The semaphorin receptor plexin-B1 specifically interacts with active Rac in a ligand-dependent manner

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Semaphorin molecules serve as axon guidance signals that regulate the navigation of neuronal growth cones. Semaphorins have also been implicated in other biological processes, including the immune response. Plexins, acting either alone or in complex with neuropilins, have recently been identified as functional semaphorin receptors. However, the mechanisms of signal transduction by plexins remain largely unknown. We have demonstrated a direct interaction between plexin-B1 and activated Rac. Rac specifically interacts with the cytosolic domain of plexin-B1, but not with that of plexin-A3 or -C1. Neither RhoA nor Cdc42 interacts with plexin-B1, indicating that the Rac/plexin-B1 interaction is highly specific. The binding of GTP and the integrity of the Rac effector domain are required for the interaction with plexin-B1. Furthermore, we have identified that a Cdc42/Rac interactive binding (CRIB) motif in the cytosolic domain of plexin-B1 is essential for its interaction with active Rac. We have also observed that the semaphorin CD100, a ligand for plexin-B1, stimulates the interaction between plexin-B1 and active Rac. Our results support a model by which activated Rac plays a role in mediating semaphorin signals, resulting in reorganization of actin cytoskeletal structure.

Semaphorins comprise a family of soluble and membrane-associated proteins that were originally characterized in the nervous system and play a critical role in axonal guidance (1, 2). In vertebrates, the first semaphorin, Sema3A/collapsin-1, was characterized as an activity that causes growth cone collapse (3). At the same time, genetic investigation in *Drosophila* indicated that semaphorins function as repulsive cues in axonal guidance (4). Further studies indicated that both secreted and transmembrane semaphorins can act as repellents in patterning the projection of a variety of classes of developing axons (5). Interestingly, it has recently been demonstrated that members of the semaphorin family can also function as attractive guidance cues for axons and apical dendrites of cortical neurons (6, 7). Here, cGMP levels control whether semaphorins act as repulsive or attractive cues, because the repulsive effect of Sema3A can be converted to an attraction by pharmacological activation of the cGMP pathway (8).

In addition to the nervous system, semaphorins have been found in a variety of other tissues and have been implicated in the immune response (9), cell migration (10), and tumor growth (11). CD100, also known as Sema4D, is a leukocyte transmembrane semaphorin that stimulates B cell aggregation and differentiation (9). Several viruses, including vaccinia virus, also contain semaphorin proteins that may act to regulate the host immune system (12, 13).

In attempts to understand the signaling mechanisms of semaphorins, neuropilins were identified as required receptors for the class 3 semaphorins (14–16). Initially, the role of neuropilins as a functional semaphorin receptor was perplexing, in that neuropilins have a very short intracellular domain and are unlikely to function alone as a signaling receptor (15, 16). It has only recently been shown that neuropilins act as coreceptors with the plexin family of proteins to transduce signals by semaphorins (17, 18).

Insights into this issue came initially with the finding that a viral semaphorin SemaVA (SemaA39R) binds to virus-encoded semaphorin protein receptor (VESPR) (12). Based on sequence homology, VESPR (or plexin-C1) belongs to the plexin family, which has at least nine identified members and can be grouped into four subfamilies (plexin-A, -B, -C, and -D) (19). This finding indicates that plexins are potential receptors for semaphorins and are likely targets for viral infection. Subsequent studies in *Drosophila* demonstrated that plexins are indeed functional receptors for transmembrane semaphorins (20). However, the *Drosophila* genome contains no neuropilin genes, which suggests that *Drosophila* semaphorin signals must be transduced without the involvement of neuropilins.

Recent reports have shown that, in vertebrates, plexin-B1 is a receptor for CD100 and that neuropilins are not required for CD100 binding to plexin-B1 (18). In addition to mediating the effects of transmembrane semaphorins, members of the plexin family are also able to form functional receptor complexes with neuropilins to mediate the effects of secreted Sema3A. Thus, a unified theme is emerging where plexins might function as signal transducers for both transmembrane (neuropilin-independent) and secreted (in the case of class III semaphorins, neuropilin-dependent) forms of semaphorins. Plexins have a large intracellular region composed of two highly conserved domains that are required for semaphorin-induced signaling (19). Unfortunately these conserved domains provide no direct clue about the signaling mechanism because of the lack of significant sequence homology to any known signaling molecules.

In response to attractive and repulsive signals, growth cone guidance is the result of continuous reorganization of actin filament structures within lamellipodia and filopodia (21–24). The Rho family of small GTPases, which includes Rho, Rac, and Cdc42, are critical for the regulation of actin structures (25). In Swiss3T3 cells, it has been well documented that activation of Rho results in stress fiber formation. Activation of Rac and Cdc42 result in formation of filopodia and lamellipodia, respectively. Previous experiments have implicated a role for Rac in growth cone guidance. Trituration of recombinant active Rac into embryonic chick dorsal root ganglion neurons increases growth cone collapse, whereas dominant negative Rac inhibits

Abbreviations: CRIB, Cdc42/Rac interactive binding; RBD, Rac-binding domain; MBP, maltose-binding protein; GST, glutathione *S*-transferase; HA, hemagglutinin; VSV, vesicular stomatitis virus; PAK, p21-activated kinase; WASP, Wiskott-Aldrich Syndrome protein; ACK, activated Cdc42-associated kinase.

