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Compensatory and Long-Range Changes in Picosecond–Nanosecond Main-Chain Dynamics upon Complex Formation: ¹⁵N Relaxation Analysis of the Free and Bound States of the Ubiquitin-like Domain of Human Plexin-B1 and the Small GTPase Rac1

S. Bouguet-Bonnet¹ and M. Buck^{1,2,3,*}

¹Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA

²Department of Neuroscience, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA

³Department of Pharmacology, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA

Abstract

The formation of a complex between Rac1 and the cytoplasmic domain of plexin-B1 is one of the first documented cases of a direct interaction between a small guanosine 5'-triphosphatase (GTPase) and a transmembrane receptor. Structural studies have begun to elucidate the role of this interaction for the signal transduction mechanism of plexins. Mapping of the Rac1 GTPase surface that contacts the Rho GTPase binding domain of plexin-B1 by solution NMR spectroscopy confirms the plexin domain as a GTPase effector protein. Regions neighboring the GTPase switch I and II regions are also involved in the interaction and there is considerable interest to examine the changes in protein dynamics that take place upon complex formation. Here we present main-chain nitrogen-15 relaxation measurements for the unbound proteins as well as for the Rho GTPase binding domain and Rac1 proteins in their complexed state. Derived order parameters, S^2 , show that considerable motions are maintained in the bound state of plexin. In fact, some of the changes in S^2 on binding appear compensatory, exhibiting decreased as well as increased dynamics. Fluctuations in Rac1, already a largely rigid protein on the picosecond–nanosecond timescale, are overall diminished, but isomerization dynamics in the switch I and II regions of the GTPase are retained in the complex and appear to be propagated to the bound plexin domain. Remarkably, fluctuations in the GTPase are attenuated at sites, including helices $\alpha 6$ (the Rho-specific insert helix), $\alpha 7$ and $\alpha 8$, that are spatially distant from the interaction region with plexin. This effect of binding on long-range dynamics appears to be communicated by hinge sites and by subtle conformational changes in the protein. Similar to recent studies on other systems, we suggest that dynamical protein features are affected by allosteric mechanisms. Altered protein fluctuations are likely to prime the Rho GTPase–plexin complex for interactions with additional binding partners.

*Corresponding author. Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106, USA. E-mail address: Matthias.Buck@case.edu..

Present address: S. Bouguet-Bonnet, Méthodologie RMN (UMR CNRS-UHP 7565), Nancy-Université, Faculté des Sciences et Techniques, B.P. 239, Boulevard des Aiguillettes, 54506 Vandœuvre-les-Nancy, France.

Supplementary Data

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protein–protein interactions; cell signaling; allostery; NMR; spin relaxation

Introduction

Proteins are dynamic molecules that often undergo extensive fluctuations and conformational changes. Some of these protein motions are essential for biological function.¹ Particularly in cell signalling, protein–protein recognition, if not binding, often involves profound changes in the structure and dynamics of the binding partners.² A number of experimental techniques are available for the detailed characterisation of such changes, including optical spectroscopies, molecular modelling, X-ray crystallography and NMR spectroscopy. The latter is unique because it yields site-specific information on multiple timescales and has become a technique of choice for the study of macromolecular dynamics.^{3,4} Specifically, backbone ¹⁵N relaxation measurements can be used not only to monitor internal motions on the fast (pico- to nanosecond) timescale, but also to indicate fluctuations on a slower (micro- to millisecond) timescale, and are used to assess the overall rotational diffusion of the molecule (nanosecond timescale).

Dynamics measurements that characterize protein–protein or protein–ligand interactions are carried out for proteins both in their free and in their bound state, providing site-specific information about the relevant molecular motions in protein recognition, complex formation and transmission of information to allosteric sites.^{5,6} Although comparative dynamics studies on free and complexed proteins are an important goal, relatively few NMR relaxation studies have been reported for protein–ligand interactions (e.g., Refs. 7–12) and even fewer on protein–protein interactions.^{13–16} The present article is to our knowledge the first that reports the main-chain dynamics of the free proteins, as derived from ¹⁵N NMR relaxation measurements, and also examines their fluctuation in the complexed state (i.e., measurements were carried out on both proteins in each of the two states).

The system we have chosen is a small guanosine 5′-triphosphatase (GTPase)–effector protein interaction. Small GTPases of the Ras superfamily are understood as on/off switches in cell signaling processes, depending on the nature of the nucleotide, guanosine triphosphate (GTP) or guanosine diphosphate (GDP), that is bound.¹⁷ When bound to GTP (or GTP analogues), GTPases are active and capable of mediating specific biochemical reactions or binding events. They remain in this state until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the protein. Regulatory proteins, GTPase-activating proteins (GAPs) and guanine exchange factors (GEFs), bind to the GTPase and affect the rate of nucleotide hydrolysis or exchange and thus switch the protein between the “on” and the “off” state. Interestingly, GTPase regions that are involved in protein–protein interactions (switch I and switch II) typically exhibit decreased mobility in the bound state relative to their free state, suggesting that protein flexibility may be required for binding. For example, the switch I and II regions of GTPase Cdc42Hs and the binding region of its effector PBD46 are unstructured and highly mobile in their free forms, but their degree of motional complexity is reduced upon binding.¹⁸ Dynamics data were only reported on the side of the GTPase in this study—although a reduction in the flexibility of the effector polypeptide has been inferred in this system¹⁷ as well as in others.¹⁹ By contrast to the well-characterized Rho GTPase Cdc42,²⁰ no NMR dynamics studies have yet been reported for the homologous protein, Rac1.²¹

The complex formation between the small GTPase Rac1 and a cytoplasmic Rho GTPase binding domain (RBD) of plexin-B1 is one of the first documented cases of a direct interaction between a small GTPase and a transmembrane receptor.^{22,23} Plexins use Rho GTPases to

signal to the actin cytoskeleton in developmental processes such as axon and blood vessel guidance.²⁴ We have found that the plexin RBD is a central part of the cytoplasmic region of the receptor and has a ubiquitin fold.²⁵ Unusual for a ubiquitin fold, the structure includes several long and flexible loops.²⁶ Mapping of the RBD residues that are in contact with active Rac1 by chemical shift perturbation and NMR cross saturation revealed two spatially adjacent segments of the structure, a short loop and β -strand 4 (residues 72–77) and a short α -helix (residues 96–99), are the primary sites of interaction with Rac1.²⁷ On the side of Rac1, previous site-directed mutagenesis data suggested that the plexin RBD may be an effector protein for active Rac1.^{22,23} Here we substantiate this finding by a surface mapping of regions of Rac1 that are in contact with the plexin domain in the complex. Because the plexin RBD is an effector domain, in principle, large changes in the protein's conformation and/or dynamics could occur upon GTPase binding.

