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# Mammalian Raf-1 is activated by mutations that restore Raf signaling in *Drosophila*

**Richard E.Cutler,Jr and Deborah K.Morrison**

Molecular Basis of Carcinogenesis Laboratory, ABL-Basic Research Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702, USA

**An interaction with the Ras proto-oncogene product is a requirement for Raf-1 activation in many signaling cascades. The significance of this interaction is demonstrated by the fact that a mutation preventing the Ras–Raf interaction severely impairs the function of both mammalian (Raf-1) and *Drosophila* (D-Raf) Raf proteins. In D-Raf, however, dominant intragenic mutations have been identified that suppress the effect of the Ras-binding site (RBS) mutation. To address the mechanism by which these mutations restore Raf signaling, we have introduced the suppressor mutations into the analogous residues of mammalian Raf-1. Here, we show that rather than compensating for the RBS mutation by restoring the Ras–Raf-1 interaction, the suppressor mutations increase the enzymatic and biological activity of Raf-1, allowing Raf-1 to signal in the absence of Ras binding. Surprisingly, we find that while one of the suppressor mutations (P181L) increases the basal kinase activity of Raf-1, it also abolishes the ability of wild-type Raf-1 to become activated by Ras. This mutation occurs in the cysteine-rich domain (CRD) of Raf-1 and demonstrates the importance of this region for a productive Ras–Raf interaction. Finally, we present evidence that the most activating suppressor mutation (G498S) increases Raf-1 activity by introducing a novel phosphorylation site into the $L_{12}$ activation loop of the Raf-1 kinase domain.**

*Keywords*: activation/mutation/Raf-1/Ras/signaling

## Introduction

The Raf-1 serine-threonine kinase plays an essential role in the transmission of many proliferative, developmental and oncogenic signals. Although the exact mechanisms responsible for activating Raf-1 in response to signaling events are not fully understood, a prerequisite for Raf-1 activation in many signaling pathways is an interaction with the Ras proto-oncogene product (reviewed by Marshall, 1994a; Moodie and Wolfman, 1994; Morrison, 1995). The involvement of Ras in Raf-1 function was first demonstrated by genetic and biochemical studies showing that in many cases the activation of Raf-1 is dependent on a functional Ras protein (Szeberenyi *et al.*, 1990; Dickson *et al.*, 1992; Troppmair *et al.*, 1992; Wood *et al.*, 1992; Han *et al.*, 1993). Subsequently, Raf-1 has been shown to interact directly with GTP-bound forms of Ras *in vitro* and *in vivo* (Finney *et al.*, 1993; Moodie *et al.*, 1993; Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993; Zhang *et al.*, 1993; Hallberg *et al.*, 1994). In mammalian Raf-1, Arg89 is a critical residue required for the Ras–Raf-1 interaction. Mutation of this site (R89L) prevents the association between Raf-1 and Ras and the Ras-dependent activation of Raf-1 (Fabian *et al.*, 1994). Arg89 is contained within the Raf-1 N-terminal regulatory domain in a conserved region that has been defined as the Ras-binding domain (RBD; residues 51–131) (Vojtek *et al.*, 1993; Scheffler *et al.*, 1994). Elucidation of the crystal structure of the Raf-1 RBD in complex with the Ras subfamily member Rap1A has revealed that Arg89 forms a direct, water-mediated interaction with two amino acid residues of Rap1A (Nassar *et al.*, 1995). In addition, studies using circular dichroism to analyze the dissociation constant of the Ras–Raf interaction have confirmed that the R89L mutation abrogates the interaction between Ras and Raf-1 (Block *et al.*, 1996). Therefore, both structural and mutational analyses have established the importance of Arg89 for the binding of Raf-1 to Ras.

In *Drosophila* Raf (D-Raf), the analogous arginine residue (Arg217) has also been shown to play a crucial role in D-Raf-mediated signal transduction. Mutation of Arg217 (R217L) markedly alters D-Raf function and causes lethality in hemizygous males (Melnick *et al.*, 1993). However, dominant intragenic mutations have been identified that suppress the lethality associated with the Ras-binding site (RBS) Arg217 mutation (Lu *et al.*, 1994). From this study, four intragenic suppressor mutations were recovered, each containing one compensatory amino acid change in either the CR1 (F290I and P308L) or CR3 (G621S and L733Q) domains of D-Raf. Of the four residues altered by mutation, three are conserved in all Raf proteins (F290I, P308L and G621S; analogous to F163I, P181L and G498S in Raf-1, respectively). Since the suppressor mutations compensate for the functional defect of the RBS mutation, it is likely that they have identified important residues involved in Raf function. Therefore, to address whether the D-Raf RBS suppressor mutations have occurred in key residues that regulate Raf activity and to determine the mechanism by which these mutations restore Raf signaling, we introduced the analogous mutations into mammalian Raf-1 and examined the activities of the resulting mutant proteins. In this study, we find that the suppressor mutations increase the biological and enzymatic activity of Raf-1 and reveal new mechanisms of Raf-1 activation.

