ErbB-2 and Met Reciprocally Regulate Cellular Signaling via Plexin-B1*

Received for publication, August 16, 2007, and in revised form, November 8, 2007 Published, JBC Papers in Press, November 19, 2007, DOI 10.1074/jbc.M706822200

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Sema4D-induced activation of plexin-B1 has been reported to evoke different and sometimes opposing cellular responses. The mechanisms underlying the versatility of plexin-B1-mediated effects are not clear. Plexin-B1 can associate with the receptor tyrosine kinases ErbB-2 and Met. Here we show that Sema4D-induced activation and inactivation of RhoA require ErbB-2 and Met, respectively. In breast carcinoma cells, Sema4D can have pro- and anti-migratory effects depending on the presence of ErbB-2 and Met, and the exchange of the two receptor tyrosine kinases is sufficient to convert the cellular response to Sema4D from pro- to anti-migratory and vice versa. This work identifies a novel mechanism by which plexin-mediated signaling can be regulated and explains how Sema4D can exert different biological activities through the differential association of its receptor with ErbB-2 and Met.

Beyond their well established role in the nervous system (1–3), mammalian semaphorins have also been involved in various other processes like the regulation of immune functions (4), morphogenetic processes during the development of the cardiovascular system (3, 5–7), as well as in tumor progression and angiogenesis (8–11). The majority of the semaphorin effects are mediated by a group of large transmembrane proteins called plexins (12, 13) that are divided into four classes, A, B, C, and D (12). Whereas semaphorins activate class A plexins in most cases indirectly through neuropilins, class B plexins are activated by direct binding of semaphorins (14).

The activation of plexins by semaphorins initiates a variety of signaling processes that involve several small GTPases of the Ras and Rho families (14, 15). Plexins possess a GTPase-activating protein (GAP)3 activity (16), and plexin-B1 has been shown to act as a R-Ras GAP when activated by Sema4D (17). Activated plexin-B1 can also bind GTP-bound Rac1, thereby preventing downstream signaling of Rac (18–20). In addition, the very C-terminal end of B-class plexins stably interacts with the PDZ domain of the Rho guanine nucleotide exchange factors PDZ-RhoGEF and LARG (21–24), and it has been shown that the plexin-B1-RhoGEF complex mediates Sema4D-induced RhoA activation. Finally, all B-class plexins can interact with the receptor tyrosine kinases Met and ErbB-2 (25–27). Upon binding of Sema4D to plexin-B1, the kinase activity of ErbB-2 and Met is increased resulting in tyrosine phosphorylation of both the kinase and plexin-B1. Met kinase has been shown to mediate Sema4D-induced cell migration and invasive growth (25), and ErbB-2 is required for activation of RhoA via plexin-B1 and PDZ-RhoGEF/LARG as well as for Sema4D-induced growth cone collapse in hippocampal neurons (26).

Activation of plexin-B1 by Sema4D results in different cellular effects. Plexin-B1 activation in NIH-3T3 and PC12 cells inhibits integrin-mediated cell attachment and cell migration (28, 29) through the activation of the R-RasGAP activity inherent to plexin-B1 (29) or through the inhibition of RhoA (30). However, activation of plexin-B1 by Sema4D stimulates the migration of SK-BR3 cells (25) and endothelial cells. The latter effect involves plexin-B1-mediated activation of RhoA and the phosphatidylinositol 3-kinase/Akt pathway (8, 31). Thus, plexin-B1 appears to be able to mediate different and sometimes opposite effects depending on the cellular context. The aim of this study was to define mechanisms, that determine the cellular responses to plexin-B1 activation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were commercially obtained: mouse monoclonal anti-Myc (9E10) and goat polyclonal LARG (Santa Cruz Biotechnology, Heidelberg, Germany); mouse monoclonal anti-VSV and mouse monoclonal anti-α-tubulin (Sigma-Aldrich); anti-ErbB-2 [Y1248], anti-Met [Y1234/1235] and anti-pAkt [S473] (Cell Signaling Technology, Frankfurt, Germany); mouse monoclonal anti-phosphotyrosine (4G10) and anti-Rac1 (Upstate Biotechnology, Millipore, Billerica); mouse monoclonal anti-RhoA and anti-ErbB-2 (Becton Dickinson, Heidelberg, Germany); mouse monoclonal anti-Met (Zymed Laboratories Inc./Innogenetics); and goat polyclonal anti-plexin-B1 (R & D Systems, Wiesbaden, Germany).