NMR relaxation measurements were also carried out in order to better understand the dynamical properties of Rac1 in its active form [bound to guanosine-5'-[(β,γ)-imido] triphosphate triethylammonium salt (GMPPNP), a non-hydrolysable GTP analogue] and in its interaction with the RBD of plexin-B1. We describe the backbone dynamics of Rac1.GMPPNP and of plexin-B1 RBD both as free proteins and in the plexin-B1 RBD/Rac1.GMPPNP complex. Molecular correlation times, extended Lipari–Szabo (LS) model-free order parameters and local correlation times, as well as chemical exchange (R_{ex}) contributions, were derived using ¹⁵N relaxation data acquired at two spectrometer frequencies. The changes observed in the dynamics upon association of the proteins are wide-ranging and complex. Surprisingly, changes in the dynamics also occur far from the site of binding and several appear to be compensatory in character. Increased dynamics in the bound state of the RBD suggest the involvement of allostery in the cell-signalling mechanism. Possible implications of the dynamics changes seen in complex formation are discussed for the plexin-B1–Rho GTPase system and comparison is made with other small GTPase–protein interactions.

Results

Resonance assignments in free and bound proteins

Resonance assignments for both proteins have recently been reported by us.^{21,25} While the assignments for the main chain of the free plexin RBD are complete, despite our best efforts a total of 28 of the 169 expected main-chain amide groups could not be assigned in Rac1.GMPPNP. Not unusual for NMR studies on small GTPases, the intractable signals almost exclusively belong to parts of the switch I and II regions (residues 27–43 and 60–75), which are known to fluctuate between a number of different conformations in unbound small GTPases. For example, 36 main-chain amide residues are not assigned in Rac1.GDP.²⁸

Assignments of resonances from the bound proteins

Amide resonances in plexin are in slow exchange on the NMR timescale, if they are affected at all by binding (Supplementary Fig. S1). Only 21 of 113 amides of the RBD are significantly affected in their chemical shift by binding to Rac1.GMPPNP. Most of these could be assigned by the standard sequential strategy employed through bond correlations as detected in 3-D heteronuclear NMR experiments.²⁷ A small number of resonances remain unassigned, most likely due to conformational exchange that exists regionally at the plexin-B1 RBD–Rac1.GMPPNP interface. Complex formation on the side of Rac1 is associated with chemical shift changes that could be followed in a titration with the plexin RBD. Eleven amides experienced large chemical exchange broadening so that they could not be traced to the spectrum of the bound state. For several GTPase-bound states, such as Rac1.GDP complexed with RhoGDI and for a peptide from p21-activated kinase (PAK) binding to Cdc42, both the switch I and switch II regions become significantly more ordered and could largely be assigned. However,

no new resonances became visible upon plexin RBD binding, which suggests that that intermediate chemical exchange persists in these two regions of the GTPase even in the complex. Neither different temperature nor pH conditions brought forth a significant number of additional resonances.

The plexin-B1 RBD is confirmed as an effector protein

Several dozen structures of complexes between Rho GTPases and regulatory [activating (GAP), exchange (GEF), exchange inhibitory GDP dissociation inhibitor (GDI)] or effector proteins are known from X-ray crystallography or NMR spectroscopy. Regions of these small GTPases that are contacted by binding proteins are well conserved and indicate the functional role of the interaction.^{29,30} Using NMR, we monitored chemical shift and intensity changes of active Rac1 GTPase²¹ in a series of ^{15}N - ^1H transverse relaxation optimized spectroscopy spectra of ^{15}N -labeled Rac1.GMPPNP as it was titrated with unlabeled plexin-B1 RBD. Supplementary Fig. S2a plots these perturbations as a function of Rac1 sequence near the end point of the titration (plexin-saturated GTPase). Shown in comparison (Supplementary Fig. S2b) is one of the contact profiles compiled by Ahmadian *et al.*, here for GTPase-effector interactions.²⁹ This is the profile that matches the sites of interaction of Rac1 with the plexin RBD most closely, suggesting that plexin is an effector protein for Rac1 and that Rac1 and most likely two other RhoGTPases, Rnd1 and RhoD, have a role in regulating the activity of plexin. There is no general assay for effector function beyond the observation of binding and conformational change, whereas the possibility of GEF, GDI and GAP activity can be tested using established biochemical tests.^{31,32} We measured the rate of GTP hydrolysis of wild-type Rac1 by itself and in the presence of a saturating concentration of plexin-B1 RBD but found no significant change in the rate of phosphate release. Similarly, the rate of GDP release (measured using fluorescent Mant-GDP) and exchange for GDP or GTP from wild-type Rac1 was not significantly increased in the presence of plexin RBD. These assays suggest that the plexin RBD does not have a regulatory function for Rac1, although such functionality may arise in the context of the full-length cytoplasmic receptor region or as a result of a posttranslational modification, such as phosphorylation.

Features of the relaxation measurements and analysis for the free and bound proteins

Four sets of relaxation data [^{15}N R_1 , ^{15}N R_2 and $\{^1\text{H}\}$ - ^{15}N heteronuclear nuclear Overhauser enhancement (NOE)]—corresponding to both proteins in the free and then in the complexed state—were recorded at ^1H resonance frequencies of 600 and 800 MHz. A single-site monomerization mutant (W90F) of the plexin-B1 RBD³³ and a single-site mutant (C178S) of truncated Rac1, loaded with non-hydrolysable GTP analogue, GMPPNP, were used throughout the study in a buffer mimicking physiological conditions (see Materials and Methods). In order to characterize the molecular motions, the relaxation measurements obtained at two spectrometer frequencies were jointly analysed by use of the extended LS formalism³⁴⁻³⁶ (see Materials and Methods). The derived global motion is found to be axially symmetric for Rac1.GMPPNP with a correlation time of 12.5 ns and an anisotropy equal to 1.14 and also axially symmetric for the free plexin-B1 RBD with a correlation time of 8.6 ns and an anisotropy equal to 1.26. When they are part of the complex, both proteins are shown to also experience an axially symmetric global motion, with an average correlation time of 24.5 ns and an anisotropy of 1.31 (Table 1). Protein dynamics is described by the LS order parameter, S^2 , for internal motion and in most cases by a value for the internal correlation time τ_{loc} (>10 ps) and for some residues also by an exchange contribution (R_{ex}). Optimized order parameters are tabulated in the Supplementary Material.