## Results

### *The RBS suppressor mutations activate the biological and enzymatic activity of Raf-1*

To determine whether amino acid changes analogous to the D-Raf suppressor mutations would act as suppressors of the R89L mutation in mammalian Raf-1, we generated

mutant Raf-1 proteins that contained both the R89L mutation and each of the suppressor mutations (F163I, P181L and G498S; Figure 1A). In addition, to determine the effect that these mutations would have on the activity of wild-type (WT) Raf-1, we also introduced the suppressor mutations into the WT protein. The resulting mutant proteins were then expressed in stage VI-arrested *Xenopus* oocytes and evaluated for their ability to promote oocyte meiotic maturation [as evidenced by germinal vesical breakdown (GVBD)]. The *Xenopus* oocyte meiotic maturation assay was chosen for this analysis because it has been previously used to identify other activated mutants of the Ras/Raf-1/MAPK pathway (Birchmeir *et al.*, 1985; Fabian *et al.*, 1993a; Therrien *et al.*, 1996). In addition, a significant advantage of this assay system is that mutations resulting in subtle changes in the biological and enzymatic activity of Raf-1 can be easily detected. As shown in Figure 1B, expression of R89L Raf-1 was unable to promote oocyte meiotic maturation. However, all of the R89L Raf-1 proteins containing the suppressor mutations (R89L/G498S Raf-1, R89L/F163I Raf-1 and R89L/P181L Raf-1) induced maturation in ~20% of the oocytes (Figure 1B). Similarly, when the suppressor mutations were introduced into WT Raf-1, an increase in biological activity was observed. While WT Raf-1 induced GVBD in only 4% of the oocytes, F163I Raf-1 promoted maturation in 41%, P181L Raf-1 in 17% and G498S Raf-1 in 85% of the oocytes. Interestingly, although the P181L mutation had an equivalent effect on both the R89L Raf-1 and WT Raf-1 proteins, both the F163I and G498S mutations resulted in a more substantial increase in the activity of WT Raf-1 (41% GVBD for F163I Raf-1 versus 20% for R89L/F163I Raf-1 and 85% GVBD for G498S Raf-1 versus 22% for R89L/G498S Raf-1).

To examine whether the increased biological activity of the mutant proteins correlated with an increase in enzymatic activity, we immunoprecipitated the WT Raf-1 and R89L Raf-1 proteins from oocyte lysates and then measured the kinase activity of the mutant proteins using MEK (also known as MKK1) as an exogenous substrate (Figure 1C). In the R89L Raf-1 background, all of the suppressor mutations resulted in a 3.5- to 4-fold increase in activity; and in the context of WT/Raf-1, a 4.8-fold increase was observed for F163I Raf-1, 3.8-fold for P181L Raf-1 and 9-fold for G498S Raf-1. These findings correlate with the results observed in the meiotic maturation assay and indicate that Raf-1 proteins containing the RBS suppressor mutations have both an elevated biological and an elevated enzymatic activity.

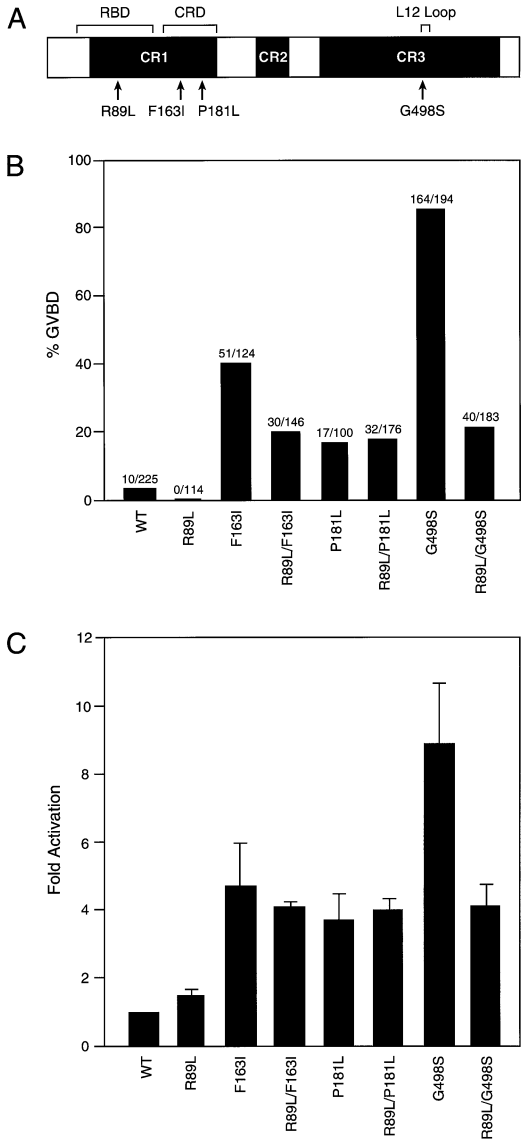


**Fig. 1.** RBS suppressor mutations increase the biological and enzymatic activity of Raf-1. (**A**) Schematic representation of Raf-1 showing the R89L RBS mutation present within RBD and the three conserved intragenic suppressor mutations analogous to D-Raf mutations (F163I, P181L and G498S). RBD, CRD and the $L_{12}$ activation loop are also indicated. (**B**) Induction of *Xenopus* oocyte meiotic maturation by the expression of Raf-1 proteins. RNA (~30 ng) encoding WT Raf-1, R89L Raf-1, F163I Raf-1, R89L/F163I Raf-1, P181L Raf-1, R89L/P181L Raf-1, G498S Raf-1 or R89L/G498S Raf-1 proteins were injected into stage VI oocytes. Oocytes were scored for GVBD within 24 h of injection. The percentage of oocytes undergoing GVBD is expressed as a solid bar and the ratio of the number of oocytes undergoing GVBD to the total number injected is displayed above each bar. The numbers obtained represent a compilation of at least five independent experiments in which equivalent amounts of the Raf-1 proteins were expressed. (**C**) *In vitro* kinase activity of the Raf-1 proteins. Raf-1 proteins were immunoprecipitated from the lysates of three injected oocytes and *in vitro* protein kinase assays were performed as described in Materials and methods, using MEK as an exogenous substrate. The amount of $^{32}P$ incorporated into MEK was then determined. The activity of WT/Raf-1 was expressed as one; and the fold activation for each mutant represents the average of three experiments.

