Improved Binding of Raf to Ras-GDP Is Correlated with Biological Activity

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The GTP-binding protein Ras plays a central role in the regulation of various cellular processes, acting as a molecular switch that triggers signaling cascades. Only Ras bound to GTP is able to interact strongly with effector proteins like Raf kinase, phosphatidylinositol 3-kinase, and RalGDS, whereas in the GDP-bound state, the stability of the complex is strongly decreased, and signaling is interrupted. To determine whether this process is only controlled by the stability of the complex, we used computer-aided protein design to improve the interaction this process is only controlled by the stability of the complex, we used computer-aided protein design to improve the interaction of interacting proteins leading to a controlled level of cellular response. Within the superfamily of small GTP-binding proteins, Ras appears to be a “master regulator” involved in cell proliferation, cell cycle progression, cell division, and apoptosis (1, 2). Attached to the inner leaflet of the cell membrane, Ras comes in two states; the inactive GDP-bound and the active GTP-bound state. The activation of Ras occurs by GDP/GTP nucleotide exchange, whereas hydrolysis of GTP leads to inactivation and interruption of signaling (3, 4). The activation of a distinct pathway occurs by the interaction of Ras with the responsible effector. Therefore, Ras has a large number of effector proteins, such as Raf kinase (5, 6), RalGDS (7), phosphatidylinositol 3-kinase (8–10), and Nore1A (11, 12), representing different signal directions. These effectors have in common the so-called Ras binding domain (RBD), enabling them to interact with Ras. Only the GTP-bound form of Ras binds strongly to effectors and leads to their activation.

Zooming more into the molecular detail of the Ras/effector interactions reveals two regions of Ras to be responsible to the nucleotide state and thereby convey specific recognition by the effectors. The flexible region switch I (residues 30–38) is mainly responsible for the interaction with the RBD of the effectors (13–15), whereas only a few effectors like Nore1A contact also the second flexible region of Ras, switch II (residues 60–67) (16). The RBDs from most effectors comprise 80–100 amino acids, and despite poor sequence homology, they all show the same topology (i.e. the ubiquitin fold) (17). In addition, the structures of various effector RBDs in complex with Ras show the same mode of binding, namely an intermolecular, antiparallel β-sheet established by β1 and β2 of the RBD and β3 and β3 of Ras (13, 18, 19). Despite the structural similarities, the range of affinities of Ras/effector spans 2 orders of magnitude. Except Nore1A with a small dissociation rate constant, \( k_{\text{off}} \) (16), the values for this constant are similar for the other effectors (\( \sim 10^{-3} \)), with the differences in affinity being governed by variations in \( k_{\text{on}} \) (17, 20–22).

Another common feature observed in all Ras-RBD complex structures is the high charge complementarity between the two proteins. The contact area of Ras is mainly negatively charged, whereas the binding interface of the RBDs is mainly positive (13, 15, 17–19, 23, 24). The general influence of the charge complementarity on protein-protein interactions was investigated in great detail by Schreiber and Fersht (29), which resulted in the concept of electrostatic steering; the process of protein complex formation is favored by complementary interactions.

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2 The abbreviations used are: RBD, Ras binding domain; mant-, 2’(3’)-O-(N-methylanthraniloyl)-Gpp(NH)p; mantGDP, 2’(3’)-O-(N-methylanthraniloyl)-Gpp(NH)p; GDP, guanosine 5’-O-(3’-O-methylanthraniloyl)-triphosphate; mantGppNHp, 2’(3’)-O-(N-methylanthraniloyl)-Gpp(NH)p; mantGDP,
charges within or near the binding areas, leading to a proper preorientation of the two proteins (25–29). The computer program PARE (predicted association rate enhancement) was developed, which allows the calculation of changes of the association rate constant for mutations by introducing or removing charges (30, 31). We have demonstrated that PARE-predicted mutations around the RBD of the effector RalGDS transformed it into a Raf-like effector, efficiently binding Ras (32). We further developed the program HyPare to scan entire protein-protein interfaces for mutations that will alter $k_{on}$ (33).

