The contribution of residues outside the Ras binding domain of Raf (RafRBD) to Ras-Raf interaction and Ras-dependent Raf activation has remained unresolved. Here, we utilize a double mutant approach to identify complementary interacting amino acids that are involved in Ras-Raf interaction and activation. Biochemical analysis demonstrates that Raf-Arg59 and Raf-Arg67 from RafRBD are interacting residues complementary to Ras-Glu37 located in the Ras effector region. Raf-Arg59 and Raf-Arg67 also mediate interaction with Ras-Glu37 in Ras-dependent Raf activation. The characteristics observed here can be used as criteria for a role of residues from other regions of Raf in Ras-Raf interaction and activation. We developed a quantitative two-hybrid system as a tool to investigate the effect of point mutations on protein-protein interactions that elude biochemical analysis of bacterially expressed proteins. This assay shows that Raf-Ser257 in the RafCR2 domain does not contribute to Ras-Raf interaction and that the Raf-S257L mutation does not restore Raf binding to Ras-E37G. Yet, Raf-S257L displays high constitutive kinase activity and further activation by Ras-G12V/E37G is still impaired as compared with activation by Ras-G12V. This strongly suggests that the RafCR2 domain is an independent domain involved in the control of Raf activity and a common mechanism for constitutively activating mutants may be the interference with the inactive ground state of the kinase.

The activation of Raf appears to be a complex multistep process (13). The regulatory N terminus of Raf contains two regions that are highly conserved between different members of the Raf family. A part of the first conserved region (CR1) encompassing amino acids 51–131 is responsible for Ras binding and has therefore been termed Ras binding domain (RafRBD).1 RafRBD constitutes an autonomous structural domain sufficient for GTP-dependent binding of Ras (14–18). The crystal structure of RafRBD complexed to the Ras homologue Rap1A has been solved (19, 20). Functional analysis of the interaction between Ras and RafRBD demonstrated that the mutation of Raf-Arg68 is sufficient to abrogate Ras-dependent Raf activation completely and that the activation of Raf correlates quantitatively with the binding affinity between Ras and RafRBD (21, 22).

Whereas the role of RafRBD in Ras binding is understood in great detail, the contribution of other domains in the Raf regulatory domain to Ras-Raf interaction and signaling is far from clear. A number of reports have suggested that the cystein-rich domain (RafCRD) and the RafCR2 domain also might be involved in Ras-Raf interaction and Ras-dependent Raf activation (10, 23–27). As to the role of the RafCR2 domain, it was found that the interaction of the Raf effector mutant Ras-E37G with Raf was restored by the Raf-S257L mutation that is localized within the Ser/Thr-rich RafCR2 domain, using the two-hybrid system (10). In addition, transformation by Ras-G12V/E37G could be achieved by cotransfection of Ras-G12V/E37G with Raf-S257L. These results suggested that the Raf-S257L mutation is capable to restore the binding of Raf to Ras-E37G and as a consequence Ras-dependent transformation was proposed to be achieved due to the activation of Raf-S257L by Ras-G12V/E37G (10). This further suggested that the RafCR2 domain might be involved in Ras-dependent Raf binding and activation. In addition, the RafCR2 region was shown to mediate interaction of Raf with 14–3–3 proteins that require phosphorylation of Raf-Ser259 (28). Interestingly, the mutation of Raf-Ser259 to alanine leads to constitutive activation of Raf demonstrating that the RafCR2 region plays an important role in the control of Raf activation (29).

Since no structural data are yet available, functional analysis of the RafCR2 domain provides the primary tool to investigate its role in Ras-Raf protein-protein interaction and Raf activation. Mutational analysis has successfully identified functional epitopes in protein-protein interaction (30, 31), such as the Ras effector domain (32). Since mutations of multiple residues within a protein-protein interface lead to additive effects on binding affinity (33), this effect has been utilized to

1 The abbreviations used are: RBD, Ras binding domain; mant-Gp(NH)p, fluorescent analogue of guanylyl-5′-yl imidodiphosphate labeled with the mant group; 2,3′-4(N-methylanthroniloyl)-CR, conserved region; CRD, cysteine rich domain; wt, wild-type; PCR, polymerase chain reaction; RSV, Rous sarcoma virus; MEK, mitogen-activated protein or extracellular signal-related kinase kinase; ERK, extracellular signal-related kinase.
analyze interacting protein interfaces by the double mutant approach to identify complementary interacting amino acids (34). Therefore, we used the double mutant approach to identify amino acids in Raf that participate in interaction with Ras and especially to probe the role of Raf-Ser257 in Ras-Raf interaction.

