Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation

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The Raf family of serine/threonine protein kinases couple growth factor receptor stimulation to mitogen activated protein kinase activation, but their own regulation is poorly understood. Using phospho-specific antisera, we show that activated Raf-1 is phosphorylated on S338 and Y341. Expression of Raf-1 with oncogenic Ras gives predominantly S338 phosphorylation, whereas activated Src gives predominantly Y341 phosphorylation. Phosphorylation at both sites is maximal only when both oncogenic Ras and activated Src are present. Raf-1 that cannot interact with Ras-GTP is not phosphorylated, showing that phosphorylation is Ras dependent, presumably occurring at the plasma membrane. Mutations which prevent phosphorylation at either site block Raf-1 activation and maximal activity is seen only when both are phosphorylated. Mutations at S339 or Y340 do not block Raf-1 activation. While B-Raf lacks a tyrosine phosphorylation site equivalent to Y341 of Raf-1, S445 of B-Raf is equivalent to S338 of Raf-1. Phosphorylation of S445 is constitutive and is not stimulated by oncogenic Ras. However, S445 phosphorylation still contributes to B-Raf activation by elevating basal and consequently Ras-stimulated activity. Thus, there are considerable differences between the activation of the Raf proteins; Ras-GTP mediates two phosphorylation events required for Raf-1 activation but does not regulate such events for B-Raf.

Keywords: B-Raf/Raf-1/Ras/serine phosphorylation/tyrosine phosphorylation

Introduction

The Raf proteins are a family of serine/threonine-specific protein kinases that form part of a conserved signal transduction module in higher eukaryotes. The module consists of receptor tyrosine kinases or heterotrimeric G-protein-coupled 7-trans-membrane receptors; the small guanine nucleotide binding proteins of the p21Ras family; the Raf proteins; the MEK (ERK activator or MAPKK) dual specificity kinases and the serine/threonine specific

extracellular signal-regulated protein kinases (ERKs) (for reviews see Davis, 1994; Marshall, 1995; Robinson and Cobb, 1997). Activated Raf proteins phosphorylate and activate MEKs, which in turn phosphorylate and activate ERKs. Active ERKs phosphorylate and regulate the activities of a number of cytosolic and nuclear proteins. Using versions of the Raf proteins whose activity can be regulated, it has been shown that weak Raf kinase activity causes cell proliferation, whereas strong kinase activity causes cell cycle arrest and senescence (Lloyd *et al.*, 1997; Woods *et al.*, 1997; Zhu *et al.*, 1998). Furthermore, in some cells, transient ERK activation is permissive for proliferation whereas sustained activation leads to differentiation or growth arrest (for review see Marshall, 1995). Thus, by regulating the magnitude or duration of ERK activity, Raf proteins can determine the fundamental responses of cells to extracellular signals. Despite this important regulatory role, the molecular mechanisms by which Raf activities are regulated are still poorly understood.

In mammals, there are three Raf proteins; Raf-1 (or c-Raf), A-Raf and B-Raf. The Rafs are highly conserved within three regions (CR1, CR2 and CR3) (see Magnuson *et al.*, 1994). The N-terminus (CR1 + CR2) encodes a regulatory function and the C-terminus (CR3) contains the kinase domain. Raf-1 is normally located in the cytosol in an inactive state and a key component to its activation is its Ras dependent association with plasma membranes. CR1 of Raf-1 contains a Ras binding domain, which has low affinity for GDP-bound Ras (Ras-GDP), but high affinity for GTP-bound Ras (Ras-GTP). In the presence of activated Ras, Raf-1 translocates to the plasma membrane, where it becomes activated (see Magnuson *et al.*, 1994; Morrison and Cutler, 1997). Indeed, it is possible to elevate Raf-1 activity simply by forcing it to associate with the plasma membrane in a constitutive fashion (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Ligands which activate Ras result in the formation of Ras-Raf-1 complexes (Hallberg *et al.*, 1994; Finney and Herrera, 1995; Marais *et al.*, 1998) and mutations in Raf-1 or Ras which disrupt the Ras-Raf-1 interaction, or agents which inhibit Ras function block Raf-1 activation (Nori *et al.*, 1992; Fabian *et al.*, 1994; Marais *et al.*, 1995, 1998; Lou *et al.*, 1997; Rodriguez-Viciano *et al.*, 1997). However, the interaction between Raf-1 and Ras is not sufficient for activation, because Ras-GTP cannot activate Raf-1 *in vitro* unless the Ras-GTP is membrane bound and an unidentified cytosolic factor is present (Traverse *et al.*, 1993; Zhang *et al.*, 1993; Dent and Sturgill, 1994; Stokoe and McCormick, 1997; Tamada *et al.*, 1997). Furthermore, membrane-targeted Raf-1 can be further activated by ligands or by activated Src independently of the Ras-Raf-1 interaction (Leevers *et al.*, 1994; Marais *et al.*, 1995, 1998). Thus, Raf-1 activation is initiated by Ras-

GTP-mediated translocation to the plasma membrane, but additional membrane-localized events are required for full activation.

There has been much interest in the role that phosphorylation plays in regulating Raf-1 activity. Even in serum starved cells, Raf-1 is phosphorylated and agents which activate Raf-1 lead to hyper-phosphorylation (see Morrison and Cutler, 1997). Sites of serine, threonine and tyrosine phosphorylation have been mapped in Raf-1 (Fabian *et al.*, 1993; Kolch *et al.*, 1993; Morrison *et al.*, 1993; Yao *et al.*, 1995), but gross hyper-phosphorylation of Raf-1 cannot be used as a marker of Raf-1 activation, but rather may be a consequence of activation of the MAPK cascade (Samuels *et al.*, 1993; Alessi *et al.*, 1995). Nonetheless, both serine/threonine and tyrosine phosphorylations appear to play a role in Raf-1 activation, because Raf-1 activity can be abolished by treating Raf-1 with serine/threonine or tyrosine specific protein phosphatases (Kovacina *et al.*, 1990; Turner *et al.*, 1993; Dent *et al.*, 1995; Jelinek *et al.*, 1996).

