Thermodynamic and Kinetic Characterization of the Interaction between the Ras Binding Domain of AF6 and Members of the Ras Subfamily*

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Cellular signaling downstream of Ras is highly diversified and may involve many different effector molecules. A potential candidate is AF6 which was originally identified as a fusion to ALL-1 in acute myeloid leukemia. In the present work the interaction between Ras and AF6 is characterized and compared with other effectors. The binding characteristics are quite similar to Raf and Raf-GEF, i.e. nucleotide dissociation as well as GTPase-activating protein activity are inhibited, whereas the intrinsic GTPase activity of Ras is unperturbed by AF6 binding. Particularly, the dynamics of interaction are similar to Raf and Raf-GEF with a lifetime of the Ras AF6 complex in the millisecond range. As probed by 31P NMR spectroscopy one of two major conformational states of Ras is stabilized by the interaction with AF6. Looking at the affinities of AF6 to a number of Ras mutants in the effector region, a specificity profile emerges distinct from that of other effector molecules. This finding may be useful in defining the biological function of AF6 by selectively switching off other pathways downstream of Ras using the appropriate effector mutant. Notably, among the Ras-related proteins AF6 binds most tightly to Rap1A which could imply a role of Rap1A in AF6 regulation.

Cellular functions like growth and differentiation are regulated by a complex network of interacting proteins with either enzymatic or adaptor functions. Ras plays a central role in this signaling network. It is bound to GDP in its resting state and bound to GTP after activation (1). Only this activated form can interact with effector molecules like Raf kinase, Raf-GEF, and PI3-kinase1 (2). A variety of incoming signals is directed to Ras activation, and this in turn triggers the activation of different signal pathways that lead to diverse consequences such as induction of DNA transcription, inhibition of apoptosis, and/or cytoskeletal rearrangements (3). Synergism with other signaling events or inhibition by competing pathways may supplement or modulate the signal amplitude resulting in a distinct effect. When Ras binds to the effectors they become activated by different and not fully understood mechanisms. This leads to the activation of other molecules located further downstream in the signal cascade. There are other small GTP-binding proteins closely related to Ras like Rap1A/2A, TC21, and R-Ras which also bind to the Ras effectors. So far little is known about their biological functions.

The effectors c-Raf-1 (4), Raf-GEF (5), and PI3-kinase (6, 7) have been shown to become activated upon Ras binding. For other possible Ras targets like Rin1/2 (8), PKCζ (9), Krit (10), or Nore1 (11) the requirements for a Ras effector have been fulfilled in that they bind in a GTP-dependent manner and do not bind to the effector mutant Ras Δ38A, but functional data are missing for these proteins. Recently, AF6 was discovered as another putative effector of Ras (12, 13). This protein was originally described as a fusion partner of ALL-1 in acute myeloid leukemia (14). AF6 is a homolog of Canoe in Drosophila which is genetically linked to the Notch cascade and other signaling pathways (15). Canoe and AF6 have the PDZ domain which in the case of AF6 was shown to bind to the Eph3B receptor (16). The amino terminus of AF6 was identified as the Ras-binding site (12, 13). ZO-1, a protein involved in the formation of tight junctions, also binds to AF6 close to the amino terminus thereby competing with Ras binding (17). These data suggest a participation of AF6 in the regulation of cell-cell contacts via a Ras-modulated interaction with ZO-1. Further evidence for this role of AF6 comes from the discovery of l-Afadin, a larger AF6 splicing variant in rat which additionally carries an actin-binding site at the carboxyl terminus (18). l-Afadin appears to serve as a linker between the actin cytoskeleton and the plasma membrane in adherens junctions. A recent finding that the de-ubiquitinating enzyme Fam (fat facets in mouse) interacts with AF6 suggests that the AF6 action is based on the turnover of participating proteins (19).

In contrast to the well characterized effectors Raf, Raf-GEF, and PI3-kinase, AF6 is a protein with no enzymatic function. It seems to serve as a scaffolding component for protein complexes. This would represent a new type of Ras effector because Ras binding in this case does not lead to the activation of the enzymatic activity of the effector. In contrast, for the regulation of Raf only transient Ras binding is necessary. Therefore in this work the Ras binding domain (RBD) of AF6 is defined, and its interaction with Ras is characterized by thermodynamic and kinetic parameters and compared with the binding behavior of the RBDs of Raf and Raf-GEF. Since for other effectors highly specific binding to different Ras proteins was demonstrated,
all members of the Ras subfamily are included in this investigation.