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the response to semaphorin (22). In contrast, Cdc42 was found to have no effect. Similar experiments performed by Kuhn *et al.* by using adenoviral-mediated expression also demonstrated the involvement of the Rho family GTPases (23). The biochemical role of Rac in semaphorin signaling is currently unknown, although these small GTPases are clearly involved in the semaphorin response.

In this report, we demonstrate that Rac1 interacts directly and specifically with the intracellular domain of the CD100 receptor, plexin-B1. This study provides an original example of an activated small GTPase that directly binds to a transmembrane receptor. The interaction between Rac and plexin-B1 depends on the GTP binding of Rac and the integrity of the Rac effector domain. In addition, the association between active Rac and plexin-B1 is enhanced by the presence of ligand CD100. These observations propose a model for plexin signaling through direct interaction with Rac.

Materials and Methods

Plasmids and Protein Expression. Glutathione *S*-transferase (GST)-Rac/N17/L61 fusion proteins were expressed from the vector pGEX-KG in *Escherichia coli* DH5α and purified as described previously (26). Maltose-binding protein (MBP)–plexin fusion constructs were obtained by PCR amplification of the intracellular domain of plexin-A3 (amino acids 1243–1871, accession no. X87852), plexin-B1 (amino acids 1512–2135, accession no. X87904), and plexin-C1 (amino acids 967-1568, accession no. AF030339) from a human brain cDNA library (19). Plexin-A3, plexin-B1, and plexin-C1 PCR fragments were cloned into the *Eco*RI/*Xba*I, *Bam*HI/*Sal*I, and *Xmn*I/*Xba*I sites, respectively, of the pMALc2 vector (New England Biolabs). MBP-plexin fusion proteins were expressed in *E. coli* DH5α and purified in buffer A [50 mM Tris·Cl (pH 7.5)/150 mM NaCl/1 mM EDTA/1 mM DTT/protease inhibitors, of which EDTA was omitted in the final washes]. MBP-B1-ΔCRIB (CRIB = Cdc42/Rac interactive binding) corresponds to a deletion of residues 1843–1889 of plexin-B1. The Rac-binding domain (RBD, amino acids 72–152) of mouse PAK-A was expressed as a GST fusion protein from pGEX-KG and purified from *E. coli* DH5α. The mammalian expression constructs pRK5-myc-cdc42/N17/L61 and pRK5-myc-Rho/N19/L63 were generous gifts from A. Hall (University College London, London). pRK5-myc-Rac/N17/L61 was subcloned from pZIP-Rac/N17/L61 provided by C. Der (University of North Carolina, Chapel Hill, NC). The RacL61 effector domain mutations (Y32F, P34G, T35S, F37G, and Y40C) were constructed by PCR mutagenesis (Stratagene). Deletion constructs of MBP-plexin-B1 were created by PCR. Point mutations of MBP-plexin-B1 were made by PCR mutagenesis (Stratagene). pCDNA3-VSV-plexin-B1 and -plexin-A1 express full length N-terminal vesicular stomatitis virus (VSV)-tagged proteins and have been described previously (18). Hemagglutinin (HA)-tagged Rap1bV12 was expressed from pCDNA3HA-Rap1b of which the cDNA for bovine Rap1bV12 was provided by P. Stork (The Vollum Institute, Portland, OR). pCDNA3HA-HRasV12 has been described previously (27).

Cell Culture. *HEK293* cells were maintained in DMEM supplemented with 10% FBS. Transfections were performed by using Lipofectamine (Life Technologies) as recommended by the manufacturer.

Binding Experiments. *HEK293* cells were lysed in mild lysis buffer [10 mM Tris·Cl (pH 7.5)/100 mM NaCl/1% Nonidet P-40/50 mM NaF/2 mM EDTA/10 mM $MgCl_2$] containing 1 mM DTT, 0.2 mM Na_2VO_4, and a protease inhibitor mixture (Boehringer Mannheim). Precleared lysates were incubated for 1–2 h with 10–20 μg MBP-plexin fusion protein prebound to amylose resin (New England Biolabs). Beads were collected by centrifugation, washed several times, and eluted in 10 mM maltose or SDS sample buffer, followed by SDS/PAGE and Western blotting. *In vitro* mixing experiments were performed by mixing MBP-plexin prebound to amylose beads with 10 μg GST-Rac/N17/L61 in Buffer M [20 Tris·Cl (pH 7.5)/100 mM KCl/1 mM EDTA/10 mM $MgCl_2$/5% glycerol/1% Triton X-100/1 mM DTT] for 30 min. Beads were collected, washed, and eluted in SDS sample buffer, followed by SDS/PAGE and Western blotting. To demonstrate the nucleotide dependence of the Rac/plexin interaction, 10 μg of GST-Rac was incubated in loading buffer [25 mM Tris·Cl (pH 8.0)/1 mM DTT/40 μg/ml BSA/4.7 mM EDTA/0.16 mM $MgCl_2$/200 μM GTPγS or GDP] for 20 min at 30°C. The reaction was stopped by adding $MgCl_2$ to 10 mM and placed on ice. Loaded Rac was incubated with 20 μg MBP-plexin-B1 bound to amylose resin in Buffer M for 20 min. The resin was washed and the sample eluted with SDS sample buffer, followed by SDS/PAGE and Western blotting.