Plasmids—Eukaryotic expression plasmids carrying the cDNAs of PDZ-RhoGEF, Sema4D, (Myc)-RhoA, and Met were described previously (21, 26). VSV-plexin-B1 was kindly provided by L. Tamagnone (University of Torino, Torino, Italy). Full-length sequences of human Met and ErbB-2 were inserted into pLNCX2 vector (Clontech).

Cell Culture and Immunoprecipitation Studies—HEK293 cells were cultured as described previously (21). MCF-7 and MDA-MB-468 cells were cultured according to American Type Cell Culture Collection protocols. For immunoprecipitation,
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HEK293 cells were collected 48 h after transfection and lysed for 30 min in ice-cold radioimmunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1 μg/ml of each leupeptin, aprotinin, and pepstatin, 1 mM 4-(2-aminoethyl)benzysulfonylfluorid-hydrochloride, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂, 10 μg/ml each of leupeptin and aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and clarified cell lysates were incubated with GST-RBD at 4°C for 50 min. The beads were then washed four times with the respective lysis buffer. The bead pellets were finally resuspended in 25 μl of Laemmli sample buffer. The proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose membrane, and GTPases were detected using a specific antibody against RhoA and Rac1, respectively.

### SDS-PAGE and Immunoblotting

Precipitated proteins and cell lysates were separated using SDS-PAGE followed by transfer onto nitrocellulose membrane (Whatman, Dassel, Germany) and blocking of nonspecific binding sites with 5% milk in TBST. The blots were probed with appropriate antibodies, and the proteins were visualized using an ECL system (GE Healthcare, Munich, Germany and Millipore, Schwalbach, Germany).

### Wound Healing Assay

Wounding was performed with a 10-μl tip that was cut longitudinally. The recordings were performed immediately after wounding and after 24–48 h, using a motor-controlled Leica DC 350 FX camera connected to the Leica Microscope CTR MIC and operated by the Leica software FW4000. For statistical analysis, the wound distance from each well was measured in duplicate at two randomly defined wound gap locations per frame recorded per experiment, and at least three independent scratch-wound experiments were used for calculations.

### Transwell Assay

Cell migration was assayed using polystyrene Transwell inserts (Greiner, Frickenhausen, Germany) with pore sizes of 0.8 μm. Cell concentration was adjusted to 2 × 10⁵ cells/ml of medium, and 500 μl were added. The inserts were then placed in wells of a 24-well plate. The plates were
then incubated at 37 °C at 5% CO₂ for 12 h (MDA-MB-468 cells) or 24 h (MCF7 cells). The inserts were removed, and the cells on top of the insert were removed. Cells that had migrated to the bottom of the inserts were then fixed in methanol, stained with 1% toluidine blue in 1% sodium tetraborate for 1 h, and counted.

Small Interfering RNAs—The target sequence for siRNAs specific to ErbB-2 and Met used in this study were published before (33, 34). The sequence of the siRNA used to suppress plexin-B1 expression was ACCACGGUCACCGGAUUC. A control siRNA, CGCUUGAGAAGAAGUUG, was purchased from IBA (Göttingen, Germany).

Northern Blot Analysis—Primer used for amplification of a plexin-B1 human cDNA fragment that served as a probe for Northern blot analysis were 5'-CCTCCGAGGCACTTGACTCT-3' (sense) and 5'-GCCGTGCATCTCCTTCAG-3' (anti-sense). The probe used for detection of PDZ-RhoGEF mRNA was a purified 941-bp fragment from a BamHI digest of PDZ-RhoGEF human cDNA. Northern blot analysis using total RNA (10 μg) derived from the respective cell lines was performed as described previously (35). Equal loading of the lanes was confirmed by comparing the intensities of the 18S ribosomal RNA bands on the denaturing agarose gel.