The role of phosphorylation in Raf-1 activation is complex because both stimulatory and inhibitory sites have been described. There are two 14-3-3 binding sites in Raf-1, the cores of which are formed by the phosphorylation of S259 and S621 (Michaud *et al.*, 1995; Muslin *et al.*, 1996). The binding of 14-3-3 to Raf-1 is essential for activity (Tzivion *et al.*, 1998) and dephosphorylation of Raf-1 disrupts 14-3-3 binding and blocks activity (Michaud *et al.*, 1995). The regulation of Raf-1 activity by 14-3-3 is complicated, because binding of 14-3-3 to the N-terminal site appears to inhibit Raf-1 activity, whereas binding to the C-terminal site is essential for activity (Morrison *et al.*, 1993; Rommel *et al.*, 1996). The levels of phosphorylation of S259 and S621 do not appear to change significantly when Raf-1 is activated by ligands (Morrison *et al.*, 1993). It is therefore not clear what role phosphorylation of S259 and S621 have in the regulated activation of Raf-1. An additional complication is raised by studies that demonstrated that phosphorylation of S621 may be involved in the negative regulation of Raf-1 by protein kinase A (Mischak *et al.*, 1996). Protein kinase A also inhibits Raf-1 activation by phosphorylation of S43 which disrupts the Ras-Raf-1 interaction (Cook and McCormick, 1993; Wu *et al.*, 1993). In some, but not all studies, phosphorylation of residue S499 or S497 by protein kinase C has been reported to increase Raf-1 activity *in vitro* and *in vivo* (Kolch *et al.*, 1993; Carroll and May, 1994; Barnard *et al.*, 1998; Schonwasser *et al.*, 1998). T268 has been reported to be an *in vitro* and an *in vivo* autophosphorylation site (Morrison *et al.*, 1993) and the adjacent amino acid (T269) appears to be the target of a ceramide activated protein (CAP) kinase (Yao *et al.*, 1995). CAP kinase may be the mammalian homologue of the kinase suppressor of Ras (Ksr), identified by genetic methods in *Drosophila melanogaster* and *Caenorhabditis elegans* (Zhang *et al.*, 1997).

The role of tyrosine phosphorylation in Raf-1 activation is also unclear. The activation of Raf-1 by activated Src requires phosphorylation of Raf-1 on Y340 and/or Y341 *in vivo* and *in vitro* (Fabian *et al.*, 1993; Chow *et al.*, 1995; Marais *et al.*, 1995; Stokoe and McCormick, 1997). Furthermore, in a number of studies in hemopoietic (Carroll *et al.*, 1991; Turner *et al.*, 1991, 1993; Popik and Pitha,

1996) and non-hemopoietic cells (Morrison *et al.*, 1989; Kasid *et al.*, 1997), tyrosine phosphorylation and activation of Raf-1 have been shown to be coincident. However, others have been unable to detect phosphotyrosine in active Raf-1 (Baccarini *et al.*, 1990; Blackshear *et al.*, 1990; Kovacina *et al.*, 1990). These data suggest that in different cells Raf-1 is activated by a variety of independent molecular mechanisms, but the results could be misleading because only a small proportion of the total Raf-1 pool is activated in ligand-stimulated cells (Hallberg *et al.*, 1994). Thus, only a small proportion of the Raf-1 will be tyrosine phosphorylated, making detection difficult. Further support for the role of Y340/341 phosphorylation in Raf-1 activation comes from the observations that substitution of Y340/341 for phenylalanines blocks Raf-1 activation by oncogenic Ras and activated Src and by ligand stimulation of cells (Marais *et al.*, 1995; Diaz *et al.*, 1997; Stokoe and McCormick, 1997; Barnard *et al.*, 1998).

Y340/341 are located at the very N-terminus of the catalytic domain, ~20 amino acids upstream of the putative ATP binding domain (see Marais and Marshall, 1996) and it is not known how their phosphorylation regulates Raf-1 activity. However, recent intriguing data have shown that mutation of the two preceding amino acids (S338 and S339) to alanine also blocks Raf-1 activation, implying that phosphorylation of these residues may also regulate Raf-1 activity (Diaz *et al.*, 1997; Barnard *et al.*, 1998). Thus, phosphorylation of this region of Raf-1 appears to be an important regulator of Raf-1 activity and it has recently been shown that Pak3 can phosphorylate S338 of Raf-1 *in vitro* and may phosphorylate S338 *in vivo* when Raf-1 is activated by oncogenic Ras (King *et al.*, 1998).

B-Raf is closely related to Raf-1 and is conserved at the positions equivalent to S338/339 of Raf-1 (S445/446 in B-Raf), but the positions equivalent to Y340/341 of Raf-1 are occupied by aspartic acids (D447/448) in B-Raf (see Marais and Marshall, 1996). B-Raf has elevated basal kinase activity compared with Raf-1, is highly responsive to oncogenic Ras and is not stimulated by activated Src (Marais *et al.*, 1997). Substitution of D447/448 with phenylalanines reduces basal activity but does not block B-Raf activation by oncogenic Ras (Marais *et al.*, 1997). Whether S445/446 are phosphorylated in B-Raf or if phosphorylation has a role in regulating B-Raf activity has not been explored.

In order to address how phosphorylation regulates the activity of the Raf proteins, we have undertaken a systematic study of the potential phosphorylation sites within this region. We show that phosphorylation of S338 and Y341 is required for full Raf-1 activity and describe phospho-specific antisera for these sites. Oncogenic Ras gives predominantly S338 phosphorylation and activated Src gives predominantly Y341 phosphorylation. Both sites must be phosphorylated for full activity, because they cooperate to activate Raf-1. The Ras-Raf-1 interaction is required for both phosphorylation events, suggesting that they occur at the plasma membrane. In contrast, the regulation of B-Raf is rather different. S445 (the B-Raf equivalent of S338 in Raf-1) is constitutively phosphorylated and this phosphorylation, together with the aspartic acids at 447/448 (equivalent to Y340/341 of Raf-1) contributes to the high basal kinase activity of B-Raf. Mutation of these residues to alanine still results in activation by

Ras, but at a much reduced level. Thus, the level of activation of both Raf-1 and B-Raf following a stimulus is dependent on phosphorylation at the N-terminal end of CR3, but the regulation of this phosphorylation differs in Raf-1 and B-Raf.

Results

Activation of Raf-1 by oncogenic Ras and activated Src requires S338 and Y341 phosphorylation

We transiently expressed myc-tagged Raf-1 (mRaf-1) in COS cells and measured its activity using an immunoprecipitation kinase cascade assay. As we have shown (Marais *et al.*, 1997), mRaf-1 activity was low in resting cells, but was strongly stimulated by oncogenic Ras and activated Src (Figure 1A). To examine the role of phosphorylation in the activation of Raf-1, we created single amino acid substitutions in mRaf-1, replacing either S338, S339, Y340 or Y341 with alanine (mRafS338A, mRafS339A, mRafY340A and mRafY341A, respectively). The substitutions at S338 or Y341 reduced activity to ~10% of wild type, whereas those at S339 or Y340 did not affect activity (Figure 1A). Similar results were obtained when Y340 or Y341 was substituted for phenylalanine; mRafY340F was activated, whereas mRafY341F was not (Figure 1A).

