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Activation of c-Raf-1 by Ras and Src through different mechanisms: activation *in vivo* and *in vitro*

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The c-Raf-1 protein kinase plays a critical role in intracellular signaling downstream from many tyrosine kinase and G-protein-linked receptors. c-Raf-1 binds to the proto-oncogene Ras in a GTP-dependent manner, but the exact mechanism of activation of c-Raf-1 by Ras is still unclear. We have established a system to study the activation of c-Raf-1 *in vitro*. This involves mixing membranes from cells expressing oncogenic H-RasG12V, with cytosol from cells expressing epitope-tagged full-length wild-type c-Raf-1. This results in a fraction of the c-Raf-1 binding to the membranes and a concomitant 10- to 20-fold increase in specific activity. Ras was the only component in these membranes required for activation, as purified recombinant farnesylated K-Ras.GTP, but not non-farnesylated K-Ras.GTP or farnesylated K-Ras.GDP, was able to activate c-Raf-1 to the same degree as intact H-RasG12V membranes. The most potent activation occurred under conditions in which phosphorylation was prohibited. Under phosphorylation-permissive conditions, activation of c-Raf-1 by Ras was substantially inhibited. Consistent with the results from other groups, we find that the activation of c-Raf-1 by Src *in vivo* occurs concomitant with tyrosine phosphorylation on c-Raf-1, and *in vitro*, activation of c-Raf-1 by Src requires the presence of ATP. Therefore we propose that activation of c-Raf-1 by Ras or by Src occurs through different mechanisms.

Keywords: c-Raf-1/membranes/phosphorylation/Ras/Src

Introduction

c-Raf-1 is a member of a protein kinase family including A-Raf and B-Raf, which play a crucial role in the transmission of signals initiated at the plasma membrane by a variety of signals, including growth factors, oncogenes and differentiating agents (reviewed in Avruch *et al.*, 1994; Rapp *et al.*, 1994). Raf proteins share three conserved regions, CR1, CR2 and CR3, the latter of which is the kinase domain itself. The N-terminus of c-Raf-1, which includes the CR1 domain, was shown to bind specifically and with high affinity to the GTP-bound form of Ras *in vitro* (Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993), and Ras/Raf complexes have been isolated from stimulated cells (Finney *et al.*, 1993; Hallberg *et al.*, 1994). Activated Raf proteins are able to phosphorylate and activate the dual specificity kinases MEKs 1 and 2 (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Wu *et al.*, 1996), which in turn phosphorylate and activate ERKs 1 and 2 (Crews *et al.*, 1992; Nakielny *et al.*, 1992; MacDonald *et al.*, 1993). Active ERKs are thought to elicit many of the effects of extracellular signals, through the phosphorylation of a number of key substrates including other protein kinases, transcription factors and cytoskeletal proteins (reviewed in Davis, 1993).

The exact mechanisms involved in the activation of c-Raf-1 by Ras and/or other signals remain inconclusive. Studies using a variety of overexpression systems have demonstrated that when full-length c-Raf-1 is expressed alone, it has relatively low activity. However, co-expression of c-Raf-1 with either Ras or activated pp60src causes a significant increase in c-Raf-1 activity, and expression of all three proteins results in a synergistic activation of c-Raf-1 activity as assayed either by autophosphorylation or phosphorylation of MEK (Williams *et al.*, 1992; Fabian *et al.*, 1993; Marais *et al.*, 1995). c-Raf-1 activation induced by activated pp60src is accompanied by tyrosine phosphorylation of c-Raf-1 residues 340 and 341 (Fabian *et al.*, 1993; Marais *et al.*, 1995), which is at least partially responsible for activation under these conditions, as mutation of these amino acids to acidic residues results in a partially active kinase which cannot be further activated by pp60src, and mutation to residues which cannot be phosphorylated results in a kinase which can no longer be activated by pp60src (Fabian *et al.*, 1993; Marais *et al.*, 1995). However, the significance of tyrosine phosphorylation in the activation of c-Raf-1 under physiological conditions remains unclear, as the appearance of tyrosine phosphorylation on endogenous c-Raf-1 following activation has been disputed (Baccarini *et al.*, 1990; Blackshear *et al.*, 1990; Kovacina *et al.*, 1990; Marais *et al.*, 1995), and may be limited to cells of hematopoietic origin (Turner *et al.*, 1994).

Less clear however, is the mechanism by which Ras activates c-Raf-1. Direct binding of the two purified proteins has been shown to be insufficient to activate c-Raf-1 (Traverse *et al.*, 1993; Zhang *et al.*, 1993). However, co-expression of the two proteins *in vivo* causes the recruitment of c-Raf-1 to the plasma membrane where activation takes place (Traverse *et al.*, 1993; Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Indeed, all of the c-Raf-1 activity isolated from such cells is associated with the plasma membrane (Stokoe *et al.*, 1994; Wartmann and Davis, 1994). Consistent with the idea that a component in the plasma membrane is responsible for the activation of c-Raf-1, targeting of c-Raf-1 to cell membranes by addition of Ras prenylation sequences, is sufficient to activate c-Raf-1 independently of Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). One possibility for the activation event could be through phosphorylation. In addition to the

two tyrosine residues mentioned above, c-Raf-1 is also phosphorylated on a multitude of serine and threonine residues *in vivo*, including Ser43, Ser259, Ser499 and Ser621 (Kolch *et al.*, 1993; Morrison *et al.*, 1993), although only phosphorylation on Ser259 has been shown to increase upon c-Raf-1 activation *in vivo* (Morrison *et al.*, 1993). This residue has subsequently been shown to be critical for the interaction of c-Raf-1 with 14-3-3 proteins (Michaud *et al.*, 1995; Muslin *et al.*, 1996), although mutation to alanine does not inhibit c-Raf-1 activity, and may actually increase c-Raf-1 activity marginally (Morrison *et al.*, 1993; Michaud *et al.*, 1995). An increase in B-Raf phosphorylation is also seen following prolonged stimulation with growth factors, and is accompanied by an upward mobility shift, but the time course of this is delayed with respect to B-Raf activation (Traverse and Cohen, 1994). Nevertheless, it has been recently suggested that activation of c-Raf-1 by Ras does occur as a result of phosphorylation, both on tyrosine and serine/threonine residues. Addition of either serine/threonine-specific or tyrosine-specific phosphatases to c-Raf-1 isolated from c-Raf-1 and Ras-infected Sf9 cells, was shown to decrease the activity of c-Raf-1, and the activity of c-Raf-1 isolated from Ras-transformed NIH3T3 cell membranes was decreased upon incubation with a tyrosine-specific phosphatase (Dent *et al.*, 1995a; Jelinek *et al.*, 1996).

Here, we establish an *in vitro* system to study the activation of c-Raf-1 by Ras. This approach gives a clearer interpretation than studying c-Raf-1 activated *in vivo*, as additional modifications may occur following several days or weeks in culture, for example via autocrine loops. These secondary modifications may exert additional levels of control on c-Raf-1 activity, while having little to do with the initial activating event. Our results suggest that the activation of c-Raf-1 by Ras is not due to phosphorylation, and that Ras is the only component in the plasma membrane required for this activation. The data obtained are consistent with a critical role of tyrosine phosphorylation in the activation of c-Raf-1 by Src; however, in the system we have developed, serine/threonine phosphorylation of c-Raf-1 seems to be more important in the down-regulation, rather than up-regulation, of its activity.

Results

Activation of Raf in vitro by Ras membranes

As shown previously (Leevers *et al.*, 1994; Stokoe *et al.*, 1994), transient transfection of epitope- (myc-) tagged c-Raf-1 into COS1 cells resulted in a predominantly cytosolic localization of c-Raf-1, which possessed low levels of activity, whereas co-transfection with HRasG12V resulted in a partial translocation of c-Raf-1 to the membrane, where c-Raf-1 was activated (Figure 1A). To determine whether this activation can be reproduced *in vitro*, the cytosolic fraction of COS1 cells transiently expressing c-Raf-1 was mixed with the membrane fraction from COS1 cells transiently expressing H-RasG12V. As shown in Figure 1B, membranes from H-RasG12V transfected cells bound a significantly greater proportion of c-Raf-1 than control membranes (lane 4 versus lane 2). Significantly, the c-Raf-1 associated with the H-RasG12V membranes, but not the control membranes, was activated (Figure 1B). Similar activation was seen whether c-Raf-1 was assayed using direct phosphorylation of 'kinase-dead' MEK, or when c-Raf-1 activity was assayed by its ability to activate recombinant MEK (our unpublished data). As the latter assay is more specific for c-Raf-1 activity it was used routinely in subsequent experiments. Comparable with the c-Raf-1 activity seen upon co-transfection with H-RasG12V (Figure 1A), the activity of c-Raf-1 activated *in vitro* was entirely associated with the H-RasG12V membranes, despite the majority of the c-Raf-1 protein remaining unbound. The specificity of the interaction was demonstrated by preincubation of the H-RasG12V membranes with a glutathione (GST) fusion protein containing the Ras binding domain (RBD) of c-Raf-1, or by preincubation with the Ras monoclonal antibody Y13-259, which competes with c-Raf-1 for binding to the effector domain of Ras. Both proteins abolished the binding and activation of c-Raf-1 by H-RasG12V membranes (Figure 2A). In contrast, pre-incubation of the H-RasG12V membranes with the Ras monoclonal antibody Y13-238 did not affect either the binding or activation of c-Raf-1. Two mutations in the N-terminus of c-Raf-1 have previously been shown to reduce the binding to Ras, namely R89L (Fabian *et al.*, 1994) and C168S (Zhang *et al.*, 1993; Hu *et al.*, 1995). As shown in Figure 2B, either of these mutations abolished the binding and activation of c-Raf-1 by H-RasG12V membranes in this system.

