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Interaction characteristics of Plexin-B1 with Rho family proteins

Eyad Kalawy Fansa, Radovan Dvorsky, Si-Cai Zhang, Dennis Fiegen [1], Mohammad Reza Ahmadian *

Institut für Biochemie und Molekularbiologie II, Medizinische Fakultät der Heinrich-Heine-Universität, 40225 Düsseldorf, Germany

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ABSTRACT

Plexin-B1 regulates various cellular processes interacting directly with several Rho proteins. Molecular details of these interactions are, however, not well understood. In this study, we examined *in vitro* and *in silico* the interaction of the Rho binding domain (B1RBD) of human Plexin-B1 with 11 different Rho proteins. We show that B1RBD binds in a GTP-dependent manner to Rac1, Rac2, Rac3, Rnd1, Rnd2, Rnd3, and RhoD, but not to RhoA, Cdc42, RhoG, or Rif. Interestingly, Rnd1 competitively displaces the Rac1 from B1RBD but not *vice versa*. Structure–function analysis revealed a negatively charged loop region, called $B1L^{31}$, which may facilitate a selective B1RBD interaction with Rho proteins.

1. Introduction

The semaphorin transmembrane receptor Plexin-B1 is involved in the regulation of several cellular processes including axonal guidance in the developing nervous system [1], cellular migration, immune response [2,3], and angiogenesis [4]. Binding of the specific ligand semaphorin-4D to the extracellular domain of Plexin-B1 induces a variety of signaling processes on the intracellular side, for which proteins of Rho family are known to be important components [1,5]. Members of the Rho family have been shown to directly associate with the Rho binding domain of Plexin-B1 (B1RBD), such as Rnd1, Rac1 and RhoD [6–10]. Structural analysis of B1RBD in complex with Rho proteins has suggested that B1RBD binds to similar regions of Rnd1 and Rac1 [9,11,12]. However, how the selectivity of the Rho protein interactions with B1RBD is determined and how Plexin-B1 discriminates between Rac1 and Rnd1, are still important open questions.

In this study, we have investigated the structure–function relationships of the interaction between B1RBD and 11 different members of the Rho family. B1RBD revealed interaction with the active forms of Rnd and Rac isoforms as well as with RhoD but not with other members of the Rho family, including RhoA, Cdc42, RhoG and Rif. We further demonstrate that Rnd1 displaces Rac1 from B1RBD. Our efforts to elucidate the selectivity-determining residues in B1RBD-Rho proteins interactions led us to focus on a loop region consisting of 31 residues (amino acids 1854–1885) in B1RBD (hereafter referred to as $B1L^{31}$), which may facilitate the B1RBD interactions with certain Rho proteins at the plasma membrane.

2. Materials and methods

2.1. Constructs

Human Plexin-B1RBD (amino acids or aa 1724–1903), B1RBD variant L1849G/V1850G/P1851A ($B1RBD^{GGA}$), B1RBD variant E1868A/E1874A/D1875A/D1877A ($B1RBD^{AAAA}$), as well as human Rho-related genes, i.e. full-length $Rac1^{wt}$, C-terminal truncated Rac1ΔC (aa 1-179), $Rac1^{A95E}$, $RhoA^{E97A}$, Rac2, Rac3, Rnd1, Rnd2ΔNΔC (aa 26-184), Rnd3, $RhoA^{wt}$, Cdc42ΔC (aa 1-178), RifΔC (aa 1-195) and RhoGΔC (aa 1-184), and mouse RhoDΔC (aa 2-193) were cloned in pGEX vectors.

2.2. Proteins

All proteins were isolated and prepared as previously described [13].

2.3. Isothermal titration calorimetry (ITC)

All measurements were performed using an isothermal titration calorimeter (VP-ITC) at 20 °C as described [14].

2.4. Analytical size exclusion chromatography (aSEC)

Experiments were carried out at 4 °C on a Superdex75 10/300 GL column (GE Healthcare).