Production and Purification of Recombinant Sema4D—Generation of Lec3.2.8.1 Chinese hamster ovary cells expressing the extracellular part of human Sema4D (residues 1–657 followed by a lysine residue and a C-terminal histidine tag for purification) was described previously (36). Recombinant soluble Sema4D was purified by metal ion affinity chromatography using nickel-nitrilotriacetic acid-agarose (Qiagen) followed by gel filtration chromatography using a Superdex 200 10/300 GL column (Amersham Biosciences, Freiburg, Germany). Concentration of purified soluble Sema4D was determined by comparison to a bovine serum albumin standard on Coomassie stained SDS-polyacrylamide gels. Activity of purified soluble Sema4D was routinely verified by measuring ErbB-2 phosphorylation in plexin-B1 transfected HEK cells by Western blot analysis after 20 min of exposure to 150 nM purified Sema4D.

Production of Soluble Sema4D—HEK cells were transfected with a construct expressing the N-terminal portion of Sema4D in a secretion vector (pSecTag2-Hygro). 3 h after transfection, the cells were placed in serum-free medium. 48 h after transfection, Sema4D-containing medium was gathered and used to then incubated at 37 °C at 5% CO₂ for 12 h (MDA-MB-468 cells) or 24 h (MCF7 cells). The inserts were removed, and the cells on top of the insert were removed. Cells that had migrated to the bottom of the inserts were then fixed in methanol, stained with 1% toluidine blue in 1% sodium tetraborate for 1 h, and counted.

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for stimulation. For control, HEK cells were transfected with "empty" pSecTag2-Hygro and processed as described above.

Retroviral Infections—Recombinant retroviruses were constructed by subcloning full-length human cDNA of ErbB-2 and Met into the retroviral expression vector pLNCX2 (Clontech), which carries a neomycin phosphotransferase cassette. A retroviral expression vector carrying the cDNA of human placenta alkaline phosphatase (pLAPSN) served as a control. The DNA constructs were transfected into the packaging cell line PT67 (Clontech) using calcium phosphate. Transfected PT67 cells were selected with 400 μg/ml Geneticin (Invitrogen) for 14 days. Viral supernatants were collected, filtrated through a 0.45-μm polyvinylidene difluoride filter (Millipore) and used to infect 3 × 10⁵ cells in a 10-cm dish in the presence of 6 μg/ml polybrene (Sigma). The infected cells were selected with 400 μg/ml geneticin for 14 days.

Statistical Analysis—The data are given as the mean values ± S.D. The statistical significance was evaluated by Student’s t test. The levels of significance are: *, <0.05; **, <0.01; ***, <0.001.

RESULTS

It has been shown that Sema4D-induced plexin-B1 activation can result either in activation or inhibition of RhoA (20, 30). As described before (21–23, 26), Sema4D stimulation of HEK cells expressing plexin-B1 and PDZ-RhoGEF and kept at 0.5 or 1.5% FBS led to a strong RhoA activation, which was accompanied by tyrosine phosphorylation of plexin-B1 and endogenous ErbB-2 and which could be blocked by the ErbB-2 inhibitor AG1478 (Fig. 1, left and middle panels). We did not detect Sema4D-induced RhoA activation in cells transfected with Met and pretreated with AG1478 at low serum concentrations (Fig. 1A, left panels), although Met became activated as indicated by its phosphorylation on tyrosine residue 1234/1235 and was able to mediate plexin-B1 phosphorylation. However, at intermediate and high serum concentrations resulting in higher basal RhoA activity, the inhibition of ErbB-2 and the expression of Met led to an inhibition of RhoA activity in response to Sema4D (Fig. 1A, middle and right panels). This inhibitory effect could be blocked by the tyrosine kinase inhibitor K252a, which has been shown to inhibit Met at nanomolar concentration (25, 37). Thus, whereas plexin-B1-mediated RhoA activation requires ErbB-2, the inhibition of RhoA activity by plexin-B1 appears to involve Met.