Immunoblots and Immunoprecipitations. HEK293 cells (in a 6-well plate) were transfected with 0.5 μg of VSV-plexin-B1, VSV-plexin-A1, neuropilin-1, Sema3A, AP-CD100 (alkaline phosphatase fusion), 0.2 μg of myc-Rac, myc-RacL61, and myc-RacN17. Two days posttransfection, cells were harvested in 200 μl immunoprecipitation buffer [10 mM Tris·Cl (pH 7.5)/100 mM NaCl/50 mM NaF/2 mM EDTA/0.5 mM Na_2VO_4/5 mM $MgCl_2$/1% Nonidet P-40/2 μg/ml leupeptin/5 μg/ml aprotinin/1 mM benzamidine/0.2 mM PMSF]. After 20 min lysis in immunoprecipitation buffer, samples were centrifuged at 13,000 rpm for 10 min. Cell lysates (150 μl supernatant) were incubated with 1 μg of anti-myc antibody for 2 h, and then 10 μl of protein G agarose was added and incubated for another hour. The immunoprecipitates were washed four times with immunoprecipitation buffer. One half of the immunoprecipitated samples was immunoblotted with anti-VSV antibody following manufacturer's recommendation. Other antibodies used in Western blotting include α-HA (Babco, Richmond, CA), α-myc9E10 (Covance, Princeton, NJ), α-GST (Zymed), α-Rac1 (Santa Cruz Biotechnology), and α-VSV (Boehringer Mannheim).

Results

Plexin-B1 Interacts Directly with Rac-GTP. Previous studies have implicated Rac in semaphorin signaling (22, 23). To investigate the underlying mechanisms of Rac in semaphorin signal transduction, we tested the interaction between the cytosolic domain of plexins and various small GTPases. The cytosolic domains of human plexin-A3, -B1, and -C1 were amplified by PCR from a human brain cDNA library and expressed as MBP fusion proteins. These proteins were expressed in *E. coli*, purified by amylose affinity chromatography and tested for interaction with the activated mutants of H-Ras, Rac1, and Rap1b, which were expressed in HEK293 cells. *In vitro* binding experiments showed that MBP-plexin-B1 specifically interacted with RacL61 (Fig. 1*A*, lane 7), but not with H-RasV12 or RapV12 (Fig. 1*A*, lanes 3 and 11). Neither MBP-plexin-A3 nor MBP-plexin-C1 interacted with RacL61 (Fig. 1*A*, lanes 6 and 8). Furthermore, we observed that the majority of RacL61 protein in HEK293 cell lysates was precipitated by MBP-plexin-B1. The interaction between plexin-B1 and RacL61 is much stronger than the interaction observed between Raf and RasV12 (data not shown). The above results demonstrate that plexin-B1 specifically interacts with Rac1.

In vivo Rac activity is regulated by binding of guanine nucleotide. Wild-type Rac normally exists in a GDP-bound form *in vivo.* On stimulation, Rac becomes activated by exchanging GDP for GTP and is subsequently able to interact with downstream effectors. To examine whether the interaction between plexin-B1 and Rac is nucleotide dependent, we tested the interaction by using wild-type Rac (mainly GDP-bound),