### *The RBS suppressor mutations enhance the biological activity of membrane-localized Raf-1*

Previous studies have indicated that Ras binding localizes Raf-1 to the plasma membrane where it becomes activated and that artificially targeting Raf-1 to the plasma membrane results in constitutive activation of the kinase (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Since membrane-localized Raf-1 proteins, such as Raf-CAAX, efficiently promote the meiotic maturation of *Xenopus* oocytes, we next investigated the effect of the suppressor mutations on the kinetics with which Raf-CAAX induced oocyte maturation. WT and mutant Raf-CAAX proteins were expressed in *Xenopus* oocytes and maturation levels were determined at times when WT Raf-CAAX had induced GVBD in 0% (T1), 15% (T2), 40% (T3) and 80% (T4) of the oocytes (Figure 2). Our results indicate that while

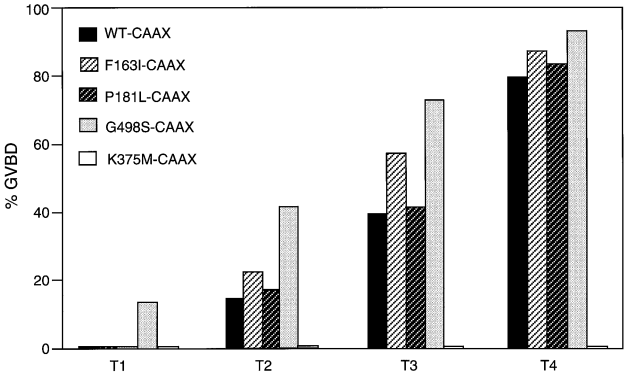


**Fig. 2.** Effect of the RBS suppressor mutations on the biological activity of membrane-localized Raf-1. *Xenopus* oocytes were injected as described in Figure 1B and the kinetics of GVBD induced by the expression of WT Raf-CAAX, F163I Raf-CAAX, P181L Raf-CAAX, G498S Raf-CAAX or K375M Raf-CAAX was determined. Maturation levels were measured at times when WT/Raf-CAAX had induced GVBD in 0% (T1), 15% (T2), 40% (T3) and 80% (T4) of the oocytes. The results shown are the average of two independent experiments.

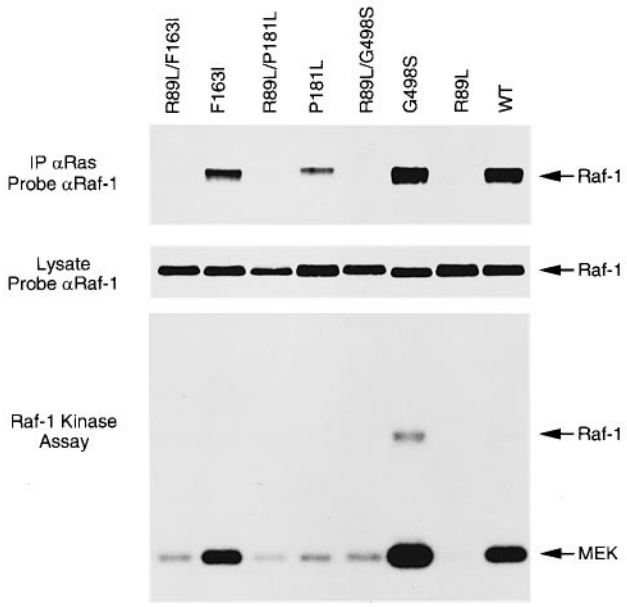


**Fig. 3.** The effect of the RBS suppressor mutations on the Ras–Raf-1 interaction. *Xenopus* oocytes were injected with RNA (~30 ng) encoding WT/Raf-1, R89L Raf-1, F163I Raf-1, R89L/F163I Raf-1, P181L Raf-1, R89L/P181L Raf-1, G498S Raf-1 or R89L/G498S Raf-1. Four hours later, oocytes were injected with ~30 ng of Ha-Ras$^{V12}$ RNA and lysates were prepared at GVBD. Top panel: Ras proteins were immunoprecipitated from oocytes lysed in NP-40 lysis buffer. The immunoprecipitates were resolved by electrophoresis on a 10% SDS–polyacrylamide gel and examined for the presence of Raf-1 by immunoblotting with antibodies to Raf-1. Middle panel: total lysates were also examined for expression of the mutant Raf-1 proteins. Bottom panel: Raf-1 proteins were immunoprecipitated from oocytes lysed in RIPA buffer. *In vitro* protein kinase assays were performed using MEK as an exogenous substrate and assays were terminated by the addition of gel-loading buffer. The phosphoproteins were resolved by electrophoresis on a 10% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and visualized by autoradiography. The amount of $^{32}$P incorporated into MEK was determined by Cerenkov counting. The immunoprecipitates were subsequently examined by immunoblotting with antibodies to Raf-1 to ensure that equivalent amounts of the Raf-1 proteins were present. In comparison with the activity of R89L Raf-1, the fold activation for WT Raf-1 expressed in the presence of Ha-Ras$^{V12}$ was: 25.3; F163I Raf-1, 22.1; R89/F163I Raf-1, 4.2; P181L/Raf-1, 4.4; R89L/P181L Raf-1, 3.8; G498S Raf-1, 41.0 and R89L/G498S Raf-1, 4.8.