Here we utilized HyPare to predict the appropriate positions on Raf-RBD that would increase the association rate constant and challenge the upper limit of Ras affinity. After gauging the surface of Raf-RBD facing Ras, we found only little scope for further improvement of binding. Nevertheless, our results allow intriguing insight into the molecular basis of Ras/effecter binding specificity and activation.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The introduction of single lysine mutations to DNA coding for Raf-RBD and Raf full-length was performed using the QuikChange<sup>TM</sup> site directed mutagenesis kit (Stratagene) using pGEX-2T vector harboring the insert coding Raf-RBD 51–131 and pCDNA3 Raf full-length (wild type) as templates, respectively. The single lysine mutants were used as templates to generate multiple lysine mutants. All mutations were analyzed by sequencing.

**Protein Expression and Purification**—Raf-RBD and Ras wild type and mutants were synthesized in bacteria and purified as described earlier (13, 15, 34, 35). The nucleotide exchange with the non-hydrolyzable GTP analogue GppNHp and mant-GppNHp, respectively, was performed according to Ref. 35. The loading of Ras with the fluorescent GDP analogue, mant-GDP, was performed in the presence of 10 mM EDTA and a 5-fold excess of mantGDP and incubated at 4 °C overnight. For the last step of purification, all proteins were subjected to size exclusion chromatography (Superdex 75, GE Healthcare) equilibrated with the buffer containing 15 mM Hepes, pH 7.4, 5 mM MgCl$_2$, and 125 mM NaCl. The protein concentration was measured by the Bradford method (36) using bovine serum albumin as a standard.

**Calculation of Association Rate Constants**—The increase of the association rates of mutant Raf-RBD-Ras-GppNHp complexes were calculated as described earlier using the program HyPare, which automatically generates all of the mutant files (33). Coordinates for the calculation were taken from the crystal structure Raps-GppNHp, a Ras homologue complexed with Raf-RBD resolved at 1.8 Å resolution (15) (Protein Data Bank entry 1GUA). The surface-exposed residues were chosen, and the lysine mutations at the appropriate positions were modeled using SwissPdbViewer (37).

**Stopped-flow Measurements**—The measurements of association rate constants were performed with an SM17 apparatus (Applied Photophysics) by rapid mixing of 0.5 μM Ras bound to either mantGppNHp or mantGDP with Raf-RBD at various concentrations. Pseudo-first order conditions were established with Raf-RBD at a minimum concentration of 2.5 μM, allowing for single exponential curve fitting. Mant-nucleotides were excited at 360 nm, and the emitted fluorescence was monitored through the 408 nm cut-off filter. The binding of Raf-RBD was detected by the fluorescence change as described earlier (20–22).

**Isothermal Titration Calorimetry**—The thermodynamic parameters of the Ras-GppNHp interaction with Raf-RBD wild type and mutants were determined using an isothermal titration calorimeter (MCS-ITC, Microcal) as described by Wise-man et al. (38). Dependent on the affinity, Raf-RBD mutant concentrations between 10 and 80 μM were placed into the cell, and Ras was placed in the syringe at a 10-fold higher concentration. The data analysis was performed by the manufacturer’s software as described earlier (39).

**Guanine Nucleotide Dissociation Inhibitor (GDI) Assay**—The GDI assay was performed as described earlier (40). Briefly, Ras loaded with either mantGppNHp or mantGDP was used for the GDI assay in a concentration of 0.05 μM in the presence of different concentrations of Raf-RBD wild type and mutants. The nucleotide dissociation was initiated by the addition of a 1000-fold excess of the non-fluorescent analogue GppNHp or GDP. The time-dependent fluorescence decrease was fitted using single exponential decay, and the observed rate constants of the nucleotide dissociation were plotted against the corresponding Raf-RBD concentration. The curves were fitted using the quadratic equation as described previously (35).

**Phosphor NMR Measurements**—$^{31}$P NMR spectra have been recorded as described earlier (48). Ras proteins were dissolved in 40 mM Hepes, pH 7.4, 5 mM MgCl$_2$, 1 mM dithioerythritol, 150 mM NaCl. 5% D$_2$O was added to obtain a lock signal, and 0.1 mM 2,2-dimethyl-2-silapentane-5-sulfonate was added for referencing. $^{31}$P NMR spectra were recorded with a Bruker Avance 500 NMR spectrometer operating at $^{31}$P frequency of 202 MHz using a 70° pulse and a total repetition time of 7 s to avoid saturation effects. Measurements were performed in a 10-mm probe using 8-mm Shigemi sample tubes. Protons were decoupled during data acquisition by a GARP sequence (41) with a strength of the B$_1$-field of 803 Hz. For referencing, the $\Xi$-value of 0.4048073561 reported by Maurer and Kalbitzer (42) was used, which corresponds to 85% external phosphoric acid contained in a spherical bulb.