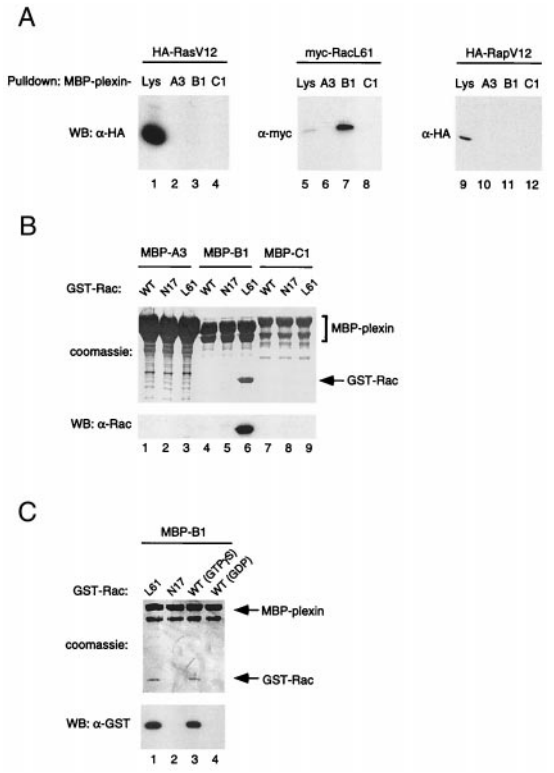

Fig. 1. (*A*) Interaction between activated Rac and plexin-B1. Active mutants of HA-RasV12 (lanes 1–4), myc-RacL61 (lanes 5–8), and HA-RapV12 (lanes 9–12) were transfected into HEK293 cells. Cells lysates (75 μl) were incubated with 5 μg of MBP-plexin prebound to amylose resin. The resin was washed and eluted in buffer containing 10 mM maltose. The eluted samples were analyzed by SDS/PAGE and detected by Western blot (WB) with the antibodies as indicated. MBP-plexin-A3, -B1, or -C1 are indicated on top of each lane. Lanes labeled Lys denote total cell lysate (7.5 μl). (*B*) Plexin-B1 interacts with constitutively active RacL61 but not with dominant negative RacN17. *In vitro* binding of purified GST-Rac/N17/L61 was incubated with MBP-plexin-A3, -B1, and -C1 prebound to amylose resin. MBP-plexins and associated proteins were isolated and stained by Coomassie (upper) or subject to WB with α-Rac antibody (lower). GST-RacL61 was quantitatively recovered by MBP-plexin-B1 pulldown (lane 6). GST-wild-type Rac (WT), RacN17 (N17), and RacL61 (L61) are indicated on top of corresponding lanes. (*C*) Plexin-B1 interacts with Rac-GTP but not Rac-GDP. Wild-type Rac was preloaded with GTPγS (a nonhydrolyzable GTP analogue, lane 3) or GDP (lane 4) before *in vitro* binding with MBP-plexin-B1. The interaction with MBP-plexin-B1 was analyzed as in *B*, with the exception that α-GST antibody was used. RacL61 (lane 1) and RacN17 (lane 2) were included as positive and negative controls, respectively.

RacN17 (constitutively GDP-bound), and RacL61 (constitutively GTP-bound). Neither the wild-type nor the N17 mutant of Rac showed any binding to plexin-B1, whereas RacL61 strongly bound to MBP-plexin-B1 (Fig. 1*B*). When an excess molar quantity of MBP-plexin-B1 was used in the *in vitro* binding experiments, we observed that GST-RacL61 was quantitatively bound to MBP-plexin-B1 (Fig. 1*B*, lane 6, Coomassie staining). Reciprocal binding experiments using immobilized GST-Rac to precipitate plexin-B1 also showed that MBP-plexin-B1 was specifically precipitated by GST-RacL61, but not by RacN17 (data not shown). To confirm the nucleotide dependence, experiments were performed with wild-type Rac loaded with GTP or GDP nucleotides. GST-Rac loaded with GTPγS, a nonhydrolyzable GTP analog, interacted with plexin-B1 (Fig. 1*C*, lane 3) whereas GST-Rac loaded with GDP did not (Fig. 1*C*, lane 4). Our results unequivocally demonstrate that the interaction between Rac and plexin-B1 depends on the GTP-binding status of Rac.

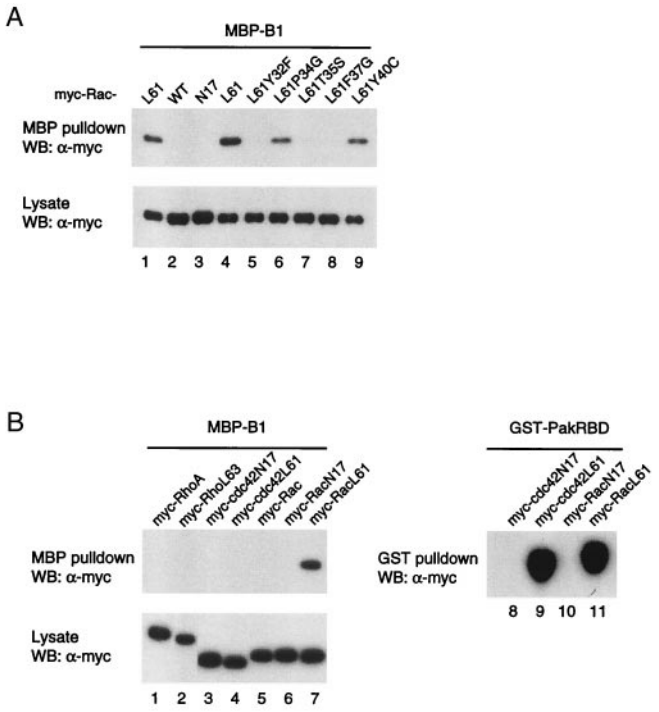

Fig. 2. (*A*) Mutations in the Rac effector domain abolish plexin-B1 interaction. Various myc-Rac mutants were transfected into HEK293 cells, and cell lysates were subjected to *in vitro* pulldown by MBP-plexin-B1. Expression of different Rac mutants in cell lysates is shown (lower). The mutations Y32F (lane 5), T35S (lane7), and F37L (lane 9) in the Rac effector domain completely abolished interaction with plexin-B1. In contrast, the mutations P34G (lane 6) and Y40C (lane 9) had no significant effect on the interaction with plexin-B1. (*B*) Cdc42 and RhoA do not interact with plexin-B1. Various mutants of myc-RhoA, -Cdc42, and -Rac were transfected into HEK293 cells. Cell lysates were used in either MBP-plexin-B1 or GST-PAK-RBD pulldown. Neither RhoL63 (lane 2) nor Cdc42L61 (lane 4) bind to plexin-B1. As a control, GST-PAK-RBD binds both RacL61 (lane 9) and Cdc42L61 (lane 11).

