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Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility

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ABSTRACT

Plexins encode receptors for semaphorins, molecular signals guiding cell migration, and axon pathfinding. The mechanisms mediating plexin function are poorly understood. Plexin activation in adhering cells rapidly leads to retraction of cellular processes and cell rounding ("cell collapse"). Here we show that, unexpectedly, this response does not require the activity of Rho-dependent kinase (ROCK) nor the contraction of F-actin cables. Interestingly, integrin-based focal adhesive structures are disassembled within minutes upon plexin activation; this is followed by actin depolymerization and, eventually, by cellular collapse. We also show that plexin activation hinders cell attachment to adhesive substrates, blocks the extension of lamellipodia, and thereby inhibits cell migration. We conclude that plexin signaling uncouples cell substrate-adhesion from cytoskeletal dynamics required for cell migration and axon extension.

Key words: semaphorin • migration • axon guidance

The plexin gene family includes two members in invertebrates and nine in vertebrates, grouped into four subfamilies (plexins-A to -D; ref 1). Plexins encode receptors for semaphorins, a wide family of molecular signals that can be either secreted/diffusible or membrane bound (2-4). Although initially identified as repelling cues in axon guidance (5, 6), semaphorins were later implicated in a variety of functions involving regulation of cell-to-cell interactions and cell migration, and including morphogenesis, immune response, angiogenesis, and cancer progression (1). Secreted semaphorins in vertebrates interact with plexins of the A subfamily, associated in receptor complexes with neuropilins (4, 7). Conversely, membrane-bound semaphorins interact directly with plexins. For example, Plexin-B1, the prototype receptor of plexin B subfamily, is the high affinity receptor of Sema4D (4).

Semaphorins have been mainly studied in vitro for their ability to induce the retraction of extending axonal processes ("axon collapse"). Moreover, it has been shown that treatment with Sema3A leads to rounding of primary neural crest cells (8), as well as COS cells transfected to

express both Neuropilin-1 and Plexin-A1 (7). The retraction of the axonal growth cone has been attributed to a localized turnover of actin filaments and microtubules, redirecting extension in more permissive directions (9, 10). Semaphorins might similarly mediate a localized retraction of pseudopodia on the leading edge of a migrating cell, thereby affecting its motility. This mechanism could explain semaphorin functions in neural crest cells and oligodendrocytes during embryo development (11), in endothelial cells during angiogenesis (12), and in leucocytes during the immune response (13).

The intracellular signaling pathways elicited by plexins are still poorly understood (14, 15). The cytoplasmic domain of plexins contains two highly conserved stretches, separated by a variable linker sequence (16). The conserved domains include motifs distantly related to GTPase activating proteins (GAPs; see ref 17); however, as yet no GAP activity associated to plexins has been demonstrated. Small GTPases of the Rho family have been identified as potential regulators of semaphorin-dependent cytoskeletal dynamics (18). However, the results are partly contradictory and the implicated molecular mechanisms are still elusive. For example, it has been consistently shown that the axonal collapse induced by Sema3A or Sema3F does not depend on RhoA signaling, (19-22; and S. Niclou and M. Driessens, personal communication). Genetic interference in Drosophila, however, indicated that plexins antagonize Rac and activate Rho signaling (23). Intriguingly, human Plexin-B1 binds and possibly sequesters activated Rac1 (24, 25), and the C-terminal sequence of plexins B associates RhoA GTP/GDP exchangers (26-29). These direct interactions, moreover, require specific sequences only found in the cytoplasmic domain of the plexins of B subfamily. Intriguingly, we and others reported that these specific sequences are implicated in the regulation of receptor localization at the cell surface (30, 31). Plexins A were reported to associate with the MICALs, a family of proteins interacting with the focal adhesion component CAS-L (32). Furthermore, 12/15-lipoxigenase was implicated in the axonal collapse induced by Sema3A, via the production of eicosanoids that release the contact of the growth cone with the adhesive substrate (33).

As alternative to a direct control of cytoskeletal dynamics, we hypothesized that plexins may regulate cell migration and axon extension by directly impinging on cell adhesion to the extracellular matrix (ECM). In fact, integrin-dependent cell-matrix adhesion is transiently induced at the leading edge of migrating cells and growth cones or during cell spreading (34-36). In adhesive structures, the cytoplasmic domain of integrins is coupled with the cytoskeleton through adaptor molecules, such as paxillin, vinculin, or talin and cytosolic tyrosine kinases such as focal adhesion kinase (FAK). These are responsible for the so-called inside-out signaling, regulating integrin avidity for the ECM components. Focal complexes are also a privileged site for the functional cross-talk between integrins and monomeric GTPases of the Rho family (37), regulating cytoskeletal dynamics. For example, the engagement of integrins with adhesive substrates promotes Rac1 activation, which in turn triggers actin polymerization and protrusion of cellular processes (lamellipodia) that push forward the leading edge. Moreover, this localized coupling between integrins and the actin cytoskeleton undergoes a rapid turnover, to allow for cell migration. In contrast, upon stable adhesion, F-actin cables connected to adhesive structures are put under tension due to a Rho-kinase and myosin light chain kinase-dependent contraction and appear as “stress fibers.” Both the impairment of adhesive complexes and the presence of stiff nondynamic focal adhesions have been shown to inhibit cell migration (38).

Recently, Pasterkamp et al. (39) and Serini et al. (40) reported opposite regulation on integrin function by Sema7A and Sema3A, respectively. Sema7A is reported to promote axonal outgrowth in a plexin-independent manner, by engaging β-integrins and eliciting MAPK signaling. Conversely, Sema3A seems to regulate angiogenesis by inhibiting integrin function in a neuropilin- and plexin-dependent manner. In this study, we show that plexin signaling rapidly induces the disassembly of integrin-based focal adhesive structures, followed by the disappearance of F-actin cables. Furthermore, we demonstrate that plexin activation inhibits integrin-dependent adhesion and cell migration on the extracellular matrix and induces Rho-kinase independent collapse of lamellipodia. We also show that the intracellular domains of both Plexin-B1 and Plexin-A1 are able to mediate these responses, suggesting that this is a common feature of the conserved cytoplasmic domain of plexins.

METHODS

Cells and reagents

Cells were cultured in standard culture media supplemented with 10% fetal bovine serum (FBS, Gibco), with the exception of NIH-3T3, which were grown in presence of 10% heat-inactivated bovine serum.

ROCK inhibitor Y-27632 was generously provided by Welfide Corporation (A. Yoshimura). The 12/15-lipoxygenase inhibitor CDC (cinnamyl-3-4-dihydroxy-α-cyanocinnamate) was purchased from Biomol, while Heregulin-β1 was from Neomarkers Inc. Other reagents were from Sigma.