**Luciferase Reporter Gene Assay**—Rabbit kidney epithelial-like RK-13 cells were grown to 25% confluence on 6-cm dishes and then transfected with DNA by the calcium phosphate co-precipitation method. 2 μg of the reporter constructs (E743-tk80-luc or tk80-luc), 0.5 μg of a β-galactosidase expression vector (pEQ176), 1.5 μg of ERK-1 expression vector (43), 1.5 μg of an Ets protein expression vector (44), and 1.5 μg of pcDNA3 expression plasmid alone or pcDNA3 expression plasmid containing a Raf mutant were used for each transfection. When indicated, 80 ng of the RSV-Ras G12V or RSV-Ras wild type plasmid were additionally transfected. 36 h after transfection, cells were harvested and lysed, and luciferase and β-galactosidase activities were determined as described (45). Relative luciferase activity was obtained by normalizing the luminescence to the β-galactosidase activity. ERK inhibitor was purchased from Calbiochem (catalogue number 328007; see supplemental Fig. 1).
predicted and experimental rate constants and binding constants for the interaction of Raf-RBD and Ras-GDP. The lysine substitutions of Asn56, Asp80, and Ala85 were mutated to increase association by a factor between 6.3 and 45.6. The multiple lysine mutants were calculated to increase the association rate constant by a factor between 1.8 and 2.8 (relative to wild type). The calculations suggested a smaller effect for the lysine substitutions of Asn56, Asp80, and Glu125 in the proximity of the binding interface and of Val69 and Met83 within the interface showed no change in the association rate constant. The lysine mutations at the positions Thr68, Asp80, and Ala85 showed a small but significant increase of the association rate constants from 29 to 67, 52, and 50 μM⁻¹ s⁻¹, respectively (Table 1). The association rate constants of all multiple mutations harboring A85K were in the range of 80 μM⁻¹ s⁻¹. Except for E125K and its combination with A85K, the trend of the change in rate constant is as predicted by the calculation, whereas the values differed significantly (Fig. 1B). The largest increase that could be reached was only 3-fold above the wild type value. As was discussed before, it seems that an upper limit for association rate increase through electrostatic steering is reached in these cases (32).

Having the Raf-RBD mutants with the potential for faster and stronger binding at hand, we measured now the affinity for Ras. To this end, the Raf-RBD-Ras-GppNHp interaction was quantified using isothermal titration calorimetry (Table 1 and Fig. 1C). Surprisingly, the only modest increase in the association rate constants is not reflected by the complex stability. Raf-RBD with the mutation A85K alone and in combination with the other lysine substitutions increased the affinity constant (reciprocal Kd value) of the complex with Ras-GppNHp by factors between 3 and 12. Because all other single mutations did not contribute significantly to an increase of complex stability, A85K in Raf-RBD was identified as the most prominent mutation to increase the association rate constant and the stability of the complex with Ras-GppNHp.

Raf-RBD Interaction with Ras-GDP—Having identified Raf mutants that bind Ras-GppNHp around 10-fold tighter than wild type, we were curious about their impact on Ras-GDP affinity toward the Raf-RBD mutants and on a possible change of specificity of Ras-GppNHp versus Ras-GDP. To address this issue, mutants binding more tightly to Ras-GppNHp (namely Raf-RBD A85K, Raf-RBD A85K/E125K, Raf-RBD N56K/A85K, and Raf-RBD N56K/D80K/A85K) were evaluated as to their binding to Ras-GDP. The dissociation constants (Kd) of Ras-GDP and Ras-GppNHp were determined using the GDI assay, because it allows us to define precisely Ras effector interactions over a range of Kd values of more than 4 orders of magnitude. This assay takes advantage of the observation that binding of effector protein inhibits nucleotide dissociation from Ras. The dissociation of the fluorescent nucleotides, mantGppNHp or mantGDP (40), leads to a decrease of fluorescence intensity, and the obtained time traces (Fig. 2A) can be fitted using a single exponential equation. The plot of the observed rate constants versus the concentration of the RBDS can be fitted using a quadratic equation (21, 35). The dissociation constants of the Raf-RBD interaction with either Ras-mantGDP (Fig. 2B–F) or Ras-mantGppNHp (data not shown) were determined at 37 °C and at buffer conditions identical to the previous kinetic and thermodynamic measurements. The lysine muta-
Binding of Raf to Ras-GDP and Biological Activity