As in NIH 3T3 cells (Marais *et al.*, 1995), in serum starved COS cells mRaf-1 was not phosphorylated on tyrosine, but was phosphorylated on serine and threonine (Figure 1B). Coexpression with oncogenic Ras and activated Src resulted in tyrosine phosphorylation in addition to serine and threonine. The levels of phosphotyrosine were not affected by the alanine substitution at Y340, but were significantly reduced by the alanine substitution at Y341. These data show that under these conditions, Y341 is the major site of tyrosine phosphorylation, whereas Y340 is at best a minor phosphorylation site which does not contribute to Raf-1 kinase activity.

The alanine substitution at S338 or S339 had insignificant effects on the total amount of phosphoserine accumulation in mRaf-1 (Figure 1B). Both unactivated and activated mRaf-1 were highly phosphorylated on serine and so we conclude that even in active mRaf-1, S338 and S339 were relatively minor sites of phosphorylation or were not phosphorylated. Since the alanine substitution at S339 did not affect mRaf-1 kinase activity, we conclude that phosphorylation of this residue was not required for kinase activity. Our data argue that phosphorylation of both S338 and Y341, but not S339 or Y340, was required for mRaf-1 activation under the conditions we used. To facilitate analysis of phosphorylation at S338 and Y341, we developed phospho-specific antibodies to these residues.

Rats were immunized with peptide S338*/Y341*, a synthetic peptide corresponding to amino acids 334–347 of Raf-1 in which both S338 and Y341 were phosphorylated (see Materials and methods). One monoclonal antibody (#2d) bound to mRaf-1 that had been activated by oncogenic Ras and activated Src, but did not bind to unactivated mRaf-1 (Figure 2A). The ability of #2d to bind to mRaf-1 was blocked by the immunizing peptide, but not by the unphosphorylated peptide (Figure 2A). A peptide in which S338 alone was phosphorylated

Fig. 1. S338 and Y341 are required for Raf-1 activation. mRaf-1 (Raf-1), mRafS338A (S338A), mRafS339A (S339A), mRafY340A (Y340A), mRafY340F (Y340F), mRafY341A (Y341A) or mRafY341F (Y341F) were transiently expressed in COS cells alone (–) or with oncogenic Ras and activated Src (+Ras/Src) as indicated. (A) Raf kinase assays. Equivalent amounts of mRaf protein were immunoprecipitated with the 9E10 monoclonal antibody for kinase activity determination. The results presented are for one experiment assayed in triplicate, with error bars to represent standard deviations from the mean. The background counts (~4600 c.p.m.; cells transfected with vector controls) were subtracted and the results are expressed relative to wild-type mRaf-1 activated by oncogenic Ras and activated Src (% of wt activity; ~54 800 c.p.m.). Similar results were obtained in five independent experiments. (B) Phosphoamino acid analysis. COS cells were metabolically labelled with [32 P]orthophosphate and the mRaf-1 proteins were immunoprecipitated for phosphoamino acid analysis. A total of 1500 c.p.m. were analysed for each sample and the labelled amino acids were identified by reference to ninhydrin stained standards (P-S, phosphoserine; P-T, phosphothreonine; P-Y, phosphotyrosine).

blocked #2d binding, whereas peptides in which either Y341 or S339 were phosphorylated did not. Finally, #2d binding was blocked by a peptide in which S338 was

Fig. 2. Raf-1 phospho-specific antisera. mRaf-1 was expressed in COS cells alone (–) or with oncogenic Ras and activated Src (+). The mRaf-1 was immunoprecipitated with 9E10 for immunoblot analysis, probing with the phospho-specific antibodies. For competition analysis, the antibodies were pre-incubated with the indicated peptides (8.5 μ M for 2 h at 4°C) prior to addition to the PVDF membranes (control: no peptide). Once the results had been obtained with the phospho-specific antibodies (upper panels), the blots were stripped and reprobed with the 9E10 monoclonal antibody (lower panels). The position of mRaf-1 is indicated by the arrowhead and the positions of standard protein markers are also indicated ($\times 10^{-3}$). (A) Monoclonal antibody #2d (pS338). (B) Serum #256 (pY341).

phosphorylated, but Y341 was substituted for an alanine. Thus, even though the immunizing peptide was phosphorylated on both S338 and Y341, #2d specifically bound to phosphorylated S338, irrespective of the amino acid at position 341. This antibody is henceforth referred to as pS338.

To generate phospho-specific antisera to Y341, rabbits were immunized with peptide Y341*, a synthetic peptide corresponding to amino acids 334–347 of Raf-1 in which only Y341 was phosphorylated. The serum from one of these rabbits (#256) did not bind to unactivated mRaf-1, but did bind to mRaf-1 activated by coexpression with oncogenic Ras and activated Src (Figure 2B). The binding of serum #256 to active mRaf-1 was blocked by the immunizing peptide but not by the unphosphorylated peptide, or when the peptide was phosphorylated on Y340 (Figure 2B). The binding was also blocked by peptides where Y341 was phosphorylated but E343 or I344 were substituted by alanine. However, a peptide in which Y341 was phosphorylated but W342 was substituted by alanine did not block binding of serum #256 to activated Raf-1. Thus, we conclude that serum #256 bound to phosphorylated Y341 in conjunction with W342 and was therefore dependent on and specific for phosphorylation at Y341; this serum is henceforth referred to as pY341. Antibody pS338 did not bind to mRafY338A and serum pY341 did not bind to mRafY341A, even when these proteins were coexpressed with oncogenic Ras and activated Src (see below; Figure 5; data not shown). Thus, each of these reagents only bound to one site in activated mRaf-1.

Phosphorylation of S338 and Y341 occurs at the plasma membrane

Oncogenic Ras and activated Src synergize to activate Raf-1 (Williams *et al.*, 1992; Fabian *et al.*, 1993, 1994; Marais *et al.*, 1995). We have previously studied the regulation of Raf-1 by oncogenic Ras and activated Src and demonstrated that Ras-GTP was required to translocate Raf-1 to the plasma membrane for tyrosine phosphorylation (Marais *et al.*, 1995). A mutant form of Raf-1 in which R89 was substituted by leucine (mR89LRaf-1) was unable to associate with Ras-GTP and so failed to translocate to the plasma membrane to undergo tyrosine phosphorylation. This deficiency was overcome by constitutive targeting of mRaf-1 to the plasma membrane with the Ras membrane localization (CAAX) motif, so that phosphorylation and activation could occur (Figure 3A; Marais *et al.*, 1995).