Antibodies

EC-6.9 monoclonal antibodies were raised in mice against the extracellular domain of human Plexin-B1. BALB/c mice were inoculated twice (at an interval of 4 wk), both subcutaneously and intramuscularly, with 100 μg of a cDNA expression vector encoding the extracellular portion of the receptor. Subsequently, antibody production was stimulated by two subcutaneous injections (at an interval of 3 wk) of 4×10^6 cells exogenously expressing Plexin-B1. Five days after final injection, splenocytes of the immunized mice were isolated and fused with P3-X63Ag8 myeloma cells using PEG, according to standard protocols (41). Hybridoma cells were selected in HAT medium and subsequently cloned by limiting dilution. Cell clones were screened for the production of antibodies specifically recognizing Plexin-B1. EC-6.9 MoAbs are of IgG2a isotype (as assessed with Mouse Monoclonal Antibody Isotyping kit, by Amersham). They can specifically immunoprecipitate Plexin-B1 from cell extracts (31). EC-6.9 antibodies bind the native receptor expressed on the surface of human cells and do not cross-react with Plexin-B2 or Plexin-B3 (tested by immuofluorescence experiments, not shown). Moreover, after 30-60 min pretreatment with 100 μg/ml EC6.9, the binding of Sema4D to cells expressing Plexin-B1 is significantly reduced (see Fig. 3*D*), as well as the functional response that normally follows receptor activation. EC-6.9 antibodies were purified to homogeneity by affinity chromatography on protein A-Sepharose columns, according to standard methods (41).

Monoclonal antibodies directed to the activated form of human β1-integrin (clone HUTS-4, MAB2079Z) were purchased from Chemicon International, Inc. Anti-VSV (clone P5D4) and anti-vinculin (V-9131) antibodies were from Sigma. Anti-paxillin monoclonal antibodies (clone

349) were from Tranduction Laboratories, antibodies to FAK were from Transduction Laboratories (clone 77), while anti-phosphotyrosine antibodies (clone 4G10) were from Upstate Biotech.

Expression of proteins in mammalian cells

Wild-type and mutated plexins were expressed in COS cells by transient transfection of cDNA expression constructs, using a DEAE-dextran method. Plexin-B1 and its mutated forms Plexin-Δcyto and Plexin-B1-R1mut (Arg_{1687}Ala, kindly provided by A. Puschel) contained a VSV-tag sequence at the N terminus of the protein. Plexin-B1/A1 chimera was obtained by substituting the sequence encoding the cytoplasmic domain of Plexin-B1 with that derived from Plexin-A1. The construct expressing Plexin-B1-Δ10 (26) was a gift from J. Swiercz, who also provided the cDNA to express a truncated form of PDZ-Rho-GEF, containing PDZ and proline reach domains and associating with the plexin (aa 1-290, FLAG-tagged, indicated as F1).

3T3 mouse fibroblasts were efficiently transduced using lentiviral vectors. To this end, the same cDNAs used in standard transfections were subcloned into the lentiviral transfer plasmid pRRLsin.cPPT.hCMV.Wpre (kindly provided by L. Naldini, University of Torino). Viral vectors were then produced in 293T packaging cells, transiently cotransfected with a mix of transfer, envelope, and core-packaging plasmids, as described previously (42). Conditioned media containing the vectors were harvested 48 h after transfection and incubated with a fresh culture of sparse mouse 3T3 fibroblasts, in the presence of 8 μg/μl Polybrene for 16 h. The percentage of cells stably expressing the transgenes was between 70-95%, judged by immunofluorescent staining. NIH-3T3 cells expressing Plexin-B1 were further transfected with a G418-selectable expression construct for RhoQ63L. Positive clones were isolated and found to display significantly reduced collapsing response to Sema4D (from 50% of positive controls to complete abrogation).

Production of purified recombinant Sema4D

Soluble Sema4D was purified from the conditioned media of cells transfected with a cDNA expression construct, generated by recombinant PCR. This construct was designed to encode the extracellular region of Sema4D (residues 1-657 of the mature peptide), followed by a lysine residue and a carboxy-terminal histidine tag for purification. The cDNA was subcloned into the glutamine synthase-encoding expression vector pEE14 (43) and used to transfect Lec3.2.8.1 Chinese hamster ovary (CHO) cells, using Pfx-8 lipids (Invitrogen). Several clones, resistant to 15 μM methionine sulfoximine, secreted Sema4D-His at high concentrations. Sema4D was purified to homogeneity using metal-ion affinity chromatography (Ni-NTA agarose, Qiagen), followed by gel filtration chromatography (Superdex S200 HR10/30, Pharmacia Biotech), and judged to be >95% pure based on Coomassie-stained SDS-polyacrylamide gel after electrophoresis.

Immunofluorescence and binding assays

For immunofluorescence analysis, cells were cultured overnight on glass coverslips, previously coated with 10 μg/μl fibronectin (Sigma). After the indicated treatments, cells were washed once with PBS and fixed with 4% paraformaldehyde (PAF) in PBS for 10 min on ice. Cells were then

permeabilized for 5 min on ice with 0.2% Triton X-100 in PBS. To reduce background signal from anti-integrin-β1 antibodies, some cells were only subjected to mild permeabilization by extensive treatment with 4% PAF. After incubation with blocking buffer (PBS supplemented with 2% goat serum, Vector Laboratories Inc.), the cells were incubated with primary antibodies for 30 min at room temperature. F-actin was stained with fluorescein-labeled phalloidin (phalloidin-FITC, Sigma). Secondary anti-mouse-IgG antibodies were conjugated with AlexaFluor-546 (Molecular Probes). Stained cells were examined, and digital images are acquired, using a Zeiss Axyoskop microscope equipped with Bio-Rad Confocal Imaging System. The numbers of collapsed NIH-3T3 (defined as rounded cells having a diameter ≤20 μm) and of cells containing focal adhesions or stress fibers were counted independently by two persons, in at least two separate wells (n>100/well).

Binding assays with alkaline-phosphatase-conjugated semaphorins (Sema4D-AP and Sema3F-AP) were performed as described previously (31). After incubation with the ligands, cells were extensively washed, then fixed, heated for 15 min at 65°C in a waterbath to inactivate endogenous phosphatases, and finally incubated with AP cromogenic substrate *p*-nitrophenylphosphate (Sigma). Absorbance was eventually measured at 405 nm.

Cell-substrate adhesion and spreading assays

Cells were washed with PBS and then incubated with 1 mM EDTA in PBS, to allow detachment from culture dishes. The harvested cells were counted, spun, and resuspended in serum-free culture medium, containing 0.2% BSA; 1-5 x 10^4 cells were seeded in 96-microtiter wells, previously coated with adhesive substrates (as indicated in the text) and blocked with 0.2% BSA. Incubation was done at 37°C in a 5% CO% atmosphere. At the indicated times, the wells were delicately washed twice with PBS, and the adherent cells were fixed and subsequently stained with crystal violet and photographed with Leica DMLB microscope coupled to Leica DC300F camera. Spread cells were counted independently by two persons, in at least two separate wells (n>100/well). To quantify cell adhesion, the dye was then eluted from adherent cells with 10% acetic acid, and the absorbance was measured at 595 nM in a microplate reader.

Cell migration assay

Cell motility was assayed using Transwell® chamber inserts (Costar) with a porous polycarbonate membrane (8 μM pore size). The lower side of the membrane was coated with 10 μg/ml fibronectin for 2 h and then blocked with 0.2% BSA. Cells were detached with 1 mM EDTA, as above, and resuspended in serum-free medium containing 0.2% BSA (for haptotactic migration) or in 5% serum-containing medium (for chemotaxis experiments). Approximately 1 x 10^5 cells were added to the upper side of the porous membrane and allowed to migrate toward the lower chamber, containing the chemoattractants or Sema 4D. Cell migration was allowed to occur for the indicated times in a cell culture incubator. Then, the cells adherent to the upper side of the filter were mechanically removed, while those that migrated to the lower side were fixed with 11% glutaraldehyde and stained with crystal violet. Cells were then photographed and the dye solubilized in 10% acetic acid to measure absorbance with a microplate reader, as described above.