A linear plot of the observed rate constants for the association of Ras-GppNHp with Raf-RBD A85K (open squares), Raf-RBD A85K/E125K (open circles), and Raf-RBD wild type (filled triangles) versus the concentration of the Raf-RBD mutants. B, double logarithmic plot of the experimental versus the calculated association rate constants (μM⁻¹ s⁻¹). C, ratio of the dissociation constants $K_d$ of the wild type to $K_d$ of the mutants determined by isothermal titration calorimetry.

Structural Requirements for Interaction Analyzed by $^{31}$P NMR Spectroscopy—By $^{31}$P NMR spectroscopy, we wanted to investigate possible structural changes in the Ras-Raf binding interface evoked by the Raf mutation. Changes of Ras switch I conformation are reported by shifts of $^{31}$P NMR resonances (47–49). Wild type Raf-RBD binds tightly only to the GppNHp-bound state of Ras, where the switch I region of Ras (residues 30–38) adopts an appropriate conformation favoring effector binding. This positioning of switch I is established by Thr³⁵ hydrogen bonds with the γ-phosphate of the nucleotide, and this conformation (state 2) is shown to be stabilized by effector binding (47–49). After hydrolysis and loss of the γ-phosphate, Ras-GDP relaxes to other conformations of switch I, leading to much lower effector binding affinity.

Above we have identified the lysine substitution at position 85 of the Raf-RBD that is able to bind not only the activated Ras, Ras-GTP, but also the inactive, GDP-loaded Ras more tightly. The $^{31}$P NMR spectrum of Ras-GDP alone shows two resonance lines with chemical shifts at −10.6 ppm and −2.0 ppm, corresponding to the α- and β-phosphates of Ras-GDP with a slight inhomogeneity in line shape of the β-phosphate resonance, as shown previously (Fig. 4A) (50). The resonance lines of the bound nucleotide are assigned by their chemical shift values because the α-phosphate groups of free metal bound nucleotide are very similar (Table 3). The chemical shift values obtained are −10.6 ppm of the Ras-GDP complex and −11.1 ppm for Ras-GppNHp T35A. The addition of Raf-RBD A85K to Ras-GDP leads to the appearance of a second resonance line of the α-phosphate at −11.2 ppm, which means a chemical shift difference of −0.6 ppm, which is similar to the Δδ in the GppNHp-bound wild type with −0.5 ppm between state 1 and the effector-bound state 2 (Table 3). The new signal becomes larger with higher concentration of Raf-RBD A85K, whereas the resonance at −10.6 ppm decreases and disappears completely at a molar ratio of 4:1 for Raf-RBD A85K and Ras-GDP (Fig. 4A). In contrast, with wild type Raf-RBD, the new α-phosphate peak at −11.2 ppm assignable to the Raf complex can only be observed at much higher concentrations (Fig. 4B). Even at a molar ratio of 6:1, the resonance at −10.6 ppm still predominates, whereas it has entirely vanished in favor of −11.2 ppm in the case of the A85K mutant. The observed chemical shift change by binding of wild type Raf is again −0.6 ppm (Table 3), indicating that the local conformational change induced by
binding is similar in the two cases. The NMR spectra are typical for a slow two-site exchange. From the slow exchange condition \(2 \pi \Delta \nu \tau_e \gg 1\), a lower limit of the exchange correlation time \(\tau_e\) of 1.3 ms can be obtained (51).