The Effector Domain of Rac Is Required for the Interaction with Plexin-B1. The switch I region, also known as the effector domain of Rac, undergoes a dramatic conformational change when Rac exchanges between GDP and GTP (28). The interaction between Rac and its physiological targets depends on an intact effector domain of Rac in the GTP-bound state. For example, mutation of tyrosine 40 in Rac eliminates the interaction with p21-activated kinase (PAK) (29). We therefore examined the requirement of the Rac effector domain for its interaction with plexin-B1. The following RacL61 effector domain mutants were created: Y32F, P34G, T35S, F37G, and Y40C. These mutants were expressed in HEK293 cells and tested for their ability to bind plexin-B1. We found that mutation of Y32F, T35S, and F37G completely eliminated the ability of Rac to bind plexin-B1 (Fig. 2*A*, lanes 5, 7, and 8). In contrast, mutation of P34G and Y40C had little effect on plexin-B1 binding (Fig. 2*A*, lanes 6 and 9). To further confirm the effector domain mutations, mutant RacL61F37G and L61Y40C were expressed in bacteria, and the recombinant proteins were purified. RacL61Y40C, but not RacL61F37G, was able to bind MBP-plexin-B1, confirming that residue F37 but not Y40 is important for plexin-B1 interaction (data not shown). The results in Fig. 2*A* clearly demonstrate that the integrity of the Rac effector domain is important for its interaction with plexin-B1.

Rac is a member of the Rho subfamily, which belongs to the Ras superfamily of small GTPases. Many downstream targets are common to the Rho subfamily members (30). For example,