RESULTS

The cellular collapse elicited by Plexin-B1 does not rely on Rho-dependent kinase signaling pathway

We analyzed the functional response mediated by Sema4D in COS7 cells transfected to express the high affinity receptor Plexin-B1. As can be seen in Fig. 1*A*, while these cells are basally spread, the activation of plexin signaling induces a generalized retraction of pseudopodia and lamellipodia, eventually leading to cell rounding. Interestingly, collapsed cells display several tiny and branched retraction processes. This functional response is induced either in the presence or in the absence of calf serum (not shown). It appears as early as after 10 min of stimulation, and it is often complete within 30 min. Moreover, we observed that the collapsed cells are not dead and recover their normal phenotype upon ligand removal (not shown). Thus we conclude that, unlike plexins of A subfamily, Plexin-B1 alone is fully functional upon activation by its specific ligand Sema4D. In fact, co-expression of Neuropilin-1 with Plexin-B1 does not change the response (not shown).

To prove that the cellular collapse is dependent on the cytoplasmic domain of Plexin-B1, we analyzed cells expressing either a truncated form of Plexin-B1, lacking the entire intracellular portion (B1-Δcyto), or a chimeric TrkA-Plexin-B1 receptor, in which the extracellular domain of Plexin-B1 is substituted with that of the NGF receptor TrkA. The results, shown in Fig. 1*A*, demonstrated that the cytoplasmic domain of Plexin-B1 is both required and sufficient to induce the collapsing response. The C′-terminal tail of Plexin-B1 has been shown to associate PDZ-Rho-GEFs, potentially implicated in signal transduction (26). However, we found that neither deletions removing the PDZ domain binding motif (Plexin-B1-Δ10, ref 26), nor the coexpression of the PDZ domain of PDZ-Rho-GEF (F1, acting as dominant negative), could interfere with the collapsing response mediated by Plexin-B1 (Fig. 1*A*). As we reported previously (31), Plexin-Δ10 is expressed poorly on the cell surface; however, we found that the cells interacting with Sema4D could collapse. These results suggest that plexin signaling can occur even without the association with Rho exchangers.

We then analyzed the cellular collapse elicited by Sema4D in 3T3 mouse fibroblasts engineered to express Plexin-B1 (Fig. 1*B*). The functional response was quite similar to that observed in COS cells, but it occurred more rapidly. Time-lapsed movies of two representative experiments are provided as supplemental data (available at http://www.fasebj.org). The cellular mechanisms mediating semaphorin-dependent cell and axon collapse are currently unclear. Interestingly, we found that the collapsing response mediated by Plexin-B1 in fibroblasts is not blocked by inhibitors of Rho-dependent kinase (Y-27632) or myosin light-chain kinase (ML-7), both involved in stress fibers contraction (Fig. 1*B*). In addition, actin depolymerization induced by cytochalasin D (cytoD) did not interfere with the cellular collapse, whereas the stabilization of F-actin cables by treatment with jasplakinolide antagonized the functional response. It is known that cytoskeletal dynamics are functionally linked to integrin-mediated adhesion and that the latter is implicated in the regulation of axon guidance and cell migration. Interestingly, we observed that cells spread on higher concentrations of fibronectin were more resistant to semaphorin-induced cellular collapse, consistent with the presence of a higher number of adhesive structures (not shown). We also found that the collapsing response was inhibited by lysophosphatidic acid (LPA), an activator of Rho signaling known to induce larger and more

abundant focal adhesions or by co-expression of constitutive active RhoQ63L (Fig. 1*B*). Moreover, the inhibition of 12/15-lipoxigenase, the eicosanoid products of which are known to release cell-substrate adhesion, effectively hampered the cellular collapse mediated by Plexin-B1 activation, consistent with previous observations that it blocks the axonal collapse induced by Sema3A. We also performed plexin-dependent COS collapse assays in the presence of each of the above described drug treatments and obtained similar results to that in fibroblasts (not shown). In sum, these data indicate that the signaling pathway of Rho-dependent kinase is not required for plexin-mediated cellular collapse and suggest the involvement of another upstream regulatory mechanism, possibly related to cell-substrate adhesion.

Plexin activation leads to fast disassembly of focal adhesions

We therefore focused our analysis on the early events affecting actin cytoskeleton and focal adhesive complexes of 3T3 mouse fibroblasts, upon Plexin-B1 activation. Cells growing in presence of serum displayed a prominent actin meshwork of stress fibers and typical clustering of focal adhesion components, such as paxillin (shown in Fig. 2*A*) or vinculin (not shown). Upon treatment with purified Sema4D, focal adhesion components were rapidly dispersed (in 1-2 min) and the actin stress fibers disappeared thereafter (after 5 min; see Fig. 2*A*, right panel). The next step was cellular retraction and rounding up, eventually leading to the overt collapsed phenotype.

We also studied cells expressing a chimeric Plexin-B1/A1 receptor, to compare the functional response elicited by the cytoplasmic domain of two different plexins. We found that the intracellular signaling elicited by Plexin-B1 or Plexin-A1 equally led to rapid disassembly of adhesive structures (compare Fig. 2*A* and *B*), indicating that this is a common functional response to plexin signaling. In addition, since the mutation of a conserved arginine residue in the cytoplasmic domain of Plexin-A1 has been reported previously to abrogate the functional response (17), we analyzed the corresponding mutated form of Plexin-B1 (Plexin-B1-R1mut; B. Rohm and A. W. Püschel, unpublished). We found that the latter lost the ability to elicit both the collapsing response and the disassembly of focal adhesions and stress fibers, further suggesting that all these events depend on the same signaling pathway (Fig. 2*B*).

In addition, the disassembly of adhesive complexes mediated by plexins was confirmed at the molecular level by decreased phosphorylation levels of focal adhesion components, such as FAK, known to become tyrosine phosphorylated upon integrin-mediated adhesion (Fig. 2*C*).

Sema4D elicits the disassembly of focal complexes in cells expressing the endogenous receptor Plexin-B1

We then analyzed the functional response to Sema4D in human cells that endogenously express the receptor Plexin-B1, such as mammary carcinoma cells SKBR3 (shown in Fig. 3*A*) and neuroblastoma cells NB100 (Fig. 3*B*). We found that focal complexes disappeared almost completely after 30 min with purified Sema4D and that the cells underwent cellular collapse after ~1 h. Moreover, by using an antibody recognizing the activated form of human β1-integrin (clone HUTS-4), we found that Sema4D induced the relocalization of the integrin from focal complexes at the cell surface into the cytoplasm. Receptor specificity of the functional response elicited by Sema4D in SKBR3 cells was further demonstrated by using anti-Plexin-B1 blocking antibodies EC6.9 (Fig. 3*C*). Production and characterization of these antibodies are described in

Methods. Figure 3*D* demonstrates the specificity of EC6.9 in blocking the interaction between Sema4D and its receptor Plexin-B1. The collapsing response mediated by Sema4D in NIH-3T3 cells was also significantly inhibited by EC-6.9 (not shown).