Ras is the only protein in the membranes required for Raf activation

The system described above utilized crude cytosol and membrane components. However, in agreement with other studies (Zhang *et al.*, 1993), addition of purified recombinant GTP-loaded K-Ras was unable to activate purified recombinant c-Raf-1 (our unpublished data). Therefore experiments were performed to determine which additional components were required for activation. Purification of c-Raf-1 away from additional cytosolic components by a variety of biochemical techniques abolished the ability to be activated by H-RasG12V membranes. Also, addition of cytosol to recombinant c-Raf-1 did not support activation by H-RasG12V membranes (our unpublished data). However, addition of purified, prenylated recombinant K-Ras loaded with GTPγS to control membranes, supported the binding and activation of c-Raf-1 in cytosol to the same extent as H-RasG12V membranes (Figure 3A, lane 3). Denaturation of integral membrane proteins by heating, protease treatment, or by addition of chloroform/methanol abolished the ability of H-RasG12V membranes to activate c-Raf-1 (Figure 3A, lanes 4 and 5). However, the same treatment of control membranes, followed by addition of K-Ras.GTPγS, resulted in binding and activation of c-Raf-1 to the same extent as intact H-RasG12V membranes (Figure 3A, lanes 6–8). These experiments excluded the possibility that an integral membrane protein was required for activation. To determine the requirements for membrane lipids, K-Ras.GTPγS was added to a reconstituted mixture of phospholipids designed to mimic the constitution of the plasma membrane. Figure 3B shows that this also resulted in the activation of c-Raf-1, whereas addition of phospholipids in the absence of K-Ras did not activate c-Raf-1. Finally, lipids were omitted entirely from

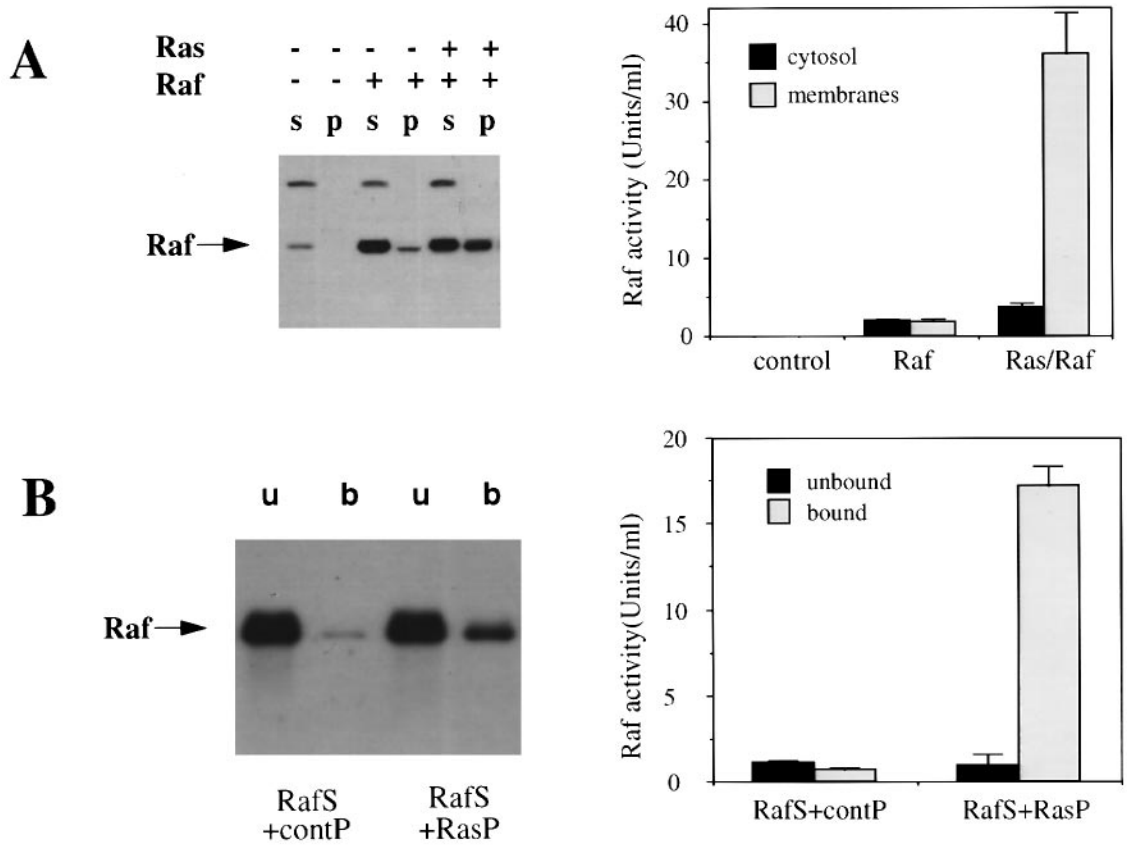

Fig. 1. Activation of c-Raf-1 by H-RasG12V *in vivo* and *in vitro*. (**A**) COS1 cells were fractionated into cytosol (indicated as s) and membrane (indicated as p) fractions, the lysates separated by SDS–PAGE, and blotted with the c-Raf-1 C-20 peptide antibody. Cells transfected with empty vector (lanes 1 and 2), cells transiently expressing epitope- (myc-) tagged c-Raf-1 (lanes 3 and 4), or cells transiently expressing c-Raf-1 and H-RasG12V (lanes 5 and 6) are shown, and the migration of c-Raf-1 is indicated by an arrow (left-hand panel). c-Raf-1 was immunoprecipitated from the cytosol and membrane fractions using the 9E10 antibody, and assayed as described in Materials and methods (right-hand panel). (**B**) Cytosol from COS1 cells transiently expressing c-Raf-1 (RafS) was mixed with membranes from either empty vector-transfected COS1 cells (contP), or COS1 cells transiently expressing H-RasG12V (RasP) as indicated. The membranes were repelleted at 100 000 *g*, and c-Raf-1 was immunoprecipitated from the membrane bound (b) and unbound (u) fractions, and examined by Western blot (left-hand panel) or by activity (right-hand panel).

the incubation and purified K-Ras.GTPγS added directly to c-Raf-1 cytosol, which also resulted in c-Raf-1 activation. Purified K-Ras loaded with GDPβS had a reduced ability to activate c-Raf-1, whereas unprenylated K-Ras.GTPγS was essentially unable to activate c-Raf-1 (Figure 3B).

Ras forms a tight complex with Raf in vitro and in vivo

Although Ras/Raf complexes have been isolated from growth factor-stimulated cells (Finney *et al.*, 1993; Hallberg *et al.*, 1994), it has been previously suggested that once c-Raf-1 has been activated by Ras at the plasma membrane, the active c-Raf-1 dissociates from Ras but still remains anchored to the plasma membrane (Leevers *et al.*, 1994). As no membrane components were required for c-Raf-1 activation in the experiments described above (Figure 3), it was important to determine whether c-Raf-1 also dissociated from Ras following activation under these conditions. Figure 4B, lane 4 shows that a portion of the c-Raf-1 protein, and the majority of the c-Raf-1 activity, was immunoprecipitated from the H-RasG12V membranes following binding and activation of c-Raf-1 *in vitro*, using the Ras monoclonal antibody Y13-238. Similar results were obtained following activation of c-Raf-1 *in vivo* by co-transfection with H-RasG12V (Figure 4A, lane 4). In contrast, no c-Raf-1 protein was immunoprecipitated by the Ras antibody when H-RasG12V was omitted from the transfection (Figure 4A, lane 3), or when c-Raf-1 cytosol was mixed with control membranes (Figure 4B, lane 3), or when the Ras antibody Y13-259 was used to immunoprecipitate H-RasG12V (Figure 4A, lane 5). The complex can also be detected by immunoprecipitation through c-Raf-1 and blotting for H-RasG12V, following activation of c-Raf-1 by H-RasG12V both *in vitro*, and *in vivo* (Figure 4C). It can be estimated from these experiments that ~10–30% of the c-Raf-1 associated with the membranes is in a complex with H-RasG12V, but that this comprises 60–90% of the c-Raf-1 activity.

Activation of Raf by Ras is independent of phosphorylation

The activation of c-Raf-1 by H-RasG12V seen in Figures 1–4 was performed in the presence of EDTA, which chelates any free Mg, thus preventing phosphorylation occurring at this stage. The lack of phosphorylation occurring on c-Raf-1 was demonstrated by adding [γ-^{32}P]ATP to the incubation mixture containing c-Raf-1 cytosol and H-RasG12V membranes (Figure 5, lanes 1

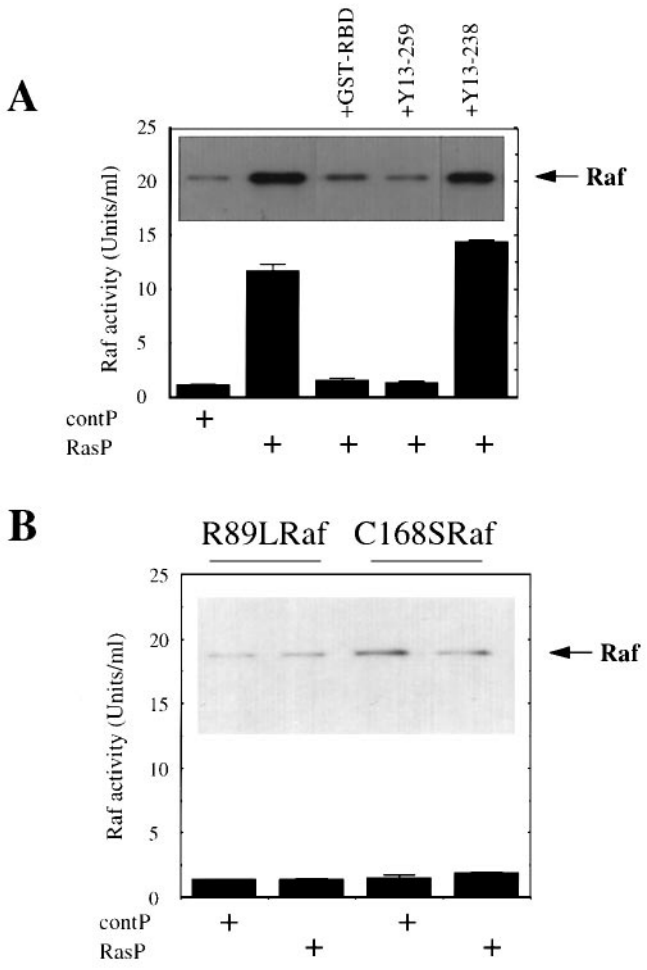

Fig. 2. Activation of c-Raf-1 by H-RasG12V *in vitro* is inhibited by interfering with the Ras effector domain, or by mutations in the Ras-binding domain of c-Raf-1. (**A**) H-RasG12V membranes were pre-incubated with either 5 μg of a fusion protein containing the Ras-binding domain of c-Raf-1, 5 μg of the Ras monoclonal antibody Y13-259, or 5 μg of the Ras monoclonal antibody Y13-238, as indicated. These membranes were then mixed with c-Raf-1 cytosol and repelleted at 100 000 *g*. The c-Raf-1 that bound to the membranes was immunoprecipitated with the 9E10 antibody, and detected either by Western blotting or by activity. (**B**) Cytosol from COS1 cells transiently expressing R89Lc-Raf-1 (lanes 1 and 2) or C168Sc-Raf-1 (lanes 3 and 4) was mixed with either membranes from empty vector-transfected COS1 cells (contP) or membranes from H-RasG12V-transfected COS1 cells (RasP) as indicated. The c-Raf-1 bound to the membranes was detected by Western blotting and activity.