P181L Raf-CAAX induced oocyte maturation at approximately the same rate as did WT Raf-CAAX, F163I Raf-CAAX and G498S Raf-CAAX induced maturation 30% and 60% faster, respectively, than did WT Raf-CAAX. In addition, oocytes expressing G498S Raf-CAAX consistently began undergoing GVBD 2 h earlier than did oocytes expressing WT Raf-CAAX. The enzymatic activity of the mutant Raf-CAAX proteins also correlated with their ability to promote oocyte maturation, in that G498S Raf-CAAX exhibited the strongest and earliest detectable kinase activity (data not shown). Therefore, in the context of the Raf-CAAX protein, the most activating mutation was the G498S substitution. This finding is consistent with the results observed for the WT Raf-1 protein and suggests that the G498S mutation has the greatest activation potential.

### *The RBS suppressor mutations do not restore the Ras–Raf-1 interaction*

To address the mechanism by which the RBS suppressor mutations have increased Raf-1 activity, we first examined the effect of these mutations on the ability of Raf-1 to interact with Ras. Activated Ha-Ras$^{V12}$ and each of the WT/Raf-1 and R89L/Raf-1 proteins were coexpressed in *Xenopus* oocytes. At the time of GVBD, Ras immunoprecipitates were prepared and examined for the presence of Raf-1 by immunoblot analysis. As expected, WT Raf-1 was detected in the Ras immunoprecipitates, but R89L Raf-1 was not. Likewise, none of the mutant R89L Raf-1 proteins were able to coimmunoprecipitate with Ha-Ras$^{V12}$, indicating that the suppressor mutations had not restored the ability of R89L Raf-1 to associate with Ha-Ras$^{V12}$. Examination of the WT Raf-1 proteins revealed that both G498S Raf-1 and WT Raf-1 associated with Ha-Ras$^{V12}$ to approximately the same extent. However, a decreased interaction between Ha-Ras$^{V12}$ and either F163I Raf or P181L Raf-1 was consistently observed, with the reduction being the greatest (>80%) for P181L/Raf-1. Thus, in the context of WT Raf-1, none of the suppressor mutations enhanced the association with Ha-Ras$^{V12}$ and, in fact, two of the mutations inhibited the Ras–Raf-1 interaction.

To extend these observations, we examined whether the RBS suppressor mutations altered the ability of Raf-1 to be activated by Ha-Ras$^{V12}$. Raf-1 proteins were immunoprecipitated from *Xenopus* oocytes coexpressing activated Ha-Ras$^{V12}$ and each of the WT Raf-1 and R89L Raf-1 proteins, and the enzymatic activity of the immunoprecipitated proteins was determined (Figure 3). Consistent with previous reports from our laboratory (Fabian *et al.*, 1994), the kinase activity of R89L Raf-1 was very low and unable to be activated by Ha-Ras$^{V12}$. The mutant R89L Raf-1 proteins all exhibited a kinase activity that was ~4-fold higher than that of R89L Raf-1, but this level of activity was equivalent to that observed when the mutant R89L Raf-1 proteins were expressed in the absence of Ha-Ras$^{V12}$ (compare Figures 1C and 3), indicating that these proteins were unable to be activated by Ha-Ras$^{V12}$. When the activity of the WT Raf-1 proteins containing the suppressor mutations was compared with the activities of the respective mutant R89L Raf-1 proteins, both the G498S Raf-1 and F163I Raf-1 had increased activity in

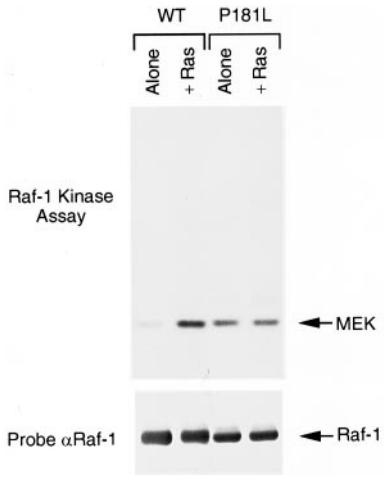


**Fig. 4.** The P181L suppressor mutation prevents the Ras-dependent activation of Raf-1 in Sf9 cells. WT Raf-1 or P181L Raf-1 was expressed in Sf9 cells alone or in the presence of Ha-Ras$^{V12}$ (+ Ras). Raf-1 proteins were immunoprecipitated from cells lysed in RIPA buffer and *in vitro* protein kinases were performed using MEK as the exogenous substrate. Assays were terminated by the addition of gel loading buffer, the samples were resolved by electrophoresis on an 8% SDS–polyacrylamide gel and the phosphoproteins were visualized by autoradiography.

response to Ha-Ras$^{V12}$, demonstrating that these mutations had not altered the ability of WT Raf-1 to be activated by Ha-Ras$^{V12}$. However, the strong activational effect of Ha-Ras$^{V12}$ appears to override and partially obscure the activational effect of the F163I and G498S mutations. Surprisingly, the activity of P181L Raf-1 was equivalent to the activity of R89L/P181L Raf-1 and was not elevated in the presence of Ha-Ras$^{V12}$, indicating that the P181L mutation had altered the ability of this protein to be activated by Ha-Ras$^{V12}$. This finding was further confirmed using the baculovirus/Sf9 cell expression system. When expressed in Sf9 cells, the basal kinase activity of P181L Raf-1 was elevated in comparison with WT Raf-1; however, no increase in activity was observed when P181L Raf-1 was coexpressed with Ha-Ras$^{V12}$ (Figure 4). These findings indicate that not only has the P181L mutation inhibited the Ras–Raf-1 interaction but it has rendered the protein unable to be activated by Ha-Ras$^{V12}$.