In analogy to that found in fibroblasts and COS cells, actin depolymerization or Rho-dependent kinase inhibition did not block the functional response to Sema4D in SKBR3, while the latter was hampered by the lipoxygenase inhibitor CDC, preventing release of cell-substrate contacts, as well as by LPA-mediated increase of focal adhesive complexes (Fig. 3*E*).

Plexin activation inhibits integrin-dependent cell adhesion and cell spreading on the extracellular matrix

We then assayed whether the disassembly of focal adhesive complexes mediated by plexin activation could hinder cell attachment to the ECM. It is known that cell-substrate adhesion depends primarily on integrin molecules. In addition, integrin coupling to the cytoskeleton in focal adhesive complexes allows lamellipodia protrusion, cell spreading, and migration on the ECM. As shown in Fig. 4*A*, at early time points, plexin activation inhibited the adhesion to fibronectin of 3T3 fibroblasts, without interfering with adhesion to the integrin-independent substrate poly-L-lysine. At later times, many cells could adhere to fibronectin also in presence of the semaphorin; however, they never spread on the substrate (Fig. 4*B*). Notably, instead of forming lamellipodia, semaphorin-treated cells displayed several fine long and branched extensions. Furthermore, Sema4D inhibited substrate adhesion and spreading of cells expressing the chimeric receptor Plexin-B1/A1 but not of cells expressing a truncated receptor lacking the cytosolic domain or the inactive receptor Plexin-B1-R1mut (Fig. 4*A–B* and data not shown). Inhibition of cell adhesion and spreading was furthermore observed on vitronectin, another adhesive substrate for fibroblasts engaging a different set of integrin receptors (not shown). Notably, the treatment with blockers of the Rho signaling pathway during adhesion assays did not interfere with semaphorin-mediated inhibition (not shown).

We also observed semaphorin-dependent inhibition of substrate adhesion and cell spreading in epithelial SKBR3 cells, expressing endogenous Plexin-B1 (Fig. 4*C*). The inhibitory effect was clear both basally, and in the presence of Heregulin-β1 (HRG), a pro-adhesive and motogenic factor for these cells (44, 45), inducing the formation of wide lamellipodia. These results suggest that plexin activation regulates the functional coupling of adhesive structures to the actin cytoskeleton, which is crucially required for cell spreading, lamellipodia extension, and cell migration.

Plexin activation inhibits cell migration

In vivo, cell-substrate adhesion is a pivotal signal to regulate cell migration, leucocyte extravasation, and cell differentiation. We therefore tested if the inhibition of integrin-mediated adhesion by plexins would also result in reduced cell migration on a gradient of ECM substrate ("haptotaxis"). As shown in Fig. 4*D*, while NIH-3T3 cells efficiently moved along a gradient of fibronectin, plexin activation inhibited this haptotactic migration. Consistently with the results on adhesion and spreading, both Plexin-B1 and Plexin-A1 cytoplasmic domains were able to mediate this response. Although in this assay SKBR3 cells do not display spontaneous motility, we found that Sema4D could efficiently inhibit their chemotactic migration elicited by an

attractive gradient of Heregulin-β1 (Fig. 4*E*). Therefore, by inhibiting cell adhesion and lamellipodia protrusion, plexin signaling also regulates directional cell migration.

DISCUSSION

Plexins are semaphorin receptors and their signaling guides cell migration and axon extension (1, 46). Although semaphorins have been extensively studied for their ability to elicit axon turning and axon retraction (known in vitro as "axonal collapse"), their functional role as regulators of cell migration has been addressed only recently. Among migrating cells responsive to semaphorins, there are neural crest cells (8, 47), oligodendrocytes (11), leucocytes (13, 48), as well as endothelial (12) and epithelial cells (49). Notably, most prominent phenotypes observed in semaphorin-deficient mice have been attributed to defective migration of neural crest cells and endothelial cells (50-52).

It has been shown in vitro that Sema3A induces the "collapse" of neural crest cells (8), as well as of COS cells transfected to express both Neuropilin-1 and Plexin-A1 (7). The cellular collapse is currently explained as a generalized retraction of extending cellular processes (lamellipodia and pseudopodia). In this study, we show that Sema4D elicits the "collapse" of cells transfected to express Plexin-B1 alone and of cells that endogenously express this receptor. We demonstrate that the intracellular domain of plexins is fully responsible for the collapsing response. Notably, both the cytoplasmic domains of Plexin-B1 and Plexin-A1 can mediate the functional responses described in this study. We also observed identical responses in cells expressing a constitutively active form of Plexin-A1 (construct described in ref. 53; data not shown). This is consistent with the high sequence conservation of the cytoplasmic domain of all plexins. Moreover, site directed mutagenesis of a conserved arginine in the intracellular domain of Plexin-B1 (Plexin-B1-R1mut, generously provided by A. Puschel) leads to functional inactivation of the receptor, consistent with that reported for Plexin-A1 (17). Importantly, Plexin-B1-R1mut retains full ligand binding ability (not shown), and, therefore, it has been further used in this paper to verify signal specificity.

The molecular mechanisms mediating plexin-dependent cell collapse are currently unknown. We have described previously how the tyrosine kinase receptor Met specifically associates with Plexin-B1 and mediates some of the functions of Sema4D (49); however, Met is not expressed in NIH-3T3 mouse fibroblasts, nor could Met signaling account for the described collapsing response. Recently, the activation of the small GTPase RhoA has been specifically implicated in the axonal collapse mediated by plexins of the B subfamily (26). The suggested mechanism is by myosin-mediated pulling on F-actin cables, triggered by the effector molecule ROCK. Surprisingly, we did not observe a functional requirement for the association with PDZ-Rho-GEF, nor did we find inhibition of the semaphorin-induced cellular collapse in presence of drugs blocking ROCK or leading to F-actin depolymerization. In addition, starved fibroblasts with no stress fibers underwent cellular collapse equally well upon plexin activation (data not shown). This is consistent with the observation that the axonal collapse elicited by Sema3A or Sema3F is ROCK independent (19, 22).

In this study, we focused on the role of plexin signaling in the guidance of cell migration. Although an analogy between the functional regulation of lamellipodia and filopodia in the leading edge of motile cells and in the growth cone of extending axons has been proposed, some

of the mechanisms appear to be different. For example, Rho and ROCK activation (e.g., mediated by LPA) induces retraction of neuronal axons, while it elicits cell spreading and cell migration in epithelial and mesenchymal cells. Conversely, the inhibition of Rho and ROCK promotes axonal extension and antagonizes collapsing signals, while it can hamper cell motility (54). Clearly, major differences subsist between the cytoskeletal organization of axons and migrating cells. For instance, microtubule- or actin-depolymerizing drugs may lead to retraction of the axonal shaft in vitro, whereas the same drugs have been extensively used to study the role of cytoskeletal components in the motility of adhering cells. In this perspective, our finding that the cellular collapse induced by semaphorins is independent from ROCK- and myosin-mediated cell contraction, or F-actin depolymerization, provides relevant information on plexin-specific signaling mechanisms.