We wished to determine how oncogenic Ras and activated Src synergized to activate mRaf-1, and so examined how mRaf-1 phosphorylation correlated with activation. Oncogenic Ras stimulated S338 phosphorylation to a greater extent than did activated Src, whereas activated Src stimulated Y341 phosphorylation, but oncogenic Ras did not (Figure 3B). For both sites, maximal phosphorylation was achieved in the presence of both oncogenic Ras and activated Src. mR89LRaf-1 was not phosphorylated on either S338 or Y341 under any of these conditions (Figure 3B). The block in phosphorylation of S338 and Y341 caused by the R89L substitution were overcome by constitutive membrane targeting. mR89LRafCAAX was phosphorylated on S338 even in serum starved cells, but this was further stimulated by oncogenic Ras or activated Src or both (Figure 3B). mR89LRafCAAX was not detectably phosphorylated on Y341 either in serum starved cells, or in cells expressing oncogenic Ras. In the presence of activated Src, however, mR89LRafCAAX was strongly phosphorylated on Y341 and this was not further enhanced by the presence of oncogenic Ras (Figure 3B).

The above data show that oncogenic Ras elevates S338 phosphorylation better than does activated Src, and conversely, activated Src elevates Y341 phosphorylation better than oncogenic Ras. We therefore tested the ability of alanine mutants at these sites to be activated by Ras or Src. Figure 3C shows that in response to oncogenic Ras both mRafS338A and mRafY341A were activated to only ~10% of wild-type Raf-1 levels. Similarly both mutants were activated to ~10% of wild-type activity in response to activated Src. Finally, membrane-localized Raf-1 in which S338 or Y341 was substituted by alanine were not activated by oncogenic Ras or activated Src or both (Figure 3A). Taken together, these data demonstrate that expression with oncogenic Ras mainly leads to S338 phosphorylation, but there is still a requirement for Y341 phosphorylation in activation. Similarly, although Y341 is preferentially phosphorylated in the presence of activated Src, Raf-1 activation still requires S338 phosphorylation. Alanine substitutions at S339 or Y340 did not suppress Raf-1 activation by oncogenic Ras or activated Src (data not shown). Thus, we conclude that the activation of Raf-1 by oncogenic Ras, activated Src or the combination of the two requires phosphorylation on both S338 and Y341.

Fig. 3. Raf-1 phosphorylation occurs at the plasma membrane. mRaf-1 (Raf-1), mR89LRaf-1 (89LRaf), mR89LRafCAAX (89L/CX), mR89LRafS338A/CAAX (89L/338A/CX), mR89LRafY341A/CAAX (89L/341A/CX), mRafS338A (S338A) or mRafY341A (Y341A) were expressed in COS cells alone (-), with oncogenic Ras (Ras), with activated Src (Src) or with both (Ras/Src) as shown. (A) Raf kinase activity assays. These were determined as in Figure 1. The background counts were ~2400 c.p.m. and mRaf-1 activated by oncogenic Ras together with activated Src gave ~30 500 c.p.m. Similar results were obtained in three independent experiments. (B) Phospho-specific antibody blots. Upper panel pair: pS338 monoclonal antibody; lower panel pair: pY341 serum. The samples were processed as in Figure 2. For each pair, the phospho-specific blot image is shown together with its appropriate 9E10 reprobe image. (C) Raf kinase activity assays. These were determined as in Figure 1. The background counts were 3200 c.p.m.; mRaf-1 activated by oncogenic Ras gave 8500 c.p.m. and mRaf-1 activated by activated Src gave 11 500 c.p.m. Similar results were obtained in two independent experiments.

Phosphorylation of S338 and Y341 is required for growth factor stimulation of Raf-1

Having determined that S338 and Y341 phosphorylation is required for both high and low levels of Raf-1 activity induced by oncogenes, we wished to address the role of these sites in growth factor induced activation of Raf-1. Endogenous Raf-1 was readily activated in COS cells treated with epidermal growth factor (EGF) or 12-*O*-

tetradecanoyl-phorbol-13-acetate (TPA) or in NIH 3T3 cells treated with platelet-derived growth factor (PDGF) (Figure 4A). Figure 4B shows that there was a low level of S338 phosphorylation on endogenous Raf-1 in serum-starved cells, but that this was greatly increased following stimulation with EGF, TPA or PDGF. This demonstrates that S338 phosphorylation on endogenous Raf-1, is increased by ligands which lead to Raf-1 activation. Mutation of S338 to alanine significantly impaired the response to growth factors showing that ligand-mediated activation of Raf-1 requires S338 phosphorylation (Figure 4C). Under the conditions where S338 phosphorylation was observed, we were unable to detect phosphorylation of Y341 with serum pY341 (data not shown). However, mRafY341A demonstrated severely impaired responses to EGF or TPA treatment of COS cells and to PDGF treatment of NIH 3T3 cells (Figure 4C). In contrast, mRafS339A and mRafY340A were activated to wild-type kinase levels or better. These results show that phosphorylation of both S338 and Y341 is required for Raf-1 activation when cells are ligand-stimulated.

A functional relationship between S338 and Y341 phosphorylation

The use of the phospho-specific antibodies revealed an interesting relationship between the phosphorylation sites. In Figure 2 we show that the pS338 antibody is able to bind to Raf-1 when S338 was phosphorylated even when Y341 was substituted by alanine. However, pS338 did not bind to mRafY341A which had been coexpressed with oncogenic Ras and activated Src showing that S338 was not phosphorylated in mRafY341A under these conditions (Figure 5A). In contrast, mRafY341A was phosphorylated on S338 when COS cells were treated with EGF or TPA (Figure 5B). This indicates that when Raf-1 is activated by coexpression with oncogenic Ras and activated Src, S338 phosphorylation requires Y341 phosphorylation, whereas when Raf-1 is activated by growth factor stimulation of cells, S338 phosphorylation occurs in the absence of Y341 phosphorylation. One interpretation of these data is that there are at least two S338 kinases, one of which requires prior phosphorylation of the Y341 site and another one which does not.