Dynamics in free Rac1.GMPPNP

The derived order parameters, S^2 , are plotted in Fig. 1a for the active form of Rac1, also mapped for visual display onto the X-ray structure of Rac1. GMPPNP [Protein Data Bank (PDB) ID 1MH1]³⁷ in Fig. 1b. In total, relaxation data for 94 main-chain amides could be fitted, with gaps due to unassigned residues especially in the switch I and II regions (indicated as grey blocks). Both the relaxation data (and consequently, the derived order parameters) show a relatively modest variation across the sequence. With the possible exception of the switch I and II regions (for which we have no data), this analysis confirms that the GTPase is overall quite a rigid protein on the picosecond–nanosecond timescale. For example, at the protein's termini, only the first and last several residues show increased mobility, indicating that the polypeptide chain becomes highly structured in the protein's fold. It should be noted, however, that the wild-type protein runs to residue 192 and that the truncated residues are highly flexible in other small GTPases. Regions for which mobility is indicated in the free protein ($S^2 < 0.7$) comprise a short loop between the β -strand 1 and α -helix 1 (residues 8–16), a region near a turn between β 2 and β 3 (residues 47–48), a short stretch between residues 81–85 connecting β 4 and α 3, and residues 114 and 149, which form hinges around α 5/ α 6 and α 7. Helix α 6 is the Rho family small GTPase specific insert helix. Residues 157 and 165 could also be hinge sites of loops connecting β 6 to α 8. The concept of hinge sites arises because they are typically located between elements of secondary structure, such as the insert helix, and are also found near the surface of the protein (Fig. 1b). There are other, apparently isolated residues, including residues 53, 90, 129 and 146, with S^2 values that are significantly lower than those of their neighbors. We confirmed the assignments of these residues, also with reference to those of unbound Rac1.GDP²⁶ and a recent complex of active Rac1 with effector PRK1.³⁸ Relatively isolated dynamic sites exist in proteins, as seen, for example, for residue 85, just following a 3_{10} helix in hen lysozyme,³⁹ and for several residues, including a hinge near the center of a long helix in adenylate kinase.⁴⁰

It should be noted that the data from free Rac1 were not easily fitted and that an extended model had to be used in all but 19 cases. The amplitude of the faster motion, S^2_f , however, is in the range of 1 to 0.8 and spans the entire protein, while τ_{loc} is below 4 ns for all except for five residues (two in the N-terminal region, see below, and three surrounding the α 5/ α 6 segment mentioned above). R_{ex} is fitted for a total of 67 amides but has a value of less than 3 Hz for the great majority of them, especially in the C-terminal half of the molecule. In the N-terminal region (up to residue 30) several values are as high as 5–6 Hz at 600 MHz.

Dynamics in the free plexin-B1 RBD domain

Relaxation data could be fitted for 99 of 113 amides yielding order parameters (Fig. 2a). In the case of 36 sites, an extended model was used (with τ_s of 1–4 ns) and 3 sites required corrections to R_2 by an exchange term, R_{ex} (residues 82–83 and 92 of ~2.5 to 5 Hz at 600 MHz). Based on sequence homology and chemical shift analysis, we previously identified and described the plexin-B1 RBD as a ubiquitin-like protein²⁵ and have recently determined its solution structure (PDB ID 2JPH).²⁶ At least 4 residues at the N-terminus and 10 residues at the C-terminus of the domain are highly flexible, if not completely unstructured. By contrast to ubiquitin, the plexin RBD has several prominent loops, L1–L3, which are shown to be also mobile by the relaxation data ($S^2 < 0.8$) (Fig. 2b). Residues immediately following β 4 (residues 78–82) have S^2 values that are noticeably lower than those of a regular secondary structure. This region has the edge strand, β 4, of the ubiquitin fold on one side (no protection of amides against exchange was observed in this strand) and a long flexible loop, L4, on the other. Similar to the case discussed above with Rac1 and with hen lysozyme, residues at the termini of secondary structure may also undergo considerable fluctuations. For example, residue 15 at the end of β 1 and residue 74 at the beginning of β 4 in the RBD are still relatively flexible. Only some residues (residues 50, 54 and 62) have lower S^2 and surround a short flexible region (residues

57–60) in an otherwise lengthy and relatively rigid loop (L2) connecting $\alpha 1$ and $\beta 3$. The amide bond vector of Asp59 undergoes especially large amplitude fluctuations. Interestingly, this residue is surrounded by Pro58 and Pro60. By contrast, a turn (residues 32–36) between $\beta 2$ and the beginning of the α -helix, as well as helix $\alpha 2$ (residues 96–99) and a turn to $\beta 5$ (residues 100–104) are, with the exception of residue 104, the most rigid parts of the free protein on the picosecond–nanosecond timescale.

Thus, more so than seen in the case of active Rac1, the plexin RBD is a protein with highly varied picosecond–nanosecond dynamics along its polypeptide chain.

Dynamics of proteins in complex

Main-chain fluctuations in Rac1.GMPPNP complexed with plexin-B1 RBD—No structure has yet been determined for the complex between Rac1.GMPPNP and the plexin-B1 RBD. However, with reference to a database of GTPase-protein contacts³⁰, the chemical shift perturbations suggest the regions that are likely to be involved on the side of the GTPase (Supplementary Figs. S1 and S2 and Fig. 3b). Rac1 binding is exquisitely sensitive to the state of the nucleotide cofactor that is bound. Rac1 complexed with GMPPNP (a non-hydrolyzable analogue of GTP) binds to the plexin RBD with a K_d of $\sim 6 \mu\text{M}$, whereas no interaction, even transient, could be detected for the inactive GDP state of Rac1 even at millimolar concentrations. Thus, it is expected that the switch regions I and II are involved in the interaction with the plexin RBD, as well as the P-loop (residues 10–17) and parts of two other regions, residues 115–118 and 158–160, which are known to contact the nucleotide (Supplementary Figs. S2 and S3). Based on chemical shift changes, the latter three regions are clearly involved in plexin binding or are affected by conformational changes that result from complex formation. The two switch regions are difficult to characterize as pointed out above. In addition, several other sites experience chemical shift changes and at present we do not know whether these differences arise as a result of direct or indirect contacts. It is clear, however, that several regions of Rac1 are not affected in their conformation by binding. No substantial chemical shifts are seen in helices $\alpha 5$, $\alpha 6$ (the insert helix), $\alpha 7$ or $\alpha 8$, or in $\beta 6$, suggesting that this side of the molecule is distant from the interaction surface.

Internal dynamics seem overall decreased in Rac1 on binding to plexin (Fig. 3a and b). This is apparent from S^2 values that are more tightly clustered around 0.9–0.95 for the C-terminal part of the GTPase in the bound state, compared to S^2 values in the free protein that are on average slightly lower (Table 2). Also in this region, τ_{loc} values are approximately 1 ns, compared to ~ 2 ns in the free protein. Furthermore, R_{ex} contributions of around 2 Hz, which are used for many residues in this segment in the free protein, are no longer needed to fit data for the majority of residues in the bound GTPase. Some of the largest changes in terms of τ_{loc} and R_{ex} occur in the N-terminal 70 residues (Table 2). Residues in this region of the protein are mostly involved in the binding interaction with plexin, as discussed below. Here, no R_{ex} terms are needed and τ_{loc} values are substantially diminished, especially for the first 30 amino acids. It is difficult to compare some of the derived order parameters between the two states on a residue-by-residue basis because not all of the data are available. However, when such comparisons are possible, Z-scores, which indicate the statistical significance of the change, are considered (see Supplementary Material). Errors on relaxation rates were estimated from baseline noise – an analysis that often underestimates the level of uncertainty in the data. However, the procedure we used in GIFA allowed us to compensate for possible underestimates: for each NH group, relaxation rates and their uncertainties were adjusted so that good fits of the data to single exponential decays were obtained with χ^2 at 95% confidence. Furthermore, the S^2 differences for all residues described below are significant, having a Z-score that gives greater than 95% confidence that the two residues have a different motional amplitude. This is true even when the uncertainty in the relaxation rates is increased (a two-