In contrast to biochemical analysis of mutations within the Raf-RBD with respect to binding affinity (22), analysis of the interaction of large parts of the Raf regulatory N terminus has been prevented by a strong tendency these proteins have to aggregate when expressed in E. coli, preventing equilibrium affinity measurements (35). In this study, we circumvented these problems by investigating of protein-protein interaction using the yeast two-hybrid system, which has been shown to discriminate between high, intermediate, and low-affinity interactions (36). We developed a quantitative two-hybrid system to measure the effect of RafCR2 mutations on Ras-Raf binding affinity. Furthermore, we investigated the effect of Raf-E37G and RafCR2 mutations on Ras-dependent Raf activation using a reporter gene assay. Using this combined approach, we thoroughly dissected the role of Raf-Ser257 and the RafCR2 domain by measuring Ras-Raf binding and activation.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The construction of the expression vector for GST-RafRBD and expression and purification of wild-type and mutant RafRBD have been described previously (18). H-Ras protein was prepared as described before (37).

Measurement of Ras-RafRBD Interaction—RafRBD acts as a nucleotide dissociation inhibitor for Ras (18). The affinity of wild-type RafRBD and RafRBD mutants for Ras was therefore determined by titrating Ras loaded with fluorescent mant-Gpp(NH)p nucleotide with RafRBD proteins. The dissociation rate constants of nucleotide dissociation from Ras were used to determine the Ras-RafRBD interaction. Dissociation constants were determined in 50 mM Tris/HCl, 5 mM MgCl2, pH 7.4, supplemented with 100 mM NaCl. For measurement of the Ras-RafRBD interaction, 25 nM Ras-mant-Gpp(NH)p complex was incubated with a 1000-fold excess of unlabeled nucleotide and different concentrations of RafRBD, and the dissociation was measured as the decrease of fluorescence. The dissociation constants were obtained by fitting the data to single exponentials. Details of this procedure have been described (18).

Plasmids—Rsv-Ras-G12V and pcDNA3-Raf plasmids were constructed as described (22). The mutant Rsv-Ras-G12V/EGST73 construct was generated by PCR mutagenesis using two subsequent PCR amplifications of the Ras-wt Ras-E37G complex. The PCR fragments were cut by MfeI site were introduced 5′ of the Kozak sequence to prevent K84A 14.0 108
R59A 3.80 29 33 2.5
K65E 0.67 5 20 1.5
Q66A 7.40 57 >100 >7
K84A 14.0 108 >100 >7

a Dissociation constants between wild-type Ras and mutant RafRBD proteins are taken from Ref. 22.

Results

Identification of RafRBD Residues Complementary to Ras-Glu37—We have previously determined the contribution of individual amino acids to Ras-Raf binding in vitro by analyzing the effect of single mutations within Raf-RBD on the Ras-Raf binding affinity (22). Since the effect of a second mutation within a protein-protein contact surface adds to the effect of the first mutation, complementary interacting amino acids can be identified by the lack of this addition character (34). To identify interacting residues in Raf-RBD that are complementary to Ras-Glu37 we have measured binding affinity between Ras-E37G and various RafRBD mutants in vitro. The assay to measure Ras-RafRBD binding exploits the fact that RafRBD behaves as a guanine nucleotide dissociation inhibitor for Ras (18).
Ras-E37G bound about 100-fold weaker to RafRBD than did Ras-wt (Table I). Amino acid exchanges at the position of Raf-K65 decreased the binding of RafRBD to Ras-E37G and to wild-type Ras only very weakly. The mutation of Raf-T65A moderately decreased the affinity to Ras-wt and to Ras-E37G 10- and 6-fold, respectively. Mutations of Raf-Gln65 and Raf-Arg59 had strong effects on the binding of Ras; however, as in the cases before, it affected both mutant Ras-E37G and Ras-wt strongly. In marked contrast, the mutation Raf-R67A and Raf-R59A reduced only the binding to Ras-wt drastically, whereas the interaction to Ras-E37G remained largely unaffected. The dissociation constant of Raf-R67A and Raf-R59A upon binding to Ras-wt increased by 16- and 29-fold respectively, while it only led to a 2.5-fold increase in $K_d$ upon binding to Ras-E37G. These results indicate that Raf-Arg59 and Raf-Arg67 bind directly to Ras-Glu37, as Raf-R59A and Raf-R67A have no additional effect on the interaction with Ras-E37G. These data show that the double mutant approach is suitable to identify amino acids in Ras effector proteins that bind to specific residues within the Ras effector region.