* Corresponding author. Address: Institut für Biochemie und Molekularbiologie II, Medizinische Fakultät der Heinrich-Heine-Universität, Universitätsstr. 1, Gebäude 22.03, 40255 Düsseldorf, Germany. Fax: +49 211 811 2726.
E-mail address: reza.ahmadian@uni-duesseldorf.de (M.R. Ahmadian).
[1] Birkendorfer Straße 65, 88397 Biberach an der Riss, Germany.

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2.5. Nucleotide quantification by high pressure liquid chromatography (HPLC)

The type and concentration of guanine nucleotides were analyzed on a reversed-phase (RP-) HPLC as described [13].

2.6. Structural analysis and molecular modeling

Modeling of the human Plexin-B1 region (aa 1854–1885) was performed, based on the crystal structure of cytoplasmic domain of Plexin-B1 in complex with Rac1 (Protein Data Bank code 3SUA), using MODELLER [15]. Structural analysis was done and electrostatic potential maps were obtained using PyMOL (Molecular Graphics System, version 1.5.0.4 Schrödinger, LLC) and APBS [16], respectively.

3. Results and discussion

3.1. B1RBD binds among the Rho family proteins selectively to Rnd and Rac isoforms as well as to RhoD in vitro

Previous reports have shown that B1RBD interacts with Rnd1, Rac1 and RhoD [6,7,10–12]. Here, we set out to investigate the interaction of B1RBD with 11 representative members of the Rho family proteins (i.e. Rac1, Rac2, Rac3, RhoG, Rnd1, Rnd2, Rnd3, RhoA, Cdc42, RhoD and Rif), using ITC. Fig. S1A shows that B1RBD binds selectively to the active (GppNHp-bound) Rac1 with an affinity in the low micromolar range (K_d = 6.6 μM) but not to the inactive (GDP-bound) Rac1. To confirm this interaction, analytical size exclusion chromatography (aSEC) was carried out. It is important to note that B1RBD eluted as a dimer in the absence and in the presence of interacting Rho protein. As shown in Fig. S1B, B1RBD co-eluted with Rac1 GppNHp, but not with Rac1 GDP.

Notably, among the Rac isoforms, Rac1 displayed the highest affinity of B1RBD considering the unprecedented K_d values of 11.0 and 30.3 μM for GppNHp-bound Rac2 and Rac3, respectively (Figs. S2 and S3; Table S1). Like Rac1 GDP, GDP-bound forms of Rac2 and Rac3 showed no interaction with B1RBD (data not shown). The reason for the lower affinity of Rac2 and particularly Rac3 as compared to Rac1 could not be linked to the B1RBD binding amino acids as analyzed below. It is rather likely that the overall dynamics of these isoforms is responsible for their functional specificities as discussed previously [17]. Several studies have shown that Rac1 binding to Plexin-B1 sequesters it from its downstream signaling [18], which in turn results in Rho activation and in the assembly of contractile stress fibers [7].

The interaction of Rnd isoforms with B1RBD was measured in the GTP-bound state, because they do not hydrolyze GTP [13]. As shown in Fig. S2 and summarized in Table S1, Rnd1 and Rnd2 bind to B1RBD with K_d values of 3.2 μM and 4.6 μM, respectively. In this study we further measured the interaction between Rnd3 and B1RBD and showed that also Rnd3, similarly to Rnd1 and Rnd2, is able to bind B1RBD with an affinity of 8.5 μM (Figs. S2 and S3; Table S1). A detailed sequence and structural analysis, using the B1RBD Rnd1 complex (PDB code 2REX) [19], showed that all three Rnd isoforms are likely to interact in a comparable manner with B1RBD. As shown in Fig. 1A, almost all B1RBD contacting residues are identical among the Rnd proteins. Deviations at two different positions, C81 in Rnd1 (A75 in Rnd2, S91 in Rnd3) and Y114 in Rnd1 (F108 in Rnd2 and F124 in Rnd3) does not seem to play a critical role in B1RBD binding as their binding affinities are in a similar range. This suggests that the association of Rnd proteins with Plexin-B1 is most likely mediated via the same mechanism that may