In order to support our hypothesis on the potential of Raf-RBD A85K to stabilize state 2 of Ras, we used the GppNHp-bound form of Ras T35A. This mutant is known to be arrested in state 1, leading to decreased effector affinity (40, 48). The \(^{31}P\) NMR spectrum of Ras-GppNHp T35A is shown in the bottom panel of Fig. 4C, where all resonance lines correspond to state 1. The addition of high concentrations of wild type Raf-RBD and its binding to Ras-GppNHp T35A leads to small changes in chemical shift values but no shift toward lines corresponding to state 2 (48). In contrast, the addition of Raf-RBD A85K leads to the appearance of a second set of resonance lines that correspond to state 2. This is most clearly visible for the \(\gamma\)-phosphate resonance of Ras-GppNHp at \(-3.29\) ppm (Fig. 4C). As described for Ras-GDP above, the up-field-shifted line at \(-3.3\) ppm increases with Raf-RBD A85K concentration, and at a molar ratio of 3:1 it strongly dominates over the line at \(-2.6\) ppm, which represents state 1 of Ras-GppNHp T35A. For the wild type Ras, the shift of the \(\beta\)-resonance is indistinguishable in the two states and is not influenced by Raf binding. The same is true for the binding of Raf-RBD A85K to Ras-GppNHp T35A.

The resonance position of the \(\alpha\)-phosphate group is shifted slightly downfield in the presence of Raf-RBD A85K. Although for the \(\gamma\)-phosphate resonance, clearly slow exchange conditions prevail because two separate lines are observable, this is not clear for the resonance of the \(\alpha\)-phosphate, since the line widths are here too large. If fast exchange conditions would prevail then at complete saturation, a much larger upfield shift would correspond to the Ras-Raf complex. Summarizing, the \(^{31}P\) NMR measurements show that Raf-RBD A85K binding to Ras leads to a conformational change of switch I in Ras-GDP as well as in Ras-GppNHp T35A, which could not be achieved by wild type Raf-RBD in the concentration range studied.

**Biological Activity of Mutant Complexes Using a Reporter Gene Assay**—To determine the biological consequences of the loss of specificity toward the GTP-bound state of Ras when using the tighter binding Raf-RBD mutants, we followed the level of expression of a reporter gene. RK13 cells were transfected with the luciferase reporter gene driven by three E74 binding sites. The E74 is a high affinity binding site for Ets transcription factors and has been shown to specifically mediate transcription upon activation of the Ras/Raf/MEK/ERK signaling pathway (45). This Ras/Raf/MEK/ERK-dependent signaling has been shown to correlate quantitatively with the affinity of the Ras-Raf interaction in vitro (43, 52).

Here, we addressed the activation of the Ras/Raf/MEK/ERK signaling cascade by the Raf-RBD A85K mutant binding more tightly to Ras-GTP and Ras-GDP. To examine the tighter binding of Raf-RBD mutants to Ras-GTP, the cells were co-transfected with the constitutively active Ras mutant, Ras G12V, and Raf kinase wild type and mutants, respectively. Fig. 5A shows immunoblots of transfected Raf mutants demonstrating similar concentration levels of the proteins in the cells. In order to compare quantitatively the luciferase activity that reports the extent of Raf activation, the luminescent readout was normalized to the enzymatic activity of co-transfected \(\beta\)-galactosidase. Inhibition of ERK phosphorylation by the addition of 40 \(\mu\)M inhibitor results in abolishment of luciferase activity (53).
Binding of Raf to Ras-GDP and Biological Activity

To test if the stronger Ras-GDP binding to Raf A85K leads also to higher activation, the cells were co-transfected with Ras wild type (Fig. 5B, hatched columns) or without Ras (Fig. 5B, gray columns). Interestingly, the activity of the reporter gene was almost the same with and without the co-transfection of Ras wild type, indicating the interaction of Raf kinase with the endogenous Ras-GDP. All of the Raf kinase mutants harboring the A85K mutation had an increased reporter gene activity relative to the Raf kinase wild type. In addition, in this type of experiment, instead of Ras WT, we co-transfected Ras S17N, which is known not to be activated, to reside constitutively in the GDP-bound state, and to suppress activation of endogenous Ras. A pull-down experiment demonstrates that no Ras GTP is detectable when Ras S17N is co-transfected (data not shown). Ras S17N leads to a similar low level of luciferase activity (16–18%) as after co-transfection with Ras WT, and Raf A85K leads to a 4-fold increase for both Ras WT and Ras S17N co-transfection (supplemental Fig. 1).

Because the reporter gene assay is known for the correlation of its activities with the affinities determined using in vitro methods like the GDI assay (43), we plotted the reporter gene activities of the Ras G12V and Ras wild type transfection versus log $K_d$ values of the respective Ras-Raf complexes (Fig. 5C). The plot shows a clear correlation between the dissociation constants determined using the GDI assay and the reporter gene activity with a correlation coefficient of $–0.97$. This linear dependence identifies the affinity as the major factor of the activation of the Ras/MEK/ERK signaling cascade.