**Raf-mediated Transactivation Induced by Ras-G12V/E37G**—Since the mutation of Ras-E37G increased the dissociation constants between Ras and RafRBD to micromolar concentrations in *vivo*, we measured the effect of this mutation on Raf activation in *vivo*. To this end, we used a transient transfection assay in RK13 cells. This assay utilizes a luciferase reporter gene driven by three E74 binding sites (40), which are high affinity sites for ETS transcription factors shown to mediate transcription upon activation of the Ras/Raf/MEK/ERK signaling pathway (44). Cotransfection of Ras-G12V/E37G with Raf reduced the luciferase activity to 30% of the activity observed upon activation of Raf by Ras-G12V (Fig. 1A). This level of luciferase activity was insufficient to measure the effects of additional mutations within RafRBD since even mutations in RafRBD that modestly decrease Ras-Raf binding affinity led to a decrease in reporter gene activity to almost background values (data not shown). To establish conditions that would allow us to test the effect of mutations within RafRBD on stimulation of Raf by Ras-G12V/E37G, we increased the amount of Ras-G12V/E37G transfected. The 2- or 4-fold amount of Ras-G12V/E37G plasmid led to an equivalent increase in luciferase activity in the presence of Raf (Fig. 1B). In the absence of Raf, no increase of reporter gene activity was observed upon stimulation with Ras-G12V/E37G. This demonstrated that the transactivation induced by Ras-G12V/E37G was clearly mediated by the activation of Raf and that Ras-G12V/E37G could not bypass Raf to activate the reporter gene. The signal intensity achieved by increasing the amount of Ras-G12V/E37G plasmid 4-fold even exceeded that originally obtained by activation of Raf by Ras-G12V. This yielded conditions suitable to test the effect of RafRBD mutants on activation by Ras-G12V/E37G.

**Analysis of RafRBD Mutations in Ras-G12V/E37G-dependent Transactivation**—The double mutant approach identified complementary interacting residues measuring the interaction of Ras with RafRBD in *vivo*. To test the activation of Raf by Ras in *vivo*, we analyzed the effect of RafRBD mutations within the full-length Raf protein on the activation of the Ras/Raf/MEK/ERK pathway by Ras-G12V/E37G. We have shown previously, that Ras/Raf-induced transactivation correlates quantitatively with the binding affinity between Ras and RafRBD (22). Using experimental conditions which result in a maximum of Raf activation by Ras-G12V/E37G (Fig. 1B) we tested RafRBD mutants for activation by Ras-G12V/E37G. Mutations of Raf-K65M, R67A, R59A, and T68A, which resulted in slight to moderate decrease in affinity of RafRBD to Ras-E37G (Table I), also led to an equivalent decrease of Raf activity in the presence of the activator Ras-G12V/E37G (Fig. 2A). Although the affinity of the Raf-Q66A mutant was strongly reduced, it still showed a significant activation of residual transcription of 5% as compared with the wild-type. Furthermore, the Raf-R89L mutant, which cannot be activated by Ras-G12V, was unable to induce transcription of the reporter gene upon cotransfection with Ras-G12V/E37G. The small effect of the Raf-R59A and the Raf-R67A mutation on the activation by Ras-G12V/E37G is in marked contrast to the effect of these mutations on the activation by Ras-G12V which is decreased by 60–70% (22). To compare the effects of various mutations within RafRBD on Ras-dependent activation, we plotted the amount of activation of Raf mutants by Ras-G12V/E37G versus activation by Ras-G12V (Fig. 2B). The plot demonstrates that residues Raf-Arg59 and Raf-Arg67 in RafRBD contribute strongly to activation of Raf by Ras-G12V, whereas they do not play a significant role in activation by Ras-G12V/E37G. These results clearly identify Raf-Arg59 and Raf-Arg67 as interacting residues complementary to the Ras-Glu37 effector residue in the Ras-dependent activation of Raf.