Phosphorylation of S445 in B-Raf is constitutive and not regulated by Ras

The above data indicate that Raf-1 requires phosphorylation of both S338 and Y341 for activation by oncogenic Ras and/or activated Src as well as by ligand stimulation of cells. B-Raf is closely related to Raf-1 and the position equivalent to S338 of Raf-1 is conserved in B-Raf (S445 in B-Raf; Figure 6A). B-Raf is not responsive to activated Src, but is activated by oncogenic Ras alone (Marais *et al.*, 1997). Since our data show that phosphorylation of S338 of Raf-1 is dependent on Ras and that the kinase is stimulated by oncogenic Ras, we wished to determine whether S445 phosphorylation was required for B-Raf activation.

When S445 and S446 in myc-tagged B-Raf (mB-Raf) were substituted by alanines (mB-Raf445/6A), the basal kinase activity was reduced to ~50% of wild-type levels, but mB-Raf445/6A was still strongly responsive to oncogenic Ras (Figure 6B). Due to the conservation of sequence

between Raf-1 and B-Raf, antibody pS338 was able to bind to mB-Raf when S445 was phosphorylated. pS338 did not bind to mB-Raf when S445 was substituted by alanine (data not shown). Surprisingly, we observed that S445 was constitutively phosphorylated in mB-Raf and there was only a slight increase in S445 phosphorylation when mB-Raf was coexpressed with oncogenic Ras (Figure 6C). We used quantitative immunoblot analysis and found that there was only a 1.5-fold (± 0.3 ; average of three independent determinations) increase in S445 phosphorylation when mB-Raf was coexpressed with oncogenic Ras compared with expression in the absence of Ras. In order to ensure that S445 phosphorylation in the absence of oncogenic Ras was not a result of mB-Raf having been overexpressed in COS cells, we examined S445 phosphorylation in another cell system. We found

that the endogenous B-Raf from serum starved PC12 cells was phosphorylated on S445 to similar levels to mB-Raf which had been expressed in serum starved COS cells (Figure 6D). We therefore conclude that B-Raf is constitutively phosphorylated on S445 and unlike S338 in Raf-1, oncogenic Ras does not stimulate S445 phosphorylation significantly in B-Raf.

The effects of the S445/6A substitutions were similar to our previous observations for the role of D447/8 in regulating B-Raf activity. We demonstrated that substitution of D447/8 for phenylalanines reduced B-Raf basal kinase activity, but did not totally block its ability to respond to oncogenic Ras (Marais *et al.*, 1997). We now show that alanine substitutions for D447/8 (mB-RafD447/8A) also reduced basal kinase activity, but did not block the response to oncogenic Ras (Figure 6B). The D447/8 to alanine substitutions reduced, but did not ablate B-Raf phosphorylation on S445 (data not shown). Finally, when all four amino acids (S445, S446, D447, D448) were substituted by alanines (mB-Raf/A₄), basal kinase activity was further suppressed, but the kinase activity of mB-Raf/A₄ was still stimulated by oncogenic Ras (Figure 6B).

Discussion

Raf-1 activation requires phosphorylation on S338 and Y341

Raf-1 activation is a highly complex process which involves interaction with Ras and 14-3-3 proteins together with phosphorylation events (Morrison and Cutler, 1997; Tzivion *et al.*, 1998). Previous work has suggested that phosphorylation of S338, S339, Y340 and Y341 may be required for activation of Raf-1 (Barnard *et al.*, 1998; Fabian *et al.*, 1993; Marais *et al.*, 1995; Stokoe and McCormick, 1997). We now show that phosphorylation of both S338 and Y341 is required for activity, while S339 or Y340 phosphorylation is not.

In mammalian cells, we find that when Raf-1 is activated by oncogenic Ras and activated Src, the major site of

Fig. 4. S338 and Y341 phosphorylation is required for Raf-1 activation when cells are treated with mitogenic ligands.

(A) Activation of endogenous Raf-1. COS cells or NIH 3T3 cells were stimulated with the indicated ligands (EGF, 100 ng/ml, 20 min; TPA, 40 nM, 60 min; PDGF, 50 ng/ml, 5 min) and the endogenous Raf-1 was immunoprecipitated with a Raf-1 monoclonal antibody for kinase activity assays. The background counts (irrelevant antibody) were ~20 000 c.p.m. Similar results were obtained in three independent assays. (B) Raf-1 is phosphorylated on S338. Endogenous Raf-1 was immunoprecipitated from serum starved cells (Cont), or from COS cells treated with EGF or TPA, or from NIH 3T3 cells treated with PDGF as shown and subjected to immunoblotting with the pS338 monoclonal antibody (upper panel). A control blot, prepared with the same samples was immunoblotted in parallel with the α-Raf-1 monoclonal antibody (lower panel). (C) mRaf-1 kinase activities. These were as determined in Figure 1. The indicated mRaf-1 proteins (see Figure 1) were transiently expressed in serum starved COS cells or NIH 3T3 cells and the cells were treated with EGF, TPA or PDGF as shown. The mRaf-1 proteins were immunoprecipitated with 9E10 for Raf kinase activity assays. The background counts (vector only controls from serum starved cells) were ~10 400 c.p.m. for the COS cells and ~11 400 for the NIH 3T3 cells; mRaf-1 activated by EGF gave ~60 000 c.p.m.; mRaf-1 activated by TPA gave ~42 400 c.p.m.; mRaf-1 activated by PDGF gave ~50 200 c.p.m. Similar results were obtained in two independent experiments.

Fig. 5. Ras and Src dependent phosphorylation of S338 requires Y341 phosphorylation. The indicated mRaf-1 proteins (see Figure 1) were transiently expressed in serum starved COS cells alone (-) or with oncogenic Ras and activated Src (Ras/Src), or the cells were stimulated with EGF or TPA as indicated. The mRaf-1 proteins were immunoprecipitated for immunoblotting with the pS338 antibody. Each blot was stripped and reprobed with the 9E10 monoclonal antibody as shown. The position of migration of mRaf-1 is shown by the arrowhead. (A) mRaf-1 coexpressed with oncogenic Ras and activated Src. (B) mRaf-1 from cells treated with EGF or TPA.

tyrosine phosphorylation is Y341. Previous work to identify tyrosine phosphorylation sites on Raf-1 over-expressed with Src in insect cells identified Y340 as the major site of tyrosine phosphorylation (Fabian *et al.*, 1993). However, our mutational analysis together with the generation of the pY341 specific antibodies demonstrates that Y341 is the major site of tyrosine phosphorylation site when Raf-1 is coexpressed with oncogenic Ras and activated Src in mammalian cells. Presumably this reflects differences in Raf-1 regulation in insect versus mammalian cells. In contrast to mammalian cells, the tyrosine phosphorylation of Raf-1 when over-expressed in insect cells is independent of the Ras-Raf interaction (Chow *et al.*, 1995).