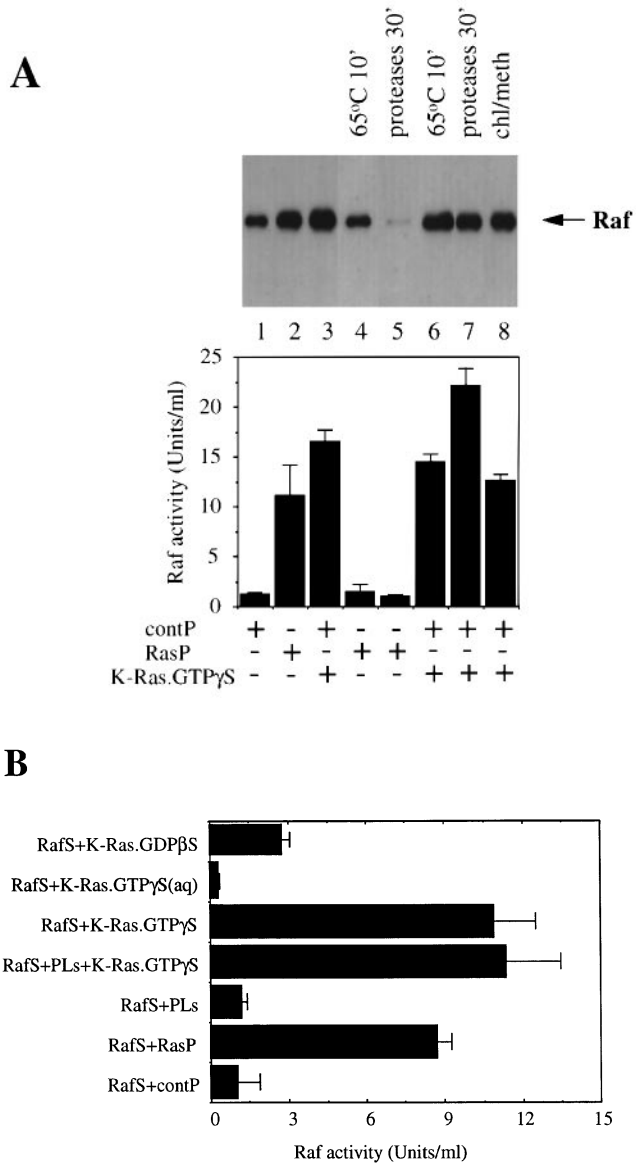

Fig. 3. Ras is the only protein at the plasma membrane required for c-Raf-1 activation *in vitro*. (**A**) c-Raf-1 cytosol was mixed with membranes from empty vector-transfected COS1 cells (contP), membranes from H-RasG12V-transfected COS1 cells (RasP), or membranes from empty vector-transfected COS1 cells to which 1 μg recombinant K-Ras.GTPγS was added as described in Materials and methods (lanes 1–3). Membranes were heated at 65°C for 10 min, treated with trypsin and chymotrypsin (5 μg each) at 30°C for 30 min, or added to a mixture of chloroform/methanol (3:1) as indicated (lanes 4–8). c-Raf-1 bound to the membranes was immunoprecipitated with the 9E10 antibody and detected by Western blotting and activity. (**B**) c-Raf-1 cytosol was added to a mixture of synthetic phospholipids, a mixture of phospholipids containing K-Ras.GTPγS, or recombinant nucleotide-bound K-Ras alone. Following immunoprecipitation with 9E10, the c-Raf-1 activity was determined as described in Materials and methods.

and 3). When Mg was added to these conditions at concentrations exceeding that of the EDTA, phosphorylation of c-Raf-1 became evident (Figure 5, lanes 2 and 4). Therefore it would appear that activation of c-Raf-1 by H-RasG12V can occur independently of phosphorylation. However, since c-Raf-1 forms a tight complex with H-RasG12V following addition of the two proteins (Figure 4), there remains the possibility that, although the initial binding step is phosphorylation-independent, activation of c-Raf-1 only occurs during the assay of c-Raf-1 activity, when ATP is added to the complex. To detect phosphorylation occurring at this stage, c-Raf-1 was immunoprecipitated following binding to H-RasG12V membranes, then incubated in the presence of Mg and [γ-^{32}P]ATP. In the c-Raf-1 immune complex, a number of bands incorporated [^{32}P]phosphate, although some were non-specific, as shown by a similar pattern of bands occurring in a c-Raf-1 K375A immune complex (Figure 6A, lanes 1 and 3). However, an ~80 kDa protein present in both the c-Raf-1 and H-RasG12V immune complexes was not present when c-Raf-1 K375A S100 was mixed with H-RasG12V membranes, immunoprecipitated, and incubated with Mg-[γ-^{32}P]ATP. Although the phosphorylated protein in lanes

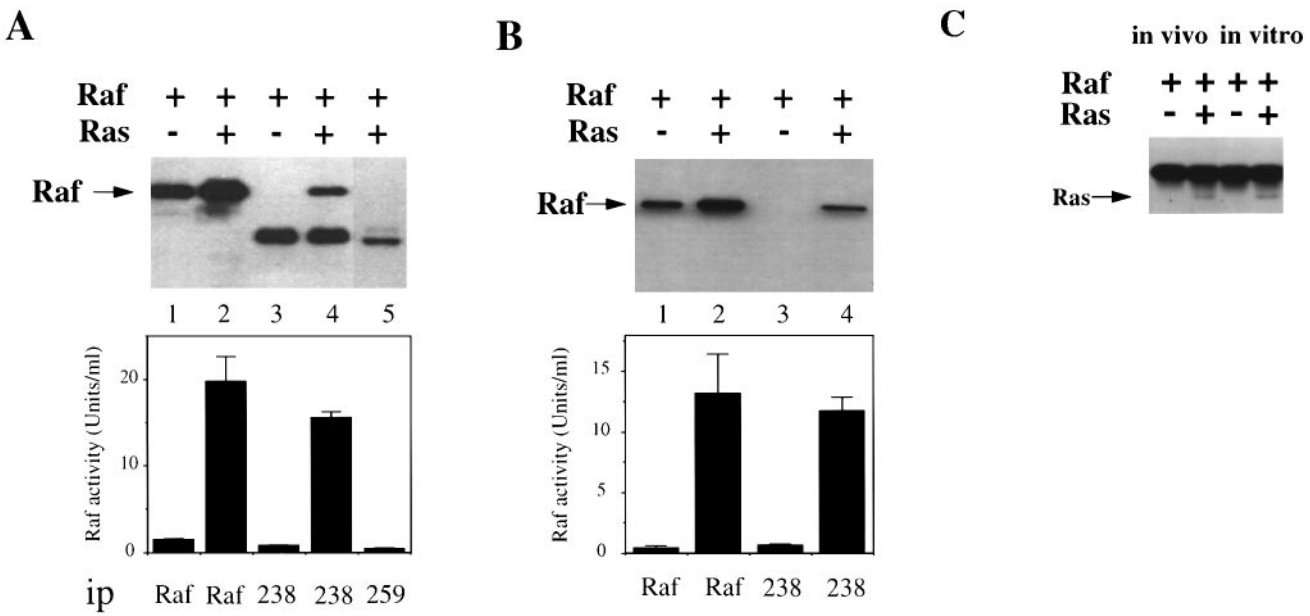

Fig. 4. c-Raf-1 remains in a complex with Ras following activation. (**A**) COS1 cells transiently expressing the indicated proteins were separated into cytosol and membrane fractions, and the c-Raf-1 immunoprecipitated from the membranes with either the 9E10 antibody (indicated as Raf), the Y13-238 Ras monoclonal antibody (indicated as 238), or the Y13-259 Ras monoclonal antibody (indicated as 259). The immune complexes were either Western blotted with the c-Raf-1 C-20 peptide antibody (upper panel) or assayed for activity (lower panel). (**B**) c-Raf-1 cytosol was mixed with control membranes or H-RasG12V membranes as indicated, the membranes repelleted, and the c-Raf-1 immunoprecipitated with either the 9E10 monoclonal antibody (indicated as Raf) or the Y13-238 monoclonal antibody (indicated as 238). The immune complexes were either Western blotted with the c-Raf-1 C-20 peptide antibody (upper panel) or assayed for activity (lower panel). (**C**) c-Raf-1 was immunoprecipitated from the membrane fraction from COS1 cells transiently expressing c-Raf-1 alone, or in combination with H-RasG12V, with the 9E10 antibody (lanes 1 and 2). Lanes 3 and 4 show c-Raf-1 immunoprecipitated from the H-RasG12V membranes following mixing with c-Raf-1 cytosol *in vitro*. The presence of Ras in the immune complex was detected using the Ras antibody Y13-259.

1 and 2 migrated at a slightly lower mobility than the 74 kDa c-Raf-1 protein visualized by immunoblotting (Figure 6B), its identity was confirmed as c-Raf-1, and not an endogenous cellular protein present in the c-Raf-1 immune complex, by performing the same set of experiments using RafCAAX. The phosphorylated protein now migrated higher than 80 kDa due to the presence of the additional CAAX motifs (our unpublished data). Phosphorylation of RafCAAX under these conditions occurred to the same extent from c-Raf-1 activated *in vitro* compared with c-Raf-1 activated *in vivo*, and occurred on the same set of sites as judged by two-dimensional phosphopeptide mapping (our unpublished data). Phosphoamino acid analysis of the ^{32}P-phosphorylated polypeptide showed the presence of phosphoserine and phosphothreonine, but no detectable phosphotyrosine (Figure 6C).