**DISCUSSION**

Cellular signal transduction through Ras is established by the interaction with many different effector proteins mediated by an RBD that is structurally but not sequence-wise conserved in all effectors. Hence, all RBDs have a similar size and share the same topology, namely the ubiquitin-like fold. In addition, there is a minimum requirement of positively charged amino acid residues located in their interface area which qualifies for binding to Ras (17). Despite these similarities, the interactions between Ras and effector proteins are characterized by $K_d$ values ranging from 0.1 $\mu M$ up to 2 $\mu M$, with, for example, Raf at the high and RalGDS at the low affinity end of this scale. In this study, we wanted to further increase the Ras-Raf binding affinity, define the structural requirements for this, and elucidate the biological consequences.

**Improving Ras-Raf Binding Affinity**—The first part of the project presented here aimed at improving Ras-Raf association, challenging the upper limit of Ras/effector binding affinity. We have previously shown that RalGDS furnished with some

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**TABLE 2**

Dissociation constants of Raf mutants complexed with Ras-mantGppNHp or Ras-mantGDP

| Raf-RBD          | $K_d$, Ras-mantGppNHp $\mu M$ | $K_d$, Ras-mantGDP $\mu M$ | $\Delta \Delta G$, Ras-mantGppNHp $kJ/mol$ | $\Delta \Delta G$, Ras-mantGDP $kJ/mol$ |
|------------------|--------------------------------|----------------------------|---------------------------------------------|-------------------------------------------|
| Wild type        | 0.13                           | 46                         | –4.83                                       | –8.12                                     |
| A85K             | 0.02                           | 1.97                       | –6.49                                       | –8.81                                     |
| N56K/A85K        | 0.0105                         | 1.51                       | –4.05                                       | –8.57                                     |
| A85K/E125K       | 0.027                          | 1.68                       | –2.47                                       | –9.24                                     |
| N56K/D80K/A85K   | 0.050                          | 1.26                       | –                        |                                          |

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**(supplemental Fig. 1)**, demonstrating that Raf activation leads to transcription of the luciferase gene mediated by the ERK pathway.

The activities from each experiment were set into relation to the activity of the co-transfection of Ras G12V with Raf wild type (100%) (Fig. 5B, blank columns). The transfection with the constitutively active Ras mutant G12V in the absence of Raf was done as a negative control, showing that the activity increase is due to the presence of the Raf kinase (43). All proteins harboring the A85K mutation showed an increased reporter gene activity in the presence of Ras G12V compared with the Raf wild type.
appropriate mutations, identified using the computer algorithm \textit{PARE}, may reach the Ras binding strength of Raf (32). These calculations are based on Coulomb energies contributed by charged residues near or within the binding interface areas of the two interacting proteins. The most promising mutations proposed were lysine residues, which were introduced into the sequence of Raf-RBD. In contrast to the case of Ras/RalGDS with good agreement between predicted and experimentally determined changes of the association rate constants (32), the comparison of theoretical and experimental results obtained here with Raf-RBD shows large discrepancies. Basically, the changes observed experimentally are much smaller than the calculated values, whereas the trend is predicted correctly. This is not due to the use of \textit{HyPare} instead of \textit{PARE} (data not shown) and may suggest that for Raf there is not much space for improvement of the association rate. Natural evolution seems to have exploited the complementary electrostatics on the two protein surfaces to reach almost an optimum rate of association. The upper limit of the rate of binding for Ras/effector association can be defined to \( k_{on} \leq 10^{8} \text{M}^{-1} \text{s}^{-1} \).