### *The RBS suppressor mutations do not alter the Raf-1–14-3-3 interaction*

Another mechanism by which the RBS suppressor mutations could increase Raf activity is to enhance the interaction of Raf-1 with an activator molecule or protein. One such protein that has been proposed to be involved in the Raf-1 activation process is 14-3-3 (Fantl *et al.*, 1994; Freed *et al.*, 1994; Irie *et al.*, 1994). Therefore, we investigated the effect of the RBS suppressor mutations on the Raf-1–14-3-3 interaction. Human 293 cells were transiently transfected with constructs encoding FLAG epitope-tagged versions of the various WT Raf-1 and R89L Raf-1 proteins. Forty-eight hours later, the FLAG-tagged Raf-1 proteins were immunoprecipitated and examined for the presence of 14-3-3. As a control, cells were also transfected with a construct expressing a Raf-1 mutant defective in its ability to associate with 14-3-3 (CRM Raf-1; Michaud *et al.*, 1995). Results from this experiment indicate that in comparison with WT Raf-1

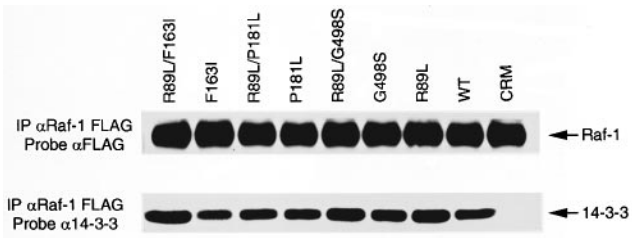


**Fig. 5.** Effect of the RBS suppressor mutations on the Raf-1/14-3-3 interaction. FLAG epitope-tagged WT Raf-1, R89L Raf-1, F163I Raf-1, R89L/F163I Raf-1, P181L Raf-1, R89L/P181L Raf-1, G498S Raf-1, R89L/G498S Raf-1 and CRM/Raf-1 proteins were transiently expressed in 293 cells. The FLAG antibody was used to immunoprecipitate the tagged Raf-1 proteins from cells lysed in NP-40 lysis buffer. The immunoprecipitates were resolved by electrophoresis on a 10% SDS–polyacrylamide gel and examined by immunoblotting with antibodies to Raf-1 and 14-3-3.

and R89L Raf-1, equivalent amounts of 14-3-3 were immunoprecipitated by the proteins containing the RBS suppressor mutations (Figure 5). Therefore, the RBS suppressor mutations do not appear to enhance the activity of Raf-1 by altering the Raf-1–14-3-3 interaction.

### *Activation of Raf-1 by a negatively charged aspartic acid residue at the 498 site*

Examination of the suppressor mutations and the amino acid context in which they are located reveals that the glycine to serine substitution at the 498 site has generated a consensus sequence of phosphorylation (RXXS) and hence introduced a potential site of phosphorylation (Pearson and Kemp, 1991). The 498 residue is contained within the $L_{12}$ activation loop of the Raf-1 kinase domain and, for other protein kinases, phosphorylation in this region has been shown to have an activational effect (reviewed by Marshall, 1994b). Thus, to address whether phosphorylation plays a role in enhancing the activity of the G498S mutants, we performed further mutational analysis of the 498 site. By site-directed mutagenesis, the glycine residue at the 498 site was changed to an alanine residue, which is unable to be phosphorylated; to an aspartic acid residue, which mimics the negative charge of a phosphorylated residue; and to a threonine or tyrosine residue, both of which are potential phosphate acceptors. The activity of these mutants was then measured using the *Xenopus* oocyte maturation assay. As shown in Figure 6, G498A Raf-1 induced maturation in 23% of the oocytes; G498T, 21%; G498Y, 31% and G498D, 61%. These findings demonstrate that although any perturbation of the 498 site increased the biological activity of Raf-1, the greatest increase was observed with the negatively charged aspartic acid substitution. This result, together with the finding that the activational effect of phosphorylation in the L12 loop can be recapitulated in MEK kinase by the substitution of negatively charged residues (Alessi *et al.*, 1994; Huang and Erickson, 1994; Mansour *et al.*, 1994, 1996), support the idea that phosphorylation plays a role in the activation of G498S Raf-1.

### *G498S Raf-1 contains a novel phosphopeptide*

To determine whether the G498S mutation has indeed resulted in a novel phosphorylation event, we compared the *in vivo* phosphorylation state of WT Raf-1 and G498S Raf-1. FLAG-tagged WT Raf-1 and G498S Raf-1 were

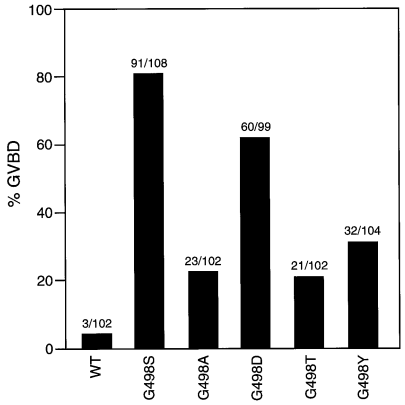


**Fig. 6.** Effect of other amino acid substitutions at the 498 site. RNA (~30 ng) encoding WT Raf-1, G498S Raf-1, G498A Raf-1, G498D Raf-1, G498T Raf-1 or G498Y Raf-1 proteins were injected into stage VI oocytes. Oocytes were then scored for GVBD within 24 h of injection. The numbers shown represent a compilation of at least four independent experiments in which equivalent amounts of the Raf-1 proteins were expressed.