Expression of increasing amounts of Met in HEK cells that endogenously express ErbB-2 resulted in a decreased interaction between plexin-B1 and ErbB-2 as determined by coprecipitation experiments (Fig. 1B). At the same time, interaction between plexin-B1 and Met increased. This was accompanied by a decrease in Sema4D-induced ErbB-2 phosphorylation and RhoA activation, whereas Sema4D-induced Met phosphorylation increased (Fig. 1C). At high Met expression levels, Sema4D induced RhoA inactivation (Fig. 1C). This suggests that ErbB-2 and Met compete for plexin-B1 and that the stoichiometry of plexin-B1, ErbB-2, and Met in the plasma membrane determines whether Sema4D-induced plexin-B1 activation involves ErbB-2 or Met and results in RhoA activation or inhibition.

Our data suggest that Sema4D-induced tyrosine phosphorylation of plexin-B1 by the receptor tyrosine kinases ErbB-2 and Met is an important regulatory mechanism in plexin-B1 signaling and that the identity of the receptor tyrosine kinase that mediates phosphorylation of plexin-B1 greatly affects the cellular response to plexin-B1 activation by Sema4D. To test whether this holds true also in cellular systems endogenously expressing the components of plexin-B1-mediated signaling systems, we analyzed the two plexin-B1-expressing breast cancer cell lines MCF-7 and MDA-MB-468, of which MCF-7 expresses ErbB-2 but not Met, whereas MDA-MB-468 expresses only Met but not ErbB-2 (Fig. 2A). Endogenous plexin-B1 interacted either with ErbB-2 or with Met in MCF-7 or MDA-MB-468 cells, respectively (Fig. 2B). In starved MCF-7 cells stimulation with Sema4D led to an activation of RhoA (Fig. 2C), which could be blocked by the ErbB-2 inhibitor AG1478 (Fig. 2C). In contrast, stimulation of Met-expressing MDA-MB-468 cells with Sema4D led to RhoA inhibition (Fig. 2D), an effect that was sensitive to the Met inhibitor K252a (Fig. 2D). The involvement of plexin-B1 in the regulation of RhoA activity in MCF7 and MDA-MB-468 cells is demonstrated by the block of Sema4D-induced RhoA regulation in both cell lines after siRNA-mediated knockdown of plexin-B1 expression (supplemental Fig. S1, A–C).

Plexin-B1 can bind activated Rac upon stimulation by Sema4D (18–20), thereby competing with other Rac-binding proteins like PAK (19). Both in MCF7 as well as in MDA-MB-468 cells, Sema4D induced a decrease in the amount of activated Rac1 bound to the Rac-binding domain of PAK (supplemental Fig. S2A). Thus, in contrast to the regulation of RhoA, the binding of active Rac was obviously not differentially influenced by ErbB-2 and Met.
The role of ErbB-2 and Met in plexin signaling was further analyzed by suppression of ErbB-2 and Met expression using siRNAs (Fig. 2, E and F). Sema4D-mediated RhoA activation in MCF-7 cells was fully abolished after suppression of ErbB-2 expression, whereas cells expressing control and Met siRNAs showed a normal response (Fig. 2E). In MDA-MB-468 cells, we found that expression of control or ErbB-2-specific siRNA had no effect on Sema4D-mediated RhoA deactivation, whereas cells in which Met expression had been suppressed did not show Sema4D-dependent, plexin-B1-mediated RhoA inhibition (Fig. 2F).

Because Met and ErbB-2 strongly influence the downstream signaling events of plexin-B1 activation, we tested whether the receptor tyrosine kinases affect the regulation of migratory activity by Sema4D using Transwell migration assays. In untreated (data not shown) and control siRNA transfected MCF-7 cells Sema4D induced an increase in migration (Fig. 3A), whereas stimulation of untreated or control siRNA transfected MDA-MB-468 with Sema4D led to an inhibition of migration (data not shown and Fig. 3B). Transfection of MCF-7 cells with Met-specific siRNA had no effect on the promigratory activity of Sema4D, whereas inhibition of ErbB-2 expression completely blocked Sema4D-induced migration of MCF-7 cells (Fig. 3A). In contrast, transfection with Met-specific but not ErbB-2-specific siRNA resulted in the complete inhibition of Sema4D-induced anti-migratory effects on MDA-MB-468 cells (Fig. 3B). In addition, we found that pharmacological inhibitors of Met and ErbB-2 had analogous effects (data not shown). Both in MCF7 and MDA-MB-468 cells, the effects of Sema4D on cell migration were completely blocked after siRNA-mediated knockdown of plexin-B1 (supplemental Fig. S1, D and E).