The largest increase of the \( k_{on} \) value by a single mutation is realized by A85K (1.7-fold), T68K (2.3-fold), and D80K (1.8-fold), whereas the others show less pronounced and insignificant changes. Intriguingly and in contrast to RalGDS in our previous study (32), the changes of the \( k_{on} \) values do not increase the binding affinity of Ras-Raf in the manner expected from pure electrostatic steering effects. Although the \( K_d \) value for D80K is similar to that for the wild type protein, it is 2 times higher for T68K and almost 5-fold lower for A85K. The contradictory effect of lysine 68 on association rate and affinity can be rationalized by a positive contribution to electrostatic steering but a negative consequence for the affinity, since this residue is located in the binding interface and leads to the loss of original contacts. In contrast, the improvement of binding affinity by lysine 85 is even stronger, as expected from its increased \( k_{on} \) value. An increased binding of Ras and Raf A85K was observed earlier when using radioactive \textit{in vitro} assays (54). As pointed out by Fridman \textit{et al.} (54), this lysine may reach into the interface area and may form a direct contact to Ras, whereas the wild type alanine side chain is too short for this. Altogether, not only electrostatic steering effects are responsible for changes in affinities of our Raf mutations. The interaction between Ras and Raf is significantly improved by A85K, and with the assistance of N56K and D80K the \( K_d \) value is decreased up to 10-fold.

**Raf with High Affinity for Ras-GDP and Lower Nucleotide Specificity**—The interaction between Ras and Raf leads to the activation of one of many possible signal pathways. The affinity of a protein-protein complex in the context of network formation with various binding partners is one side of the coin, whereas the specificity meaning relative affinities is the other. Therefore, we wondered about the impact of our Raf mutations on the interaction with the “non-active” form of Ras (i.e. bound to GDP). Compared with Ras-GppNHp, a 1000-fold higher \( K_d \) value was reported for the interaction between Raf-RBD and Ras-GDP (40). Here a 350-fold difference was measured using a buffer with a salt concentration close to the physiological ionic strength. We focused our attention on the Raf mutations containing A85K, which show higher affinity to Ras. In Table 2, the

![Figure 4](http://www.jbc.org/)

**FIGURE 4.** \( ^{31} \text{P} \) NMR spectroscopy. Shown is the spectrum of Ras-GDP at 20 °C alone and in the presence of different concentrations of Raf-RBD A85K (A) or Raf-RBD wild type (B). C. \( ^{31} \text{P} \) NMR spectrum at 5 °C of Ras T35A-GppNHp alone and in the presence of Raf-RBD A85K. The ratio of Raf-RBD to Ras is indicated. The resonances are assigned to the phosphate groups (\( \alpha, \beta, \text{ and } \gamma \)), and (1) and (2) refer to the two conformational states of Ras (see text). Chemical shift values are summarized in Table 3.
The lysine mutations of Raf have reached a $K_d$ value of 1 $\mu M$ for Ras-GDP, which is even slightly smaller than observed for Ras-GppNHp and the effector Racl (3.5 $\mu M$) (19). The question we are addressing is this: Can Ras-GDP activate Raf A85K? In fact, in contrast to Raf wild type, the lysine mutants of Raf lead to activation (60%) of the reporter gene when Ras wild type (or S17N) is co-transfected rather than Ras G12V (which is constitutively in the active GTP-bound form and which corresponds to 100% activity).

Strikingly, the same activities are observed without co-transfection of Ras wild type, meaning the concentration of endogenous Ras is high enough to activate the transfected Raf mutants, as shown in Fig. 5B, and thus does not influence the outcome of our experiments. The reporter gene activities observed here for the lysine mutants of Raf and Ras-GDP (open squares in Fig. 5C) correspond to the values observed in the earlier study for the alanine mutants of Raf and Ras-GTP with similar $K_d$ values (filled circles in Fig. 5C). Notably, there are high affinity Raf mutants leading to higher reporter gene activation with Ras-GDP compared with low affinity mutants in the presence of Ras G12V. Taking all of these observations together, it seems that the state of Ras, GDP versus GDP, is not relevant for the activation of Raf; only binding affinity counts.

**A85K Mutant of Raf Induces the High Affinity Conformation of Ras Switch I**—It has been shown earlier that Ras-GppNHp exists in two conformational states, with state 1 a low affinity state for effectors and state 2 a high affinity effector binding state (47–49). Ras mutations of Thr$^{35}$ lead to a shift of the switch 1 conformational equilibrium to state 1, resulting in lower affinity for Ras or other effectors, as was shown for different Ras-nucleotide complexes (48, 49, 55, 56). It is assumed that only in state 2 does switch 1 occur in the correct conformation for effector binding. Intriguingly, although in Ras T35S this equilibrium can be shifted back to state 2 by Raf binding, this is not observed in Ras T35A even at high concentrations of Raf (48). This is rationalized by a stronger shift of the equilibrium in T35A to state 1, which cannot be overcome by Raf binding. Hence, Raf binds to the weak binding state 1 of Ras T35A and therefore shows lower affinity, similar to wild type Ras-GDP (40, 41).
In line with this interpretation, the more tightly binding Raf-RBD mutant A85K was found here to force Ras T35A to adopt the effector binding state 2, where switch I is in the high affinity conformation. Hence, a structural switch of Ras evoked by lysine 85 of Raf-RBD rather than an additional, local interaction between two amino acid side chains leads to stronger binding.