What then are the molecular mechanisms eventually leading to the collapsing response mediated by semaphorins? One intriguing suggestion emerged from the fact that GSK-3β, a mediator of Sema3A-induced collapse (55), is silenced during axon outgrowth and cell migration by integrin-linked kinase (ILK), active into ECM-adhesive structures (56). Interestingly, we found that the cellular collapse elicited by Sema4D is prevented by inhibiting 12/15-lipoxigenase, the products of which release cell-substrate adhesion (57); and by LPA, which activates RhoA and ROCK and leads to increased stress fibers and stiffer focal adhesions (58). In fact, in this study we demonstrate that the intracellular signaling mediated by different plexins first leads to the disassembly of focal adhesive structures, followed by pseudopodia retraction and cellular collapse. The mechanistic role of the inhibition of cell-substrate adhesion mediated by plexins is strongly suggested by the fact that increasing the number and stability of adhesive structures by multiple and distinct mechanisms (high substrate concentrations, 12/15 lipoxygenase inhibition, F-actin stabilization by jasplakinolide, Rho activation) results in partial or complete block of semaphorin-induced cellular collapse.

Cell adhesion to the ECM is mediated by adhesive complexes containing the integrins (34). In fact, on engagement with extracellular substrates, integrins recruit intracellular adaptors and signal transducers, as well as cytoskeletal components, leading to the formation of the so-called “focal complexes” and “focal adhesions.” Focal adhesive structures not only link integrins to the cytoskeleton (outside-in signaling) but also are pivotal in the regulation of integrin affinity and avidity for the ECM (inside-out signaling). Perturbation of integrin-mediated adhesion is known to cause cell rounding and cell detachment, and this may result from (largely unknown) regulatory mechanisms impinging on the cytoplasmic domain of integrins. For example, it has been reported that the tyrosine kinase receptor EphA negatively regulates cell adhesion by recruiting the tyrosine phosphatase SHP-2, which in turn dephosphorylates FAK (59). We did not find a specific association of plexins with FAK or SHP2, nor did we observe any functional block by treatment with the tyrosine phosphatase inhibitor sodium ortovanadate (not shown), suggesting that a distinct pathway is triggered downstream to semaphorins. Another example is cell rounding that occurs before mitosis, recently shown to require ROCK-mediated cell contraction (60). However, the collapsed phenotype induced by semaphorins, with the presence of several fine and branched cellular processes, seems quite distinct from that of mitotic cells. In addition, we have shown here in different cell types that semaphorin-mediated cell rounding is ROCK-independent. Furthermore, it is known that F-actin depolymerization weakens cell-substrate adhesion and may eventually lead to cell rounding. However, we have shown that adhering fibroblasts without stress fibers are still capable of undergoing the collapsing response

mediated by plexin activation, suggesting that actin rearrangement is a consequence rather than a cause of loss of adhesion. At this stage, our conclusion is that plexin signaling negatively regulates integrin-mediated adhesion via a still undetermined mechanism. Interestingly, we found that plexin activation (even when sustained for hours) attenuated cell-substrate adhesion but rarely caused cell detachment. We thus postulate that this mechanism may serve as a molecular clutch, putting adhesion-dependent protrusion of cellular processes "on hold," and may allow redirecting cell migration or axonal extension.

In this study, we also show that plexin activation inhibits integrin-mediated adhesion of suspended cells to ECM substrates. These results are consistent with those recently obtained in endothelial cells treated with Sema3A (40). Sema3A seems to control endothelial cell motility and vascular morphogenesis by regulating integrin-mediated adhesion. Here we describe that this is a major common functional response mediated by different plexins and in various cell types; a function that specifically depends on the cytoplasmic domain of plexins and can be blocked by point mutations in conserved residues. Importantly, we further show that plexin signaling blocks the extension of lamellipodia, responsible for cell spreading, even when cell adhesion is not abolished. This suggests that plexin signaling impinges on the functional coupling between integrin-mediated adhesion and cytoskeletal dynamics occurring in focal complexes. Moreover, the extension of lamellipodia is required for cell migration, and we found that plexin signaling could efficiently inhibit both haptotactic and chemotactic migration.

Intriguingly, it was recently reported that another semaphorin, Sema7A, is capable of engaging β-integrins and eliciting MAPK-activation and axonal outgrowth (39). It should be noted that this function is not mediated by the interaction of the semaphorin with its putative receptor Plexin-C1. This is similar to that reported for Sema4D, and its low affinity receptor CD72, and suggests that certain semaphorins may interact with low affinity receptors to trigger plexin-independent signaling pathways.

In conclusion, semaphorins regulate several biological events, including guidance of migrating cells and extending growth cones; however, the cellular mechanisms primarily mediating these effects have not been elucidated. We found that the conserved cytoplasmic domain of plexins mediates the disassembly of focal adhesive structures, leading to inhibition of cell-substrate adhesion and hampering the extension of lamellipodia and cell motility. Although further studies will be required to establish the functional role in vivo of this mechanism, our findings strongly indicate that it could account for the regulation of cell migration and axon guidance by semaphorins.