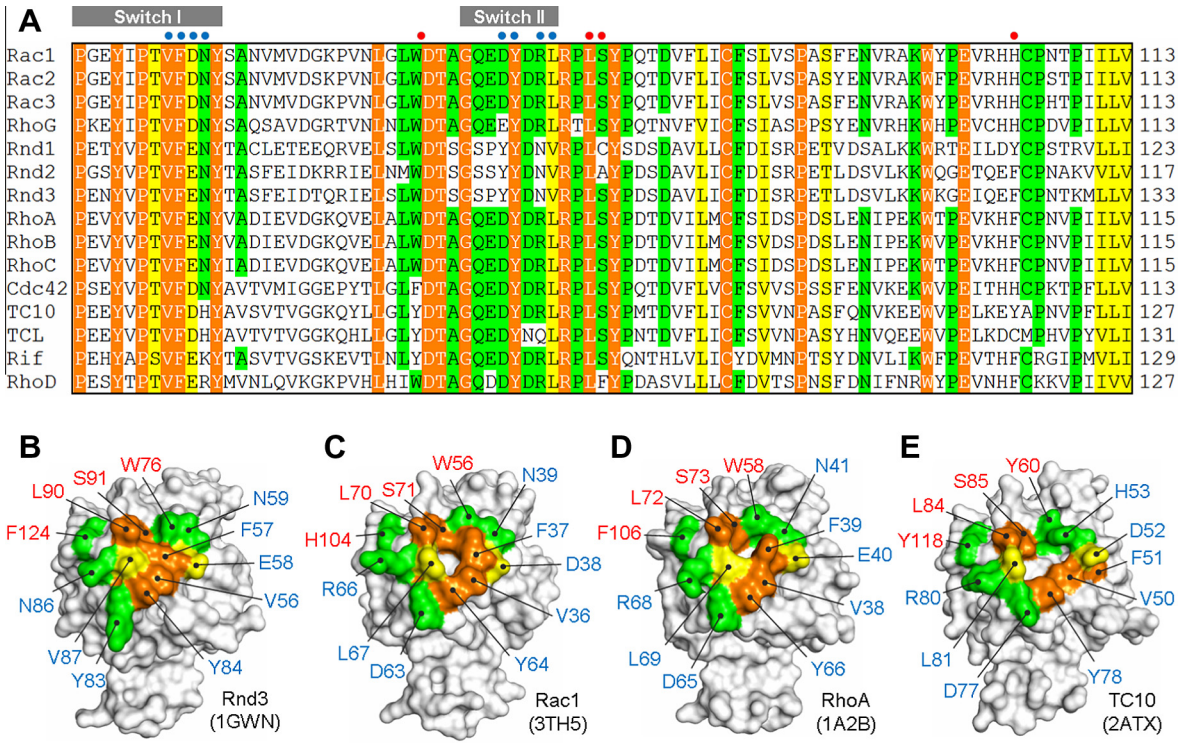

Fig. 1. Conserved B1RBD contact regions on Rnd1 and Rac1. (A) Rho protein sequences were aligned using the ClustalW program and the alignment was edited by GeneDoc. Highlighted are conserved residues in yellow, identical residues in white and orange background, and identical residues in at least 11 proteins in green. Dots indicate B1RBD binding residues of Rnd1 and Rac1, inside the switch regions in blue and outside switch regions in red. (B–E) Surface representation of 3D structures of major representatives of Rho proteins subgroups in their active forms, including Rnd3 [28], Rac1 [27], RhoA [29] and TC10 [30], highlighted are the conserved B1RBD binding residues of Rnd1 and Rac1. The color code of surface patches and amino acid labels correspond to A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

have different cellular effects, including inactivation of R-Ras [20], and sequestration of Rnd proteins as a modulators of actin Dynamics [21,22]. Rnd2 binding to Plexin-D1 has been shown to inhibit axon outgrowth of cortical neurons and down-regulation of R-Ras activity [23].