To examine further the possibility of tyrosine phosphorylation on c-Raf-1 following activation by H-RasG12V, immunoblotting with tyrosine-specific monoclonal antibodies was performed. Figure 7 shows that c-Raf-1 isolated from COS1 cells expressing c-Raf-1 alone, or from COS1 cells co-expressing c-Raf-1 and H-RasG12V, did not contain any detectable tyrosine phosphorylation (Figure 7A, lanes 1 and 2), which was also unaffected following activation by H-RasG12V membranes *in vitro* (lane 6). To demonstrate the effectiveness of the phosphotyrosine antibodies, c-Raf-1 isolated from COS1 cells co-expressing Y527FSrc, or H-RasG12V and Y527FSrc, was also examined. In Figure 7, lanes 3 and 4 show that co-expression of Y527FSrc caused tyrosine phosphorylation of c-Raf-1, which was dramatically enhanced by co-expression with H-RasG12V. In these cells, co-expression

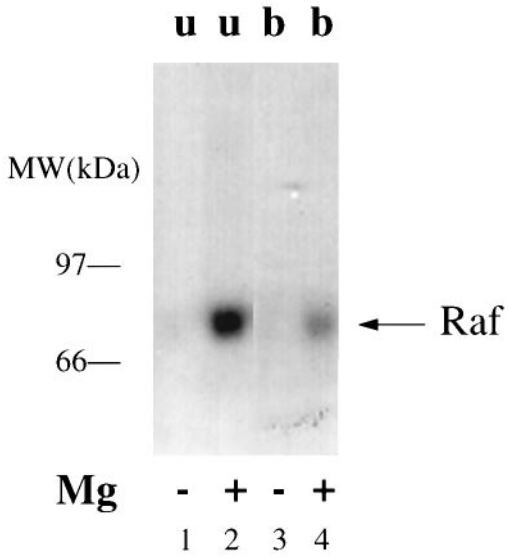

Fig. 5. c-Raf-1 does not become phosphorylated during incubation with H-RasG12V membranes. Cytosol from c-Raf-1-transfected COS1 cells was mixed with membranes from H-RasG12V-transfected COS1 cells and [γ-^{32}P]ATP, either in the absence or presence of excess $MgCl_2$ as indicated. The mixture was subjected to centrifugation at 100 000 *g*, and c-Raf-1 immunoprecipitated from the membrane bound (b) and unbound (u) fractions, and visualized by autoradiography.

of c-Raf-1 with Y527FSrc did not significantly stimulate the activity of c-Raf-1 (Figure 7C, lane 3), whereas co-expression of c-Raf-1 with both H-RasG12V and Y527FSrc caused a 4- to 8-fold enhancement of c-Raf-1 activity compared with that seen with H-RasG12V alone

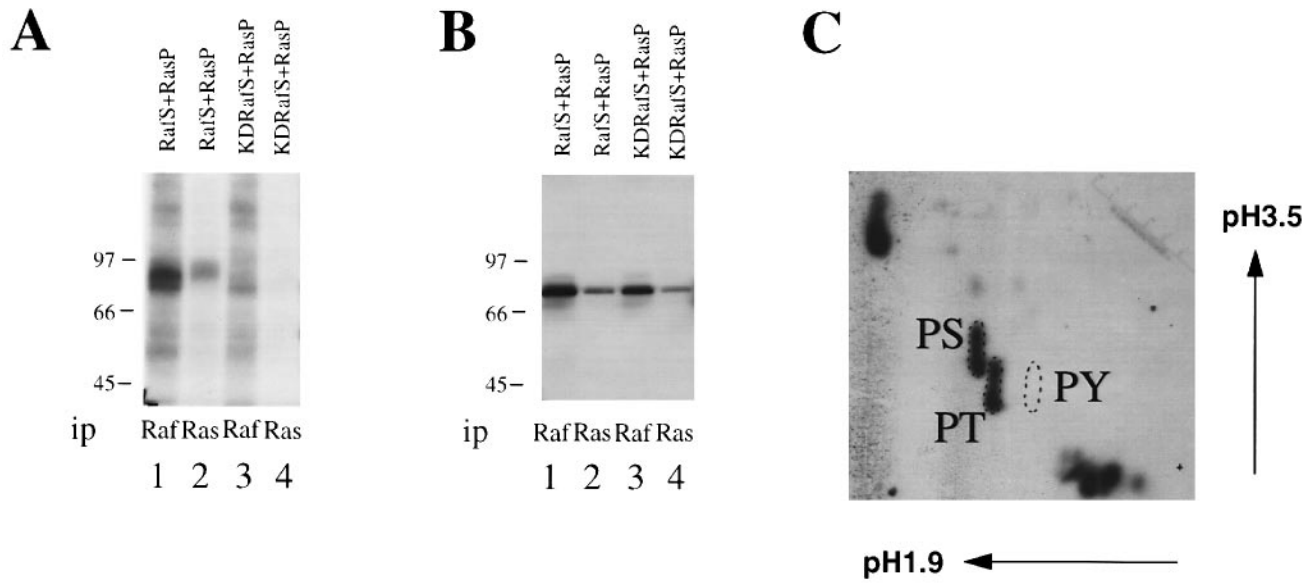

Fig. 6. Phosphorylation of c-Raf-1 following activation by H-RasG12V and incubation with Mg-ATP occurs on serine and threonine residues. Cytosol from c-Raf-1-transfected COS1 cells, or cytosol from epitope- (glu-glu-) tagged c-Raf-1 K375A-transfected COS1 cells, was mixed with membranes from H-RasG12V-transfected COS1 cells. Following centrifugation at 100 000 *g*, bound proteins were immunoprecipitated with either 9E10 or glu-glu as appropriate (indicated as Raf) or Y13-238 (indicated as Ras). The immune complexes were run on SDS–PAGE, transferred to PVDF, and analyzed by autoradiography (**A**) or by blotting with the c-Raf-1 C-20 peptide antibody (**B**). (**C**) The ^{32}P-labeled c-Raf-1 protein from the Y13-238 immune complex in (A) was excised from the membrane, and subjected to phosphoamino acid analysis as described in Materials and methods.

(Figure 7C, lane 4), in agreement with previous studies (Williams *et al.*, 1992; Fabian *et al.*, 1993; Marais *et al.*, 1995).

To further test the possibility of H-RasG12V causing activation of c-Raf-1 through tyrosine phosphorylation, the effect of mutations at the previously identified sites of tyrosine phosphorylation were examined. As previously reported, mutation of tyrosine residues 340 and 341 to aspartate resulted in a c-Raf-1 polypeptide that exhibited constitutive activity which, in contrast to c-Raf-1 activated by H-RasG12V, was entirely cytosolic (Figure 8). However, this mutant still retained the ability to be substantially activated by H-RasG12V, and the additional activity bestowed on c-Raf-1 by H-RasG12V was entirely at the plasma membrane (Figure 8). In contrast, this mutant was unable to be further activated by H-RasG12V and Src, compared with the activation by H-RasG12V alone (Figure 8). This further demonstrates that H-RasG12V is causing activation of c-Raf-1 through mechanisms other than tyrosine phosphorylation on residues 340 and 341, and Src is most likely exerting its effect through phosphorylation of these residues.

Activation of Raf by Ras and Src in vitro

To investigate whether the enhanced c-Raf-1 activation occurring in COS1 cells expressing c-Raf-1, H-RasG12V and Y527FSrc, relative to cells expressing c-Raf-1 and H-RasG12V, could also occur *in vitro*, c-Raf-1 cytosol was mixed with membranes from COS1 cells expressing H-RasG12V and Y527FSrc. In the presence of EDTA, the H-RasG12V/Y527FSrc membranes activated c-Raf-1 to the same extent as H-RasG12V membranes alone (Figure 9C). To measure any potential effect of Y527FSrc on c-Raf-1 activity, the effect of EDTA in preventing phosphorylation was overcome by the addition of an ATP-regenerating system to the incubation. Figure 9C shows that addition of an ATP-regenerating system to c-Raf-1 cytosol and H-RasG12V membranes caused a dramatic inhibition in the activation of c-Raf-1, which was accompanied by an upward mobility shift of the c-Raf-1 which remained unbound to the H-RasG12V membranes (Figure 9B). This mobility shift was due to phosphorylation as incubation with a serine/threonine-specific phosphatase reversed the mobility shift (our unpublished data). The reduction in c-Raf-1 activation by H-RasG12V membranes in the presence of the ATP-regenerating system was also accompanied by a reduction in the amount of c-Raf-1 binding to the H-RasG12V membranes (Figure 9B, compare lane 4 with lane 3). Membranes from COS1 cells expressing Y527FSrc alone were unable to activate c-Raf-1 either in the presence or absence of ATP (our unpublished data). However, although membranes from COS1 cells expressing both H-RasG12V and Y527FSrc activated c-Raf-1 to a similar degree in the presence of EDTA, when an ATP-regenerating system was added to the incubation, there was a 2-fold increase in c-Raf-1 activation by these membranes relative to activation by H-RasG12V membranes (Figure 9C), which was associated with an increase in tyrosine phosphorylation (Figure 9A, lanes 6). This suggests that, in the presence of ATP, there is an inhibitory event which occurs to c-Raf-1 activated by both H-RasG12V and H-RasG12V/Y527FSrc membranes, and also a stimulatory, tyrosine phosphorylation-related event which occurs only in the presence of H-RasG12V/Y527FSrc membranes.

Active Raf is inactivated by autophosphorylation

Although Figures 6–9 demonstrate that tyrosine phosphorylation of c-Raf-1 is not required for activation by H-RasG12V, there remained the possibility that serine/

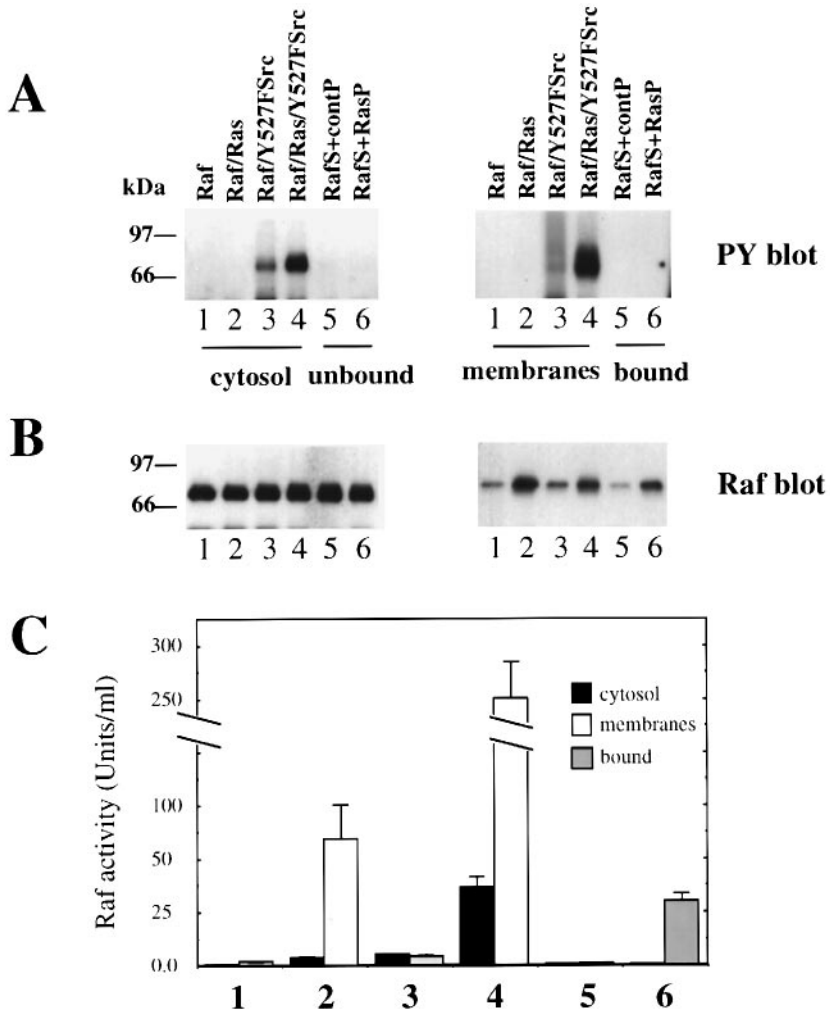

Fig. 7. c-Raf-1 activated by H-RasG12V is not phosphorylated on tyrosine residues. COS1 cells were transfected with either c-Raf-1 alone (lanes 1), with c-Raf-1 and H-RasG12V (lanes 2), with c-Raf-1 and Y527FSrc (lanes 3), or with c-Raf-1, H-RasG12V and Y527FSrc (lanes 4). Cytosol and membrane fractions were prepared, and the c-Raf-1 immunoprecipitated from each fraction with the c-Raf-1 C-12 peptide antibody, and analyzed either by Western blotting with phosphotyrosine-specific antibodies (**A**), with the c-Raf-1 C-20 peptide antibody (**B**), or by activity (**C**). c-Raf-1 activated *in vitro* by H-RasG12V membranes was analyzed in a similar manner (lanes 6).