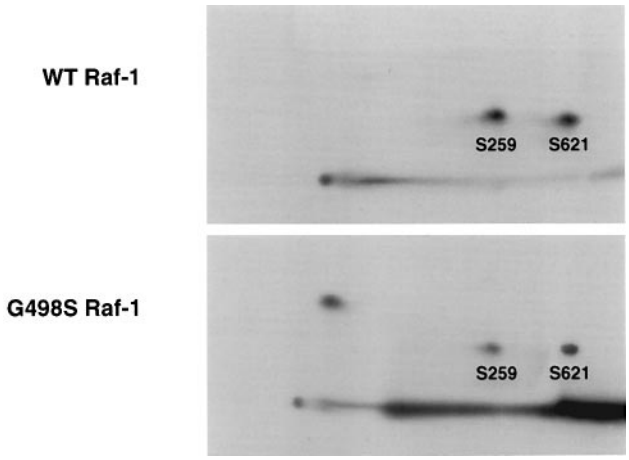


**Fig. 7.** G498S Raf-1 contains a novel phosphopeptide. 293 cells expressing Ha-Ras$^{V12}$ and either WT Raf-1 or G498S Raf-1 were labeled *in vivo* with [$^{32}$P]orthophosphate. $^{32}$P-labeled Raf-1 proteins were then isolated and digested with trypsin. The phosphopeptides were resolved in two dimensions on TLC plates by electrophoresis (horizontal axis) and by chromatography (vertical axis). Shown are phosphopeptide maps of WT Raf-1 and G498S Raf-1. Peptides containing the phosphorylated Ser259 and Ser621 residues are indicated.

expressed in 293 cells with Ha-Ras$^{V12}$, and the cells were labeled *in vivo* with [$^{32}$P]orthophosphate. The $^{32}$P-labeled Raf-1 proteins were then isolated and subjected to two-dimensional tryptic phosphopeptide mapping analysis (Figure 7). Examination of the WT Raf-1 peptide map revealed two major phosphopeptides representing the phosphorylation of serine residues 259 and 621 (Morrison *et al.*, 1993). In comparison, the map of G498S Raf-1 contained a novel third phosphopeptide whose migration was consistent with the predicted mobility of the peptide containing the G498S site (based on the charge, mass and hydrophobicity of the peptide; Boyle *et al.*, 1991). Therefore, the findings that substitution of a negatively charged residue at the 498 site had the greatest activation effect and that the increased activity of G498S Raf-1 correlates with the presence of a major novel phosphopeptide provide strong evidence that phosphorylation is the mechanism by which the G498S mutation enhances Raf-1 activity.

## Discussion

In many signaling pathways, an interaction with Ras is a critical early step in the Raf-1 activation process. For both mammalian and *Drosophila* Raf proteins, an arginine mutation in the Raf RBD severely compromises the ability of these proteins to mediate cell signaling. However, in *Drosophila*, the effect of this mutation can be suppressed by several intragenic amino acid substitutions in D-Raf. In this report, the RBS suppressor mutations identified in D-Raf were introduced into mammalian Raf-1 and their effect on Raf-1 activity was determined.

Using the *Xenopus* oocyte maturation assay to measure biological activity, we found that all of the suppressor mutations activate Raf-1 (Figure 1). When introduced into Raf-1 proteins containing the RBS mutation (R89L Raf), the suppressor mutations resulted in an equivalent increase in activity, with all the mutant R89L Raf-1 proteins inducing maturation in ~20% of the oocytes. In addition, the enzymatic activity of the mutant R89L Raf-1 proteins was elevated 3- to 4-fold above that of WT Raf-1 and R89L Raf-1. The activational effect of the suppressor mutations on the R89L Raf-1 protein was more subtle than has been observed for other activating Raf-1 mutations (Fabian *et al.*, 1993b; Michaud *et al.*, 1995). However, this result is consistent with the observation that, in *Drosophila*, the suppressor mutations restore R217L D-Raf signaling but do not induce a dominant activated phenotype (Lu *et al.*, 1994). In the context of WT Raf-1, the suppressor mutations also increased the enzymatic and biological activity of Raf-1, but the degree to which these mutations altered Raf-1 activity was not equivalent. The G498S mutation was found to be the most activating, followed by the F163I and P181L mutations, respectively. The observed differences in activation levels may simply reflect some deleterious effect caused by the R89L mutation (such as a structural alteration) that cannot be overcome by the suppressor mutations. Alternatively, since the WT Raf-1 proteins are still competent to bind Ras, some degree of activation may be due to an interaction with the endogenous *Xenopus* Ras protein that is continually cycling between the GDP- and GTP-bound states. In this scenario, because Ras binding localizes Raf-1 to the plasma membrane where it becomes activated, the suppressor mutations would be expected to have a similar effect on both WT Raf-1 and membrane-localized Raf-CAAX. This is indeed the case, since the activity of both the WT Raf-1 and Raf-CAAX proteins was most affected by the G498S mutation followed by the F163I mutation. Further support for the idea that an interaction with endogenous Ras contributes to the increased activity of the WT Raf-1 mutants, comes from the finding that the P181L mutation, which severely inhibits the Ras–Raf-1 interaction and renders WT Raf-1 unable to be activated by Ras, has the same activational effect on both WT Raf-1 and R89L Raf-1. Irrespective of the different levels of activation, however, all of the suppressor mutations clearly increase both the enzymatic and biological activity of Raf-1.