We then evaluated the role of ErbB-2 and Met in plexin-B1-mediated regulation of cell migration using a scratch wound healing assay. Interestingly, Sema4D stimulated wound closure in MCF-7 monolayers (Fig. 4, A–D), whereas it inhibited closure of wounds in MDA-MB-468 cell monolayers (Fig. 4, E–H). The stimulatory effect of Sema4D in MCF-7 cells was completely blocked in cells transfected with ErbB-2-specific siRNA (Fig. 4, B and D), whereas transfection with control or Met-specific siRNA had no effect (Fig. 4, C and D). In contrast, Sema4D induced inhibition of wound healing in MDA-MB-468 cell monolayers transfected with siRNA directed against ErbB-2 (Fig. 4, F and H), and this effect could be blocked by the suppression of Met expression (Fig. 4, G and H). Thus, Sema4D not only regulates migratory activity of single cells but...
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FIGURE 5. Exchange or ErbB-2 and Met converts cellular effects of Sema4D in MCF-7 and MDA-MB-468 cells. A, MCF-7 and MDA-MB-468 cells were infected with viruses carrying alkaline phosphatase cDNA as a control (MCF-C and 468-C) or Met (MCF-M) or ErbB-2 (468-E) cDNAs. After 10 days of selection, expression of ErbB-2 and Met was analyzed using the indicated antibodies (IB). B, MCF-M and 468-E cells were transfected with control siRNA (ctrl) or with siRNAs specific for ErbB-2 and Met. 72 h after transfection, the cells were lysed, and expression of ErbB-2 and Met was analyzed using indicated antibodies. C, Met-infected MCF-7 cells (MCF-M) and ErbB-2-infected MDA-MB-468 cells (468-E) were lysed, and the lysates were incubated in the absence (−) or presence of an anti-plexin-B1 antibody (+ anti-plexin-B1 Ab) with protein A-Sepharose, and Sepharose beads were pelleted. Shown are autoradiograms of Western blots prepared from beads (IP) or lysates using the indicated antibodies (IB). D and E, MCF-C and MCF-M (D) or 468-C and 468-E cells (E) were transfected with control (ctrl), ErbB-2-specific, or Met-specific siRNA as indicated. 72 h after transfection, the cells were transfected to medium containing 1.5% FBS. After overnight incubation cells were incubated in the absence (−) or presence of 150 nm Sema4D (+). After cell lysis, active RhoA was precipitated as described and visualized by immunoblotting using anti-RhoA antibodies.

also influences collective cell migration. Interestingly, in both cases, the presence of ErbB-2 appears to be required for Sema4D-induced stimulation of migratory activity, whereas Sema4D inhibits migration in the presence of Met.

To confirm the critical role of either ErbB-2 or Met in determining the cellular response to Sema4D, we exchanged the receptor tyrosine kinases between MCF-7 and MDA-MB-468 cells. ErbB-2-expressing MCF-7 cells were infected with retroviruses carrying a control cDNA or wild type Met cDNA, whereas Met expressing MDA-MB-468 cells were infected with viruses carrying control or wild type ErbB-2 cDNAs (Fig. 5A). We then suppressed the expression of the receptor tyrosine kinases present in native cells using siRNAs directed against ErbB-2 and Met, respectively (Fig. 5B). In Met-infected MCF-7 cells and ErbB-2-infected MDA-MB-468 cells, plexin-B1 interacted with both receptor tyrosine kinases. However, after siRNA-mediated suppression of the expression of the endogenous kinase, plexin-B1 interacted with Met in MCF-7 and with ErbB-2 in MDA-MB-468 cells (Fig. 5C). Consistent with our previous observations, Sema4D induces RhoA activation or inactivation in control infected MCF-7 and MDA-MB-468 cells, and its effects on RhoA activity were blocked by knockdown of ErbB-2 or Met, respectively (Fig. 5, D and E). However, when MCF-7 cells were manipulated to express Met instead of ErbB-2, the effect of Sema4D was inverted, and Sema4D induced RhoA deactivation (Fig. 5D). Similarly, substitution of Met by ErbB-2 in MDA-MB-468 changed the Sema4D effect from RhoA inactivation to activation (Fig. 5E).