Our interaction analysis revealed that yet RhoD was able to bind B1RBD in its active form (K_d = 14.4 μM), but not other members of the Rho family, i.e. RhoA, Cdc42, RhoG, and Rif (Fig. S2; Table S1). RhoD has been shown to exhibit repulsive effects on axons by interfering with the Rnd1 binding to Plexin-A1 [24]. Nevertheless, it is interesting and surprising that B1RBD binds RhoD but not, for example, RhoG which shares the highest sequence homology with Rac isoforms [13].

3.2. Rnd1 displaces Rac1 from its complex with B1RBD

The fact that B1RBD binds to functionally distinct groups of Rho proteins raises the question, whether these interactions control different processes or are rather reciprocal. Data from crystal structures of Plexin-B1 complexes with Rac1 and Rnd1 (PDB codes 3SU8 and 2REX) [9,19] have suggested that Rac1 and Rnd1 share the same binding site on B1RBD. To examine whether and how Rac1 and Rnd1 compete for the same interacting surface on B1RBD, we performed a combination of ITC measurements along with aSEC analysis. First, we successively combined two ITC experiments in row. In the first step, we titrated in two separate experiments Rac1 GppNHp and Rnd1 GTP on B1RBD, which resulted in the respective formation of the expected complexes (Fig. 2A and C, left panels). In the second step, we subsequently titrated to the formed complexes Rnd1 GTP and Rac1 GppNHp, respectively. As shown in the right panels of Fig. 2A and C, addition of Rnd1 to Rac1 GppNHp B1RBD resulted in an additional complex formation that was not observed when Rac1 GppNHp was added to Rnd1 GTP B1RBD. The differences in heat capacity confirm that Rnd1 and Rac1 in fact compete for an overlapping binding site on B1RBD and that Rnd1 displaces Rac1 from its complex with B1RBD but not *vice versa*.

To further confirm this result we monitored the complex formation of B1RBD with Rac1 or/and Rnd1 by aSEC followed by RP-HPLC. Fig. 2B clearly shows that loading a mixture of Rnd1 GTP and Rac1 GppNHp B1RBD complex on Superdex 75 (10/300)