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Fig. 1A

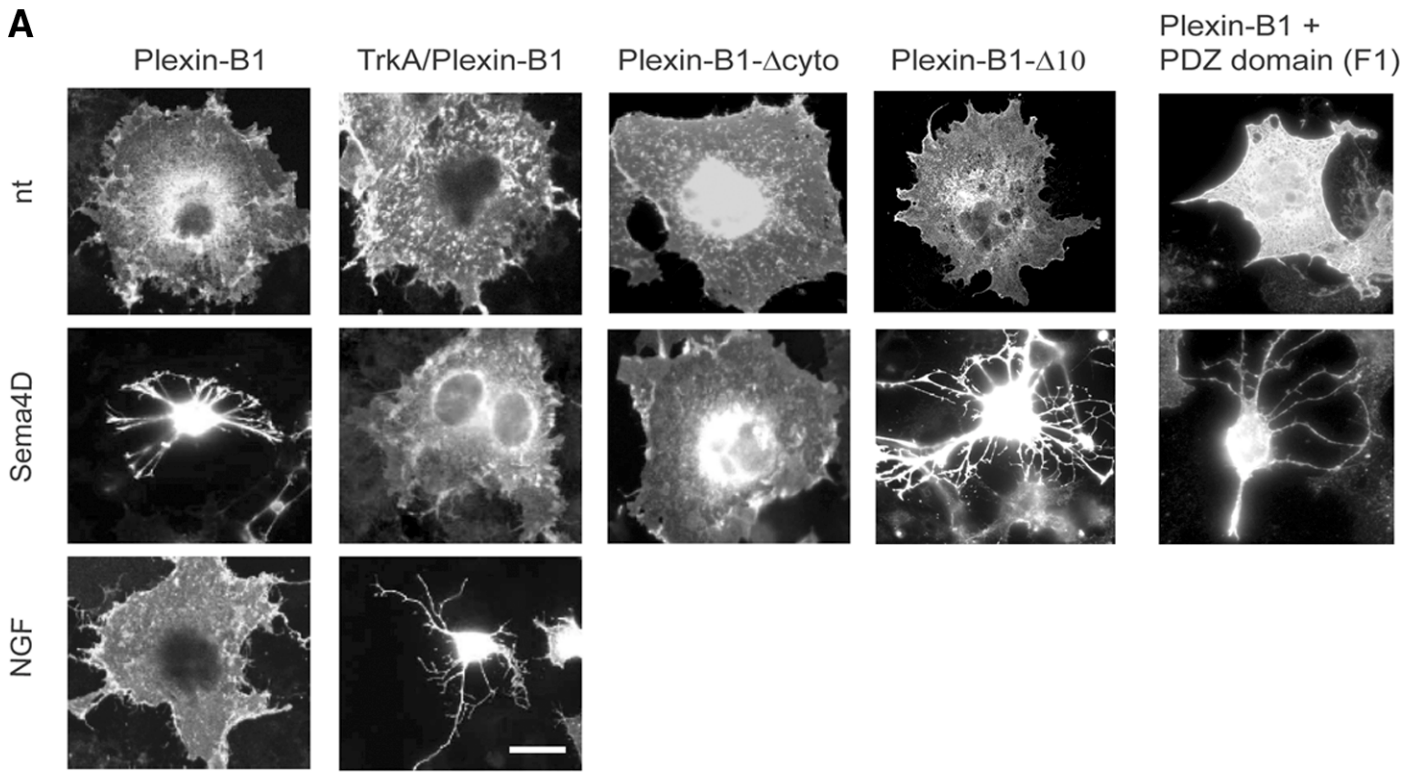

Figure 1A. Cellular collapse elicited by cytoplasmic domain of Plexin-B1 is not due to ROCK-mediated cell contraction. COS7 cells, transfected to express the indicated proteins, were treated for 45 min with Sema4D, or NGF, as indicated. Pictures show representative fields of immunofluorescence analysis using antibodies directed against the receptors (anti-VSV), with the exception of the 2 rightmost pictures, where anti-FLAG antibodies revealed the coexpression of the PDZ domain of PDZ-RhoGEF in collapsed cells (that also express Plexin-B1, not shown). Scale bar: 20 μm. The cytoplasmic domain of Plexin-B1 is required and sufficient to trigger cellular collapse, but its association with PDZ-domain containing signal transducers does not seem to be functionally required.

Fig. 1B

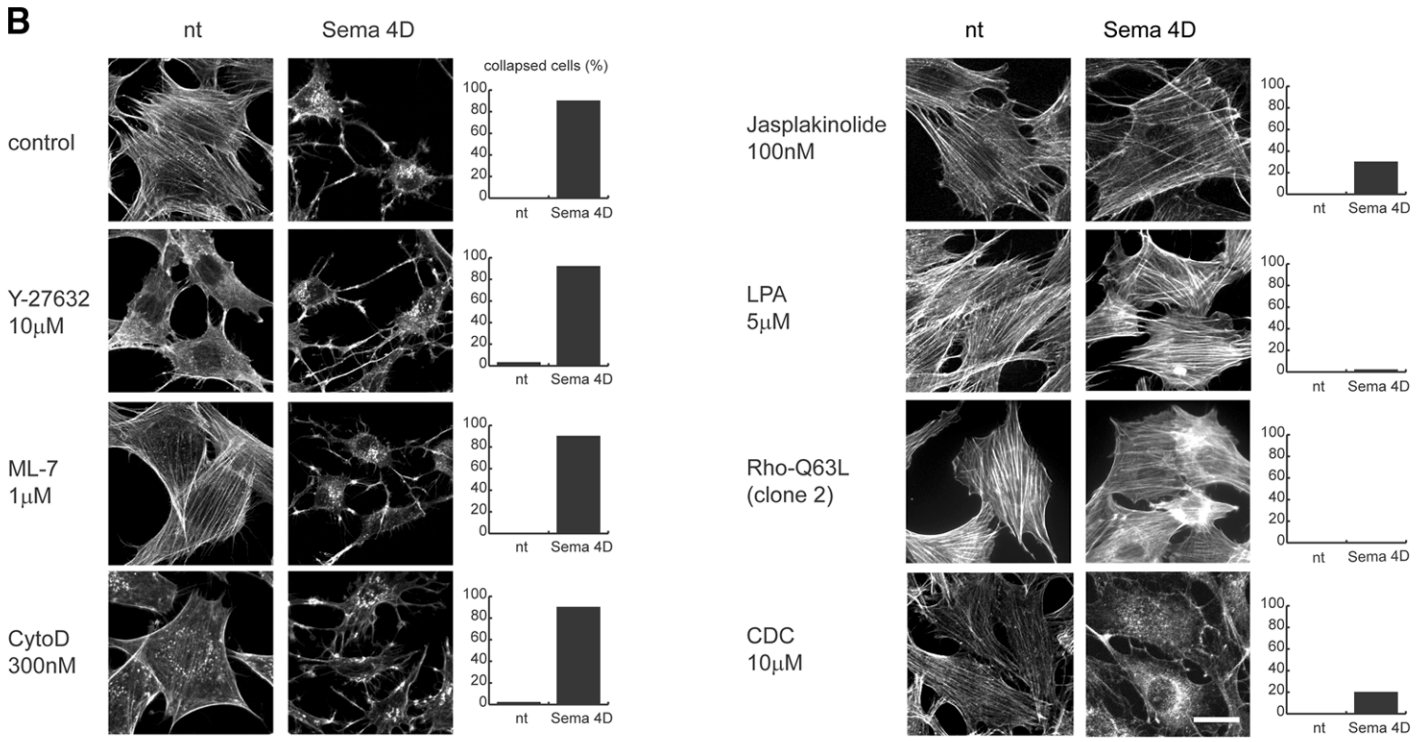

Figure 1B. Cellular collapse elicited by cytoplasmic domain of Plexin-B1 is not due to ROCK-mediated cell contraction. NIH-3T3 cells expressing Plexin-B1, either in the presence of the indicated drugs or transfected to coexpress constitutively active RhoQ63L, were incubated with 1 nM Sema4D for 15 min and then fixed and probed with phalloidin-FITC. Scale bar: 20 μm. Percentage of collapsed cells for each condition is indicated on right by graph bars. Preventing stress fibers contraction by inhibiting ROCK (with Y-27632) or myosin-light chain kinase (with ML-7), as well as depolymerizing F-actin (with cytochalasin D) did not interfere with the collapsing response. In contrast, stabilization of actin cables and adhesive structures (with jasplakinolide, LPA, or CDC), as well as coexpression of constitutively active Rho inhibited fibroblast cell collapse. Data are representative of at least 3 experiments displaying consistent results. Time-lapse movies of collapsing response in mouse fibroblasts are provided as supplementary material (available at http://www.fasebj.org).