Because Sema4D has recently been shown to activate the phosphatidylinositol 3-kinase/Akt signaling pathway via Rho and Rho-kinase (38), we tested the ability of Sema4D to induce Akt activation. Interestingly, Sema4D was able to induce Akt phosphorylation only in MCF7 cells but not in MDA-MB-468 cells (supplemental Fig. S2B). Akt activation in MCF7 cells could be blocked by treatment of cells with an siRNA directed against ErbB-2. This indicates that activation of the phosphatidylinositol 3-kinase/Akt pathway by Sema4D requires ErbB-2.

We then tested whether the exchange of receptor tyrosine kinases also leads to a conversion of Sema4D-induced effects on migration. The promigratory effect of Sema4D on control MCF-7 cells was blocked by siRNA-mediated knockdown of ErbB-2 (Fig. 6A). When expression of ErbB-2 was suppressed in MCF-7 cells stably expressing Met, Sema4D induced an inhibition of migratory activity (Fig. 6A). Similarly, the inhibitory effect of Sema4D on migration of control infected MDA-MB-468 cells was converted into a stimulatory effect in MDA-MB-468 cells expressing ErbB-2 and transfected with Met-specific siRNA (Fig. 6B). Finally, we evaluated the effects of ErbB-2 and Met exchange on plexin-B1-mediated regulation of cell migration in the wound healing assay. In Met-transfected MCF-7 cells that are depleted of ErbB-2 using ErbB-2-specific siRNA, we observed an inhibition of wound closure in response to Sema4D (Fig. 6C), whereas the inhibitory effect of Sema4D on migration of MDA-MB-468 cells was converted into a stimulatory effect in cells expressing ErbB-2 and transfected with Met-specific siRNA (Fig. 6D). These data show that Sema4D can have opposite effects on Rho activity and cell migration, which are mediated by the specific interaction of plexin-B1 with receptor tyrosine kinases ErbB-2 and Met. The replacement of ErbB-2 by Met converts Sema4D from a
RhoA-stimulating, promigratory mediator into a RhoA-inhibiting antimigratory mediator and vice versa.

DISCUSSION

The cellular effects of semaphorins are quite versatile, and in many cases it has been reported that a particular semaphorin can induce different and sometimes even opposing effects depending on the cellular context. One possible mechanism underlying the versatility of semaphorin functions is the ability of their receptors to interact with various proteins to form multimolecular receptor complexes. In recent years it has been shown that the Sema4D receptor plexin-B1 can interact with the receptor tyrosine kinases Met and ErbB-2 and that both plexin-B1 and the receptor tyrosine kinases become tyrosine phosphorylated upon activation by Sema4D. Here we show that plexin-B1-mediated activation and inactivation of the small GTPase RhoA requires ErbB-2 and Met, respectively. In breast carcinoma cells, which express plexin-B1, the cellular effects of Sema4D were determined by the identity of the expressed receptor tyrosine kinase. Although the presence of ErbB-2 resulted in Sema4D-induced RhoA activation and stimulation of migratory activity, opposite effects of Sema4D were observed in cells expressing Met.

There is much evidence that Sema4D binding to plexin-B1 induces RhoA activation through the RhoGEF proteins PDZ-RhoGEF and LARG, which stably interact with the C terminus of plexin-B1 (21–23). However, it has also been shown that plexin-B1 can mediate inhibition of RhoA via the Sema4D-dependent recruitment of p190RhoGAP into the semaphorin receptor complex (30). We could confirm that Sema4D-dependent plexin-B1 activation can lead to opposing effects on RhoA activity, and we demonstrate that the identity of the receptor tyrosine kinase determines the net effect on RhoA activity. Although ErbB-2 is required for RhoA activation (26), preferential interaction of plexin-B1 with Met results in the inhibition of RhoA. It is currently not known how p190RhoGAP and PDZ-RhoGEF/LARG activities are regulated by plexin-B1. In the case of p190RhoGAP, the ligand-dependent association with plexin-B1 may be an important step (30). It has been shown that PDZ-RhoGEF and LARG can be regulated by direct tyrosine phosphorylation (39); however, we have previously shown that tyrosine phosphorylation is unlikely to be involved in plexin-B1-mediated RhoGEF activation (26). How phosphorylation of plexin-B1 regulates activation of PDZ-RhoGEF/LARG remains to be clarified.