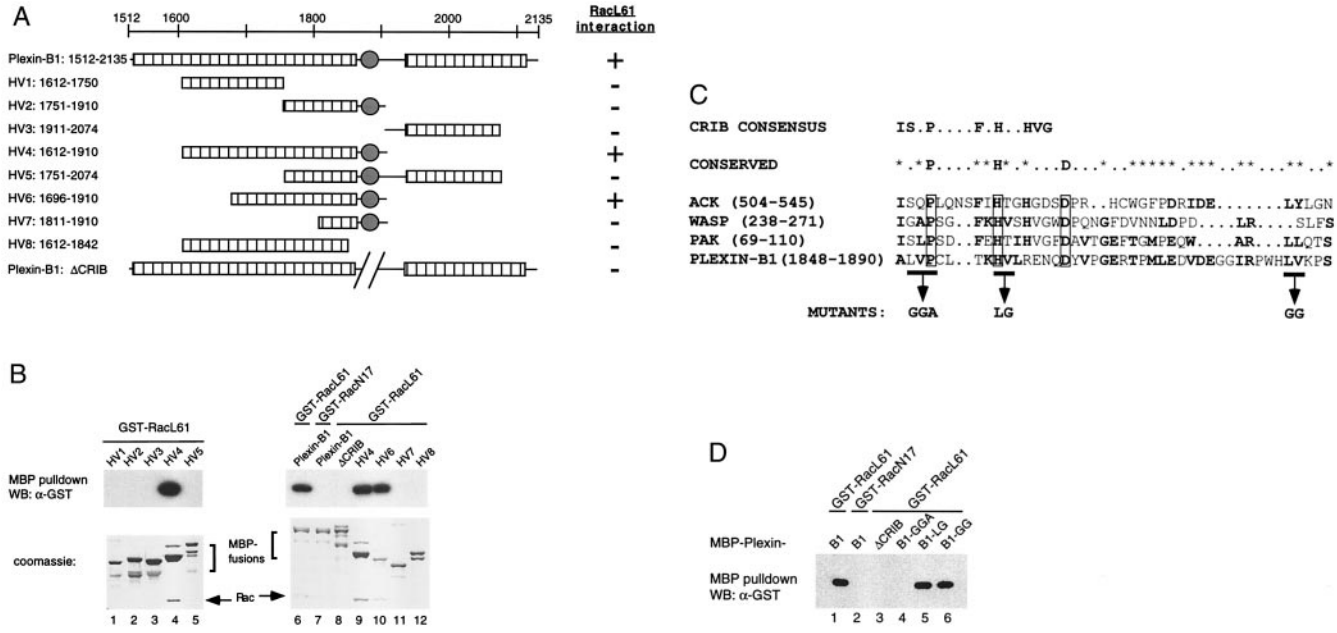

Fig. 3. (*A*) Mapping the Rac binding domain in plexin-B1. The intracellular domain of plexin-B1 (residues 1512–2135) contains two conserved regions as depicted by the hatched bars. The filled circle denotes the CRIB motif found in plexin-B1, which is not conserved in other members of the plexin family. The results of the *in vitro* interaction are summarized in the right column. (*B*) Interaction between RacL61 and deletion mutants of plexin-B1. Various MBP-plexin-B1 deletion proteins were expressed and purified from *E. coli* and used for *in vitro* interaction with GST-RacL61. The samples were stained with Coomassie (lower) or detected by α-GST WB (upper). (*C*) Sequence alignment of the CRIB domain of plexin-B1. The CRIB consensus defined by Burbelo *et al.* is shown (upper) (31). Residues conserved among ACK, WASP, PAK, and plexin-B1 are shown (lower). The invariable P, H, and D are boxed whereas * denotes partially conserved residues. (*D*) Mutation in the CRIB motif disrupts interaction with plexin-B1. MBP-plexin-B1 fusion proteins, containing mutations in the CRIB domain as indicated, were expressed and purified from *E. coli* and used for *in vitro* interaction with GST-RacL61. The samples were analyzed by α-GST WB.

PAK can interact with both Rac and Cdc42. Therefore, we tested whether other members, including RhoA and Cdc42 can interact with plexin-B1. Various mutants of RhoA, Cdc42, and Rac1 were expressed in HEK293 cells and assayed for their ability to bind MBP-plexin-B1. Our results demonstrated that both RhoL63 and Cdc42L61 fail to interact with plexin-B1 (Fig. 2*B*, lanes 2 and 4). As a control, the RBD of PAK efficiently binds to both active Rac and Cdc42 (Fig. 2*B*, lanes 9 and 11). Therefore, our data show that plexin-B1 selectively interacts with Rac and not with other members of the Rho family.

The CRIB-Like Motif in Plexin Is Required for Interaction with Rac. The intracellular sequence of plexins contains two highly conserved regions found in all members of the plexin family (Fig. 3*A*). To determine which region of plexin-B1 is responsible for interaction with Rac, several deletion constructs were created and expressed as MBP fusion proteins. *In vitro* binding with GST-RacL61 showed that the second conserved domain of plexin-B1 is not required for interaction with RacL61 because HV4 (residues 1612–1910) and HV6 (1696–1910) retain full ability to bind Rac (Fig. 3 *A* and *B*). Similarly, the first 184 residues of the intracellular domain of plexin-B1, including the N-terminal half of the first conserved domain, are dispensable for Rac interaction. The minimal Rac binding domain was mapped to 215 residues between 1696–1910. Further deletion from either the N or C terminus of this 215-aa sequence abolished interaction with Rac (comparing HV6 with HV2 or HV8, Fig. 3 *A* and *B*). We named the Rac binding domain PRB (for plexin-Rac binding).

The CRIB motif is commonly found in many Rac/Cdc42-interacting proteins, including PAK, Wiskott-Aldrich Syndrome protein (WASP), and activated Cdc42-associated kinase (ACK) (31). We thus examined the plexin-Rac binding sequence in plexin B1 and found that a putative CRIB motif exists between residues 1848–1890 (Fig. 3 *A* and *C*). To test the importance of this putative CRIB motif in plexin-B1, the CRIB motif (residues 1843–1889) was deleted to create plexin-B1:ΔCRIB (Fig. 3*A*), and its interaction with Rac was examined. As expected, plexin-B1:ΔCRIB lost its interaction with RacL61 (Fig. 3*B*, lane 8).

The three-dimensional structures of Cdc42 in complex with the CRIB motifs of ACK, WASP, and PAK predict that several residues in the CRIB motif make direct contacts with Cdc42 and play an important role for the interaction with Cdc42 (32–34). For example, the conserved Pro-72 in PAK directly interacts with Cdc42 and is important for Cdc42 interaction (33, 35). Similarly, Phe-75 and His-77 of PAK interact with Tyr-40 of Cdc42 (conserved in Rac). We tested the possibility that the corresponding residues in plexin-B1 may be responsible for interaction with Rac. Mutation of Pro-1851 (corresponding to Pro-72 in PAK) in plexin-B1 (mutant GGA) completely abolished the interaction with Rac (Fig. 3*D*, lane 4). Interestingly, mutation of His-1856 (mutant LG, corresponding to His-77 in PAK) did not affect the interaction (Fig. 3*D*, lane 5). This result is consistent with the fact that Tyr-40 is not required for plexin-B1 interaction (Fig. 2A), although Tyr-40 in Rac is essential for PAK binding (29). As mentioned above, His-77 in PAK likely contacts Tyr-40 in Rac. Finally, mutation of Leu-1886Val-1887, corresponding to Leu-96Leu-97 of PAK, had little effect on the interaction with Rac. Mutations of these residues in PAK also do not disrupt its interaction with Cdc42 (36). Our *in vitro* binding experiments with the CRIB motif mutations support the notion that the CRIB motif in plexin-B1 is directly involved in interaction with Rac. The above results are also consistent with data in Fig. 2*A* that Tyr-40 in Rac is not important for plexin-B1 interaction. It is worth noting that the CRIB motif in plexin-B1 is located in the nonconserved region found among members of the plexin family (Fig. 3*A*). The lack