to threefold increase in relaxation error would lead to an increase in S^2 of around 1.5). Dynamics in the switch I and II regions of active Rac1 bound to the plexin-B1 RBD remain complicated, with chemical exchange precluding an NMR analysis in and around these regions. However, when comparisons are possible between the free and bound states in other regions, several observations can be made. Two C-terminal dynamic residues, 129 and 146, stand out with S^2 values lower than those of the surrounding residues in the free protein, but the S^2 values for both residues are increased by approximately 0.2 on binding. Several other residues, which function as hinge sites in the free protein with lower than average S^2 values (residues 149 at the end of $\alpha 8$, 157 in $\beta 6$ and 165 just before $\alpha 9$), no longer experience picosecond–nanosecond dynamics in the bound protein. Furthermore, motions for the majority of residues in the region that includes $\alpha 5$ (residues 117–120), the insert helix $\alpha 6$ (residues 123–130) as well as helices $\alpha 7$ and $\alpha 8$ are diminished on binding, possibly because the hinge residues, mentioned above, are no longer flexible. Residue 114 also shows significantly diminished dynamics ($\Delta S^2=0.27$, Z-score 5.3). These results are surprising because the insert helix, $\alpha 6$, is not part of the region that contacts the plexin RBD. However, residues close to its N-terminal hinge (residues 116) are perturbed in both chemical shift and intensity upon binding, as is residue 162 (both residues are close to the P-loop/GTP binding site). Such subtle conformational changes may allow altered protein dynamics to be propagated to the surrounding regions, specifically to the back of the protein ($\alpha 5$ – $\alpha 8$).

A region that shows diminished dynamics and is located at the binding interface comprises residues 39–55. These residues experience a nearly uniform shift by around 0.05 in S^2 to higher values on complex formation (note that only ΔS^2 for residues 46–48 is statistically significant). Not all residues at the binding interface undergo a rigidification of picosecond–nanosecond dynamics, however. For example, residues 20 and 78 have S^2 values that are substantially lower than in the free protein, suggesting that fluctuations at these sites are increased. Since we do not have the structure of the complex, mapping of the changes in S^2 onto the crystal structures of the unbound proteins may be misleading. Specifically, residue 24 also has considerable flexibility and it is not clear whether helix- $\alpha 1$, which is adjacent to the binding interface, is still fully formed in the complex. It is apparent, however, that some of the changes in the fast internal dynamics are compensatory: upon binding, an increase in the dynamics in one part of the protein is accompanied by an attenuation of motions in another region (Fig. 3b).

Main-chain fluctuations in the plexin-B1 RBD complexed with Rac1.GMPPNP—

Similarly to the free protein, relaxation data for almost all of the amide resonances could be recorded and fitted in the bound state of the plexin-B1 RBD when in complex with Rac1.GMPPNP. The changes that occur in S^2 upon binding will be discussed in detail below. As with the free protein, many residues required fitting with the extended LS model. However, the range of the resulting correlation time, τ_{loc} , is similar to that found in the free protein for most regions. Exceptions are several residues in the first long loop (residues 15–26) and sites near the C-terminal (105, 108 and beyond residue 113), which in the bound state have τ_{loc} values greater than 4 ns (Table 3). Slower motions were already indicated in these two regions in the free protein with a correlation time of 1 ns. Also in the free protein, 16 residues required an exchange term to adjust R_2 during the fitting (all but 3 were less than 1 Hz at 600 MHz), whereas 54 residues required such a term for the bound state. Except for the N-terminus, the first long loop, the segment of residues 64–78 and the latter part of the third long loop (87–94), all other regions contained residues at least some of which have considerable R_{ex} values (>2 Hz). Remarkably, R_{ex} contributions are almost constant across several regions, for example, $\beta 1$ and $\beta 5$, the two central β -strands with values of 3–4 Hz. Two regions with the highest R_{ex} values (4–8 Hz), the N-terminus of the $\alpha 1$ -helix and the $\alpha 2$ -helix, are also close to each other in space, although only $\alpha 2$ is directly involved in GTPase binding (Fig. 4b), as determined by NMR cross saturation.²⁵ Although R_{ex} contributions for the latter part of this binding region (residues 92–99) are high, essentially no R_{ex} contribution is needed for the

second region in contact with Rac1 (residues 64–75, which form a short loop and β -strand 4 in the plexin RBD). The relaxation data show, therefore, that interactions of Rac1 with this latter binding site lead to relatively rigid contacts. Interestingly, residues adjacent to both binding regions experience conformational exchange on the microsecond–millisecond timescale. This observation suggests that they could be involved in non-specific or alternative contacts that are sampled locally in the bound state.

Regarding changes in S^2 , several flexible regions of the plexin RBD, such as residues in the first long loop, residues 16–26 and those at the N- and C-termini of the protein, are not extensively perturbed by binding (but see below). These regions are distant from the contact interface with active Rac1. Two regions in the plexin RBD show reduced fluctuations in terms of S^2 upon binding. The first (residues 77–84) is at the end of β -strand 4 and at the beginning of a long loop. The second region comprises the latter part of another long loop, L2 (residues 50–58), with residues 50, 54, 57 and 62 particularly affected. The first of these two regions is close to one of the Rac1 binding sites, while the second is near the N-terminal end of β 3 whose C-terminus is affected by binding to the loop that connects to β 4. Since residues following β 4 are also rigidified, it is possible that the strand is extended by making additional contacts with β 3, thus explaining why β 3 also becomes slightly more rigid. This observation again implies that binding is communicated beyond the region of immediate contact, this time on the side of plexin's RBD.

As with Rac1, several regions experience a decrease in S^2 values upon complex formation. Due to overlap and missing peaks, there are only few data points for one of the GTPase binding regions (residues 64–75): These data indicate, however, that the amplitude of the fluctuations is unchanged, if not increased (residue 74 appears highly flexible in the bound state). Similarly, flexibility of part of the other binding region (residues 92–99) appears to be increased (residues 91, 93, 96 and 100 have statistically significant changes in S^2). Moreover, part of a long loop that links both of these binding regions and that is one of the most flexible regions in the protein appears to undergo greater fluctuations in the complexed state, especially in the region of residues 85–87. Remarkably, this segment follows a region (residues 77–82) that has become more rigid on binding, as noted above.