Cell migration is a complex process that can be regulated at various levels. It involves the detachment of adhesions, the formation of polarized cellular protrusions, the formation of new adhesive structures, as well as the actomyosin-based cell body contraction (40). The Rho family GTPases Rho and Rac have been shown to play key roles in the regulation of cellular migration (41, 42). Although the mechanisms regulating RhoA activity during cell migration are not well described, RhoA activity appears to be stimulated and inhibited in a coordinated and spatially restricted fashion during cell migration, and it has been recently shown that RhoA activity is particularly high in the leading edge as well as in the retracting tail of migrating cells (43). Sema4D can influence cell migration in a dual fashion. Under certain conditions, Sema4D inhibits cell migration through the inhibition of integrin function, a process that involves R-RasGAP activity or p190RhoGAP-dependent RhoA inhibition (28–30). Recent studies have shown that plexin-B1 has promigratory effects that are independent of R-RasGAP activity but require the activation of RhoA and the phosphatidylinositol 3-kinase/Akt pathway (8, 31). In breast carcinoma cells expressing either ErbB-2 or Met, we observed that Sema4D had promigratory or antimigratory effects, respectively. This is consistent with the observation that the promigratory effects of Sema4D on endothelial cells were Met-independent (8). The promigratory effect of Sema4D in MCF7 cells was accompanied by Sema4D-induced RhoA and Akt activation. This would be consistent with a model in which RhoA-
mediated signaling results in Akt activation to promote cellular migration as recently demonstrated for the prominatory effect of Sema4D on endothelial cells (38). Interestingly, the response to Sema4D could be converted by exchanging the two receptor tyrosine kinases, indicating that the expression of ErbB-2 or Met alone determines the effect of Sema4D on migratory activity. In cells expressing both receptor tyrosine kinases, ErbB-2 and Met may compete for mediating Sema4D effects, and their relative expression level may decide on the cellular net effect of Sema4D. The ability of Sema4D to stimulate migration in the presence of ErbB-2 correlated with its ability to activate RhoA, whereas in the presence of Met Sema4D induced preferentially an inhibition of RhoA. It is not clear whether the differential regulation of RhoA activity mediates the opposite effects of Sema4D on migration in the presence of ErbB-2 and Met. It is also conceivable that the downstream signaling cascades of the respective tyrosine kinases that are induced by Sema4D (25, 26) or unknown signaling components that may be recruited to plexin-B1 through ErbB-2- and Met-specific phosphorylation of particular tyrosine residues are involved in the differential regulation of cell migration by Sema4D.

There is increasing evidence that extracellular mediators can induce diverse cellular effects depending on the composition of their heteromultimeric receptor complexes. Netrin1 for instance functions as a chemoattractant by activating homodimers of its receptor DCC (deleted in colorectal cancer). However, when DCC dimerizes with another Netrin1 receptor, Unc5, binding of Netrin1 has chemorepellent effects (44). The ability to induce opposite effects in various cells has also been described for several semaphorins (see Introduction and Refs. 45–47); however, the molecular mechanisms underlying the opposing cellular responses to particular semaphorins are ill defined. Recently, Sema6D was shown to induce promigratory and anti-migratory effects through its receptor plexin-A1 that depend on the association of plexin-A1 with either the vascular endothelial growth factor receptor type 2 or the receptor tyrosine kinase-like transmembrane protein Off-track (48). Our data show that Sema4D exerts different biological activities because of the differential association of its receptor plexin-B1 with the receptor tyrosine kinases Met and ErbB-2. The formation of heteromultimeric receptor complexes in a combinatorial manner together with the employment of different signaling pathways allows an increase in the versatility of the ligand response and may also provide a mechanism for signal integration whose complexities are just beginning to be understood.

Acknowledgments—We thank Evelyne Jones (London) for kindly providing Lec3.2.8.1 Chinese hamster ovary cells expressing part of human Sema4D. We thank H.-P. Gensheimer for technical assistance and R. LeFaucheur for help with the preparation of the manuscript.

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