**Quantitative Assessment of Ras-Raf Binding Affinity in Vivo**—Ras-G12V/E37G was found to be unable to interact with Raf in the two-hybrid system (10). Surprisingly, mutation of Raf-S257L within the RafCR2 domain had been reported to restore binding of Raf to the Ras-G12V/E37G mutant, suggesting that the RafCR2 domain and in particular Raf-Ser257 plays a role in Ras-Raf interaction (10). Since bacterially expressed Raf fragments comprising the complete regulatory domain have a strong tendency to aggregate (35) it was not possible to determine the contribution of the RafCR2 domain to the Ras-Raf interaction using *in vitro* binding affinity measurements by equilibrium methods. Therefore, we decided to investigate this issue employing the two-hybrid system. Using the GAL4 based two-hybrid system (39) we cotransformed the Y190 yeast strain with a plasmid encoding Ras-G12V fused to the GAL4 DNA binding domain in combination with the regulatory domain of Raf in the GAL4 activation domain fusion vector. Cotransformation of Ras-G12V with Raf-wt led to His-prototrophy and
isolated RafRBD proteins and the relationship between binding affinities measured in vitro. We observed a semilogarithmic quantitative comparison of Raf/MEK/ERK-mediated transactivation of Raf mutants induced either by Ras-G12V or by Ras-G12V/E37G. The fitted function was calculated omitting the values for Raf-S259A and Raf-S257A.

This raised the question of whether the side chain of Raf-Ser257 contributes to Ras-Raf binding affinity at all. To test the quantitative contribution of individual amino acids to Ras-Raf binding affinity, we established a quantitative two-hybrid assay. We calibrated the two-hybrid assay using the RafRBD mutants observed here, no interaction between Ras-G12V/E37G and Raf-wt, Raf-S257A, or Raf-S257L could be detected (Fig. 3B). Therefore, we were unable to confirm that Raf-S257L can restore binding of Ras-G12V/E37G and Raf, using the two-hybrid system as described.

Activation of Raf by Mutations within RafCR2—The lack of contribution of Raf-Ser257 to Ras-Raf binding affinity at all. To test the quantitative contribution of individual amino acids to Ras-Raf binding affinity, we established a quantitative two-hybrid assay. We calibrated the two-hybrid assay using the RafRBD mutants for which the contribution to Ras-Raf binding affinity had been determined previously (22). When these RafRBD mutants were tested in the context of the complete Raf regulatory domain, we observed a semilogarithmic quantitative relationship between binding affinities measured in vitro with isolated RafRBD proteins and the β-galactosidase activity determined with the two-hybrid assay (Fig. 3C). Western blotting of full-length Raf mutants has shown equal amounts of expression of these constructs in the two-hybrid system (data not shown). This demonstrates that the two-hybrid system can be used as a quantitative tool to determine the effect of single amino acid exchanges of Raf that are not accessible for affinity measurements in vitro. We measured the β-galactosidase activity induced by the Raf-S257A mutant. The activity induced by the S257A mutant was identical to that of the Raf-wt regulatory domain (Fig. 3D). This shows that mutation of Raf-S257A does not affect Ras-Raf binding affinity and strongly suggests that the side chain of Raf-Ser257 does not contribute to the interaction between Ras and Raf.

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assay under the conditions used, we reduced the amount of Raf-S257L plasmid (Fig. 5B). The resulting reporter gene activity was within the activity range that is achieved by Ras-dependent Raf activation under standard conditions. This allowed us to investigate the effect of Ras on Raf-S257L activity. Upon stimulation with Ras-G12V, the activity of Raf-S257L was significantly enhanced (Fig. 5C) showing that mutation of Raf-S257L can be further activated by Ras-G12V. In comparison with the activation of Raf-S257L by Ras-G12V, the activation of Raf-S257L by Ras-G12V/E37G was still impaired. While this demonstrates that the Raf-S257L mutant does not restore the capability of Raf to be activated by Ras-G12V/E37G, it clearly leads to a drastic increase of basal Raf activity.

**DISCUSSION**

It is still unclear how amino acids that are not part of the Ras-Raf contact surface function in Ras-Raf interaction and activation of Raf. Thus, we investigated this question by first identifying complementary interacting residues within the Ras-Raf contact surface using double mutant analysis. The result of this approach was then utilized as a criterion to judge the contribution of individual residues that are located outside RafRBD to the Ras-Raf binding affinity. Since it had been reported that the loss of Raf binding by the Ras-E37G mutation in the Ras effector region could be complemented by mutation of Raf-S257L in the RafCR2 domain (10), we focused on functional analysis of the role of amino acids Ras-Glu37 and Raf-Ser257 in Ras-Raf interaction and activation.