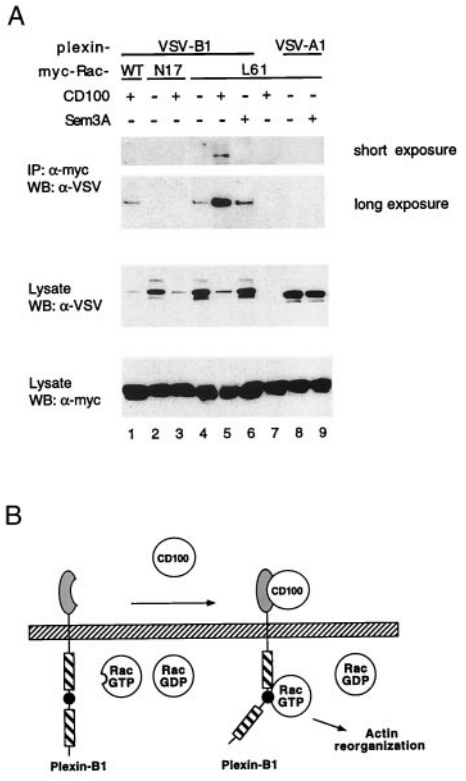

Fig. 4. (*A*) CD100 stimulates the interaction between plexin-B1 and RacL61. VSV epitope-tagged plexin-B1 or plexin-A1 (0.5 μg) was cotransfected with myc-Rac (0.2 μg) and/or semaphorin (0.5 μg) in HEK293 cells as indicated. Cells were lysed and immunoprecipitated with anti-myc antibody. The immunoprecipitated samples were probed with anti-VSV antibody. Expression of Rac (*Bottom*, detected by anti-myc) and plexin (second panel from bottom, detected by anti-VSV) are shown. VSV-Plexin-B1 coimmunoprecipitated by myc-Rac is shown in the top two panels (for short and long exposure). Cotransfection of CD100 and Sema3A is indicated. Lanes 8 and 9 also were cotransfected with neuropilin-1 (0.5 μg). (*B*) A proposed model for Rac-GTP in CD100-stimulated plexin-B1 signaling. The ability of Rac-GTP to bind the plexin-B1 receptor is enhanced on stimulation with CD100. Active Rac binds the receptor CRIB motif (denoted as a filled circle) and is predicted to affect actin reorganization.

of the CRIB motif may explain why plexin-A3 and plexin-C1 do not directly bind to Rac.

CD100 Stimulates the Association Between Plexin-B1 and Rac. The above *in vitro* biochemical studies were performed by using the intracellular domains of plexins. Therefore, we attempted to examine the interaction between Rac and the full-length plexin-B1 receptor and furthermore to determine the effect of CD100, a ligand for plexin-B1, on this interaction. Epitope tagged VSV-plexin-B1, which contains the human hepatocyte growth factor signal sequence followed by the VSV epitope fused to the N terminus of plexin-B1, was cotransfected with myc-RacL61. Cell lysates were immunoprecipitated with anti-myc antibody followed by immunoblot with anti-VSV antibody. Fig. 4*A* shows that a low level of plexin-B1 can be coimmunoprecipitated with RacL61, but not with RacN17 (Fig. 4*A*, lanes 2 and 4, second panel from top). Cotransfection with CD100 significantly enhanced the interaction between RacL61 and plexin-B1, whereas no interaction was observed between RacN17 and plexin-B1 even in the presence of CD100 (Fig. 4*A*, lanes 3–5, second panel from top). As a control, cotransfection of Sema3A, a ligand for plexin-A1/neuropilin-1, had no effect on the association between RacL61 and plexin-B1 (Fig. 4*A*, lane 6). The enhancement of plexin-B1/RacL61 interaction is very dramatic if one considers the fact that CD100 cotransfection significantly decreased the level of plexin-B1 (Fig. 4*A*, third panel from top). Approximately 15% of total VSV-plexin-B1 was immunoprecipitated by myc-RacL61 in the presence of CD100. We consistently observed a dramatic decrease of plexin-B1 protein when CD100 was cotransfected, whereas cotransfection of semaphorin 3A had no effect on plexin-B1 protein levels. The reduction of plexin-B1 protein is likely explained by the continuous binding of CD100, resulting in internalization and thus degradation of plexin-B1. It is also worth noting that low levels of plexin-B1 can be coimmunoprecipitated with wild-type Rac in the presence of CD100 (Fig. 4*A*, lane 1). No plexin-B1 was present in the immunoprecipitation of wild-type Rac in the absence of CD100 (data not shown). In addition, the specificity of the Rac-plexin-B1 interaction is supported by the observation that plexin-A1 was not immunoprecipitated with RacL61 even in the presence of ligand semaphorin 3A (the coreceptor neuropilin-1 was cotransfected in both lanes 8 and 9 in Fig. 4*A*). The above data indicate that the interaction between Rac and plexin-B1 receptor is stimulated by the CD100 ligand.