threonine phosphorylation is required, as suggested by early studies on c-Raf-1 activation (Blackshear *et al.*, 1990; Morrison *et al.*, 1993), as well as more recent experiments (Dent *et al.*, 1995a). As shown in Figure 5, c-Raf-1 did not become phosphorylated when c-Raf-1 cytosol was mixed with H-RasG12V membranes in the presence of EDTA. However, an activating phosphorylation may have occurred when ATP was added to assay c-Raf-1, as phosphorylation on c-Raf-1 did occur at this stage (Figure 6A). This possibility was investigated by preincubating the isolated c-Raf-1/H-RasG12V complex with Mg-ATP, before assaying c-Raf-1 activity, to see whether this would increase the subsequent activity of c-Raf-1 in the assay. Figure 10A shows that preincubation with Mg-ATP actually causes a rapid inactivation of c-Raf-1 activity, which occurs both with c-Raf-1 activated by H-RasG12V *in vitro*, and c-Raf-1 activated by H-RasG12V *in vivo*. This inactivation does not occur in the absence of Mg-ATP, showing that it is not a general destabilization of c-Raf-1 caused by incubation at 30°C. This inactivation is substantially reduced when c-Raf-1 activated by H-RasG12V and Y527FSrc is incubated in the presence of Mg-ATP (Figure 10B), or when c-Raf-1 containing acidic mutations at tyrosine residues 340 and 341 is incubated in the presence of Mg-ATP (Figure 10C).

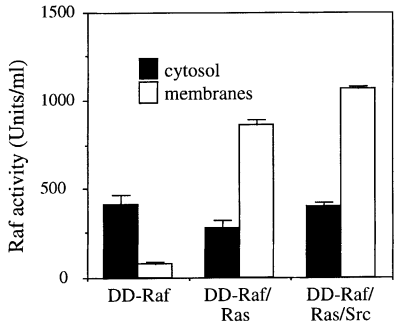

Fig. 8. c-Raf-1 tyrosine phosphorylation site mutants are unable to be further activated by Y527FSrc, but retain the ability to be activated by H-RasG12V. COS1 cells were transfected with c-Raf-1 YY340,1DD alone, in combination with H-RasG12V, or in combination with H-RasG12V and Y427FSrc. Cytosol and membranes were prepared and the c-Raf-1 activity associated with each fraction measured following immunoprecipitation with 9E10.

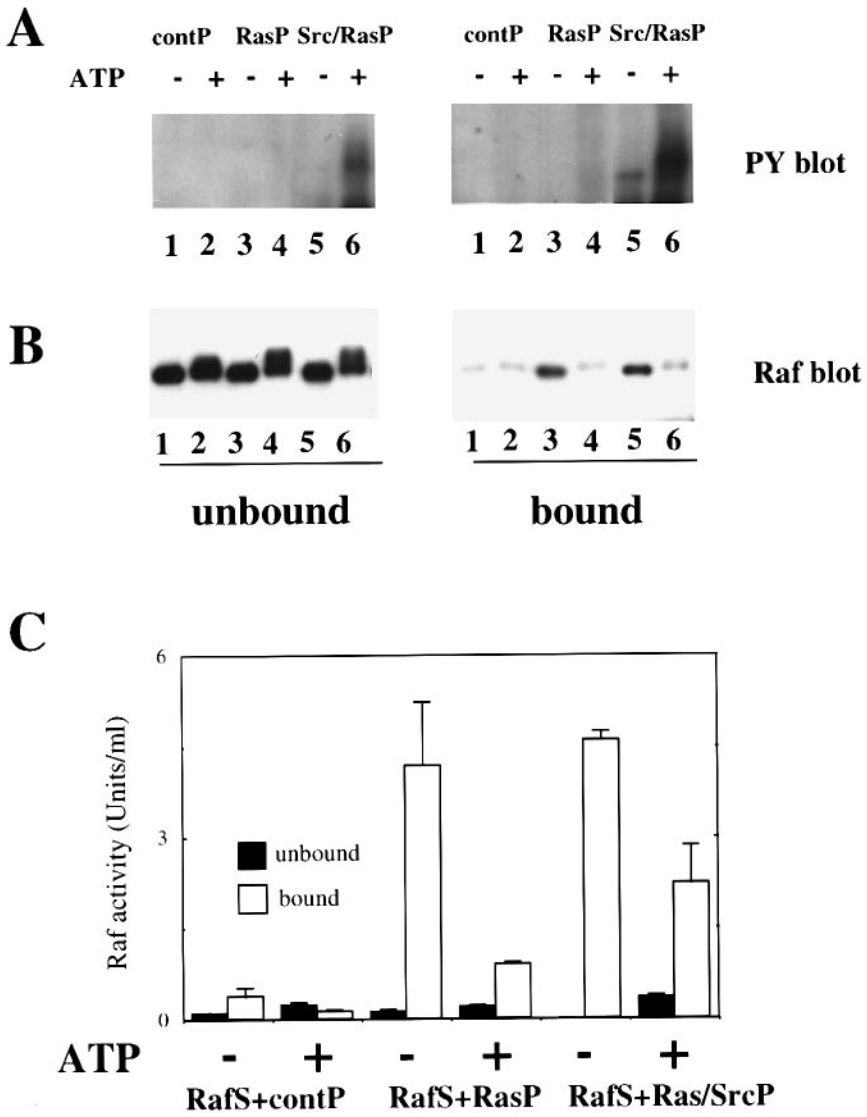

Fig. 9. Activation of c-Raf-1 by H-RasG12V and Y527FSrc *in vitro*. c-Raf-1 cytosol was mixed with either H-RasG12V membranes or H-RasG12V/Y527FSrc membranes, either in the absence or presence of an ATP-regenerating system. Following centrifugation at 100 000 *g*, c-Raf-1 was immunoprecipitated from the supernatant (unbound) or the pellet (bound), and blotted with phosphotyrosine-specific antibodies (**A**), the c-Raf-1 C-20 peptide antibody (**B**), or assayed by activity (**C**) as described in Materials and methods.

Discussion

We have established a system for the activation of c-Raf-1 by H-RasG12V, or by H-RasG12V and Src, *in vitro*, using cytoplasmic and membrane extracts from COS1 cells overexpressing these proteins. An overexpression system is not crucial for activation, however, as similar results have been obtained using Jurkat T-cell cytosol as an endogenous source of c-Raf-1 (our unpublished data). To our knowledge, only one other group has reported activation of c-Raf-1 by Ras *in vitro* (Dent and Sturgill, 1994; Dent *et al.*, 1995b). Using recombinant epitope-tagged c-Raf-1 purified from Sf9 cells, and membranes isolated from H-RasG12V- and Src-transformed NIH3T3 cells, they found a significant activation of c-Raf-1, which was abolished by pre-incubation of the membranes with an N-terminal fragment of c-Raf-1. One significant difference between our findings and theirs, is in the requirement of ATP for activation—they find that activation by H-RasG12V/Src membranes requires the presence of Mg-ATP, whereas we find that maximal activation by H-RasG12V requires the presence of EDTA to chelate free Mg, and introduction of Mg-ATP in the form of an ATP-regenerating system actually significantly inhibits the activation. One possible reason for this discrepancy could be in the use of a mixture of H-RasG12V and Src membranes in the previous studies, compared with just H-RasG12V-expressing cell membranes, or purified K-Ras, as used in the present study. We have also been unable to activate purified c-Raf-1 isolated from Sf9 cells by purified K-Ras or H-RasG12V membranes, either in the presence or absence of Mg-ATP, and rather have been limited to using c-Raf-1 in the presence of crude cytosol. The reason for this is unknown, but it may reflect the requirement of another cytosolic factor, in addition to c-Raf-1, for activation. This possibility will be explored in future work.

The finding that a homogeneous preparation of K-Ras.GTP in the absence of additional membrane com-

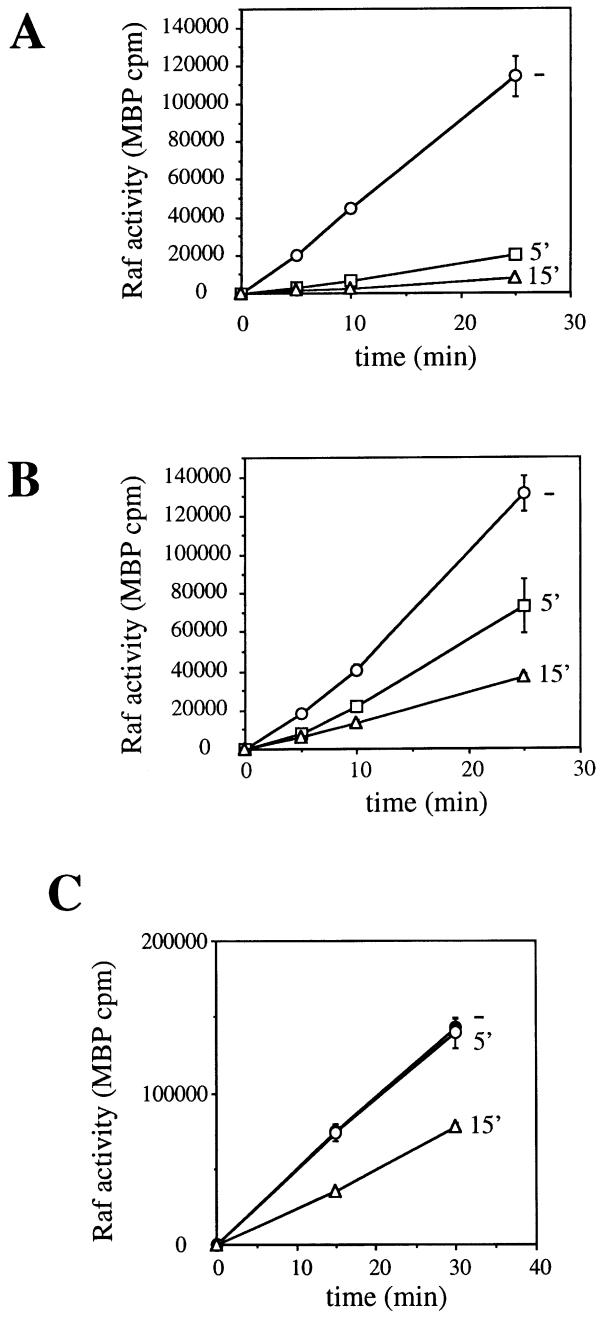

Fig. 10. c-Raf-1 activated by Ras is inactivated by autophosphorylation. c-Raf-1 was immunoprecipitated from the membranes of COS1 cells transiently expressing c-Raf-1 and H-RasG12V (**A**), c-Raf-1, H-RasG12V and Y527FSrc (**B**), or c-Raf-1 YY340,1DD and H-RasG12V (**C**). The immunoprecipitates were incubated at 30°C either in the absence of Mg-ATP for 15 min (–), or in the presence of Mg-ATP for 5 min and 15 min as indicated, and the c-Raf-1 activity determined as described in Materials and methods.