Discussion

Comparison of order parameters with those published for homologous proteins

The order parameters calculated here for unbound active Rac1 can be compared with those derived for the homologous protein, Cdc42. Although both Rac1 and Cdc42 belong to the Rho family of small GTPases and are highly homologous in sequence (73% sequence identity; 82% sequence similarity) as well as in structure (main-chain RMSD of <1 Å for common carbon atoms), only few of the regions with lowered S^2 in active Rac1 also show enhanced flexibility in active Cdc42. A total of 27 order parameters in active Rac1 are lower than 0.8, whilst only 4 are lower than this value in Cdc42. The latter are located at the N- and C-termini and in the switch I region. Overall, the correlation between the two S^2 data sets is not statistically significant. The comparison between the value and the location of R_{ex} and τ_{loc} values is also poor. Interestingly, despite these differences, the overall global motion is nearly identical for these two homologous proteins ($\tau_R = 12.6$ ns for Cdc42 and 12.5 ns for Rac1 both at 25 °C and 1 mM protein concentration). In summary, with the possible exception of the switch I and II regions (which could not be followed), the internal dynamics of these two highly similar GTPases are overall considerably different. Cdc42 appears as a highly rigid structure on the picosecond–nanosecond timescale, while Rac1 shows more considerable internal dynamics. These fluctuations are, however, modest in magnitude, at least compared to the RBD.

The RBD has a ubiquitin-like fold and it is thus interesting to compare also the main-chain dynamics to those seen in human ubiquitin, a model system for many NMR dynamics studies. The plexin RBD has three long loops and these are either short loops or tight turns in ubiquitin. Similar LS analyses of picosecond–nanosecond motions in ubiquitin have shown that the only highly flexible ($S^2 < 0.6$) region of the molecule is found at the C-terminus.^{41,42} However, careful inspection of the order parameters derived by Ernst and coworkers⁴² for example, reveals that even the short loops between strands $\beta 1$ and $\beta 2$ and between the C-terminus of helix $\alpha 1$ and strand $\beta 4$ (see above) have S^2 values lower than those associated with most regions of secondary structure. Additionally, two residues prior to the start of the β -sheet internal strand, $\beta 5$, also experience increased dynamics. Thus, even though the sequence identity between the plexin RBD and ubiquitin is only modest,²⁵ we find good agreement between the locations of the more flexible regions. However, the amplitude of the motions (i.e., the extent to which S^2 is decreased) clearly depends on the length of the loops.

On association, both plexin and Rac1 experience complex changes in their picosecond–nanosecond main-chain dynamics. Many of the changes are located in regions that are involved in the formation of the complex or are close to the binding interface (Figs. 3 and 4), but some are not (see below). The changes in order parameters seen in Rac1 upon binding of the plexin effector can be compared to those that are observed in Cdc42 (loaded with GTP analogue GMPPCP) upon binding to a 46-residue effector peptide from PAK.¹⁷ The peptide contacts strand $\beta 2$ and a portion of switch I. The switch I region experiences diminished fluctuations upon binding, as does the insert helix and helix $\alpha 3$. The apparent correlation of binding with a change in the flexibility of $\alpha 3$, which is adjacent to the insert helix ($\alpha 6$), is also noted in our Rac1 study (with S^2 for residues 84–90 significantly affected on binding), although in both cases these regions make no direct contacts with the effector protein upon binding. Both findings suggest that a “network” of amino acid contacts exists in the proteins, which serves to couple these fluctuations possibly in all Rho family small GTPases. The existence of such networks has been proposed for several proteins from relaxation measurements. For example, a long-range coupling is seen for allosteric sites in calmodulin⁴³ and has been suggested from computational/bioinformatics modeling, for example, in the G-protein, Gs α (which shares structural homology with the small GTPase Ras).⁴⁴ For Gs α , a network model explained the allosteric connection between nucleotide binding and affinity for GTP/GDP-conformation-specific binding partners via connections of switch I with the more nucleotide distant switch II region. The findings for Cdc42 and here for Rac1 suggest a network that involves hinge regions around residues 84/90, 114, 149 as well as 157 and 165. Such a network allows a more extensive dynamic coupling, propagating conformational specificity from the switch I to the switch II region and then beyond to the insert helix ($\alpha 6$), to $\alpha 7$ and $\alpha 8$.

Compensation and long-range propagation of dynamics

Considering the statistical significance of the changes in S^2 (see Supplementary Material and Materials and Methods), an overall, albeit small net decrease of main-chain fluctuations is observed for regions in both proteins (Figs. 3 and 4), particularly for several regions such as residues 68–73 in the RBD and strands $\beta 1$ and $\beta 2$ in Rac1. However, a number of residues show increased picosecond–nanosecond dynamics, such as $\alpha 1$ and $\beta 4$ in Rac1 and helix $\alpha 2$ and residues 74 and 86/87 in the plexin RBD. These regions are also located in, or are adjacent to the interaction interface. Complex changes in protein dynamics are also observed in other systems. For instance, when the Rac1 homologous protein Cdc42 binds to a PAK peptide, the main-chain dynamics of both are largely decreased, indicating an overall decrease in main-chain entropy.^{17,18} Thermodynamically, this decrease in entropy is often balanced by the enthalpy gained due to the new protein–protein interactions and from the release of water from the binding surfaces. In case of Cdc42 binding, remarkably, an increase in side-chain entropy was observed from [¹³C]methyl relaxation studies.⁴⁵ Thus, there is also the possibility of a

direct compensation in terms of protein dynamics.¹¹ For example, several studies reported a “residual” entropy that persists, or is generated in proteins upon binding.⁴⁶ This entropy is associated with residues at or near the binding site that either remain highly dynamic.^{47,48} A similar effect is seen in several regions at or near the binding site in both the plexin RBD and in Rac1 that show increased fluctuations in the bound state. Such compensatory changes in dynamics have also been seen in other protein–protein and protein–ligand interactions.^{13–15, 11,49,50} The relaxation measurements presented here do not allow us to say to what extent such changes are coupled with those of neighboring protein regions. However, such relationships are likely, as seen from a growing literature of computational modeling of domain and subdomain motions (e.g., Ref. 40). In the case of the $\alpha 2$ helix and of other segments of the RBD, the change appears to be collective in that similar values (and change in values) are apparent for τ_{loc} and R_{ex} over a wider region of residues.