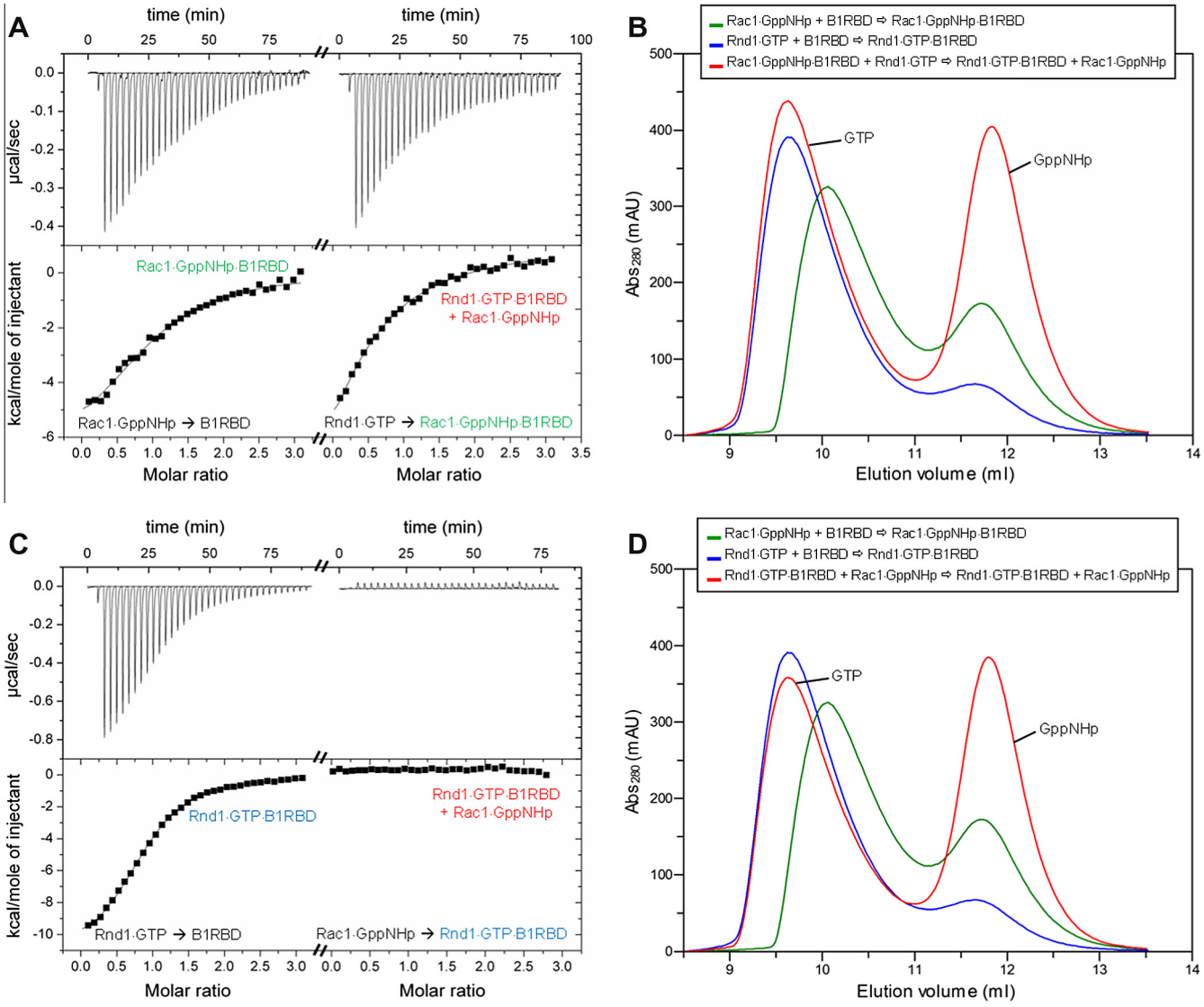

Fig. 2. Displacement of Rac1 from its complex with B1RBD by Rnd1. (A and B) Successive ITC measurements were performed by first titrating Rac1 (300 μM) to B1RBD (20 μM) and second Rnd1 (300 μM) to the Rac1-B1RBD complex. aSEC profiles of various protein complexes are shown in B, in which equivalent amount of Rnd1 GTP displaced Rac1 GppNHp from its complex with B1RBD after incubation for 5 min on ice before loading on the aSEC column. (C and D) In contrast, Rac1 is not able to displace Rnd1 from its complex with B1RBD. Comparable conditions and protein concentrations were used as in A and B. For (B) and (D) peak fractions were analyzed by RP-HPLC for detecting the nucleotide-bound state.

column resulted in co-elution of the Rnd1 GTP B1RBD complex and the release of Rac1 GppNHp. To distinguish between Rnd1- and Rac1-containing fractions, we quantified the peak fractions on a RP-HPLC system regarding the bound nucleotide, e.g. GTP or GppNHp. In contrast, a gel filtration of Rac1 GppNHp mixed with the Rnd1 GTP B1RBD complex did not displace Rnd1 from the complex (Fig. 2D). These data strongly support the notion that Rnd1 facilitates Rac1 release from B1RBD and thus may antagonize its cellular activity.

An important question to be addressed is whether the displacement of Rac1 from B1RBD by Rnd1 releases Rac1 for signaling *via* other effectors or dislodges Rac1 from its signal transduction. A previous study by Negishi and coworkers demonstrated that Rac1 activity is required for the Rnd1-induced neurite formation and that Rac1 is an integral element of the signaling pathway downstream of Rnd1 [25].