ponents is as effective as H-RasG12V membranes in activating c-Raf-1 was initially surprising, as we (Stokoe *et al.*, 1994) and others (Leevers *et al.*), had previously shown that targeting c-Raf-1 to the plasma membrane was sufficient to cause activation which was independent of H-RasG12V. These experiments suggested the presence of an additional factor in the plasma membrane which was required for c-Raf-1 activation, and the role of H-RasG12V in this process was to bring c-Raf-1 to this location for activation to take place (Hall, 1994). One possible explanation for this apparent discrepancy could be the presence of small amounts of plasma membrane components, even in a 100 000 *g* supernatant, although if this were the reason, the necessary components would need to be required in extremely non-limiting amounts in the cell. Another possibility is the presence of plasma membrane components in the preparation of processed K-Ras, although this is also unlikely as the preparation is homogeneous by protein staining (our unpublished data), and was washed extensively in the presence of detergent. An alternative explanation could be that recruitment of c-Raf-1 to the plasma membrane may more accurately reflect activation by another mechanism other than by H-RasG12V. This could include tyrosine phosphorylation by a membrane-associated tyrosine kinase (see Marais *et al.*, 1995), local clustering resulting in c-Raf-1 oligomerization (Farrar *et al.*, 1996; Luo *et al.*, 1996), or more intriguingly, an interaction of the farnesyl group of RafCAAX mimicking the interaction of the Ras prenyl group, resulting in activation. This latter possibility is supported by recent findings showing a crucial role of the Ras farnesyl group in the interaction with Raf (Hu *et al.*, 1995; Luo *et al.*, 1997), and an intact zinc finger of Raf was required for this interaction (Luo *et al.*, 1997). This latter hypothesis is supported by the observation that RafCAAX containing a mutation in the zinc finger is also compromised in its activity (our unpublished data). Experiments involving the isolation of non-farnesylated RafCAAX, followed by farnesylation *in vitro* should help to resolve the mechanism of activation of RafCAAX, although initial experiments in this laboratory using this approach have proved to be technically difficult.

The activation of c-Raf-1 by recombinant processed GTP-loaded K-Ras (Figure 3B), and the absolute requirement for post-translational processing, is very reminiscent of the activation of B-Raf that occurs *in vitro* following addition of processed K-Ras (Itoh *et al.*, 1993; Yamamori *et al.*, 1995) or processed H-Ras (McGeady *et al.*, 1995; Okada *et al.*, 1996). Interestingly, McGeady and colleagues showed that bacterially expressed H-Ras, which is unable to activate B-Raf, can be converted to a form fully able to activate B-Raf solely by farnesylation, showing that additional Ras processing (carboxy-methylation, proteolysis and palmitoylation) is not required. Ras mutants which are farnesylated, but not palmitoylated, retain the ability to transform cells (Hancock *et al.*, 1990), albeit at reduced potency. This may suggest that Ras effectors other than Raf require modifications on Ras in addition to farnesylation, for example the Rac pathway, which is required in addition to Raf activation to elicit full transformation by Ras (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). An essential role for palmitoylation of Ras has been demonstrated in *Xenopus* oocytes, where H-Ras mutants which are farnesylated and methylated, but not palmitoylated, are unable to induce meiotic maturation (Dudler and Gelb, 1996).

In experiments to determine whether phosphorylation plays a role in the activation of c-Raf-1 by H-RasG12V, we were surprised to find that inclusion of Mg-ATP at two different stages in the protocol had a profound inhibitory effect on c-Raf-1 activity. However, it is currently unclear whether the mechanisms involved are identical. Inclusion of Mg-ATP in the initial mixing step

in the presence of cytosol results in an upward mobility shift on c-Raf-1 (Figure 9B) that can be reversed by treatment with a bacteriophage serine/threonine-specific phosphatase (our unpublished data). This upward mobility shift is similar to that seen upon prolonged growth factor stimulation (Traverse and Cohen, 1994), or upon activation of kinases downstream of Raf (Samuals *et al.*, 1993). A potential negative regulatory role for this phosphorylation has been suggested by experiments utilizing a compound that inhibits the activation of MEK, PD98059 (Alessi *et al.*, 1995; Dudley *et al.*, 1995). Stimulation of Swiss3T3 cells with PDGF (Alessi *et al.*, 1995), or Rat1 cells with EGF (our unpublished data), causes a transient activation of c-Raf-1, which is rapidly reduced to baseline levels within 30 minutes, concomitant with an upward mobility shift. Prior incubation of PD98059 in the culture medium abolishes the mobility shift of c-Raf-1, and prolongs the activity to several hours (Alessi *et al.*, 1995, and our unpublished data). Although these data may indicate a role for the hyperphosphorylation of c-Raf-1 in negatively regulating its activity, and indicate a kinase downstream of Raf, an alternative explanation may lie in the ability of ERK to phosphorylate and down-regulate Sos, thereby reducing GTP levels on Ras (Porfiri *et al.*, 1996). The possibility that ERK is involved in Raf phosphorylation and down-regulation was explored more directly by inhibiting the ability of ERK to phosphorylate c-Raf-1 *in vitro*. The inclusion of kinase-dead MEK, or a synthetic inhibitor of ERK, caused a reduction in the c-Raf-1 band shift, and slightly restored the ability of c-Raf-1 to become activated by H-RasG12V membranes in the presence of Mg-ATP (our unpublished data). However, this restoration was relatively minor, and PD98059 had no effect on restoring c-Raf-1 activation (our unpublished data), suggesting that additional mechanisms were preventing c-Raf-1 activation in the presence of Mg-ATP, for example, the autophosphorylation and inactivation of purified Raf (Figure 10, also discussed below).

Addition of Mg-ATP to c-Raf-1 following affinity purification results in a rapid inactivation of c-Raf-1 (Figure 10A) which, under these circumstances, is not accompanied by an upward mobility shift in the majority of the c-Raf-1 protein (our unpublished data). This may reflect autophosphorylation of c-Raf-1, or phosphorylation by a contaminating kinase in the immune complex. The latter possibility seems less likely, as only c-Raf-1 incorporates ^{32}P-phosphate under these conditions (Figure 6A). Also, addition of a specific peptide inhibitor of protein kinase A to the incubation, or a synthetic inhibitor of ERK, had no effect on the inactivation of c-Raf-1 (our unpublished data), excluding the possibility of contamination of the c-Raf-1 immune complexes by either of these kinases. Autophosphorylation and inactivation of Raf has also been noted by other investigators (Mischak *et al.*, 1996), and autophosphorylation and inactivation of at least two other protein kinases has also been previously described. Wang and co-workers (1994) showed that prior incubation of glycogen synthase kinase-3α or β with Mg-ATP, caused a marked inactivation against a peptide substrate. This was probably due to phosphorylation on Ser9, a residue previously implicated in the down-regulation of glycogen synthase kinase-3 activity (Sutherland *et al.*, 1993; Cross *et al.*, 1995), as incubation of N-terminally truncated glycogen synthase kinase-3 was not subject to down-regulation (Wang *et al.*, 1994). Autophosphorylation and inactivation of DNA-dependent protein kinase has also been observed (Chan and Lees-Miller, 1996). This phenomenon may reflect a general control mechanism whereby a protein kinase remains active while there is substrate present, but upon depletion of substrate or relocation to a different location in the cell, it can turn off its activity. Evidence for phosphorylation playing a role in Raf inactivation has also been suggested from studies in the eye development pathway in *Drosophila*. In this system, a mutant of the catalytic subunit of *Drosophila* PP2A was shown to suppress the rough eye phenotype induced by an activated Raf allele. Moreover, the same mutation in PP2A enhances the phenotype induced by a mutation in Raf which causes a lower level of wild-type Raf transcription (Wassarman *et al.*, 1996). Taken together, these results suggest that dephosphorylation of Raf, at least in *Drosophila*, can be associated with positive regulation of Raf activity. One caveat regarding inactivation of c-Raf-1 following incubation with Mg-ATP, is that its activity could not be restored by treatment with a serine/threonine-specific phosphatase (our unpublished data). Whether this reflects an irreversible inhibition of c-Raf-1, or whether the phosphatase is removing additional phosphates required for activity remains to be determined.

There has been much confusion regarding the mechanism of activation of c-Raf-1, and most, if not all, recent published results suggest a role for phosphorylation in the activation of c-Raf-1, both on tyrosine residues (Fabian *et al.*, 1993; Dent *et al.*, 1995a; Jelinek *et al.*, 1996), and on serine/threonine residues (Kolch *et al.*, 1993; Dent *et al.*, 1996). Our results using an *in vitro* system to study the mechanism of c-Raf-1 activation in detail, have provided strong evidence that activation by Ras can occur in the absence of phosphorylation, and is most likely through a conformational change mediated by direct binding of the farnesyl group of Ras to c-Raf-1, perhaps to the cysteine-rich moiety, as suggested in Luo *et al.* (1997). Phosphorylation in these experiments is accompanied by a rapid inactivation of c-Raf-1 activity. The sites of phosphorylation occurring under different circumstances are currently being investigated. In contrast, the activation by Src *in vitro* requires Mg-ATP and is accompanied by tyrosine phosphorylation, consistent with previous observations on the role of Src in c-Raf-1 activation. To what extent these different mechanisms controlling c-Raf-1 activity occur *in vivo* under different conditions remains to be elucidated. The question of which mechanism is most important in the initiation and maintenance of the transformed phenotype is clearly much more difficult to resolve, though will be of the most importance therapeutically. Optimistically, one could suggest that, the more ways that c-Raf-1 can be shown to be activated, the more potential ways there are to inhibit its activity in tumor cells. Hence, compounds which inhibit oligomerization, tyrosine phosphorylation, or interaction of c-Raf-1 with Ras farnesyl group, could all be considered as potential therapies.