The lysine substitution at position 85 of the Raf-RBD is able to bind not only the GTP-activated Ras but also the inactive, GDP-loaded Ras more tightly. Now it was interesting, of course, to understand the molecular mechanism for this observation and for the decrease in specificity of Ras-GTP versus Ras-GDP. Since $^{31}$P NMR chemical shift changes are mainly due to local structural changes (mainly torsional strain of bonds and polarization effects), they can be qualitatively interpreted in structural terms. Binding of Raf-RBD A85K to Ras-GppNHp T35A leads to chemical shift changes similar to those observed earlier for the binding of wild type Raf-RBD to Ras-GppNHp T35S. The latter chemical shift changes were interpreted as a shift of the conformational equilibrium to state 2. The same interpretation holds probably also for the complex of Raf-RBD A85K with Ras-GppNHp T35A. Different chemical shifts of the resonance of the $\alpha$-phosphate group of bound GppNHp in the two states indicate different environments (mainly coordination patterns and/or bond polarizations) of the $\alpha$-phosphate groups in the two states. The same general description holds also for GDP. The $^{31}$P data suggest that the environment of the $\beta$-phosphate group of bound GDP is not changed after Raf binding, but that of the $\alpha$-phosphate group is clearly changed after binding. The chemical shifts of the $\alpha$-phosphates of GppNHp as well as GDP are moderately shifted after binding to Ras (Table 3). They are rather similar in Ras-GppNHp T35A (state 1) and Ras-GDP, which suggests that the environment of the $\alpha$-phosphate group may also be similar in the two complexes. Analogously, the chemical shifts after Raf-RBD binding (state 2 in Ras-GppNHp) are again similar. This could mean that, concerning the $\alpha$-phosphate, Ras-GDP is predominantly in a state 1-like environment, and after Raf-RBD interaction, it is in a state 2-like environment.

In conclusion, higher affinity between Ras and Raf achieved by mutation led to stronger activation of the mitogen-activated protein kinase pathway. This suggests that nature has evolved the residues within and around the Ras-Raf interface to an optimum as to fast association and strong binding on the one hand and specific recognition of the active state of Ras on the other.

**FIGURE 5. Luciferase reporter gene assay.** A, abundance of c-Raf WT and mutants in RK13 cells. Cells were lysed 48 h after transfection and analyzed by immunoblot. Total ERK was analyzed by immunoblot as loading control. Representative blots are shown. c-Raf intensities on immunoblots were quantified with ImageJ software, and the average values are shown in the lower panel. B, results of the luciferase reporter gene assay of Raf activity with RSV-Ras G12V as co-transfectant (blank columns), with RSV-Ras WT (hatched columns), and without RSV-Ras WT (gray columns), respectively. The luciferase activity of the co-transfection of Raf wild type with RSV-Ras G12V was set to 100%. C, relative luciferase activities from B plotted versus the negative logarithm of the corresponding experimental dissociation constants from the GDI assay, c-Raf WT and mutants co-transfected with RSV-Ras G12V (open circles) and with RSV-Ras WT (open squares), respectively. In addition, three data points of alanine mutants of c-Raf from Ref. 43 were included for comparison (filled circles), from left to right: K65M, T68A, and R59A.
Binding of Raf to Ras·GDP and Biological Activity

hand. Raising the affinity (e.g. by Raf mutation A85K) leads to further improvement of the interaction, particularly with Ras-GDP. This is achieved by lysine 83-mediated positioning of switch I in a Ras-GTP-like conformation. Thus, the specificity drops, and the $K_d$ value for Ras-GDP falls into a range that leads to effector activation. Our results show that lysine at position 85 of Raf would be unfavorable for the biological function because signaling requires high specificity and was therefore discarded by evolution. Not strong binding alone but rather a sensitive balance of the affinities of all partner proteins participating in a complex protein network is required to make it operate smoothly.

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