 Raf-1 phosphopeptide or the corresponding unphosphorylated peptide. Complexes were purified by binding to glutathione-Sepharose and subsequently analysed by Western blotting with the indicated antibodies.

**Table 1 Inhibition of CR3 kinase activity by the phosphoserine 621 Raf-1 peptide**

COS-7 cells transiently expressing Myc-tagged full-length Raf-1 or wild-type CR3 together with v-Src, wild-type CR3 or the constitutively active YY340/341DD mutant CR3 proteins were lysed and incubated at 4 °C for 1 h with 0.5 mM S621 phosphopeptide corresponding to Raf-1 sequence 613–627 or the identical unphosphorylated peptide (control). Myc-tagged Raf-1/CR3 proteins were immunoprecipitated and assayed for kinase activity, which was normalized to the amount of Raf-1/CR3 in the immunoprecipitates and standardized to v-Src-stimulated Raf-1 activity in the presence of the control peptide (set to 100%). Average counts from a representative experiment performed in duplicate are also shown. Percentage inhibition for each sample was calculated from the percentage activities in the presence of the phosphopeptide relative to the control peptide.

| Protein(s) expressed | Percentage activity relative to Raf-1 + v-Src (+ control peptide) | Percentage activity relative to Raf-1 + v-Src (+ S621 phosphopeptide) | Percentage inhibition by phosphopeptide |
|---|---|---|---|
| Raf-1 + v-Src | 100% (457360 c.p.m.) | 46.8 ± 8.6% (239800 c.p.m.) | 53% |
| Wild-type CR3 + v-Src | 10.1 ± 2.7% (35120 c.p.m.) | 2.6 ± 0.2% (7880 c.p.m.) | 74% |
| Wild-type CR3 | 1.3 ± 0.2% (6047 c.p.m.) | 0.015 ± 0.1% (422 c.p.m.) | 99% |
| CR3[YY340/341DD] | 121.8 ± 13.0% (548830 c.p.m.) | 11.8 ± 1.3% (60060 c.p.m.) | 90% |

length Raf-1. Cell lysates were prepared from COS-7 cells co-expressing GST-tagged 14-3-3 and Myc-tagged full-length Raf-1 or CR3. The complexes were purified by incubation with glutathione-Sepharose, and the relative amounts of epitope-tagged Raf-1, CR3 and 14-3-3 proteins present in the isolated complexes were determined by immunoblotting (Figure 4). Similar amounts of Myc-tagged Raf and CR3 proteins were detected in the total cell lysates, and also equivalent amounts of GST-14-3-3 were present in the complexes. We observed that 14-3-3 associated strongly with full-length Raf-1 but only weakly with wild-type CR3, possibly due to the instability of the complexes in the absence of the phosphoserine 259 site in CR3. Furthermore, no binding of 14-3-3 was detected to either the CR3 S621A or S621D 14-3-3-binding-site mutants, confirming that serine 621 is required by CR3 for 14-3-3 association.

A third, more sensitive experimental approach was taken to further clarify the role of 14-3-3 in maintaining CR3 activity. Incubation of full-length Raf-1 with a phosphopeptide containing Raf-1 residues 613–627 (LPKINRSApSEPSLHR, phosphoserine at position 621) has been demonstrated previously to efficiently compete with 14-3-3 for binding to phosphoserine 621 [14,33]. We also observed that the phosphoserine 621 peptide displaced approx. 70% of bound Raf-1 and 85% of bound CR3[YY340/341DD] from GST-tagged 14-3-3 when these proteins are co-expressed in COS-7 cells (Figure 5). Displacement of wild-type CR3 was also achieved following incubation with the phosphopeptide (results not shown). We subsequently used this phosphoserine 621 peptide to displace endogenous 14-3-3 from activated CR3 and measured the net effect on kinase activity. Wild-type or superactive YY340/341DD mutant CR3 proteins, or v-Src with either full-length Raf-1 or wild-type CR3 were transiently expressed in COS-7 cells. Cell lysates were incubated with either the phosphoserine 621 peptide or the identical unphosphorylated peptide. The Raf-1 and CR3 proteins were then immunoprecipitated and assayed for kinase activity. As shown in Table 1, the phosphoserine 621 peptide inhibited the activity of all the Raf proteins tested; in contrast, incubation with the control peptide did not inhibit kinase activity (results not shown). As reported previously, the phosphopeptide significantly reduced v-Src-stimulated activity of full-length Raf-1 [14]. Similarly, the phosphopeptide reduced the kinase activity of v-Src-stimulated wild-type CR3 by more than 70%. Furthermore, greater than 90% inhibition of the intrinsic kinase activities of wild-type CR3 and the superactive YY340/341DD mutant