Materials and methods

Cell culture and fractionation

COS1 cells were cultured in 25×15 cm flasks containing 30 ml Dulbecco's Modified Eagle's Medium supplemented with 100 U/ml

penicillin, 100 μg/ml streptomycin and 2 mM glutamine in the presence of 10% Donor Calf Serum in a humidified atmosphere with 10% CO_2 at 37°C. When the cells reached 80–90% confluency, they were trypsinized from the flask, washed with HEBS buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and resuspended at 12×10^6 cells/ml in HEBS buffer. cDNAs of interest (10 μg each), cloned into the expression vector pEXV, were transfected into 3×10^6 cells by electroporation in the presence of 100 μg salmon sperm DNA as carrier. Cells and DNA were placed in a 0.4 cm electroporation cuvette (Bio-Rad) and pulsed with 250 V/125 mF giving a time constant of 5–6 ms. Following electroporation, cells were seeded onto 10 cm dishes containing 10 ml of serum-containing medium for 48 h, then switched to serum-free medium 18 h before harvesting. Cells were washed once with phosphate-buffered saline, before scraping into 0.4 ml/dish hypotonic lysis buffer (HLB: 10 mM Tris–HCl, 1 mM EDTA, 25 mM NaF, 1 mM NaVn, 1 mM DTT, 10 μg/ml aprotinin, 10 μg /ml soybean trypsin inhibitor, 10 μg /ml leupeptin and 1 mM pefabloc). The cells were lysed by 30 strokes in a Dounce homogenizer, subjected to centrifugation at 1500 *g* to pellet nuclei and unbroken cells, followed by centrifugation of the supernatant at 100 000 *g* for 20 min. The supernatant (~400 μl) was collected (S100 fraction) and the pellet resuspended in 100 μl HLB (P100 fraction). Throughout the manuscript, the terms cytosol and membranes have been substituted for S100 and P100.

When K-Ras was added to control membranes, the membranes were further purified by centrifugation through a sucrose gradient containing 1 mM NaVn, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin and 1 mM pefabloc. 400 μl of a 15% (w/v) sucrose solution was carefully layered on top of 400 μl of a 45% (w/v) sucrose solution in a 11×34 mm polycarbonate centrifuge tube (Beckman). 50 μl of control membranes were then carefully layered on top of this, and the tube centrifuged at 54 000 r.p.m. in a TLS 55 swing out rotor (Beckman) for 30 min at 4°C. Membranes were collected from the 15%/45% interface, unless the membranes were stripped of proteins, in which case they were collected at the 0%/15% interface. The membranes were washed once in 500 μl HLB, repelleted at 100 000 g, and resuspended in 50 μl HLB.

Purification of epitope-tagged proteins from Sf9 cells

Processed and unprocessed epitope- (EYMPME, referred to as Glu-Glu) tagged K-Ras proteins were purified from the aqueous and detergent phases of Sf9 cells expressing K-Ras(4B) as previously described (Porfiri *et al.*, 1995), with the substitution of 0.5% (w/v) sodium cholate with 1.2% (w/v) *n*-octyl-β-D-glucopyranoside in the buffers used to purify processed K-Ras. Glu-Glu-tagged MEK1 proteins were purified from Sf9 cells, and GST–ERK1 was purified from *Escherichia coli*, essentially as previously described (MacDonald *et al.*, 1993).

Preparation of unilamellar phospholipid vesicles

10 nmol each of phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and 5 nmol phosphatidylcholine (PC) were dried down under nitrogen in a glass vial, and resuspended in 100 μl HLB by three consecutive treatments of alternate bath sonication and vortexing (15 s each).

In vitro activation of Raf by Ras, or Ras and Src

200 μl cytosol from epitope- (myc or glu-glu) tagged c-Raf-1-transfected COS1 cells was mixed with 50 μl membranes from H-RasG12V-transfected COS1 cells, or 50 μl membranes from COS1 cells transfected with H-RasG12V and Y527FSrc, for 10 min at ambient temperature on a rotating platform, followed by centrifugation at 100 000 *g* for 20 min. The supernatant was removed, adjusted to 1% NP-40, and c-Raf-1 was immunoprecipitated as described below. The pellet was resuspended in 100 μl HLB containing 1% NP-40, vortexed, left on ice for 10 min, vortexed again, and centrifuged at 13 000 *g* for 5 min to remove insoluble material. c-Raf-1 was immunoprecipitated from the supernatant as described below. When recombinant K-Ras was added to c-Raf-1 cytosol, 1 μg processed or unprocessed K-Ras was loaded with 5 mM GTPγS or GDPβS in the presence of 2 mM EDTA in a volume of 10 μl, for 10 min at ambient temperature. This was then either added to 50 μl control membranes, or 50 μl phospholipid vesicles for 10 min on ice, followed by re-purification of the membranes or vesicles on sucrose gradients.

In indicated experiments ATP was added to the incubation in the form of an ATP-regenerating system: 10 mM creatine phosphate, 5 mM $MgCl_2$, 2 mM ATP, and 50 μg/ml creatine kinase (final concentrations) were added to the cytosolic and membrane fractions immediately before mixing.

Immunoprecipitation and immunoblotting

Epitope- (myc or glu-glu) tagged full-length c-Raf-1 was immunoprecipitated using either protein G–Sepharose pre-conjugated with the monoclonal myc antibody 9E10 (Evan *et al.*, 1985) or a glu-glu monoclonal antibody (Grussenmeyer *et al.*, 1985), or with 10 μg of a polyclonal C-12 peptide c-Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, California), followed by addition of protein A–Sepharose beads. Incubation was at 4°C for 1 h on a rotating platform. The beads were collected by centrifugation at 13 000 *g* for 1 min, and washed twice with 1 ml ice-cold wash buffer (20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1 mM NaVn). The beads were drained of wash buffer using a Hamilton syringe, and resuspended in 30 μl kinase buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 75 mM NaCl, 1 mM DTT, 1 mM NaVn). Two 5 μl aliquots of this immunoprecipitate were used for a duplicate kinase assay, and the remaining 20 μl was resolved by SDS–PAGE on 1.5 mm gels (Novex, San Diego, CA) and transferred electrophoretically to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA) at 80 mA/gel for 1 h in a semi-dry blotting apparatus (Pharmacia, Uppsala, Sweden). The blots were blocked for 1 h in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% dried milk (Marvel), washed 3×5 min with PBST, then incubated for 1 h with blocking solution containing a polyclonal C-20 peptide c-Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, California) at 1:3000 (for c-Raf-1) or the rat monoclonal antibody Y13-259 at 1:2000 (for Ras). The primary antibody was removed and the blots washed 3×5 min with PBST, before incubation with the appropriate goat secondary antibody coupled to horseradish peroxidase (Bio-Rad, Hercules, California) diluted 1:10 000 in blocking buffer for 1 h. The blots were then washed 3×5 min with PBST and immunoreactive proteins visualized by enhanced chemiluminescence. When antiphosphotyrosine antibodies 4G10 and PY20 (UBI, Lake Placid, New York) were used as primary antibodies, the procedure was identical, except that the milk in the blocking solutions was replaced by 2% BSA. The blots were stripped of antibodies by incubating in a solution containing 62.5 mM Tris–HCl, pH 6.8, 2% SDS and 100 mM 2-mercaptoethanol at 55°C for 30 min with occasional shaking, replacing the buffer once during the incubation, as described by the manufacturer (Millipore, Bedford, MA).

Assay of Raf activity

5 μl of c-Raf-1 immunoprecipitate was added to 15 μl of kinase buffer containing 2 μg of purified GST–ERK1 and 0.5 μg of purified glu-glu-tagged MEK1. The reaction was initiated by the addition of 5 μl of a solution containing 50 mM $MgCl_2$ and 500 μM ATP, and was placed in a shaking heater block maintained at 30°C. After 30 min (for the standard assay), a 2 μl aliquot was removed and diluted into 40 μl ice-cold kinase buffer containing 16 μg myelin basic protein. 10 μl of a solution containing 50 mM $MgCl_2$, 500 μM ATP and 2.5 μCi [γ-^{32}P]ATP, was then added to start the reaction. After 10 min at 30°C, a 40 μl aliquot was removed and spotted onto a 2×2 cm square of phosphocellulose P81 paper (Whatman, Maidstone, UK). The papers were washed four times in 400 ml 75 mM phosphoric acid, rinsed once in acetone, dried, and the incorporated ^{32}P-phosphate measured in a scintillation counter in the presence of liquid scintillant. c-Raf-1 activity is expressed in Units as previously defined (Alessi *et al.*, 1994), and was diluted to a concentration that was on the linear scale, as determined by assaying at 15 min in addition to 30 min. c-Raf-1 activity stated in the graphs is that which was recovered from one dish of transfected COS1 cells, and the immune complex resuspended in 30 μl of kinase buffer. The data shown are the average of duplicate points from one experiment. Each experiment shown was performed at least three times with similar results.

Phosphoamino acid analysis

20 μl of the Ras Y13-238 immunoprecipitate was incubated for 30 min at 30°C in the presence of 10 mM $MgCl_2$, 100 μM ATP and 2.5 μCi [γ-^{32}P]ATP. The beads were washed three times in ice-cold wash buffer, and then hydrolyzed in 50 μl 6 M HCl at 100°C for 2 h. Following centrifugation at 13 000 *g* for 5 min, the supernatant was collected, dried under vacuum, and dissolved in 2 μl pH 1.9 electrophoresis buffer [formic acid (88%):acetic acid:water, 50:156:1794 (v/v)]. Phosphoamino acids were resolved on a thin-layer cellulose plate in two dimensions as previously described (Cooper *et al.*, 1983). Standards were visualized by staining with 0.2% ninhydrin in acetone and ^{32}P-labeled residues were detected by autoradiography overnight.

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References

Aelst,L.V., Barr,M., Marcus,S., Polverino,A. and Wigler,M. (1993) Complex formation between RAS and RAF and other protein kinases. *Proc. Natl Acad. Sci. USA*, **90**, 6213–6217.

Alessi,D.R., Saito,Y., Campbell,D.G., Cohen,P., Sithanandam,G., Rapp,U., Ashworth,A., Marshall,C.J. and Cowley,S. (1994) Identification of the sites in MAP kinase kinase-1 phosphorylated by $p74^{raf-1}$. *EMBO J.*, **13**, 1610–1619.

Alessi,D.R, Cuenda,A., Cohen,P., Dudley,D.T. and Saltiel,A.R. (1995) PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, **270**, 27489–27494.

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.

Baccarini,M., Sabatini,D.M., App,H., Rapp,U.R. and Stanley,E.R. (1990) Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. *EMBO J.*, **9**, 3649–3657.