Discussion

Small GTPases have been previously implicated as functional mediators of semaphorins in regulating cytoskeletal reorganization (22–24). For instance, Rac has been shown to be critical for the growth cone collapsing activity of secreted Sema3A. It is possible that similar cytoskeletal modifications may also underlie other semaphorin-mediated processes, such as lymphocyte chemotaxis. On the other hand, small GTPases may also execute other functions independent of cytoskeletal modifications in response to semaphorin stimulation. However, how semaphorins regulate small GTPases or other intracellular signaling molecules is largely unknown. Here we have demonstrated that active Rac can directly interact with plexin-B1. Importantly, this interaction is enhanced by CD100, a known ligand of plexin-B1. Our data provide a biochemical mechanism by which a semaphorin binds a specific cell surface plexin receptor and stimulates its interaction with a small GTPase, thus leading to cytoskeletal reorganization (Fig. 4*B*). Our results argue for a physiological significance of the interaction between Rac and plexin-B1. First, the interaction between plexin-B1 and Rac is very strong *in vitro*. *In vitro*, active Rac can be almost quantitatively recovered in complex with plexin-B1. Furthermore, in cotransfected cells, a significant fraction of plexin-B1 can be coimmunoprecipitated with RacL61 in the presence of CD100. Second, similar to other physiological Rac targets, the interaction between Rac and plexin-B1 depends on the GTP-bound conformation of Rac. Moreover, the integrity of the effector domain of Rac is essential for its interaction with plexin-B1. Third, plexin-B1 contains a CRIB motif, which is consistent with a model for direct interaction. Mutation or deletion of the CRIB motif abolishes the interaction between plexin-B1 and active Rac. Fourth, the interaction between the full-length plexin-B1 and active Rac is enhanced by ligand stimulation, indicating that the interaction is modulated under physiological conditions. In addition, Alan Hall's laboratory has also observed the interaction between active Rac and plexin-B1 and has demonstrated the ability of plexin-B1 to regulate actin cytoskeletal structure (A. Hall, University College London, personal communication).

Small GTPases transduce their signals via direct interaction with downstream effector molecules. It is well established that Rac stimulates PAK activity by direct interaction via the effector domain of Rac (37). We would like to propose a model that, on semaphorin stimulation, plexin binds and regulates Rac function. It is possible that plexin-B1 may modulate the guanine nucleotide loading of Rac although direct evidence is not available. We have not detected any effect on Rac-GTP levels in

cells cotransfected with plexin-B1 and CD100 (unpublished observation). However, it is possible that plexin-B1 regulates only a fraction of intracellular Rac activity and that a small change of Rac-GTP was not detectable in our experiments, which examined total cellular Rac. Alternatively, plexin-B1 may modulate the localization of active Rac to the cell membrane of the growth cone, where Rac can regulate cytoskeletal structures. Conversely, plexin-B1 may bind to Rac-GTP and inhibit the function of Rac by sequestering its ability to act on other downstream targets. In any event, our study demonstrates an example of a small GTPase directly interacting with a receptor. Currently, we cannot exclude the possibility that active Rac may modulate plexin-B1 signaling, whereby plexin-B1 would serve as a downstream effector of Rac. Future experiments are aimed to elucidate whether plexin-B1 acts as an upstream regulator or downstream effector of Rac.

We have shown that the CRIB motif in plexin-B1 is essential for its interaction with Rac. However, deletion analyses indicate that the plexin-Rac binding domain is significantly larger than the CRIB motif. Furthermore, the CRIB motif alone is not sufficient for Rac binding (Fig. 3 *A* and *B*). Surprisingly, the CRIB motif in plexin-B1 is localized in a region that is not conserved among plexin family members (Fig. 3*A*; refs. 19 and 20). These results are consistent with the *in vitro* binding specificity of the plexin family members tested. However, this binding specificity is in contrast to *in vivo* experiments that have implicated Rac to play a role in signal transduction by semaphorin 3, which functions through the plexin-A/neuropilin complex (22, 23). Although we did not observe a direct interaction between the intracellular domain of plexin-A3 and Rac, it is possible that Rac may function downstream of plexin-A/neuropilin without interacting with the receptor complex directly. It is also possible that additional intracellular components are required for Rac and plexin-A/neuropilin interaction. Alternatively, plexin-A3 may act as a negative regulator of semaphorin activity. The Rho family of small GTPases contains the subfamilies of Rho, Cdc42, and Rac, of which each has multiple members (25). It is likely that different members of the plexin family may specifically interact with different members of the Rho family and hence impart specificity to semaphorin signaling. Future studies hope to elucidate the role of Rho family small GTPases and the mechanism of signal transduction downstream of semaphorin-stimulated plexin receptors.

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