In addressing the mechanisms by which the suppressor mutations enhance the activity of Raf-1, we find that

none of the mutations restore or increase the Ras–Raf-1 interaction. In coimmunoprecipitation experiments using proteins expressed in *Xenopus* oocytes (Figure 3), as well as in 293 cells and in Sf9 cells (data not shown), no interaction between activated Ras and any of the R89L Raf-1 proteins was detected. Consistent with these findings, the RBS suppressor mutations have not been found to restore the binding of R217L D-Raf and *Drosophila* Ras in the two-hybrid interaction system (Hou *et al.*, 1995; N.Perrimon, personal communication). Furthermore, in the context of WT Raf-1, none of the suppressor mutations enhanced the interaction with Ras and, in fact, the F163I and P181L mutations inhibited the Ras–Raf-1 interaction. Interestingly, both the F163I and P181L mutations are located within the N-terminal cysteine-rich domain (CRD) of Raf-1 that has been identified to be a second Ras-binding domain (Brtva *et al.*, 1995; Hu *et al.*, 1995; Drugan *et al.*, 1996). The recent solution structure of CRD reveals that the F163 residue is located in a β-strand that forms a β-sheet structure while the P181 residue is located at the end of a region that displays an α-helical character (Mott *et al.*, 1996). Like the RBD, the CRD is highly conserved and it is likely that the amino acid changes resulting from the P181L and/or F163I suppressor mutations have altered the structure of this domain, decreasing or eliminating its ability to bind or stabilize the interaction with Ras. Other mutations in this region have been shown to reduce the interaction with Ras (Zhang *et al.*, 1993; Chow *et al.*, 1995); however, the P181 mutation represents the first report of a single point mutation in the CRD that prevents the Ras-dependent activation of Raf-1. This finding, together with previous analyses of the CRD (Brtva *et al.*, 1995; Hu *et al.*, 1995; Drugan *et al.*, 1996; Mott *et al.*, 1996), demonstrates the importance of this domain for a productive Ras–Raf interaction.

It is intriguing that while the F163I and P181L mutations inhibit the interaction with Ras, they also increase the enzymatic and biological activity of Raf-1. Our data have not elucidated the precise mechanism by which these mutations increase Raf activity, but this effect may also be attributed to their location in the CRD. Not only has this region been shown to be involved in Ras binding, it has also been implicated in the interaction of Raf-1 with other proteins and ligands, such as phosphatidylserine and 14-3-3 (Ghosh *et al.*, 1994; Michaud *et al.*, 1995). Our results demonstrate that these mutations do not alter the Raf-1–14-3-3 interaction, although it is possible that the F163I and P181L mutations may enhance the interaction of Raf-1 with some other activator molecule or reduce the interaction with an inhibitor. Alternatively, it is also possible that these mutations have induced structural changes in Raf-1 that increase its enzymatic activity. The N-terminal domain, where the CRD and these mutations are located, is thought to function by suppressing the catalytic activity of the kinase domain (Morrison, 1995). Thus, in this case, any alteration that reduces the suppressive effect of the N-terminal domain would be expected to increase the activity of Raf-1. Determining which, if either, of these hypotheses is correct awaits further analysis.

Our results do, however, indicate that phosphorylation may be the mechanism whereby the G498S suppressor mutation increases Raf-1 activity. The G498S mutation generates a potential site of phosphorylation in a region of the Raf-1 catalytic domain that has been shown to play a key role in regulating the activity of a number of other kinases (Knighton *et al.*, 1991a,b; Zhang *et al.*, 1994). This region is called the $L_{12}$ activation loop and for several serine-threonine and tyrosine kinases, phosphorylation of residues within this region results in enzymatic activation (reviewed by Marshall, 1994b). In addition, for MEK kinase, substitution of a negatively charged residue within the $L_{12}$ loop (which mimics the effect of phosphorylation) results in constitutive activation of the kinase (Alessi *et al.*, 1994; Huang and Erickson, 1994; Mansour *et al.*, 1994, 1996). In our studies, we found that when several other amino acid substitutions were made at the 498 site, introduction of a negatively charged residue had the greatest activational effect. In addition, the increased activity of G498S Raf-1 was found to be correlated with the presence of a major, novel phosphopeptide not present in WT Raf-1. Together, these results provide strong evidence that phosphorylation plays a role in the activational effect of the G498S mutation. The RXXS motif generated by the G498S mutation is a consensus site of phosphorylation for several kinases, such as protein kinase C, cAMP-dependent kinase and Rsk (Hanks and Quinn, 1991). Although we have not addressed which kinase is responsible for phosphorylating G498S Raf-1, the finding that the G498S mutation has the greatest effect on WT Raf-1, which is competent to bind Ras at the plasma membrane, and on the membrane-localized Raf-CAAX raises the question of whether the responsible kinase may be located at the plasma membrane. Finally, it is interesting to note that the phosphopeptide map of WT Raf-1 expressed in the presence of activated Ha-$Ras^{V12}$ only contained two major phosphopeptides, representing the phosphorylation of Ser259 and Ser621 (Morrison *et al.*, 1993). This result appears to suggest that phosphorylation of the $L_{12}$ loop does not normally play a role in the Ras-dependent activation process. However, our results do not rule out the possibility that under other conditions phosphorylation in this region may contribute to Raf-1 regulation.