3.3. Illuminating the B1RBD selectivity for Rho proteins

One major interest of this study was to explore the selectivity of the interaction between B1RBD and Rho proteins. Importantly, our data showed that B1RBD binds to the active GTP-bound forms of Rho proteins. This clearly demonstrates that B1RBD contacts the switch regions of Rho proteins, which are known to be involved in the recognition of and interaction with various binding partners [26]. In this regard, we investigated sequence–structure–function relationships between the Rho family proteins and B1RBD (Fig. 1). Multiple sequence alignment (Fig. 1A) did not reveal any significant differences in the B1RBD interacting amino acids within the switch regions of Rho proteins. Moreover, analysis of the key amino acids in the binding interface of B1ICDRac1 and B1RBDRnd1 complexes (PDB codes 3SU8 and 2REX, respectively) [9,19] showed that the amino acids responsible for the binding of Rac1 and Rnd1 to B1RBD are remarkably conserved in all Rho proteins (Fig. 1A, dots). To further analyze the overall location and orientation of the B1RBD interacting amino acids, we used crystal structures of Rnd3, Rac1, RhoA, and TC10 (PDB codes 1GWN, 3TH5, 1A2B and 2ATX, respectively) [27–30]. There is currently no structure of Cdc42 in its active form available (see Table S1 in [13]). Therefore, we used in this study the structure of Cdc42-like TC10 [30]. Fig. 1B–E illustrate that all B1RBD binding residues are accessible and arranged in similar positions on the surface of Rac1 and Rnd3 as well as RhoA and TC10, which do not bind B1RBD. A detailed comparison of Rac1 and Rnd1 (Fig. 1B and C) revealed obvious differences regarding the shape and relative orientation of some B1RBD interacting amino acids when compared to the equivalent residues in RhoA and TC10 (Fig. 1D and E). However, these differences in the switch regions cannot account for the disability of RhoA and Cdc42-like TC10 in binding B1RBD because these regions are highly flexible and can adopt specific ordered conformations upon interaction with downstream effectors [31]. This analysis strongly suggests that the differences in B1RBD interacting amino acids may not *per se* determine the selectivity of the B1RBD-Rho protein interactions.

The comparison of the structures available for Rho proteins in complex with various regulators and effectors has revealed common characteristics of the interactions between Rho proteins and their binding partners [26]. Accordingly, the switch regions are most likely the recognition sites and different binding partners contact other regions that are specific to their function [26]. In this context, B1RBD may utilize other contact sites on the guanine nucleotide binding (G) domain of Rho proteins and also in the C-terminal hypervariable region (HVR). Accordingly, a C-terminal truncated Rac1 exhibited a significant reduction in the binding affinity for B1RBD (Table S1, Figs. S2 and S3). This is particularly interesting because HVR is a juxtamembrane region of prenylated Rho proteins and may be a significant contact site for transmembrane proteins, including Plexins.

3.4. Structural and functional insights into $B1L^{31}$, a non-structured region in B1RBD

We next addressed the question which regions of B1RBD determine the selective binding to Rac, Rnd, and RhoD proteins, but not to RhoA, Cdc42, Rif, or RhoG. An interesting B1RBD variant is the $B1RBD^{GGA}$ (L1849G, V1850G, and P1851A), which was shown to be defective in Rac1 and Rnd1 binding in cell-based experiments [6,32]. Here, we investigated the $B1RBD^{GGA}$ variant in detail *in vitro*. Interestingly, experiments under the same conditions as in Fig. S1 showed that $B1RBD^{GGA}$ is disabled in associating with Rac1 GppNHp and Rnd1 GTP using ITC and aSEC/RP-HPLC (data not shown). Additionally, CD measurements of $B1RBD^{wt}$ and $B1RBD^{GGA}$ showed that both proteins exhibit almost identical secondary structure elements (data not shown). Structural analysis of B1RBD complexes with Rnd1 and Rac1 (PDB codes 2REX and 3SU8) [9,19] showed that the region containing the ^{1849}LVP^{1851} sequence is not in direct contact with Rho proteins. Notably, these residues are directly followed by a disordered region (amino acids 1854–1885), which is not visible in any B1RBD-related crystal structures. This raises an important question to what extent this region contributes to a selective interaction of B1RBD with the Rho proteins.