**MATERIALS AND METHODS**

**Cloning**—Rat and human AF6 fragments were amplified via standard polymerase chain reaction methods, ligated into pGEX and yeast plasmids, respectively, and transformed into B21 and DH5α. Mutations were introduced in Ras by standard polymerase chain reaction protocols (20). All clones were verified by sequencing (ABI-Prism, Perkin-Elmer).

**Yeast Two-hybrid Assay**—Yeast two-hybrid studies were performed in *Saccharomyces cerevisiae* Y190 according to the system of Chevray and Nathans (21). Briefly, competent Y190 cells were prepared as described (22) and cotransformed with 1 µg of each of the two-hybrid vectors. Efficient transformation was checked on synthetic medium lacking leucine and tryptophan (−Leu/−Trp). Transformed yeast cells were grown on synthetic medium lacking leucine, tryptophan, and histidine containing 25 mM 3-amino-1,2,3-triazole (−Leu/−Trp/His, Sigma) for the indicated time in order to detect interaction between Ras and the AF6 constructs (Fig. 1). 2% glucose was used in all media as the carbon source.

**Proteins**—The *Escherichia coli* strain B211 was grown in standard medium (Merck), and the expression of the pGEX plasmid encoding the glutathione S-transferase/AF6-RBD fusion protein was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside after OD₆₀₀ = 0.6 was reached. The culture was incubated at 30 °C for 4 h and then centrifuged. The resuspended cell pellet was sonicated, and the lysate was cleared by centrifugation (32,000 × g). The insoluble and soluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis indicating that the glutathione S-transferase/AF6-RBD fusion protein (42 kDa) is readily soluble (Fig. 2, lane 1 and 2). The glutathione S-transferase fusion was purified according to the standard procedure by gel filtration (Fig. 1, lane 3–6). The AF6-RBD construct (aa 1–170) as described by van Aelst et al. (12) showed a second order of association to Ras and Raf-RBD which are known to interact strongly with its amino-terminal end, respectively, improved binding significantly (Fig. 1, panel B). The X-Gal response is similar to that of Ras and Raf-RBD which are known to interact strongly (27). Under the same conditions the sequence of the RBD of AF6 (aa 36–206) reported earlier (13) did not support growth in a buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol. The proteins were transferred into other buffers using Nap-5 columns (Amersham Pharmacia Biotech).

**Experimental Techniques**—Protein concentrations were measured using the dye assay described by Bradford (25). Bovine serum albumin was used for calibration. Nucleotide concentrations in the GTPase assay were quantified by HPLC (Beckman) using a reversed phase C18 column (ODS Ultrasphere, Beckman). For further details see Lenzen et al. (26). Fluorescence measurements were carried out on a spectrophotometer (Fluoromax, Spex) equipped with a thermostating device. For stopped-flow measurements an SM-17 apparatus (Applied Photophysics) was used. Rate constants can reliably be measured up to 500 s⁻¹ since the dead time for the mixing of two solutions is in the range of 1–2 ms. Ras-mant-GppNHp was mixed with AF6-RBD in more than 5-fold molar excess in order to have pseudo-first order conditions for the association. According to the fluorescence traces monitored, were fitted with an exponential curve yielding kₚ. Mant nucleotides were excited at 360 nm, and the fluorescence was monitored through a 408-nm cut-off filter. If not indicated otherwise, all experiments were carried out in a buffer containing 20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM dithiothreitol. The proteins were transferred into other buffers using Nap-5 columns (Amersham Pharmacia Biotech).
the Y190 strain. A similar result was obtained with another amino-terminal truncated sequence (aa 30–141) which contains all residues from the predicted RBD of AF6 (28). The latter showed only small colonies after 9 days of growth which gave a poor X-Gal stain after overnight incubation (data not shown).