Fig. 2

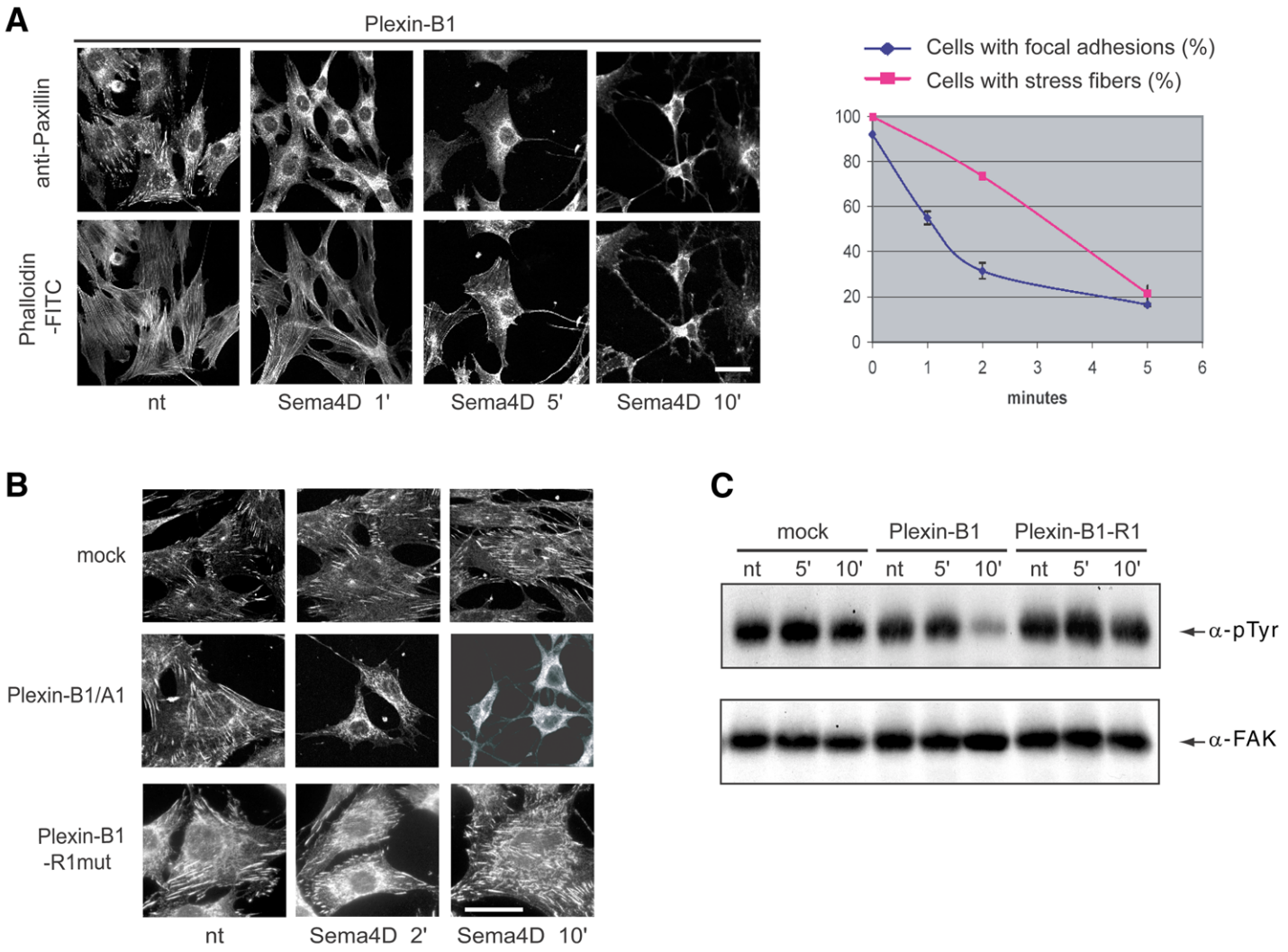

Figure 2. Plexin activation leads to disassembly of focal adhesions and F-actin cables. ***A)*** NIH-3T3 fibroblasts expressing Plexin-B1 were treated with 5 nM Sema4D for the indicated times. Focal adhesions and F-actin cables are revealed by immunofluorescence with anti-paxillin antibodies and staining with phalloidin-FITC, respectively. Focal adhesions were dispersed after 1 min, while F-actin cables disappeared after 5 min. Cellular collapse ensued after 10 min. As shown on the right, quantitation of effects was done by scoring the presence of focal adhesions and stress fibers in 2 duplicated wells (n>100). Scale bar: 40 µm. ***B)*** NIH-3T3 fibroblasts, either mock transfected or expressing the indicated plexin receptors, were treated with Sema4D for 2 or 10 min and analyzed by immunofluorescence staining with anti-paxillin antibodies. Scale bar: 40 µm. ***C)*** NIH-3T3 fibroblasts expressing indicated proteins were treated with 5 nM Sema4D for indicated times. Immunopurified focal adhesion kinase (FAK) was then analyzed by Western blotting with anti-phosphotyrosine antibodies. Filters were subsequently probed with anti-FAK antibodies to verify protein levels.

Fig. 3(A-B)

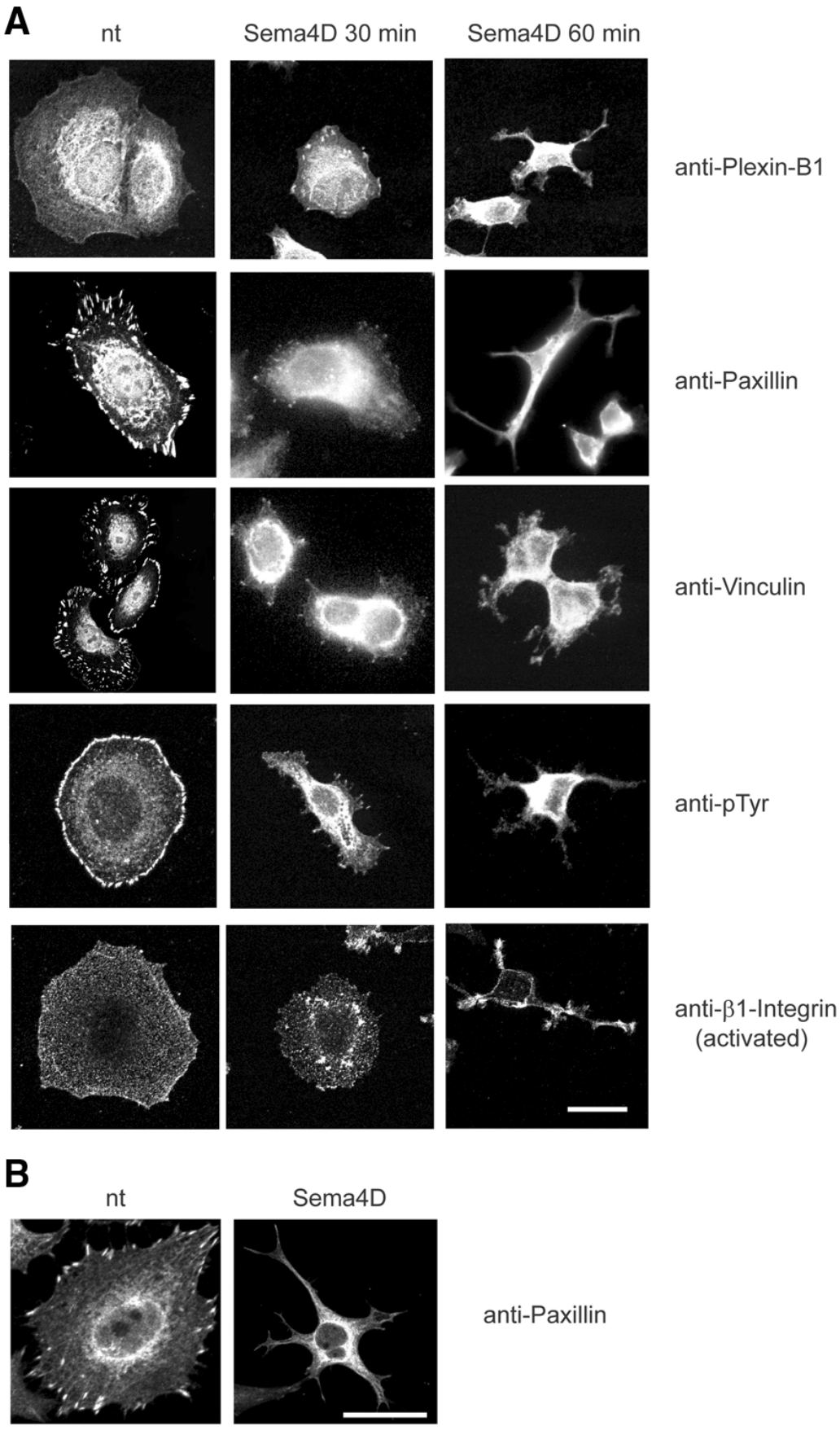
A
nt
Sema4D 30 min
Sema4D 60 min
anti-Plexin-B1
anti-Paxillin
anti-Vinculin
anti-pTyr
anti-β1-Integrin
(activated)
B
nt
Sema4D
anti-Paxillin