First, we identified residues in Raf that contribute to Ras-Raf binding affinity by interacting with Ras-Glu37, and which therefore constitute complementary interacting residues of Ras-Glu37. Biochemical analysis clearly revealed that Raf-Arg59 and Raf-Arg67 of RafRBD are functionally interacting residues complementary to Ras-Glu37. Structural analysis had shown that Raf-Arg67 is at hydrogen bonding distance to Rap1A-Glu37 in the Rap1A-RafRBD complex (19) and that Raf-Arg59 is juxtaposed to Rap1A-Glu37 in the complex of Rap1A-D30E/K31E with RafRBD (20). Consistent with these observations, we now demonstrate by functional analysis that both Raf-Arg59 and Raf-Arg67 contribute approximately equally strong to the interaction with Ras-Glu37.

The Ras-E37G mutation had been reported to inhibit Ras-Raf-dependent signaling and transformation (10). We tested the effect of the Ras-E37G mutation on signaling via Raf-wt and Raf mutants using a reporter gene assay that reflects Ras-Raf binding affinity (22). Transactivation of the reporter gene via Raf was decreased to about 30% upon activation by Ras-G12V/E37G as compared with activation by Ras-G12V. This is in good agreement with the 32% residual ERK activity induced by Ras-G12V/E37G that were detected before (10). Apparently, Ras-E37G retains some ability to activate the Raf/MEK/ERK pathway. It should be stressed that the effect of the Ras-E37G mutation on Ras-Raf interaction and activation is clearly less dramatic than the effect of the Raf-R89L mutation that shuts down Raf/ERK-mediated signaling completely (21, 22).

To enhance the absolute level of Ras-dependent Raf signaling and to measure the effect of Raf mutations, the amount of Ras-G12V/E37G activator plasmid was increased. Testing the activation of RafRBD mutants by Ras-G12V/E37G under these conditions showed that the effect of RafRBD mutations on Ras-RafRBD binding affinity is completely in agreement with the effect on Ras-dependent Raf signaling in vivo. Especially, residues Raf-Arg59 and Raf-Arg67, which we found to be complementary to Ras-Glu37 by in vitro affinity measurements, also behaved complementary to Ras-Glu37 in Ras-dependent Raf activation. This became evident when relative activation of Raf mutants by Ras-G12V were plotted against activation by Ras-G12V/E37G. The relative amounts of transactivation of Ras-G12V or Ras-G12V/E37G correlated with each other when mediated by Raf mutants with mutations that do not affect the binding to Ras-Glu37. In contrast, mutation of Raf-R59A and R67A only affected activation by Ras-G12V, whereas activation by Ras-G12V/E37G was largely unchanged. Thus, the behavior of Raf mutants in the Ras-dependent trans-

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**Fig. 4. Activation Raf-S257A by Ras-G12V and Ras-G12V/E37G.** Transactivation mediated by Raf-S257A by either Ras-G12V or Ras-G12V/E37G was measured with a E743 binding site-driven promoter, as shown in Fig. 1, under standard conditions transfecting 80 ng of RSV-Raf plasmid and 1.5 μg of pcDNA3-Raf plasmid.

**Fig. 5. Effect of the Raf-S257L mutation on Raf activity and Ras-G12V/E37G-induced transactivation.** A, transactivation induced by the standard amount of 1.5 μg of either Raf-S257L or Raf-S259A. Transactivation mediated via the MEK/ERK pathway was measured as described in Fig. 1. B, titration of the amount of Raf-S257L plasmid to a lower level of Raf-S257L-induced transactivation using 1.5, 0.4, or 0.1 μg of pcDNA3-Raf-S257L plasmid. C, transactivation mediated by Raf and the Raf-S257L mutant induced by standard amounts of 80 ng of either RSV-Ras-G12V or RSV-Ras-G12V/E37G. The amount of pcDNA3-Raf-wt or pcDNA3-Raf-S257L plasmid used here was 0.1 μg, as derived in panel B.
activation assay can be used as a general criterion to judge the contribution of individual amino acid side chains to Ras-binding and hence to Ras-dependent Raf activation in vivo.