In conclusion, our study examining the mechanism of rescue of the D-Raf RBS suppressor mutations has revealed that all of the suppressor mutations increase the enzymatic and biological activity of Raf-1, allowing Raf-1 to signal in the absence of Ras binding. Since the function of Raf proteins is highly conserved, it is therefore likely that the suppressor mutations have also resulted in an increase in the enzymatic activity of R217L D-Raf, which may account for both the enhanced biological activity and the suppressor activity associated with these mutations in *Drosophila*.

## Materials and methods

### *Antibodies*

The Raf-1 antibody used in this study was a mouse monoclonal antibody generated against human Raf-1 (Transduction Laboratories). The FLAG antibody was the M2 mouse monoclonal antibody (Eastman Kodak Co.) and the 14-3-3 antibody was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.). Ras antibodies used in this study include a mouse monoclonal antibody generated against the entire 21 kDa human Ras (Ha-Ras) protein (Transduction Laboratories) and the rat monoclonal antibody Y13-238 (Oncogene Science).

### Construction of Raf-1 plasmids

Raf-1 mutant constructs were generated by site-directed mutagenesis using a human *Raf-1* cDNA clone (*pKS:cRaf*) and the appropriate oligonucleotides to introduce the desired base changes. For the *FLAG-Raf-1* construct, sequences encoding the FLAG epitope tag were inserted proximal to the N-terminal methionine as previously described (Michaud *et al.*, 1995), and for the *Raf-CAAX* construct, sequences encoding the C-terminal 17 amino acids of K-Ras were added to the C-terminus of *Raf-1* (Stokoe *et al.*, 1994). The specific base changes in all mutant constructs were confirmed by sequence analysis. cDNA fragments encoding the entire WT and mutant Raf-1 proteins were isolated and inserted into the pSP64T transcription vector for expression in *Xenopus* oocytes (Krieg and Melton, 1984), into the pLNCX retroviral vector for expression in 293 cells, and into the pVL941 baculovirus vector for expression in Sf9 cells.

### Oocyte injection and analysis

Oocytes were isolated and defolliculated as previously described (Fabian *et al.*, 1993b). Eight to twelve hours after isolation, oocytes were injected with ~30 ng of *in vitro* transcribed RNA encoding the various Raf-1 proteins. In some cases, the oocytes were injected 4–8 h later with ~30 ng of *Ha-Ras*$^{V12}$ RNA. Oocytes were scored for GVBD, as evidenced by the appearance of a white spot on the animal pole. This observation was verified by manual dissection of oocytes after fixation in 8% trichloroacetic acid (TCA).

### Expression of recombinant proteins in 293 and Sf9 cells

For transient expression of Raf-1 proteins in 293 cells, the pLNCX-FLAG-Raf-1 constructs were transfected into 293 cells by the calcium phosphate method (Wigler *et al.*, 1978). Cells were lysed 48 h after transfection. For protein production in Sf9 cells, $2\times10^6$ cells were infected with the desired recombinant baculoviruses at a multiplicity of infection of 10 and lysed 48 h after infection.

### Preparation of cell lysates and immunoprecipitation assays

*Xenopus* oocytes were lysed by titration with a pipette tip in either Nonidet P-40 lysis buffer [20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.15 U/ml), 20 μM leupeptin, 5 mM sodium vanadate] or RIPA buffer [20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 1 mM PMSF, aprotinin (0.15 U/ml), 20 μM leupeptin, 5 mM sodium vanadate] (10 μl per oocyte). 293 and Sf9 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed for 20 min at 4°C in 500 μl of NP-40 or RIPA lysis buffer. Insoluble material was pelleted by centrifugation at 14 000 *g* for 10 min at 4°C and cell lysates were equalized for protein expression by immunoblot analysis. Immunoprecipitation assays were performed by incubating cell lysates with the appropriate antibody for 12–16 h at 4°C. Coimmunoprecipitation assays were performed using cells lysed in NP-40 lysis buffer and Raf-1 immunoprecipitates for kinase assays were prepared using cells lysed in RIPA buffer. The antigen–antibody complexes were collected with protein G–Sepharose beads (Pharmacia Biotechnology). The immunoprecipitates were then washed four times with cold NP-40 lysis buffer before analysis by SDS–PAGE.

### In vitro protein kinase assays

Raf-1 kinase activity was determined from the lysates of either three oocytes or $2\times10^6$ Sf9 cells. Raf-1 proteins were immunoprecipitated and washed three times with NP-40 lysis buffer and once with kinase buffer [30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4), 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2–5 μM ATP]. The complexes were then incubated at 25°C for 15 min in 40 μl of kinase buffer containing 20 μCi of [$\gamma$-$^{32}$P]ATP and 0.1 μg of purified 5′-*p*-fluorosulfonyl-benzoyladenosine (FSBA)-treated MEK (Dent *et al.*, 1994). Kinase assays were terminated by the addition of gel loading buffer (4% SDS, 80 mM dithiothreitol, 10% glycerol), the samples were resolved by SDS–PAGE, and phosphoproteins were visualized by autoradiography.

### Phosphopeptide mapping analysis

The phosphopeptide mapping was performed as described by Morrison *et al.* (1993). $^{32}$P-labeled proteins were separated by SDS–PAGE, eluted from the gel matrix and TCA precipitated. The isolated protein was then subjected to enzymatic digestion with trypsin. Labeled peptides were separated on TLC plates by electrophoresis followed by ascending chromatography.

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