On the basis of the B1ICD-Rac1 complex structure (PDB code 3SU8) [9], we modeled different conformations of this region consisting of 31 residues (referred to as $B1L^{31}$). We visually analyzed all obtained models using the PyMOL program and selected those conformations that could potentially interact with Rac1 (Fig. 3A). In this way, we identified a patch of negatively charged amino acids (E1868, E1874, D1875 and D1877) in $B1L^{31}$ (Fig. 3A). Such prevalence of negative charges causes significant negative electrostatic potentials around the $B1RBD^{wt}$, as can be seen from electrostatics map calculated for one conformation of $B1L^{31}$ (Fig. 3B, left panel). For a comparison, a model of the $B1RBD^{AAAA}$ variant lacking these four negative residues, does not exhibit such a pronounced negative potential (Fig. 3B, right panel). Strikingly, A95 in Rac isoforms, located in a very close proximity of $B1L^{31}$, is variable among the members of the Rho family (Fig. 3A, magnified view and C). Interestingly, Rnd proteins contain a lysine and most of the other Rho proteins a glutamic acid at the corresponding position. We thus proposed that replacement of Ala95 in Rac1 with glutamic acid or the negatively charged residues of $B1L^{31}$ with alanine, may impair B1RBD-Rac1 interaction. To examine this idea, $B1RBD^{AAAA}$ lacking the four negative residues (E1868, E1874, D1875 and D1877; Fig. 3B, right panel) along with $Rac1^{A95E}$ and as control $RhoA^{E97A}$ were generated and tested using ITC. As shown in Fig. 3D, the association constant for the interaction of $Rac1^{wt}$ with B1RBD was slightly changed when the negative residues were substituted by alanine ($B1RBD^{AAAA}$). However, $Rac1^{A95E}$ binding to $B1RBD^{wt}$ was reduced by 2.5-fold as compared to that of $Rac1^{wt}$. On the other hand, no difference was observed for the binding of $Rnd1^{wt}$ and $RhoA^{E97A}$ to $B1RBD^{AAAA}$ compared to $B1RBD^{wt}$ (Table S2). Closer look at the B1ICD-Rac1 complex structure (PDB code 3SU8) [9] further revealed that the C-terminal HVR of the B1RBD binding Rho proteins contains a set of positively charged residues, which can in principle bind to $B1L^{31}$. To investigate this idea, we next measured the interaction between $B1RBD^{AAAA}$ and Rac1ΔC. Fig. 3D shows clearly that Rac1 HVR does not undergo any interaction with $B1L^{31}$ of B1RBD. All ITC data of the $B1RBD^{AAAA}$ variant are summarized in Table S2. Taken together, our data support the notion that $B1L^{31}$ facilitates to some extent the B1RBD-Rho proteins interaction and thereby might slightly influence the selectivity for distinct Rho protein. This loop region that very likely resides in a close proximity of the lipid membrane and Rac1