The DNAs encoding different AF6 constructs, namely residues 1–84, 13–133, 30–141, and 1–141, were transferred into pGEX-4T3 vectors and expressed in the bacterial strain BL21 in order to check the integrity and the solubility of the protein fragments. The two former fragments were poorly soluble and were partly degraded in E. coli. From the two latter protein fragments 30–141 bound much less strongly to Ras than 1–141 (assay see below). From these observations we concluded that the Ras binding domain (RBD) of AF6 requires residues 1–141 for full integrity and strong binding to Ras. This fragment is defined as AF6-RBD and is used for further biochemical experiments in this study. By size exclusion chromatography and comparison to the elution volumes of standard proteins (data not shown), it was demonstrated that AF6-RBD is a homogeneously monomeric protein.

To check the stability of AF6-RBD and to show that it constitutes a stable folding domain, it was further characterized by differential scanning calorimetry. A solution of AF6-RBD was heated from 20 to 80 °C at 70 °C/h, and unfolding was monitored with the change in the heat capacity \( C_p \). The reversibility of the unfolding transition in a solution at pH 9.0 was reasonably high for a thermodynamic analysis, i.e. 85% recovery of folded protein after each scan. This analysis was carried out under the assumption of a two-state equilibrium, i.e. the protein is either in the native, folded state, or in the unfolded state, and no intermediates occur (29). Such a behavior is seen for most small proteins which constitute a single cooperativity unit. The fit to the data yielded \( \Delta H_u = 62.7 \text{ kcal/mol} \) for the enthalpy of unfolding and \( T_m = 56.1 ^\circ C \) for the melting temperature. The scanning rate had no influence on the reversibility of unfolding observed, respectively, which can be explained by the different times the unfolded protein was kept at high temperature. The quasi-reversible unfolding transition may be taken as additional evidence for the integrity of AF6-RBD.

**AF6-RBD Binding to Ras Proteins**—In our earlier studies on the interaction of Ras proteins with effector RBDs, we made use of the inhibition of nucleotide dissociation from Ras upon effector binding (30). AF6-RBD also inhibits nucleotide dissociation from Ras. In this GDI assay Ras is quantitatively loaded with the non-hydrolyzable GTP analog with a fluorescent label attached to the ribose moiety. The dissociation of this nucleotide mant-GppNHp is initiated by adding a 1000-fold excess of unlabeled GppNHp. The fluorescence decay due to quasi-irreversible mant-GppNHp dissociation is monitored and fitted by an exponential function. The obtained rate constants are plotted versus AF6-RBD concentration and a curve fitted according to Equation 2 yields the dissociation constant \( K_d \) as defined in Equation 1. Equation 2 may be applied since the equilibration of the Ras-AF6 complex is fast compared with the nucleotide dissociation as shown below. The Ras protein, the nucleotide, and the effector AF6-RBD are abbreviated by \( R \), \( N \), and \( E \), respectively, and \( k_{-1} \) and \( k_{-2} \) represent the nucleotide dissociation rate constants from Ras alone and from the Ras-effector complex, respectively. Index 0 denotes total concentrations.

\[
K_d = \frac{[R - N] \cdot [E]}{[R - N - E]} \quad \text{(Eq. 1)}
\]

\[
k_{obs} = k_{-1} - (k_{-1} - k_{-2}) \left( \frac{[R]_0 + [E]_0 + K_d}{2 \cdot [R]_0} \right) - \sqrt{([R]_0 + [E]_0 + K_d)^2 - 4 \cdot [R]_0 \cdot [E]_0} \quad \text{(Eq. 2)}
\]
It was also possible to measure the affinity between Ras and AF6-RBD by means of direct fluorescence titration. At 20 °C the fluorescence of Ras-mant-GppNHp drops by 12% on saturation with AF6-RBD as shown in Fig. 5, upper panel. In the lower panel a curve is fitted to the titration data according to Equation 2 where \( k_{-1} \) and \( k_{-2} \) are replaced by the maximum and minimum fluorescence values, respectively, and \( k_{obs} \) is replaced by the relative fluorescence. The \( K_d \) value of 1.8 \( \mu M \) obtained from this fit is in good agreement with the result of 3.0 \( \mu M \) from the GDI assay regarding the difference in temperature. At 37 °C the titration is impossible since there is almost no change in fluorescence detectable. On addition of 100 \( \mu M \) AF6-RBD to Ras-mant-GDP no change in fluorescence is seen (Fig. 5, upper panel). As for the GDI data this is explained by the lack of binding at this concentration which means that the affinity of AF6 to the GDP form of Ras is at least 33-fold lower compared with the GTP form.