Binding processes, protein–protein or protein–ligand associations are increasingly characterized in terms of a so-called equilibrium-shift mechanism, although it is not clear to what extent such models differ from classical “lock-and-key” formulations.⁵¹ In either description of structural and dynamic changes, protein conformations equivalent to the bound structures already exist in the ensemble of the unbound structures of the proteins.^{12,43,48} Binding then selects one of these structures, shifting the equilibrium in favor of the bound state. The isomerization that is observed in large parts of the small GTPase switch I and II regions is likely to be such a fluctuation that provides access to bound-like conformations. In several small GTPase–protein interactions these backbone fluctuations appear to be diminished as the protein ensemble “shifts” to populate one of the conformational substates (specifically, this is manifest by decreased exchange contributions allowing resonance assignments in one or both GTPase switch regions in the bound state). It should be noted that the majority of studies on equilibrium shift behavior involve analysis of microsecond–millisecond fluctuations and that the LS model-free analysis does not provide quantitative information on the structures involved or of their populations. However, in the case of the Rac1/plexin-B1 complex, isomerization persists in the switch regions of the GTPase. Our data, in fact, suggest increased dynamics in the bound state for several residues at or near the site of interaction in both the plexin RBD and in Rac1. For instance, complex formation increases exchange contributions, R_{ex} , to large regions of plexin, in particular to helix $\alpha 2$, a segment that is dynamically rigid in the unbound protein. Faster picosecond–nanosecond timescale motions are also increased in this region as seen by a slight decrease in S^2 . These changes are not in the direction typically seen in equilibrium-shift mechanisms, which usually involve a selection of one from several substates and results in an overall reduction of flexibility. Formally, if equilibrium shift is defined in thermodynamic terms, an increase in flexibility may not present an inconsistent concept, provided that the more dynamic bound ensemble is also sampled in the unbound state (which may occur at least at some level of low population).

It has been suggested in several systems that changes in dynamics originating from site-directed mutagenesis or from ligand binding can be propagated to more distant sites.^{52,53} We also see perturbations in the main-chain dynamics at sites that are significantly distant from the interaction surface in both plexin and Rac1. Such changes in dynamics can reflect subtle structural alterations or may occur without substantial changes in protein structure, given that the protein structure is already a dynamic ensemble of subtly different conformations that interconvert.^{46,54,55} To address this issue, we investigated a possible correlation between changes in chemical shift and picosecond–nanosecond protein dynamics, but found no significant correlation. It should be noted that in the case of protein–protein interactions such a comparison may not be straightforward, as chemical shifts are also substantially perturbed by the new protein–protein contacts, which may occur without any change in internal protein dynamics. It is remarkable that some of the more dynamic main-chain amides, particularly in the mostly rigid Rac1, are located at the borders of secondary structural units and may be

regarded as hinge sites.^{40,56} Several of these residues also undergo the largest changes in dynamics, suggesting a possible mechanism of propagation of dynamic changes along secondary structural units (especially helices), while alteration of β -strand dynamics is likely to be accompanied by changes in neighboring strands.⁵⁷

Possible implications of dynamics changes for the biological function of plexin-B1 RBD/Rac1 complex

The altered dynamics of both proteins in the bound state may be biologically relevant in that it points to a mechanism for signal transduction in the plexin–GTPase system. Alterations in the structure and/or dynamics occur in several regions of both proteins. A long loop (L3) is freed from its involvement in dimerization of the unbound plexin RBD.³³ Several GTPases, including active Rac1, RhoD, and Rnd1, bind to a common region adjacent to this loop and destabilize the RBD dimer.²⁵ GTPase binding to a region adjacent to the loop causes a conformational change and increased dynamics in at least part of it, suggesting a role for this loop that extends beyond dimerization. The reason for the increased dynamics may be a thermodynamic one (a compensatory entropy change, especially from the dimeric state that helps binding). The altered loop may also form a new binding site for another, as yet unknown, intra- or intermolecular interaction with the plexin RBD–GTPase complex. The dynamics study here suggests an allosteric change in the plexin domain through longer range conformational perturbations, if not changes in protein dynamics. Similarly to other systems, we have shown in another report that oncogenic point mutations, such as Leu75, influence the structure of the domain well beyond the immediate region of an altered residue.²⁶

New binding interfaces could also be created by altered dynamics on the side of Rac1, and most likely in other small GTPases. Our data suggest diminished dynamics for regions $\beta 5$ – $\beta 6$ (especially in $\beta 5$ and $\alpha 6$) in the C-terminal half of the protein. An analysis of relaxation data from different states of Cdc42 showed that nucleotide-dependent changes in the dynamics of the switch I and II regions have significant effects on the binding of effector proteins.¹⁷ This study also inferred that such changes are propagated to regions quite distant from the nucleotide binding and effector binding sites, specifically to the insert helix. In the present study, our relaxation data show a dynamical tightening of several “hinge” residues in Rac1 upon plexin RBD binding. This is accompanied by diminished dynamics particularly in the insert helix $\alpha 6$ in Rac1. These findings suggest the possibility of an allosteric mechanism by which binding on one side of the GTPase is propagated to the other side of the protein and that can be used in the recognition of another protein. Thus, the dynamic signature of the insert helix region, for example, could be determined by a synergy between the type of nucleotide bound and by effector binding. This combination would provide additional specificity for the association with other proteins.

Further experimental work is needed in order to uncover the allosteric role of some of the plexin loops and GTPase segments suggested by the present study of picosecond–nanosecond dynamics. Further biophysical and computational studies will also establish the molecular basis of the coupling mechanism that is employed to propagate dynamical information across the proteins. The plexin–small GTPase system offers the opportunity for these studies, promising to provide a detailed description of a signal transduction mechanism.

Materials and Methods

Sample preparation

Escherichia coli BL21(DE3) was used for production of the C178S K184Stop Rac1 mutant and the W1830F plexinB1-RBD mutant (RBD, human protein residues 1742–1862 plus two N-terminal lysines)³³ both expressed from pet11a plasmids. Several residues beyond the

structured region were included at the RBD C-terminus due to the possibility of their involvement in Rac1 binding as a putative CRIB motif. We later found that this C-terminal region is not involved in binding.²⁷ (For convenience, residues are numbered from the start of the RBD construct at residue 1743 throughout this report.) For both proteins, ¹⁵N samples were prepared by growing cells at 32 °C in minimal media M9 supplemented with ¹⁵NH₄Cl as the sole nitrogen source. Protein expression was induced at an OD₆₀₀ of 0.6 AU by addition of 1 mL of 1 mM IPTG/L and the cells were harvested after 6 h. Weak ion exchange, hydrophobic interaction, and gel filtration chromatographies were used for purification. The Rac1 samples were then loaded with a GTP analogue by nucleotide exchange in the presence of excess GMPPNP and alkaline phosphatase, followed by gel filtration. One common set of solution conditions was used for NMR studies at 25 °C. We did not observe a concentration dependence of either chemical shift (>0.02 ppm in ¹H or >0.1 ppm in ¹⁵N) or resonance intensity (less than ±5% peak height relative to the amide resonance of residue 122 in plexin-B1 RBD or residue 183 in Rac1, loaded with non-hydrolyzable GTP analogue GMPPNP) in ¹⁵N–¹H heteronuclear single quantum coherence spectra of any of the four samples comparing the proteins at concentrations of 100 μM and 1.0 mM. This suggests that the possibility of non-specific associations affecting the relaxation data is not a factor in the present study. This finding is significant because wild-type plexinB1-RBD and Rac1.GMPPNP were observed to be prone to weak dimerization and to slight aggregation, respectively, but mutation Trp90 to Phe in the plexinB1-RBD and Cys177 to Ser, and truncation at Lys184 in Rac1.GMPPNP as well as a careful choice of buffer conditions eliminated this concern.^{21, 58} At the same time, it can be shown by NMR and by isothermal titration calorimetry that the binding affinities of plexinB1-RBD and Rac1.GMPPNP for one another are not significantly affected by these amino acid changes (P. Hota and M. Buck, unpublished data).