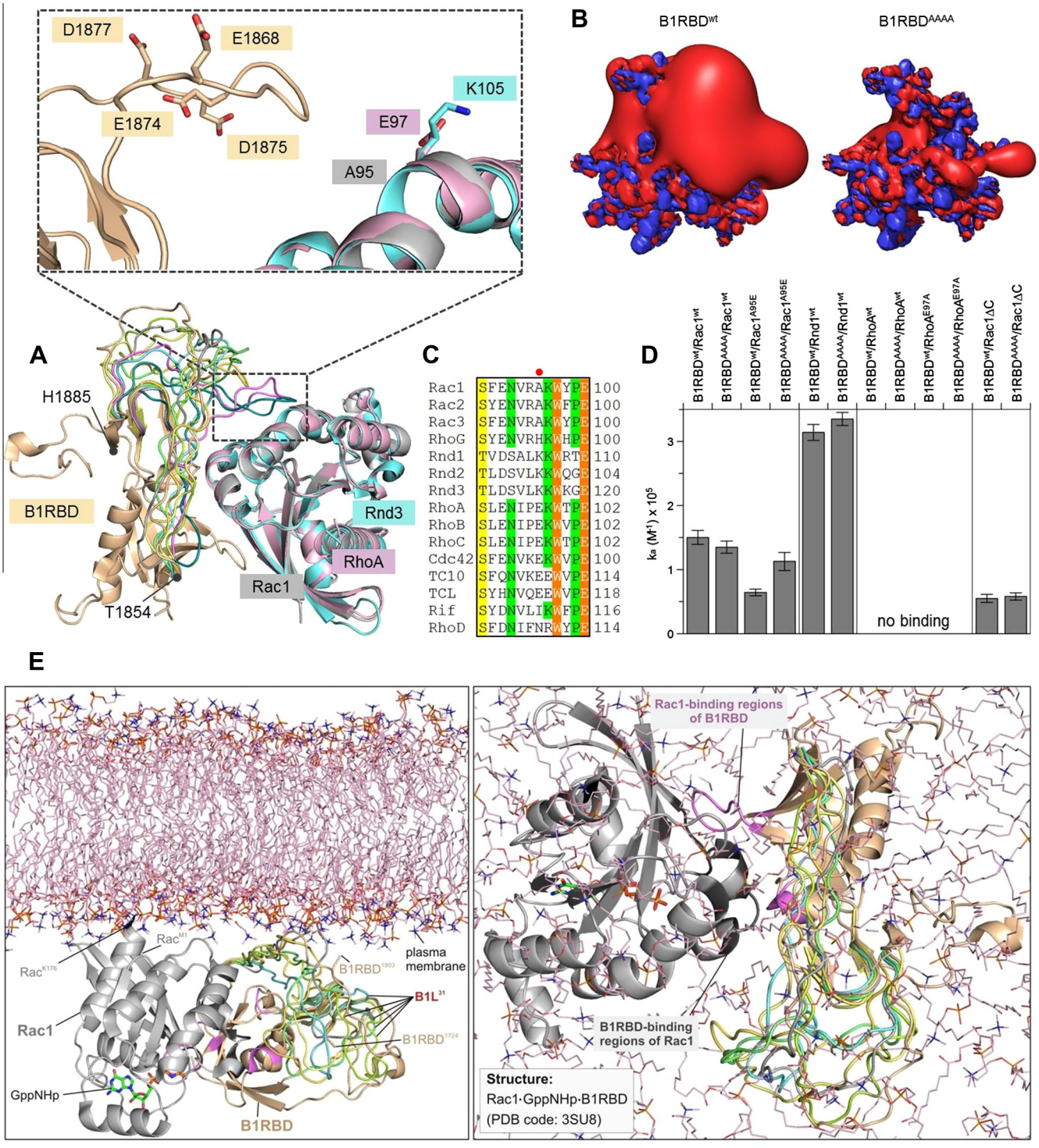

Fig. 3. Impact of modeled B1L^{31} on the B1RBD interaction with Rac1 and the plasma membrane. (A) Visualization of the B1L^{31} region (residues 1854–1885) of Plexin-B1 modeled on the basis of previous structure [9-11]. RhoA and Rnd3 crystal structures [29,30] were superimposed with Rac1 in the complex with B1ICD. Chosen conformation of modeled loop (magnified view) shows a detailed view of negatively charged B1L^{31} region that is in a close vicinity to potential interacting residues, such as Ala95 in Rac1 and Lys105 in Rnd1, which are located at the equivalent position to Glu97 in RhoA. (B) B1L^{31} with an isosurface of negative electrostatic potential. Electrostatic potential maps of B1RBD^{wt} and B1RBD^{AAAA} are represented by their isosurfaces at $-3k_bT/e_c$ (red) or $+3k_bT/e_c$ (blue) (C) Multiple sequence alignment of Rho proteins. Red dot represents the position of A95 of Rac1, Lys105 in Rnd1, and Glu97 in RhoA. (D) B1L^{31} as a potential Rho proteins contact region. ITC measurements of wild-type and variants of B1RBD, Rac1, Rnd1 and RhoA were performed to examine the impact of the B1L^{31} of B1RBD on a selective interaction with Rho proteins. For more clarity K_a values are shown (calculated K_d values are summarized in Table S2). (E) Illustrative positioning of B1RBD-Rac1 complex structure [9] at the plasma membrane with respect to Rac1 C-terminus, which associates with lipid bilayer via posttranslational modification (side view left and top view right). Six different conformations of B1L^{31} (aa 1854–1885) are shown that may approach the lipid membrane and are in the proximity of Rac1 are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3E), may provide a supportive mechanism to regulate the activity of Plexin-B1 in the cellular context.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.012.

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