Evidence has accumulated during the last few years that Ras signaling branches out into several distinct pathways (for review see Ref. 30) the output of which may depend on the type of the cell or on the synergism with other stimuli. By cell transformation studies it was shown that certain mutations in the Ras effector region lead to abrogation of signaling to distinct effectors. The mutants T35S, E37G, and Y40C appear to exert a specific effect on Ras transformation studies it was shown that certain mutations in the Ras effector region lead to abrogation of signaling to distinct effectors. The mutants T35S, E37G, and Y40C appear to exert a specific effect on Ras signaling by not fully understood mechanisms. Although Ras binds tightly to Raf, it was shown that these proteins form a short-lived complex (41, 42). This highly dynamic behavior of the Ras-Raf complex could be important for the activation of Raf kinase activity or for the down-regulation of Ras by GAP. For the Ras-RalGEF complex similar interaction dynamics are observed.2

Effect on GTP Hydrolysis—As AF6-RBD has an influence on the nucleotide binding to Ras, it was worthwhile to look at the effect on GTP hydrolysis activity of Ras. At 37 °C 50 \( \mu M \) Ras loaded with GTP was incubated in the absence and in the presence of saturating amounts of AF6-RBD (100 \( \mu M \)). The time course of GTP hydrolysis by Ras was monitored by HPLC analysis of the nucleotides. The data obtained in Fig. 6 were fitted according to first order kinetics. For both experiments the same rate constants were calculated (0.03 min\(^{-1}\)) demonstrating that AF6-RBD binding to Ras does not change its GTPase activity.

**TABLE I**

| **K\(_d\) values for AF6-RBD and members of the Ras subfamily** |
|------------------|
| **Ha-Ras** | 3.0 |
| **Ha-Ras (GDP)** | >100 |
| **Ha-Ras E31K** | 2.3 |
| **Ha-Ras T35S** | >100 |
| **Ha-Ras E37G** | 33 |
| **Ha-Ras D38A** | 36 |
| **Ha-Ras Y40C** | >100 |
| **Ha-Ras E62A** | 7.5 |
| **Ha-Ras E63A** | 7.2 |
| **Rap1A** | 0.25 |
| **Rap1A (GDP)** | >100 |
| **Rap2A** | 3.7 |
| **R-Ras** | 16 |
| **TC21** | 16 |

2 T. Linnemann and C. Herrmann, unpublished observations.
proteins as described above (Fig. 5) was used as detection signal. For Ras as well as for Rap1A the observed rate constant $k_{\text{obs}}$ showed a linear dependence on the concentration of AF6-RBD up to 15 μM. From the linear slope fitted to the data $k_{\text{on}}$, the apparent association rate constant, is obtained, and the intercept corresponds to the dissociation rate constant $k_{\text{off}}$. The dissociation rate constant can be measured more accurately by displacement experiments. Ras-mant-GppNHP is displaced from the complex with AF6-RBD by addition of a large molar excess of non-labeled Ras-GppNHP. In a typical experiment a solution containing 0.5 μM Ras-mant-GppNHP and 6 μM AF6-RBD was mixed with 60 μM Ras-GppNHP. The observed rate constant in this case is governed by the dissociation process. The dissociation rate constants obtained by this method agree with the intercepts mentioned above. In the case of Rap1A the dissociation rate constant is smaller than the experimental error for the intercept. Here the dissociation rate constant can only be obtained from the displacement experiments. At 26 °C lifetimes of the AF6-RBD complexes with Ras and Rap1A of 10 and 100 ms are calculated, respectively. This is similarly short-lived as the Ras-Raf-RBD complex (42). The results for the temperature range between 10 and 26 °C are listed in Table II together with the ratios $k_{\text{off}}/k_{\text{on}}$ which agree well with the $K_d$ values reported in the upper section.

From the temperature dependence of the rate constants the energies of activation for complex dissociation and association $E_a^{\text{off}}$ and $E_a^{\text{on}}$, respectively, can be obtained. According to the Arrhenius equation the energies of activation $E_a^{\text{off}} = 16$ kcal/mol and $E_a^{\text{on}} = 14$ kcal/mol for Ha-Ras and $E_a^{\text{off}} = 9.5$ kcal/mol and $E_a^{\text{on}} = 10$ kcal/mol for Rap1A are calculated, respectively.