Blackshear,P.J., Haupt,D.M., App,H. and Rapp,U.R. (1990) Insulin activates the Raf-1 protein kinase. *J. Biol. Chem.*, **265**, 12131–12134.

Chan,D.W. and Lees-Miller,S.P. (1996) The DNA-dependent protein kinase is inactivated by autophosphorylation of the catalytic subunit. *J. Biol. Chem.*, **271**, 8936–8941.

Cooper,J.A., Sefton,B.M. and Hunter,T. (1983) Detection and quantitation of phosphotyrosine in proteins. *Methods Enzymol.*, **99**, 387–402.

Crews,C.M., Alessandrini,A. and Erikson,R.L. (1992) The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science*, **258**, 478–480.

Cross,D.A.E., Alessi,D.R., Cohen,P., Andjelkovich,M. and Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*, **378**, 785–789.

Davis,R. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.*, **268**, 14553–14556.

Dent,P. and Sturgill,T.W. (1994) Activation of (His)6-Raf-1 *in vitro* by partially purified plasma membranes from v-Ras-transformed and serum-stimulated fibroblasts. *Proc. Natl Acad. Sci. USA*, **91**, 9544–9548.

Dent,P., Haser,W., Haystead,T.A.J., Vincent,L.A., Roberts,T.M. and Sturgill,T.W. (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and *in vitro*. *Science*, **257**, 1404–1407.

Dent,P., Jelinek,T., Morrison,D.K., Weber,M.J. and Sturgill,T.W. (1995a) Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. *Science*, **268**, 1902–1906.

Dent,P., Reardon,D.B., Morrison,D.K. and Sturgill,T.W. (1995b) Regulation of Raf-1 and Raf-1 mutants by Ras-dependent and Ras-independent mechanisms *in vitro*. *Mol. Cell. Biol.*, **15**, 4125–4135.

Dent,P., Reardon,D.B., Wood,S.L., Lindorfer,M.A., Graber,S.G., Garrison,J.C., Brautigan,D.L. and Sturgill,T.W. (1996) Inactivation of Raf-1 by a protein-tyrosine phosphatase stimulated by GTP and reconstituted by $G_{\alpha i/o}$ subunits. *J. Biol. Chem.*, **271**, 3119–3123.

Dudler,T. and Gelb,M.H. (1996) Palmitoylation of Ha-Ras facilitates membrane binding, activation of downstream effectors, and meiotic maturation in *Xenopus* oocytes. *J. Biol. Chem.*, **271**, 11541–11547.

Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686–7689.

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

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

Fabian,J.R., Vojtek,A.B., Cooper,J.A. and Morrison,D.K. (1994) A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. *Proc. Natl Acad. Sci. USA*, **91**, 5982–5986.

Farrar,M.A., Alberola-Ila,J. and Perlmutter,R.M. (1996) Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. *Nature*, **383**, 178–181.

Finney,R.E., Robbins,S.M. and Bishop,J.M. (1993) Association of pRas and pRaf-1 in a complex correlates with activation of a signal transduction pathway. *Curr. Biol.*, **3**, 805–812.

Grussenmeyer,T., Scheidtmann,K.H., Hutchinson,M.A., Eckhart,W. and Walter,G. (1985) Complexes of polyoma virus medium T antigen and cellular proteins. *Proc. Natl Acad. Sci. USA*, **82**, 7952–7954.

Hall,A. (1994) A biochemical function for Ras – at last. *Science*, **264**, 1413–1414.

Hallberg,B., Rayter,S.I. and Downward,J. (1994) Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. *J. Biol. Chem.*, **269**, 3913–3916.

Hancock,J.F., Paterson,H. and Marshall,C.J. (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize $p21^{ras}$ to the plasma membrane. *Cell*, **63**, 133–139.

Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P. and Marshall,C.J. (1992) Activation of the MAP kinase pathway by the protein kinase Raf. *Cell*, **71**, 335–342.

Hu,C.-D., Kariya,K., Tamada,M., Akasaka,K., Shirouzu,M., Yokoyuki,S. and Kataoka,T. (1995) Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. *J. Biol. Chem.*, **270**, 30274–30277.

Itoh,T., Kaibuchi,K., Masuda,T., Yamamoto,T., Matsuura,Y., Maeda,A., Shimizu,K. and Takai,Y. (1993) A protein factor for ras p21-dependent activation of mitogen-activated protein (MAP) kinase through MAP kinase kinase. *Proc. Natl Acad. Sci. USA*, **90**, 975–979.

Jelinek,T., Dent,P., Sturgill,T.W. and Weber,M.J. (1996) Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation. *Mol. Cell. Biol.*, **16**, 1027–1034.

Khosravi-Far,R., Solski,P.A., Clark,G.J., Kinch,M.S. and Der,C.J. (1995) Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. *Mol. Cell. Biol.*, **15**, 6443–6453.

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

Kovacina,K.S., Yonezawa,K., Brautigan,D.L., Tonks,N.K., Rapp,U.R. and Roth,R.A. (1990) Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation. *J. Biol. Chem.*, **265**, 12115–12118.

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

Leevers,S.J., Paterson,H.F. and Marshall,C.J. (1994) Requirement for Ras in Raf activation is overcome by targetting Raf to the plasma membrane. *Nature*, **369**, 411–414.

Luo,Z., Tzivion,G., Belshaw,P.J., Vavvas,D., Marshall,M. and Avruch,J. (1996) Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. *Nature*, **383**, 181–185.

Luo,Z., Diaz,B., Marshall,M.S. and Avruch,J. (1997) An intact Raf zinc finger is required for optimal binding to processed Ras and for Ras-dependent Raf activation in situ. *Mol. Cell. Biol.*, **17**, 46–53.

MacDonald,S.G., Crews,C.M., Wu,L., Driller,J., Clark,R., Erikson,R.L. and McCormick,F. (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. *Mol. Cell. Biol.*, **13**, 6615–6620.

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.

McGeady,P., Kuroda,S., Shimizu,K., Takai,Y. and Gelb,M.H. (1995) The farnesyl group of H-Ras facilitates the activation of a soluble upstream activator of mitogen-activated protein kinase. *J. Biol. Chem.*, **270**, 26347–26351.

Michaud,N.R., Fabian,J.R., Mathes,K.D. and Morrison,D.K. (1995) 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. *Mol. Cell. Biol.*, **15**, 3390–3397.

Mischak,H., Seitz,T., Janosch,P., Eulitz,M., Steen,H., Schellerer,M., Philipp,A. and Kolch,W. (1996) Negative regulation of Raf-1 by phosphorylation of serine 621. *Mol. Cell. Biol.*, **16**, 5409–5418.

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.

Muslin,A.J., Tanner,J.W., Allen,P.M. and Shaw,A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.

Nakielny,S., Cohen,P., Wu,J. and Sturgill,T. (1992) MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. *EMBO J.*, **11**, 2123–2129.

Okada,T., Masuda,T., Shinkai,M., Kariya,K. and Kataoka,T. (1996) Post-translational modification of H-Ras is required for activation of, but not for association with, B-Raf. *J. Biol. Chem.*, **271**, 4671–4678.

Porfiri,E. and McCormick,F. (1996) Regulation of epidermal growth factor receptor signaling by phosphorylation of the Ras exchange factor hSOS1. *J. Biol. Chem.*, **271**, 5871–5877.

Porfiri,E., Evans,T., Bollag,G., Clark,R. and Hancock,J.F. (1995) Purification of baculovirus-expressed recombinant Ras and Rap proteins. *Methods Enzymol.*, **255**, 13–21.

Qiu,R.-G., Chen,J., Kirn,D., McCormick,F. and Symons,M. (1995) An essential role for Rac in Ras transformation. *Nature*, **374**, 457–459.

Rapp,U.R., Troppmair,J., Beck,T. and Birrer,M.J. (1994) Transformation by Raf and other oncogenes renders cells differentially sensitive to growth inhibition by a dominant negative c-jun. *Oncogene*, **9**, 3493–3498.

Samuals,M.L., Weber,M.J., Bishop,J.M. and McMahon,M. (1993) Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. *Mol. Cell. Biol.*, **13**, 6241–6252.

Stokoe,D., Macdonald,S.G., Cadwallader,K., Symons,M. and Hancock,J.F. (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science*, **264**, 1463–1467.

Sutherland,C., Leighton,I.A. and Cohen,P. (1993) Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.*, **296**, 15–19.

Traverse,S. and Cohen,P. (1994) Identification of a latent MAP kinase kinase in PC12 cells as B-raf. *FEBS Lett.*, **350**, 13–18.

Traverse,S., Cohen,P., Paterson,H., Marshall,C., Rapp,U. and Grand,R.J.A. (1993) Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of *ras*-transformed retinal cells. *Oncogene*, **8**, 3175–3181.

Turner,B.C., Tonks,N.K., Rapp,U.R. and Reed,J.C. (1994) Interleukin 2 regulates Raf-1 kinase activity through a tyrosine phosphorylation-dependent mechanism in a T-cell line. *Proc. Natl Acad. Sci. USA*, **90**, 5544–5548.

Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.

Wang,Q.M., Fiol,C.J., DePaoli-Roach,A. and Roach,P.J. (1994) Glycogen synthase kinase-3β is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. *J. Biol. Chem.*, **269**, 14566–14574.

Warne,P.H., Viclana,P.R. and Downward,J. (1993) Direct interaction of Ras and the amino-terminal region of Raf-1 *in vitro*. *Nature*, **364**, 352–355.

Wartmann,M. and Davis,R.J. (1994) The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.*, **269**, 6695–6701.

Wassarman,D.A., Solomon,N.M., Chang,H.C., Karim,F.D., Therrien,M. and Rubin,G.M. (1996) Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in *Drosophila*. *Genes Dev.*, **10**, 272–278.

Williams,N., Roberts,T.M. and Li,P. (1992) Both p21ras and pp60v-src are required, but neither alone is sufficient, to activate the raf-1 kinase. *Proc. Natl Acad. Sci. USA*, **89**, 2922–2926.

Wu,X., Noh,S.J., Zhou,G., Dixon,J.E. and Guan,K.-L. (1996) Selective activation of MEK1 but not MEK2 by A-Raf from epidermal growth factor-stimulated HeLa cells. *J. Biol. Chem.*, **271**, 3265–3271.

Yamamori,B., Kuroda,S., Shimizu,K., Fukui,K., Ohtsuka,T. and Takai,Y. (1995) Purification of a Ras-dependent mitogen-activated kinase kinase kinase from bovine brain cytosol and its identification as a complex of B-raf and 14-3-3 proteins. *J. Biol. Chem.*, **270**, 11723–11726.

Zhang,X., Settleman,J., Kyriakis,J.M., Takeuchi-Suzuki,E., Elledge,S.J., Marshall,M.S., Bruder,J.T., Rapp,U.R. and Avruch,J. (1993) Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature*, **364**, 308–313.

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