Using phospho-specific antibodies for S338 and Y341, we have shown that when Raf-1 was activated by oncogenic Ras and Src, both S338 and Y341 were phosphorylated. We were also able to detect S338 phosphorylation when Raf-1 was activated by oncogenic Ras, or activated Src, or when activated following ligand stimulation of COS or NIH 3T3 cells. We could not detect Y341 phosphorylation on Raf-1 activated by oncogenic Ras alone or when cells were stimulated with a series of ligands. However, mutation of Y341 to phenylalanine blocked activation of Raf-1 by oncogenic Ras and activated Src and the alanine mutation blocked activation by all stimuli. While the effect of amino acid substitution at Y341 could be structural, this is unlikely given the conservative nature of the change to phenylalanine. Furthermore, substitution of the adjacent residue, Y340, with alanine or phenylalanine does not block kinase activity

Fig. 6. B-Raf is phosphorylated on S445. (A) Comparison of the amino acid sequences of Raf-1, B-Raf and Src. The amino acid sequence of Raf-1 from amino acid 334 to 345 is shown, together with the corresponding sequence from B-Raf (amino acids 441-452) and Src (amino acids 252-263; taken from Hesketh, 1994). S338 and Y341 of Raf-1 are indicated with * and the amino acid identities between Raf-1 and B-Raf are shown in bold. The amino acid identities between B-Raf and Src are underlined in the Src sequence and tryptophan 260 of Src is marked by Δ₂₆₀. (B) B-Raf kinase activity assays. These were performed as for mRaf-1 (see Figure 1). mB-Raf (mB-Raf), mB-RafS445/6A (445/6A), mB-RafD447/8A (447/8A) or mB-RafS445/6/D447/8A (B-Raf/A₄) was expressed in COS cells alone (-) or together with oncogenic Ras (+) and immunoprecipitated with 9E10 for kinase activity assay. Background counts were ~2200 c.p.m. and mB-Raf activated by oncogenic Ras gave ~60 500 c.p.m. (C) mB-Raf is phosphorylated on S445. mB-Raf was transiently expressed in COS cells and immunoprecipitated with 9E10 for immunoblotting with antibody pS338 (upper panel). The blot was stripped and reprobed with the 9E10 monoclonal antibody (lower panel). (D) Endogenous B-Raf is constitutively phosphorylated on S445. Exogenously expressed mB-Raf was immunoprecipitated from COS cells and endogenous B-Raf was immunoprecipitated from PC12 cells, using a B-Raf specific polyclonal antiserum. Identical sets of samples were separated on SDS gels for immunoblot analysis with the pS338 monoclonal antibody (upper panel) or the B-Raf specific polyclonal antiserum (lower panel).

and when Y341 is substituted with aspartic acid Raf-1 is readily activated by Ras (Marais *et al.*, 1995). These data argue that Y341 phosphorylation does occur when Raf-1 is activated by stimuli other than Src, but that these levels of phosphorylation were below our limits of detection. The difficulty in detecting Y341 phosphorylation may result from the activity of phosphatases, since it could

Fig. 7. A model to illustrate how Raf-1 is activated. (A) In resting cells, Raf-1 is located in the cytosol, is not phosphorylated on S338 or Y341 and is inactive. (B) In the presence of oncogenic Ras, Raf-1 is recruited to the plasma membrane and is highly phosphorylated on S338. However, the endogenous tyrosine kinases have low activity and so Y341 phosphorylation is low. (C) When only activated Src is present, Raf-1 translocation to the membrane relies on endogenous Ras, which is activated by Src. S338 phosphorylation is low, but Y341 phosphorylation is high on the Raf-1 proteins which are localized at the membrane. (D) When oncogenic Ras and activated Src are both present, Raf-1 is translocated to the plasma membrane efficiently and tyrosine kinase activity is high. Thus, both S338 and Y341 are phosphorylated to high levels and Raf-1 is fully active.

readily be detected when cells were treated with sodium pervanadate (C.S.Mason, S.Griffiths and R.Marais, unpublished data).

Both S338 and Y341 phosphorylation require Ras–Raf-1 interaction and membrane localization

We have previously shown that tyrosine phosphorylation of Raf-1 in the presence of activated Src is dependent on the Ras–Raf-1 interaction to recruit Raf-1 to the plasma membrane (Marais *et al.*, 1995). Therefore, as expected, Y341 phosphorylation was dependent on the Ras–Raf-1 interaction (Figure 3). Similarly when Raf-1 was prevented from associating with the plasma membrane by introduction of the R89L mutation, S338 phosphorylation was prevented. Targeting R89LRaf-1 to the plasma membrane with a CAAX motif restored S338 phosphorylation (Figure 3B) suggesting that S338 phosphorylation takes place at the plasma membrane. While the S338 kinase appeared to have some basal activity in COS cells since R89LRafCAAX was phosphorylated even in serum starved cells, the kinase could be further stimulated by oncogenic Ras (Figure 3). Activated Src also enhanced S338 phosphorylation. These results suggest that the activity of the S338

kinase is itself regulated. Recently, it has been shown that Pak3 is a candidate S338 kinase and that dominant negative versions of this protein will block Raf-1 activation by oncogenic Ras *in vivo* (King *et al.*, 1998). Pak3 is activated by the plasma membrane-localized small GTP-binding proteins cdc42 and Rac and so our data showing that S338 phosphorylation takes place at the membrane support the idea that Pak3 may be a S338 kinase. The observation that S338 phosphorylation requires Y341 phosphorylation when Raf-1 is activated by oncogenic Ras and activated Src, but not when cells are stimulated with ligands suggests that there may be multiple S338 kinases (Figure 5). We are currently examining whether Pak3 is the kinase that phosphorylates S338 in ligand-stimulated cells.