At room temperature and at concentrations higher than 20 μM $k_{\text{obs}}$ is too large to be measured by means of the stopped-flow technique. However, at 10 °C saturation of Ras/AF6 binding kinetics is observed at higher concentrations which is an indication of a two-step binding process. The formation of an initial weak complex is followed by a conformational rearrangement leading to tight binding. Under the assumption that the dissociation of the initial complex is fast compared with its conformational rearrangement, the observed rate constant for the two-step binding process is described by Equation 3. $K_1$ is the association constant for the initial weak complex, and $k_{\text{off}}$ and $k_{\text{on}}$ represent the rate constants of the forward and backward conformational rearrangements, respectively. Note that at concentrations far below $1/K_1$ Equation 3 can be approximated by a linear equation (see above).

$$k_{\text{obs}} = \frac{k_{\text{on}}}{1 + K_1 \cdot [\text{RBD}]} + k_{\text{off}}$$

(Eq. 3)

### Table II

| Temperature | $k_{\text{on}}$ | $k_{\text{off}}^a$ | $k_{\text{off}}^b$ | $K_d$ |
|-------------|----------------|-------------------|-------------------|-------|
| °C | μM⁻¹ s⁻¹ | s⁻¹ | μM |
| Ras | 10 | 6.4 | 20.8 | 15.3 | 2.4 |
| 16 | 11.5 | 30.8 | 28.4 | 2.5 |
| 19 | 14.5 | 40.8 | 35.5 | 2.4 |
| 23 | 22.9 | 39.3 | 53.8 | 2.3 |
| 26 | 26.0 | 77.6 | 72.9 | 2.8 |
| Rap1A | 10 | 11.9 | 2.6 | 0.22 |
| 14 | 19.0 | 3.5 | 0.18 |
| 18 | 19.7 | 4.6 | 0.23 |
| 22 | 22.1 | 5.6 | 0.25 |
| 26 | 34.8 | 7.0 | 0.29 |

| TABLE II

**Stopped-flow kinetics**

Rate constants are given for the interaction of AF6-RBD with Ras-mantGppNHP and Rap.mantGppNHP, respectively, obtained by stopped-flow experiments.

| Temperature | $k_{\text{on}}$ | $k_{\text{off}}^a$ | $k_{\text{off}}^b$ | $K_d$ |
|-------------|----------------|-------------------|-------------------|-------|
| °C | μM⁻¹ s⁻¹ | s⁻¹ | μM |
| Ras | 10 | 6.4 | 20.8 | 15.3 | 2.4 |
| 16 | 11.5 | 30.8 | 28.4 | 2.5 |
| 19 | 14.5 | 40.8 | 35.5 | 2.4 |
| 23 | 22.9 | 39.3 | 53.8 | 2.3 |
| 26 | 26.0 | 77.6 | 72.9 | 2.8 |
| Rap1A | 10 | 11.9 | 2.6 | 0.22 |
| 14 | 19.0 | 3.5 | 0.18 |
| 18 | 19.7 | 4.6 | 0.23 |
| 22 | 22.1 | 5.6 | 0.25 |
| 26 | 34.8 | 7.0 | 0.29 |

- $^a$ Obtained from the intercept (see text).
- $^b$ Obtained from displacement experiments. $K_d$ values are calculated from the ratio of $k_{\text{off}}$ from the displacement experiments and $k_{\text{on}}$.  

In Fig. 7 the observed rate constants measured under pseudo-first order conditions are plotted versus AF6-RBD concentration. From the fit to the data according to Equation 3 a value of $K_d = 25,000$ μM⁻¹ is obtained which corresponds to a dissociation constant of the initial encounter complex of 40 μM. The maximum $k_{\text{off}}$ is reached at 310 s⁻¹. For Rap1A no sign of saturation of AF6-RBD binding kinetics could be observed up to 50 μM where the upper limit for stopped-flow experiments of $k_{\text{obs}} = 500$ s⁻¹ is reached (data not shown).