proteins was observed in the presence of the phosphopeptide, demonstrating the importance of serine 621 phosphorylation for activity. Taken together, these results demonstrate that binding of 14-3-3 to serine 621 is required for maintaining the activities of full-length Raf-1 and wild-type CR3 as well as the constitutively active CR3[YY340/341DD] proteins.

## DISCUSSION

Despite laborious efforts to identify the mechanisms that govern the regulation of Raf-1, an unambiguous model of control remains elusive. The available information is often considered controversial and dependent upon the particular experimental system employed. Conclusions are complicated further by the fact that Raf-1 regulation is a complex, multi-step process, which involves relief of the inhibitory effect of the regulatory domain as well as activation of the catalytic kinase domain. In addition, each of these steps apparently involves multiple protein–protein interactions and phosphorylations. In this study, we specifically addressed how the catalytic domain of Raf-1 is regulated by phosphorylation and 14-3-3 association in the absence of the N-terminal regulatory domain. The individual phosphorylation sites examined within the Raf-1 catalytic domain were those shown previously to be inducible by growth signals or required for 14-3-3 association. Specifically, we examined the role of serines 338, 339, 497, 499, 621 and tyrosines 340 and 341 in the regulation of the Raf-1 CR3 fragment.

When expressed as a catalytic fragment, CR3 has detectable catalytic activity, which in fibroblasts is sufficient to induce morphological transformation. This low constitutive activity may reflect the unrepressed, basal catalytic activity of Raf-1 or may be the result of stimulation by the normal cellular signalling machinery already active in growing cells. We observed that the basal activity of CR3 was moderately impaired, but not eliminated, by the loss of the serine 338/339 or tyrosine 340/341 phosphorylation sites. This is consistent with a prior report that a CR3[YY340/341FF] mutant remained capable of transforming fibroblasts [12]. Loss of serine 621 also resulted in only a partial reduction in basal CR3 activity. Alanine substitution of serines 497 and 499 in the activation loop had no effect on the basal CR3 activity. Taken together, these results suggest that a significant portion of the basal CR3 activity measured in COS-7 cells depends upon phosphorylation of residues 338–341 and 621, but not activation-loop phosphorylation.

Full-length Raf-1 can be activated by plasma-membrane-associated Src-family non-receptor tyrosine kinases. Src activation of Raf-1 requires association with Ras at the plasma membrane [21]. It has been suggested that the primary role of Ras in this pathway is to co-localize Raf-1 with active Src-family protein kinases. How tyrosine phosphorylation of Raf-1 actually functions to activate the kinase is unclear. Tyrosines 340 and 341 are distant from the catalytic cleft of the enzyme, suggesting an indirect effect on the catalytic domain, possibly through accessory-protein interactions or distinct conformational effects. Our observation that v-Src is capable of stimulating the free catalytic fragment demonstrates that phosphorylation of one or both of these tyrosines regulates the Raf-1 catalytic domain directly rather than indirectly by promoting the release of the inhibitory N-terminus. The inability of Src to stimulate the activity of the CR3[YY340/341FF] mutant confirmed that these tyrosines are in fact the critical substrate residues of Src (or a Src-dependent tyrosine kinase) within the CR3 domain. We also observed that the presence of the serine 338/339 and serine 621 phosphorylation sites were required for full v-Src stimulation of CR3. The CR3[SS338/339AA] mutant was in fact partially stimulated by vSrc. This result and others indicate that the effects of phosphorylation on serine 338 and tyrosines 340/341 are additive or synergistic. As observed with full-length Raf-1, substitution of tyrosines 340 and 341 with aspartic acids, which mimic constitutively phosphorylated tyrosines, increased the intrinsic activity of the CR3 fragment more than 30-fold. The striking basal activity of this mutant in the absence of any overt external stimulus suggests that tyrosine phosphorylation is the limiting factor for full activation of CR3 in the COS-7 system. How the phosphorylation of serines 338/339 and tyrosines 340/341 mechanistically contribute to Raf-1 regulation remains unknown. Although genetic analysis suggests distinct functions for each of these phosphorylation sites, it is possible that the sequential phosphorylation of these residues is required. In support of this idea, phosphorylation of serine 338 in full-length Raf-1 activated by co-expression of oncogenic Ras and activated Src was recently found to require prior phosphorylation of tyrosine 341 [22]. Once phosphorylated, residues 338–341 might then participate in intra- or intermolecular protein interactions. The CR3[S621A] mutant was observed to be completely insensitive to Src stimulation, even though it possessed moderate basal activity. If the primary function of serine 621 is to provide an anchor point for 14-3-3, this result suggests that the presence of 14-3-3 is essential for stimulation of the basal activity of CR3 by Src.