Four NMR samples were used to measure ¹⁵N relaxation: two corresponding to proteins in their free states (¹⁵N uniformly labeled Rac1.GMPPNP and ¹⁵N uniformly labeled plexinB1-RBD) and two corresponding to the complex form (¹⁵N uniformly labeled Rac1.GMPPNP in complex with unlabeled plexinB1-RBD and ¹⁵N uniformly labeled plexinB1-RBD in complex with unlabeled Rac1.GMPPNP). As described before, we used buffer components that mimic physiological conditions,⁵⁸ including 4 mM DTT and 4 mM MgCl₂, at pH 6.8 in 10% D₂O/90% H₂O. The final protein concentrations were 1.0 mM for the ¹⁵N-labeled proteins (ratios 1:1.2 of labeled to unlabeled proteins were used for measurements of the proteins in the complex).

NMR spectroscopy

All spectra were acquired at 298 K on Bruker DRX spectrometers (14.1 and 18.1 T) equipped with cryoprobes and running XWINNMR software. In all experiments, the ¹H carrier frequency was centered on the water resonance; spectral widths of 12 ppm were used for ¹H and 40 ppm for ¹⁵N. For the indirect dimension, the carrier frequency was set to the middle of the amide region at 117 ppm.

Spin relaxation measurements and dynamics analysis

¹⁵N longitudinal relaxation rates (R_1), ¹⁵N transverse relaxation rates (R_2), and steady-state heteronuclear {¹H}–¹⁵N NOE values were determined, for each protein in the free and the bound state, at 14.1 and 18.8 T (600 and 800 MHz ¹H resonance frequency, respectively). Experiments were performed as described by Farrow *et al.*⁵⁹ For the R_1 and R_2 measurements, 2048 (¹H) and 256 (¹⁵N) complex data points were collected with 12 transients per increment and a recycle delay of 5 s. For proteins in their free states, 10 delays (between 20 ms and 1.8 s) were used during the inversion–recovery period of the R_1 experiments, whereas 8 delays (between 16 and 144 ms) were used during the Carr–Purcell–Meiboom–Gill (CPMG) period of the R_2 experiments. The ¹⁵N pulses used in the CPMG train were adjusted so that the Radio

Frequency field strength was tolerated by the cryoprobe.⁶⁰ For relaxation measurements of proteins in their bound states, 12 delays between 20 ms and 2.9 s were used for the R_1 experiments, and 8 delays between 8 and 88 ms were used for the R_2 experiments. Raw data were processed and analysed with the GIFA software package.⁶¹ Experimental uncertainties were initially determined from baseline noise by integrating regions of spectra containing no cross-peaks. The fitting and error analysis was carried out in GIFA⁶¹, which allows the user to adjust the uncertainty on the relaxation data so that good χ^2 (95% confidence) values are obtained using the nonlinear least-squares method (the baseline was multiplied for several residues by up to a factor of 2.5 in order to obtain such fits). In addition, all the fits were inspected graphically. The uncertainties were propagated to R_1 and R_2 via Monte Carlo sampling.⁶¹ Heteronuclear ^1H – ^{15}N NOE experiments were carried out in an interleaved manner, with and without a proton saturation period of 4.5 s applied before the start of the ^1H – ^{15}N correlation experiment and repeated twice under identical conditions. Forty-eight transients were collected for each experiment. $\{^1\text{H}\}$ – ^{15}N NOE are deduced from the ratios of peak intensities obtained with and without proton saturation. The ^{15}N experimental relaxation data were first used as input, together with the unbound protein structures (PDB files), for the *RotDif* program³⁵ to obtain the global motion. This way, after residues that exhibit either significant conformational exchange (high R_2) or rapid motion on the fast timescale (low NOE factor) are excluded, the remaining R_2/R_1 ratios were used to determine the rotational diffusion tensors that describe the overall tumbling.³⁵ It should be noted that the relative orientation of proteins in the complex is not required for the tensor analysis. The structures of the proteins in their free states were used in order to assess the overall motion of each protein. Results show that when they are part of the complex, both proteins experience a similar axially symmetric global motion (see Table 1), the rotational diffusion tensors are nearly superimposable, indicating the relative position of protein in the complex. The average values for the global correlation time and of the anisotropy were then used to perform the LS analysis. Using the *Dynamics* program,⁶² we derived values for the generalized order parameter (S^2) and the effective correlation time for internal motions (τ_{loc}) for each residue. An extended motion model,³⁶ needed for several residues, introduces an additional order parameter, namely S^2_{s} , which reflects the contribution of a slower (nanosecond timescale) motion to the spectral density. The procedure also takes into account possible exchange contributions (R_{ex}) that are associated with movements in the microsecond to millisecond timescale domain. The amide bond length was fixed to 1.02 Å and a mean ^{15}N chemical shift anisotropy value of –170 ppm was used during the fitting procedure. Comparison of dynamics parameters between the free and complexed proteins was done taking into account the estimated uncertainties. The significance is indicated by the Z-score. An additional calculation of the order parameters was carried out with uncertainties in the relaxation rates that are increased by a factor of 2.5. This confirmed that an increase in the uncertainty does not affect the main differences in S^2 for the residues that are discussed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

GAP, GTPase-activating protein; GEF, guanine exchange factor; GDI, GDP dissociation inhibitor; GDP, guanosine diphosphate; GMPPNP, guanosine-5'-[(β,γ)-imido]triphosphate triethylammonium salt; GTP, guanosine triphosphate; GTPase, guanosine 5'-triphosphatase; LS, Lipari-Szabo; PAK, p21-activated kinase; RBD, Rho GTPase binding domain.

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Fig. 1.

(a) Order parameters S^2 obtained for free Rac1.GMPPNP as a function of the residue number. Switch I and II regions (residues 27–43 and 60–75, respectively) are shaded grey and the location of secondary structure is indicated. (b) Ribbon structure of Rac1 (PDB ID 1MH1) showing secondary structure superimposed on the extent of dynamics represented by the diameter and color of the ribbon with radius proportional to $0.25/(S^2)^3$. The structure is rotated anti-clockwise by 90° around the y-axis shown. The value of S^2 for residue 146 was cut at 0.5.

Fig. 2.