Phosphorylations on S338 and Y341 cooperate to activate Raf-1

Our data show that oncogenic Ras leads predominantly to S338 phosphorylation, whereas activated Src leads predominantly to Y341 phosphorylation. However, when either S338 or Y341 phosphorylation were prevented by the introduction of non-phosphorylatable amino acids Raf-1 activity was reduced to ~10% of wild-type Raf-1

levels. We have argued above that mutation of Y341 to phenylalanine is unlikely to disrupt the Raf-1 structure and similar considerations hold for the substitutions at S338. Raf-1 was able to tolerate a serine for alanine substitution at position 339 and substitution of S338 with aspartic acid residues leads to Raf-1 forms with elevated basal kinase activity that can still be activated by Ras (our unpublished data; Diaz *et al.*, 1997). These data argue against the S338 and Y341 substitutions causing gross structural changes in the Raf-1 protein. Thus we conclude that phosphorylation of both S338 and Y341 are required for activation of Raf-1 by all the stimuli we have examined. Since phosphorylation of both S338 and Y341 is required for Raf-1 activation, we propose that they cooperate to give Raf-1 activation. The levels of kinase activity will therefore depend on the proportion of Raf-1 molecules that are phosphorylated at both sites. We suggest that for activators such as Ras, Y341 phosphorylation is the limiting step to activation, while for activators such as Src, S338 phosphorylation is the limiting step (see Figure 7). Only in the presence of both Ras-GTP and activated Src will a high proportion of Raf-1 molecules be phosphorylated at both S338 and Y341 (Figure 7). This may explain why Ras and Src synergize to activate Raf-1 (Williams *et al.*, 1992; Fabian *et al.*, 1993, 1994; Marais *et al.*, 1995). Ras activates the S338 kinase and recruits Raf-1 to the plasma membrane to permit its phosphorylation at S338 and Y341. In previous work we have shown that the activation of A-Raf by Ras and Src resembles the pattern seen with Raf-1 rather than B-Raf (Marais *et al.*, 1997). Preliminary studies suggest that A-Raf activity is regulated by phosphorylation in a similar manner to Raf-1 (C.S.Mason and R.Marais, unpublished data).

Since both S338 and Y341 phosphorylations are required for activation of Raf-1, it is interesting to consider how B-Raf is activated. Unlike Raf-1, B-Raf does not require tyrosine phosphorylation for activation, and does not have tyrosines at the positions equivalent to Y340/341 of Raf-1; in B-Raf, aspartates occupy these positions (D447 and D448; Figure 6A). These acidic residues seem to regulate some aspect of B-Raf activity since replacing them with phenylalanine or alanine reduces B-Raf activation by Ras (Figure 6; Marais *et al.*, 1997). S445 in B-Raf is equivalent to S338 in Raf-1 and we have shown S445 to be phosphorylated. However, unlike Raf-1, phosphorylation at S445 in B-Raf is constitutive and if stimulated by Ras, the effect is small. Furthermore, whereas mutation of S338 of Raf-1 to alanine destroys its ability to be activated, mutation of S445 in B-Raf still permits a degree of activation. The striking effect of the S445 and D447/448 substitutions in B-Raf is the reduction they cause in basal kinase activity (Figure 6), which is normally 15–20 times higher than that of an equivalent amount of Raf-1 (Marais *et al.*, 1997). Thus phosphorylation at S445 and the aspartic acids at positions 447/448 are responsible for the high basal kinase activity of B-Raf. However, phosphorylation at S445 is required for maximal B-Raf activity since the activity of B-Raf will be determined both by basal kinase activity and stimulation by Ras.

These studies identify similarities and differences between the regulation of Raf-1 and that of B-Raf. Maximal activation of both kinases requires negative

charges in a region at the N-terminus of the catalytic CR3 domain. This region is located 15–18 amino acids before the start of the glycine-rich nucleotide binding P-loop (Hanks subdomain I; Hanks *et al.*, 1988). We call this the Negative-charge regulatory region or N-region. Our studies show that in Raf-1, these negative charges are provided by Ras dependent phosphorylation at S338 and Y341, while in B-Raf negative charges are provided by aspartic acid residues at D447/448 and constitutive, Ras-independent, S445 phosphorylation. At least one other kinase is regulated by phosphorylation within a similar region. In the TGF- β type I receptor (TGF β R-I), the GS motif is located immediately N-terminal to the kinase domain and its phosphorylation by TGF β R-II is required for activation of the TGF β R-I kinase domain (Franzen *et al.*, 1995; Wieser *et al.*, 1995). As in Raf-1, when serine residues in the GS motif are mutated to aspartic acid to mimic phosphorylation, the receptor has elevated kinase activity (Wieser *et al.*, 1995). Thus, Raf-1/B-Raf and TGF β R-I are regulated by phosphorylation within the same region, but how do these phosphorylation events regulate kinase activity? A clue to this may come from an intriguing conservation of sequence between B-Raf and the non-receptor tyrosine kinase Src.

Structural studies with Src kinase family members have shown that in inactive Src, residue Y527 is phosphorylated and so forms an intramolecular interaction with its own SH2 domain (Sicheri *et al.*, 1997; Williams *et al.*, 1997; Xu *et al.*, 1997). This allows the SH3 domain to interact with proline residues within the SH2-kinase linker. Tryptophan 260 (W260) of Src is located at the C-terminal end of the SH2-kinase linker and interacts directly with the C α -helix which is located within the small lobe of the kinase domain (Gonfloni *et al.*, 1997). These interactions keep Src in a 'closed conformation', constraining the C α -helix so that the glutamate at position 310 (E310) is unable to coordinate with the lysine at position 295 (K295). Residues equivalent to E310 and K295 are conserved in all kinases and their correct alignment is required for the phosphotransfer reaction. For inactive Src to become active, this requires realignment of the C α -helix, which involves displacement of the SH3 domain and is favoured by phosphorylation of Y416 and dephosphorylation of Y527 (Gonfloni *et al.*, 1997; Moarefi *et al.*, 1997; Sun *et al.*, 1998). Together with the aid of phosphorylation at Y416, the C α -helix is repositioned to give a productive interaction between E310 and K295.

When their primary amino acid sequences are aligned, the SH2-kinase linker region of Src and the GS motif of TGF β R-I are located in precisely the same position, relative to the kinase domain, as the N-regions in the Raf proteins (see Hesketh, 1994). Recently, the solution of the crystal structure of the TGF β R-I has revealed that phosphorylation of the GS region is likely to affect the position of the C α -helix (Huse *et al.*, 1999). Furthermore, there is considerable sequence identity between B-Raf and Src within this region and importantly, the position equivalent to W260 of Src is conserved in the Raf proteins (see Figure 6A). We therefore propose that the N-regions of the Raf kinases fulfill a similar function to the SH2-kinase linker region of Src. In our model, negative charges in the N-region of Raf-1/B-Raf favour the reorientation of their C α -helices to permit productive interaction

between the Raf equivalents of Src residues E310 and K295 (E393 and K375 of Raf-1). Thus in inactive Raf-1, when S338 and Y341 are not phosphorylated, the region undergoes alternative interactions that favour the inactive state of the catalytic domain. In B-Raf, however, the negative charges provided by the aspartic acids and constitutive phosphorylation of S445 favour a more productive orientation of the C α -helix. This may account for the high basal activity of B-Raf.