The phorbol ester PMA is a potent tumour promoter that can generate mitogenic signals through the activation of PKC. Both *in vivo* and *in vitro* studies have shown that PMA activates mitogen-activated protein kinase through PKC and Raf [34,35]. Activation of full-length Raf-1 by PMA requires Ras association [17] and may be only partially affected by phosphorylation of the activation loop. In this study, PMA was shown to be a potent activator of CR3, indicating that phorbol esters can activate Raf-1 directly as well as by increasing Ras-GTP levels. Treatment of CR3-expressing COS-7 cells with PMA increased the wild-type CR3 kinase activity more than 7-fold, which is twice the stimulation achieved by v-Src co-expressed with CR3. PKC was reported previously to activate Raf-1 by directly phosphorylating Raf-1 at serines 497 and 499, located in the L12 activation loop [23,35]. Phosphorylation of the activation loop has been observed to activate many protein kinases, including PKA (cAMP-dependent protein kinase), CDK (cyclin-dependent kinase), RSK (ribosomal S6 kinase) and ERK2 [36,37]. In this study, the CR3[SS497/499AA] mutant had the same intrinsic and v-Src-stimulated activity as wild-type CR3, indicating that phosphorylation of these sites is not universally required for Raf-1 activity. We expected to see a dramatic reduction of PMA-induced activity with this mutant since eliminating these two phosphorylation sites removed the only known PKC sites in the activation loop. Surprisingly, these substitutions only partially reduced the ability of PMA to stimulate CR3 activity, implicating the involvement of other critical residues. Accordingly, the loss of either serines 338/339 or tyrosines 340/341 greatly reduced the ability of PMA to stimulate CR3. We recently reported similar results for full-length Raf-1. As observed with v-Src, the CR3[S621A] mutant was not significantly stimulated by PMA, suggesting dependence upon 14-3-3 or another S621 function. These results are indicative of additive effects of multiple phosphorylation sites within the catalytic domain of Raf-1, all contributing to the total measured catalytic activity. Confirming that PMA was not simply increasing phosphorylation of CR3 by endogenous Src, we found that the activated CR3[YY340/341DD] mutant was further activated 2-fold by exposure to PMA. However, PMA did not further stimulate the CR3-[SYY338/340/341ADD] mutant, raising the possibility that

PMA treatment might lead to an increase in the phosphorylation of S338 through the activation of Pak.

As stated above, we observed that the mutational loss of the C-terminal 14-3-3-binding site, serine 621, resulted in a CR3 mutant with moderate basal activity that was not stimulated further by either v-Src or PMA. Phosphorylation of serine 621 has also been reported to be necessary for the phosphorylation of the adjacent serine at 624, which may also be necessary for full Raf-1 activation [38]. The role of phosphorylated serine 624 is unknown and may in fact be distinct from 14-3-3 association. In our efforts to determine whether or not 14-3-3 was essential for regulation of CR3 in the absence of the N-terminus of the kinase, we directly compared the association of 14-3-3 with CR3 proteins relative to full-length Raf-1. We found that 14-3-3 associated strongly with full-length Raf-1, weakly with wild-type CR3 and not at all with the CR3 14-3-3-binding-site mutants, S621A and S621D. As depicted in Figure 4, the molar ratio of 14-3-3/Raf-1 would be at the most 2:1 assuming that 14-3-3 binds to Raf as a dimer. By densitometric analysis, the molar ratio of 14-3-3/CR3 is approx. 10-fold less or maximally 0.2:1, suggesting that the absence of the high-affinity S259 binding site in CR3 decreases the stability of the CR3–14-3-3 complex, thus compromising detection. This conclusion is supported by earlier evidence showing that 14-3-3 associated strongly with the full-length Raf-1 S621A mutant but not with the Raf-1 S259A mutant [39,40]. We observed that the phosphorylated Raf-1 613–627 peptide (S621), shown previously to displace 14-3-3 from full-length Raf under a variety of conditions and which we showed displaced Raf-1 or CR3[YY340/341DD] from 14-3-3 when the proteins were co-expressed in COS-7 cells, inactivated Src-stimulated CR3 and the active CR3[YY340/341DD] mutant, suggesting a functional role for phosphorylation at the S621 site.