The criteria for complementary behavior also have to be fulfilled for mutations of Ras interacting residues in Raf outside of RafRBD. Since it had been reported that the Raf-S257L mutation restored binding to Ras-E37G and Ras-G12V/E37G-dependent transformation (10), we tested the effect of Raf-Se257 mutations on Ras-Raf binding and Ras-dependent Raf activation. As expected, Raf-wt and the Raf-S257A and the Raf-S257L mutant showed strong interaction with Ras-G12V, but we were unable to confirm a reconstitution of the interaction with Ras-E37G by the Raf-S257L mutant using the two-hybrid system. The reason for this discrepancy to the work of others (10) might be due to the different sensitivities of the two-hybrid assay systems used. However, we had been able to calibrate our two-hybrid system for affinities between 0.13 μM (Raf-wt) and 4 μM (Raf-R59A). Therefore, the affinity of Ras-E37G to Raf-S257L must be below this range. It appears safe to suggest that the Raf-S257L mutation will not restore the interaction anywhere near to wild-type level.

We established the two-hybrid system as a quantitative method to determine the binding affinities of the Ras-Raf interaction. The necessary calibration of this quantitative two-hybrid system was done with well-characterized RafRBD mutants. When measured within the context of the complete regulatory region, the β-galactosidase activity induced by RafRBD mutants was correlated in a semilogarithmic fashion with the dissociation constants of these mutants measured in vitro. Through this correlation, we had established that the two-hybrid system can be used to determine the effect of point mutations on the affinity of Ras-Raf interaction and possibly of other protein-protein interactions as well.

When tested in this setup, the Raf-S257A mutant displayed β-galactosidase activity identical to Raf-wt. Since we had demonstrated that this method is able to distinguish between small changes in affinity, our observation shows that the serine hydroxyl group does not contribute to the Ras-Raf interaction, e.g. by hydrogen bonding. This result also ruled out that the side chain of Raf-Se257 contributes energetically to the Ras-Raf interaction. Our observations using the two-hybrid system are in good keeping with the results we obtained with our reporter gene assays which measured the Ras-dependent Raf activation. Like in the quantitative two-hybrid assay, Raf-S257A had the same activation characteristics as Raf-wt. In contrast to this, we observed a strong constitutive activity of the Raf-S257L mutant which was also noted in the Raf-S259A mutant, another mutation in the RafCR2 domain. Although already strongly constitutively active, the activity of Raf-S257L was further increased by Ras-G12V. Most interestingly however, this increase of Raf activity was clearly weaker when Ras-G12V/E37G was used instead. Therefore, none of our results supports the notion that Raf-S257L rescues the interaction with Ras-E37G and even more importantly they appear to rule out a direct interaction between the two- residues.

Still, others have observed transforming activity of Ras-G12V/E37G via Raf-S257L when cotransfected (10) which could again be indicative for the rescue of Ras-Raf interaction by the Raf-S257L mutant. As we can rule out that Raf-S257L reconstitutes Ras-Raf interaction, we suggest that Ras-G12V/E37G and the constitutively active Raf-S257L activate different signaling pathways. The activation of multiple pathways in turn could lead to transformation; however, this would be independent on Ras-Raf interaction.

Taken together, the RafCR2 domain appears to be an independent regulatory domain that can integrate further input signals into the Raf signaling pathway in addition to Ras stimu- luli. One additional input signal could be the stimulatory phosphorylation of Raf-Thr259 within RafCR2 by Src/CRK kinase (45, 46). Such a model of Raf regulation would be similar to the activation of the Src and of the HCK tyrosine kinase, of which the structures have recently been solved (47, 48). In these cases, the regulatory domain exerts constraints on the conformation of the kinase domain, keeping the kinase domain inactive. The release of these constraints by different stimuli then activates the kinase. We suggest this to be a more general model that also applies to Raf, in which different domains within the regulatory region of the protein kinase may funnel multiple input signals into kinase activation. Thus, it is conceivable that all constitutively activating point mutations in the regulatory region of Raf, like Raf-S257L or S259A, act via a common mechanism by simply interfering with the inactive ground state of this kinase.

Acknowledgments—We thank D. Vogt for providing Ras-E37G protein and R. Wothhus for yeast two-hybrid plasmids.

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