### 31P NMR Studies on the Ras/AF6-RBD Complex—By 31P NMR experiments it was shown previously that the GppNHP-bound form of Ras exists in two conformational states (43) which is reflected by the band splitting of the α- and β-phosphorus resonances in the bottom panel of Fig. 8. The higher populated state (61%) was interpreted as the conformation with the effector loop (switch I) in close proximity to the phosphate groups exhibiting a ring current shift of the Tyr-32 side chain onto the phosphorus atoms (state 2). In the other state Tyr-32 is pointed away from the nucleotide (state 1). From the bottom to the top panel of Fig. 8 increasing amounts of AF6-RBD are added to a Ras-GppNHP solution. The change of the α- and β-phosphorus resonances reflects a shift from state 1 to state 2. At saturation only state 2 is populated indicating a stabilization of this conformation by AF6-RBD binding.

**DISCUSSION**

Previously, AF6 was identified as a potential Ras target (12, 13) which would add to the list of well established effectors like Raf kinase, RasGEF, and PI(3)kinase. It was also demonstrated...
that AF6 binds to tight junctions in epithelial cells via the interaction of the AF6 amino terminus with ZO-1 which appears to be regulated by Ras (17). This mechanism would further increase the complexity of Ras signaling and could explain the perturbation of cell-cell contacts induced by activated Ras. Activation of some Ras effectors by Rap, R-Ras, or TC21 was also demonstrated (44–46). Therefore, quantitative knowledge about the interaction of AF6 with all putative partners is necessary in order to estimate physiological consequences like inhibition or synergism in the context of the cell where all Ras proteins can be present. Furthermore, information about the dynamics of protein-protein interactions could be an important criterion for the assessment of signaling or scaffolding complexes. In this work the interactions between AF6 and members of the Ras subfamily have been characterized by biophysical methods. A stable protein fragment constituting the RBD of AF6 was defined, and its binding specificity and the dynamic behavior of Ras interaction were investigated.

Apart from Ras and Rap1A (12, 13) no other Ras proteins had been reported to interact with AF6 so far. The data presented here demonstrate that the closely related Rap2A, TC21, and R-Ras also bind to AF6-RBD. Moreover, like the RBDs of the effectors Raf and RalGEF, the RBD of AF6 showed different affinities to the various Ras proteins. Intriguingly, AF6 forms the most stable complex with Rap1A which is more than 10 times tighter than with Ras, Rap2A, TC21, and R-Ras, respectively, as shown in Table I. For Rap1 an influence on the regulation of cell morphology via the cytoskeleton was shown in various organisms like yeast (47, 48), Neurospora crassa (49), Dictostelium (50), and in the Swiss 3T3 cell line (51). It is uncertain whether the affinity of the Ras-effector complex correlates with its physiological importance, but it will be worthwhile to investigate the biological function of the Rap1A-AF6 interaction.

When specificity is discussed it should be stressed that Rap2A and Ras have the same affinity. This is surprising since throughout the effector region (residues 20–45) the homology is higher between Rap1A and -2A than between Rap1A and Ras (see Fig. 9). Residues that are different between Rap1A and -2A are the same for Rap1A and Ras, and residues different between Rap1A and Ras are the same for Rap1A and -2A. So, the observed affinities could be due to subtle differences of the Rap1A and -2A conformations that are not obvious from the primary sequence. The involvement of switch II of Ras in AF6-RBD binding is unlikely since two mutants in this region, E62A and E63A, do not alter significantly the affinity that is also observed for Raf and RalGEF binding.2

Partial loss of function mutations of Ras were reported from various investigators. These particular mutants have been found to bind and activate only distinct effectors and so they are useful for reducing the complexity of Ras signaling in biological experiments. The affinities of these Ras mutants to AF6-RBD were quantified (Table I), and together with the affinities to the different Ras proteins one may say that the binding specificity of AF6 has a similar profile as RalGEF. Exceptions though are represented by the Ras/Rap residue 31 and by D38A. These findings may be helpful for the design of experiments addressing the biological function of AF6. By the appropriate design of a Ras or Rap1A mutant, AF6 binding may be preferred (e.g. E31K, D38A) or specifically blocked (e.g. T35S, Y40C), respectively.