In summary, we have shown that in the absence of the inhibitory N-terminal regulatory domain, phosphorylation sites shown previously to be important for the activation of full-length Raf-1 are required for regulating the Raf-1 catalytic kinase domain. Specifically, serines 338, 339 and 621 and tyrosines 340/341, but not serines 497/499, were found to be required for the intrinsic, v-Src and phorbol ester-stimulated activity of CR3, suggesting that phosphorylation of these sites may regulate kinase activity directly. Alternatively, these sites may affect the interaction of CR3 with regulatory proteins or be required for maintaining an active conformation through intramolecular interactions. It will be important to identify the specific functions of these critical phosphorylation residues to gain more insight into the many complexities of Raf-1 regulation.

We are grateful to Bruce Diaz for his suggestions and technical assistance and to Joseph Avruch for helpful discussions. This study was supported by the American Cancer Society and a research grant from Eli Lilly Co.

## REFERENCES


1 Avruch, J., Zhang, X. F. and Kyriakis, J. M. (1994) Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem. Sci. **19**, 279–283

2 Marshall, M. S. (1995) Ras target proteins in eukaryotic cells. FASEB J. **9**, 1311–1318

3 Heidecker, G., Huleihel, M., Cleveland, J. L., Kolch, W., Beck, T. W., Lloyd, P., Pawson, T. and Rapp, U. R. (1990) Mutational activation of c-raf-1 and definition of the minimal transforming sequence. Mol. Cell. Biol. **10**, 2503–2512

4 Braselmann, S. and McCormick, F. (1995) Bcr and Raf form a complex *in vivo* via 14-13-3 proteins. EMBO J. **14**, 4839–4848

5 Conklin, D. S., Galaktionov, K. and Beach, D. (1995) 14-13-3 proteins associate with cdc25 phosphatases. Proc. Natl. Acad. Sci. U.S.A. **92**, 7892–7896

6 Xing, H., Kornfeld, K. and Muslin, A. J. (1997) The protein kinase KSR interacts with 14-13-3 protein and Raf. Curr. Biol. **7**, 294–300

7 Vincenz, C. and Dixit, V. M. (1996) 14-13-3 proteins associate with A20 in an isoform-specific manner and function both as chaperone and adapter molecules. J. Biol. Chem. **271**, 20029–20034

8 Aitken, A., Howell, S., Jones, D., Madrazo, J., Martin, H., Patel, Y. and Robinson, K. (1995) Post-translationally modified 14-13-3 isoforms and inhibition of protein kinase C. Mol. Cell. Biochem. **149**, 41–49

9 Bonnefoy-Berard, N., Liu, Y.-C., von Willebrand, M., Sung, A., Elly, C., Musteline, T., Yoshida, H., Ishizaka, K. and Altman, A. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 10142–10146

10 Li, S., Janosch, P., Tanji, M., Rosenfeld, G. C., Waymire, J. C., Mischak, H., Kolch, W. and Sedivy, J. M. (1995) Regulation of Raf-1 kinase activity by the 14-13-3 family of proteins. EMBO J. **14**, 685–696

11 Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-13-3 not BCL-XL. Cell **87**, 619–628

12 Morrison, D. K. (1995) Mechanisms regulating Raf-1 activity in signal transduction pathways. Mol. Reprod. Devel. **42**, 507–514

13 Morrison, D. K., Heidecker, G., Rapp, U. R. and Copeland, T. D. (1993) Identification of the major phosphorylation sites of the Raf-1 kinase. J. Biol. Chem. **268**, 17309–17316