(a) Order parameters S^2 obtained for the free plexin-B1 RBD as a function of the residue number, the location of secondary structure is indicated. (b) Ribbon structure of plexin-B1 RBD (2JPH) showing secondary structure superimposed on the extent of dynamics represented by the diameter and color of the ribbon with radius proportional to $0.25/(S^2)^{1.5}$. The N-terminal three and C-terminal six residues are removed for clarity.

Fig. 3.

(a) Order parameters S^2 obtained for Rac1.GMPPNP in complex with plexin-B1 RBD as a function of the residue number. Secondary structure and residues of interest are indicated as above; the data for the free protein are shown as a reference. (b) Change in S^2 upon binding mapped onto the X-ray structure of Rac1.GMPPNP: decrease in dynamics upon binding shaded in blue and increases in red. In order to increase the number of residues that can be compared, small gaps in the data (one neighboring data point) were estimated by extrapolation for illustrative purposes (not included in Table 2). The structure is rotated anti-clockwise by 90° around the y-axis shown. The interaction region of Rac1 with the plexin RBD (indicated by surface mapping shown in Supplementary Figs. S2 and S3) is shaded in light green.

Fig. 4.

Order parameters S^2 obtained for plexin-B1 RBD in complex with Rac1.GMPPNP as a function of the residue number. Secondary structure and residues of interest are indicated as above, the data for the free protein are shown as a reference. (b) Change in S^2 upon binding mapped onto the NMR average structure of the plexin-B1 RBD: decrease in dynamics upon binding shaded in blue and increases in red. In order to increase the number of residues that can be compared, small gaps in the data (one neighboring data point) were estimated by extrapolation for illustrative purposes (not included in Table 3). The interaction region of the plexin RBD with Rac1 is shaded in light green.

Table 1

Global motion parameters obtained from the relaxation measurements

	Free Rac1.GMPPNP	Rac1.GMPPNP bound to plexin-B1 RBD	Plexin-B1 RBD bound to Rac1.GMPPNP	Free plexin-B1 RBD
τ_R (ns)	12.52	25.05	24.05	8.6
($\pm$)	(0.51)	(1.01)	(0.99)	(0.43)
$D_{\parallel}/D_{\perp}$	1.14	1.30	1.32	1.26
($\pm$)	(0.09)	(0.09)	(0.08)	(0.12)
α ($^{\circ}$)	133	155	110	113
($\pm$)	(20)	(20)	(17)	(15)
β ($^{\circ}$)	62	73	80	112
($\pm$)	(25)	(16)	(18)	(19)

For all proteins, motion is shown to be axially symmetric. The correlation time (τ_R in nanoseconds), the anisotropy ($D_{\parallel}/D_{\perp}$) and the orientation (α , β) of the rotation–diffusion tensor in the laboratory frame (PDB coordinates) are represented.

Table 2Averaged S^2 , τ_{loc} , R_{ex} (at 14.1 T) and S^2_f over regions of interest for Rac1.GMPPNP

Residue range	Free Rac1	Bound Rac1	Difference
S^2			
6–16 (6/6)	0.75	0.92	0.17
43–55 (11/9)	0.84	0.94	0.10
78–95 (12/11)	0.87	0.91	0.05
100–110 (8/9)	0.95	0.93	−0.02
114–132 (13/14)	0.81	0.93	0.12
142–155 (13/13)	0.85	0.92	0.07
165–176 (8/8)	0.87	0.94	0.07
τ_{loc} (ns)			
6–16 (6/6)	3.03	0.76	−2.27
43–55 (11/9)	1.29	1.04	−0.25
78–95 (12/11)	2.00	0.94	−1.06
100–110 (8/9)	0.96	1.41	0.44
114–132 (13/14)	2.50	0.88	−1.62
142–155 (13/13)	1.32	0.97	−0.35
165–176 (8/8)	1.61	1.41	−0.20
R_{ex} (s ^{−1})			
6–16 (6/6)	4.42	0.00	−4.42
43–55 (11/9)	0.90	0.00	−0.90
78–95 (12/11)	1.14	3.61	2.48
100–110 (8/9)	1.25	4.80	3.55
114–132 (13/14)	1.65	1.41	−0.23
142–155 (13/13)	1.26	1.75	0.48
165–176 (8/8)	1.13	1.92	0.79
S^2_f			
6–16 (6/6)	0.87	1.00	0.13
43–55 (11/9)	0.94	1.00	0.06
78–95 (12/11)	0.95	0.97	0.02
100–110 (8/9)	0.99	1.00	0.01
114–132 (13/14)	0.91	0.99	0.08
142–155 (13/13)	0.93	1.00	0.07
165–176 (8/8)	0.94	1.00	0.06

Table 3Averaged S^2 , τ_{loc} , R_{ex} (at 14.1 T) and S_f^2 over regions of interest for plexin-B1 RBD

Residue range	Free plexin RBD	Bound RBD	Difference
S^2			
9–14 (6/6)	0.92	0.95	0.03
16–26 (10/10)	0.41	0.37	−0.04
32–48 (12/14)	0.96	0.97	0.01
50–57 (7/7)	0.83	0.90	0.07
61–68 (6/6)	0.88	0.96	0.08
78–84 (6/6)	0.75	0.91	0.16
85–92 (8/7)	0.58	0.50	−0.08
94–103 (7/8)	0.96	0.91	−0.06
105–112 (5/5)	0.94	0.94	0.00
τ_{loc} (ns)			
9–14 (6/6)	0.00	0.97	0.97
16–26 (10/10)	1.27	3.86	2.59
32–48 (12/14)	0.30	1.02	0.72
50–57 (7/7)	1.40	0.34	−1.06
61–68 (6/6)	0.50	1.05	0.55
78–84 (6/6)	2.55	0.35	−2.20
85–92 (8/7)	0.61	1.88	1.27
94–103 (7/8)	0.00	2.25	2.25
105–112 (5/5)	0.00	3.37	3.37
R_{ex} (s^{-1})			
9–14 (6/6)	0.20	3.73	3.23
16–26 (10/10)	0.00	0.18	0.18
32–48 (12/14)	0.08	2.79	2.71
50–57 (7/7)	0.00	1.79	1.79
61–68 (6/6)	0.08	2.54	2.46
78–84 (6/6)	1.68	2.13	0.44
85–92 (8/7)	0.35	0.88	0.52
94–103 (7/8)	0.20	4.43	4.23
105–112 (5/5)	0.32	3.05	2.73
S_f^2			
9–14 (6/6)	1.00	1.00	0.00
16–26 (10/10)	0.91	0.83	−0.08
32–48 (12/14)	1.00	1.00	−0.01
50–57 (7/7)	0.96	1.00	0.04
61–68 (6/6)	1.00	1.00	0.00
78–84 (6/6)	0.98	0.95	−0.03
85–92 (8/7)	0.98	0.91	−0.08
94–103 (7/8)	1.00	0.97	−0.03
105–112 (5/5)	1.00	0.99	−0.01

Residue range	Free plexin RBD	Bound RBD	Difference