Proteins in general are constituted of largely independent building blocks with distinct functions like catalytic activity or docking to other proteins (e.g. Refs. 52 and 53). Our studies were done with only a fragment of the AF6 protein implying that only this part of the protein is responsible for Ras binding. Support for this assumption may be taken from our earlier findings with Raf where the binding constants of several RBD mutants showed a good correlation with the activation of Raf by Ras and with the binding of Ras to full-length Raf carrying the same mutations as probed by a reporter gene assay in a mammalian cell line and by the double hybrid method in yeast cells, respectively (54, 55).

From kinetic studies with Raf-RBD it was concluded that the association with Ras occurs in two steps (42). The formation of an initial complex with low stability is followed by a conformational rearrangement of the proteins leading to tighter binding (induced fit). This is indicated by the saturation of the binding kinetics at high concentrations where the rate of the second step becomes rate-limiting. This behavior is also observed for the association of Ras and AF6-RBD. In summary, the mechanism of Ras binding is similar for AF6-RBD and Raf-RBD, and particularly, the complexes are similarly short-lived. The differences in nature of an enzymatically acting effector like Raf or RalGEF and a potential scaffolding protein like AF6 is not reflected in the dynamics of complex formation and dissociation of their RBDs.

Further evidence for a similar interaction is taken from the influence of the Ras-AF6-RBD interaction on the dynamics of the effector region as probed by 31P NMR spectroscopy. Notably, no shift of the phosphorus resonances occurs when AF6-RBD binds to Ras which indicates that AF6-RBD at-

[Fig. 8. 31P NMR spectra of a titration series of AF6-RBD to Ras-GppNHp. 500 MHz spectra of RasGppNHp at 1 mM and AF6-RBD from bottom to top panel at 0, 0.2, 0.5, and 1 mM, respectively, at 5 °C. The resonances between −12 and −11 ppm correspond to Ps (where P indicates phosphate), between −4 and −2 to Pβ, and at −0.3 to Pγ. The peak at 2.3 ppm originates from free phosphate ions.]

[Fig. 9. Primary sequence alignment of the effector region of Ras-like proteins. The one-letter code for amino acids is used to compare the residues of the Ras relatives in the effector region (residues 32–40). Dots represent conserved amino acids.

Rap2A and Ras have the same affinity. This is surprising since throughout the effector region (residues 20–45) the homology is higher between Rap1A and -2A than between Rap1A and Ras (see Fig. 9). Residues that are different between Rap1A and -2A are the same for Rap1A and Ras, and residues different between Rap1A and Ras are the same for Rap1A and -2A. So, the observed affinities could be due to subtle differences of the Rap1A and -2A conformations that are not obvious from the primary sequence. The involvement of switch II of Ras in AF6-RBD binding is unlikely since two mutants in this region, E62A and E63A, do not alter significantly the affinity that is also observed for Raf and RalGEF binding.2

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Further evidence for a similar interaction is taken from the influence of the Ras-AF6-RBD interaction on the dynamics of the effector region as probed by 31P NMR spectroscopy. Notably, no shift of the phosphorus resonances occurs when AF6-RBD binds to Ras which indicates that AF6-RBD at-
taches to Ras remote from the nucleotide-binding site like the RBDs of Raf and RapGEF. Moreover, after addition of saturating Rap6-RBD concentrations, the band splitting disappears in favor of the state where Tyr-32 is located in closer proximity to the nucleotide. The same stabilization of this conformational state was reported for the Ras-Raf and Ras-RALGEF interactions.

In conclusion, the members of the Ras superfamily recognize AF6 in a manner characteristic for all effectors, and Rap1A shows the highest affinity. Whereas the RBDs of Raf and RapGEF have the same size and despite their low sequence homology show the same structural fold, the RBD of AF6 with 141 residues is significantly larger. Nevertheless, the binding characteristics as probed by mutagenic and kinetic studies as well as by 31P NMR spectroscopy are quite similar for all these effector RBDs. The influence of AF6 on the nucleotide dissociation from Ras and the competition of AF6 and GAP strongly suggest the same type of interaction as Raf and RapGEF. Specific differences are observed, however, such as the different contributions to binding by residues 31 and 38 from Ras. Structural results must be waited for in order to compare the Ras-AF6 interaction on an atomic level to the other RBDs. It will be interesting to see if AF6 is another effector with the same RBD topology as Raf and RapGEF, how the additional residues are accommodated, and how ZO-1 binding to AF6 or the interaction with other proteins is regulated by Ras or its relatives.

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