14 Tzivion, G., Luo, Z. and Avruch, J. (1998) A dimeric 14-13-3 protein is an essential cofactor for Raf kinase activity. Nature (London) **394**, 88–92

15 Thorson, J. A., Yu, L. W., Hsu, A. L., Shih, N. Y., Graves, P. R., Tanner, J. W., Allen, P. M., Piwnica-Worms, H. and Shaw, A. S. (1998) 14-13-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol. Cell. Biol. **18**, 5229–5238

16 Roy, S., McPherson, R. A., Apolloni, A., Yan, J., Lane, A., Clyde-Smith, J. and Hancock, J. F. (1998) 14-13-3 facilitates Ras-dependent Raf-1 activation *in vitro* and *in vivo*. Mol. Cell. Biol. **18**, 3947–3955

17 Barnard, D., Diaz, B., Clawson, D. and Marshall, M. (1998) Oncogenes, growth factors and phorbol esters regulate Raf-1 through common mechanisms. Oncogene **17**, 1539–1547

18 King, A. J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S. and Marshall, M. S. (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature (London) **396**, 180–183

19 Fabian, J. R., Daar, I. O. and Morrison, D. K. (1993) Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol. Cell. Biol. **13**, 7170–7179

20 Diaz, B., Barnard, D., Filson, A., MacDonald, S., King, A. and Marshall, M. (1997) Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol. Cell. Biol. **17**, 4509–4516

21 Marais, R., Light, Y., Paterson, H. F. and Marshall, C. J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. **14**, 3136–3145

22 Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J. and Marais, R. (1999) Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J. **18**, 2137–2148

23 Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U. R. (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature (London) **364**, 249–252

24 Carroll, M. P. and May, W. S. (1994) Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. J. Biol. Chem. **269**, 1249–1256

25 Morrison, D. K., Kaplan, D. R., Rapp, U. and Roberts, T. M. (1988) Signal transduction from membrane to cytoplasm: growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. **85**, 8855–8859

26 Schonwasser, D. C., Marais, R. M., Marshall, C. J. and Parker, P. J. (1998) Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Mol. Cell. Biol. **18**, 790–798

27 Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell **45**, 721–732

28 Chu, G., Hayakawa, H. and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res. **15**, 1311–1326

29 Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R. and Avruch, J. (1992) Raf-1 activates MAP kinase-kinase. Nature (London) **358**, 417–421

30 Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. **5**, 3610–3616

31 Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F. and Marshall, C. J. (1998) Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science **280**, 109–112

32 Erratum (1998) Science **280**, 987
33 Muslin, A. J., Tanner, J. W., Allen, P. M. and Shaw, A. S. (1996) Interaction of 14-13-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell **84**, 889–897
34 Marquardt, B., Frith, D. and Stabel, S. (1994) Signalling from TPA to MAP kinase requires protein kinase C, raf and MEK: reconstitution of the signalling pathway *in vitro*. Oncogene **9**, 3213–3218
35 Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K. and Ohno, S. (1996) Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J. Biol. Chem. **271**, 23512–23519
36 Marshall, C. J. (1994) Hot lips and phosphorylation of protein kinases. Nature (London) **367**, 686
37 Cutler, Jr, R. and Morrison, D. K. (1997) Mammalian Raf-1 is activated by mutations that restore Raf signaling in *Drosophila*. EMBO J. **16**, 1953–1960
38 Ferrier, A. F., Lee, M., Anderson, W. B., Benvenuto, G., Morrison, D. K., Lowy, D. R. and DeClue, J. E. (1997) Sequential modification of serines 621 and 624 in the Raf-1 carboxyl terminus produces alterations in its electrophoretic mobility. J. Biol. Chem. **272**, 2136–2142
39 Rommel, C., Radziwill, G., Lovric, J., Noeldeke, J., Heinicke, T., Jones, D., Aitken, A. and Moelling, K. (1996) Activated Ras displaces 14-13-3 protein from the amino terminus of c-Raf- 1. Oncogene **12**, 609–619
40 Michaud, N. R., Fabian, J. R., Mathes, K. D. and Morrison, D. K. (1995) 14-13-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-13-3- and Ras-independent manner. Mol. Cell. Biol. **15**, 3390–3397

Received 7 February 2000/26 June 2000; accepted 25 July 2000