Although phosphorylation of S338 and Y341 in the N-region of Raf-1 is Ras dependent, Ras has at least one other role in Raf-1 activation. When S338 and Y341 are replaced with aspartic acid residues to mimic phosphorylation Ras still activates the substituted kinase (C.S.Mason and R.Marais, unpublished data). In effect these aspartic acid substitutions in Raf-1 mimic the situation in B-Raf and like B-Raf, Raf-1 with aspartic acid residues substituted for S338 or Y341 has high basal kinase activity (Marais *et al.*, 1995; Diaz *et al.*, 1997). Thus we propose that negative charge in the N-region has a permissive role to provide the correct alignment of residues within the kinase domain so that when Raf-1 interacts with Ras-GTP, the kinase can be activated. It is not clear how the interaction with Ras elevates Raf-1 activity; it may depend on membrane recruitment since Ras has only a small effect on the activation of membrane-bound RafCAAX (Marais *et al.*, 1995). This membrane-associated event may be phosphorylation independent as postulated by Stokoe and McCormick and involve lipid interactions with Raf, possibly within the cysteine-rich domain, or within the catalytic domain (Ghosh *et al.*, 1994; Mott *et al.*, 1996; Stokoe and McCormick, 1997).

Materials and methods

Cell culture and Raf assays

The expression vectors for mRaf-1, mB-Raf, oncogenic Ras [Ha-Ras(G12R)] and activated Src [Src(Y527F)] have been described (Marais *et al.*, 1997). Alanine and phenylalanine substituted expression constructs were generated by using the PCR with appropriate primers (Ho *et al.*, 1989); all mutants were verified by BigDye™ Terminator Cycle Sequencing on an ABI Prism™ 377 DNA sequencer (PE Applied Biosystems). COS cells were maintained and transfected with lipofectAMINE™ (Life Technologies Inc) as described (Marais *et al.*, 1997). Cell extractions were as described (Marais *et al.*, 1997). mRaf-1 was immunoprecipitated with the 9E10 monoclonal antibody (Evan *et al.*, 1985); endogenous Raf-1 was immunoprecipitated with a Raf-1 monoclonal antibody (#R19120, Transduction Laboratories); mB-Raf or endogenous B-Raf was immunoprecipitated with either 9E10, or a rabbit polyclonal antibody specific for a unique peptide from B-Raf (K.Maycroft and R.Marais, unpublished). Raf kinase assays were performed by immunoprecipitation kinase cascade assay using G α -Mek, G α -ERK and myelin basic protein together with [γ -³²P]ATP as sequential substrates as described (Marais *et al.*, 1997). For phosphoamino acid analysis, transfected cells were metabolically labelled with [³²P]orthophosphate and the ³²P-labelled mRaf-1 proteins were analysed as described previously (Marais *et al.*, 1995).

Peptides and antibodies

Peptides were synthesized on an ABI 433A batch peptide synthesizer (Applied Biosystems Inc) using solid phase methodology on *p*-alkoxybenzyl alcohol resin using fluorenylmethoxycarbonyl (Fmoc)-based protocols, according to the manufacturer's instructions. Phosphorylated amino acid precursors (Bachem, Switzerland) were incorporated at the appropriate positions during synthesis. The peptides were analysed by electrospray mass spectrometry (TSQ 700 Triple sector quadrupole system fitted with an electrospray ion source, Finnigan Mat, USA) and by matrix assisted laser desorption/ionization mass spectrophotometry on a Finnigan LAZERMAT 2000 (Finnigan Mat, UK). The amino acid compositions and peptide concentrations were verified by amino acid

analysis on a 420A Amino Acid Derivatizer (PE-Applied Biosystems). The sequences of the peptides used are (single amino acid code where Y* and S* are phosphorylated tyrosine and serine, respectively):

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un-phos:      GQRDSSYYWEIEAS
Y341*:        GQRDSSYY*WEIEAS
Y340*:        GQRDSSYY*YWEIEAS
Y341*/W342A: GQRDSSYY*AEIEAS
Y341*/E343A: GQRDSSYY*WAIEAS
Y341*/I344A: GQRDSSYY*WEAEAS
S338*/Y341*  GQRDS*SY*WEIEAS
S338*:        GQRDS*SY*WEIEAS
S339*:        GQRDSS*YYWEIEAS
S338*/Y341A: GQRDS*SY*WEIEAS
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Peptides Y341* and S338*/Y341* were coupled to keyhole limpet haemocyanin (#374817, Calbiochem) using glutaraldehyde (G-5882, Sigma) for immunogens in standard protocols (Harlow and Lane, 1988).

Immunoblotting

The relative concentrations of the mRaf proteins were determined by quantitative immunoblotting using the 9E10 monoclonal antibody and developed with [¹²⁵I]protein A (#IM 144, Amersham International) in conjunction with a PhosphorImager (Molecular Dynamics). Equal amounts of mRaf proteins were immunoprecipitated with 9E10 and separated on SDS gels for blotting to Immobilon-P membranes (#IPVH00010, Millipore) as described previously (Marais *et al.*, 1995). The membranes for probing with pS338 were blocked in 5% fat-free milk powder (Marvel) in Tris-buffered saline (TBS)/Triton (20 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100; overnight, 4°C) and incubated with pS338 (2 µg/ml, 2 h, room temperature). They were then incubated with rabbit α -rat antibodies (R-9255, Sigma) and either developed with α -rabbit-HRP and ECL (RPN 2106, Amersham International) or with [¹²⁵I]protein A for quantitation on a PhosphorImager. The membranes for probing with pY341 were blocked in 1% bovine serum albumin (A-7638, Sigma) in TBS/Triton (overnight, 4°C), incubated with serum pY341 (diluted 1:500, 2 h, room temperature) and developed with donkey α -rabbit-HRP (#711-035-152, Stratech Scientific) and ECL. For re-probing with the 9E10 monoclonal antibody, the membranes which had been developed by ECL were incubated in strip buffer (2% w/v SDS, 62.5 mM Tris-HCl, 100 mM 2-mercaptoethanol pH 6.8) at 55°C for 1 h, washed briefly in TBS/Triton and re-incubated in 5% fat-free milk powder and developed with 9E10.

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