Fig. 3(C-E)

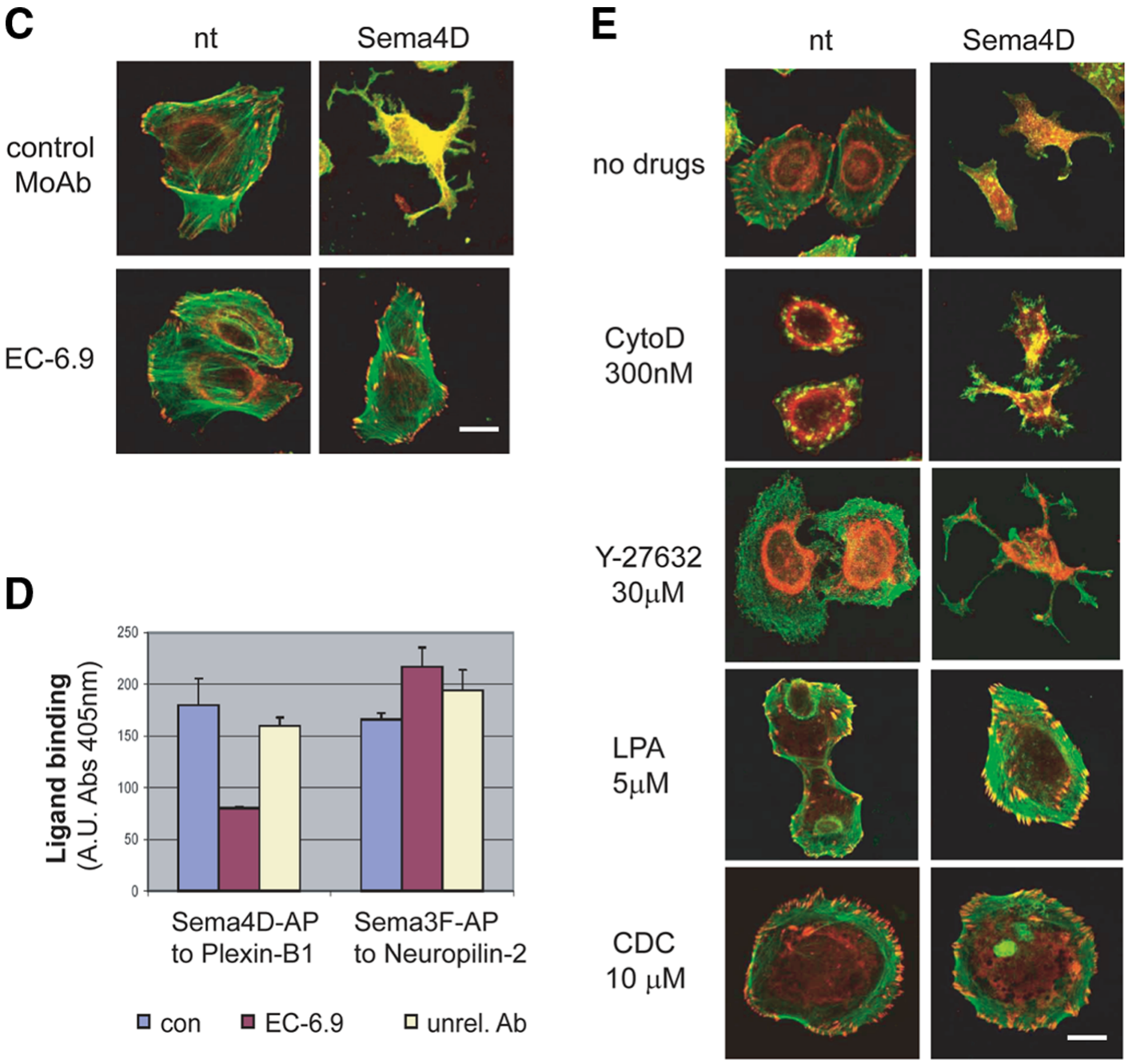

Figure 3. Sema4D elicits disassembly of focal complexes in cells expressing the endogenous receptor. ***A***) SKBR3 human mammary carcinoma cells were treated with 10 nM Sema4D for the indicated times and then analyzed by immunofluorescence using the indicated monoclonal antibodies. Scale bar: 40 μm. ***B***) NB100 neuroblastoma cells were treated with Sema4D for 60 min and analyzed by immunofluorescence with anti-paxillin antibodies. Scale bar: 20 μm. ***C***) Inhibition of the functional response induced by Sema4D by pretreating SKBR3 cells for 60 min with 100 μg/ml EC-6.9 monoclonal antibodies, directed against the extracellular domain of Plexin-B1. Scale bars: 40 μm. ***D***) Functional blocking activity of EC-6.9 MoAbs was tested by binding assays in COS cells overexpressing Plexin B1 or Neuropilin-2 (NP-2). Cells were pretreated for 30 min with 50 μg/ml of either affinity purified EC-6.9 or an unrelated antibody and subsequently incubated with the respective ligands Sema4D and Sema3F, fused to alkaline phosphatase (AP), in the presence of the antibodies. Interaction was measured as cell-bound AP activity, as described previously (31). Binding of Sema4D to Plexin-B1, but not that of Sema3F to NP-2, was efficiently inhibited. ***E***) Sema4D-induced collapse of SKBR3 in the presence of the indicated inhibitors. Results are consistent with that observed for 3T3 fibroblasts (shown in **Fig. 1*B***). Note that stabilization of cell-substrate adhesion (due to LPA or lipoxigenase inhibitor CDC) prevents cellular collapse. Scale bar: 40 μm.

Fig. 4

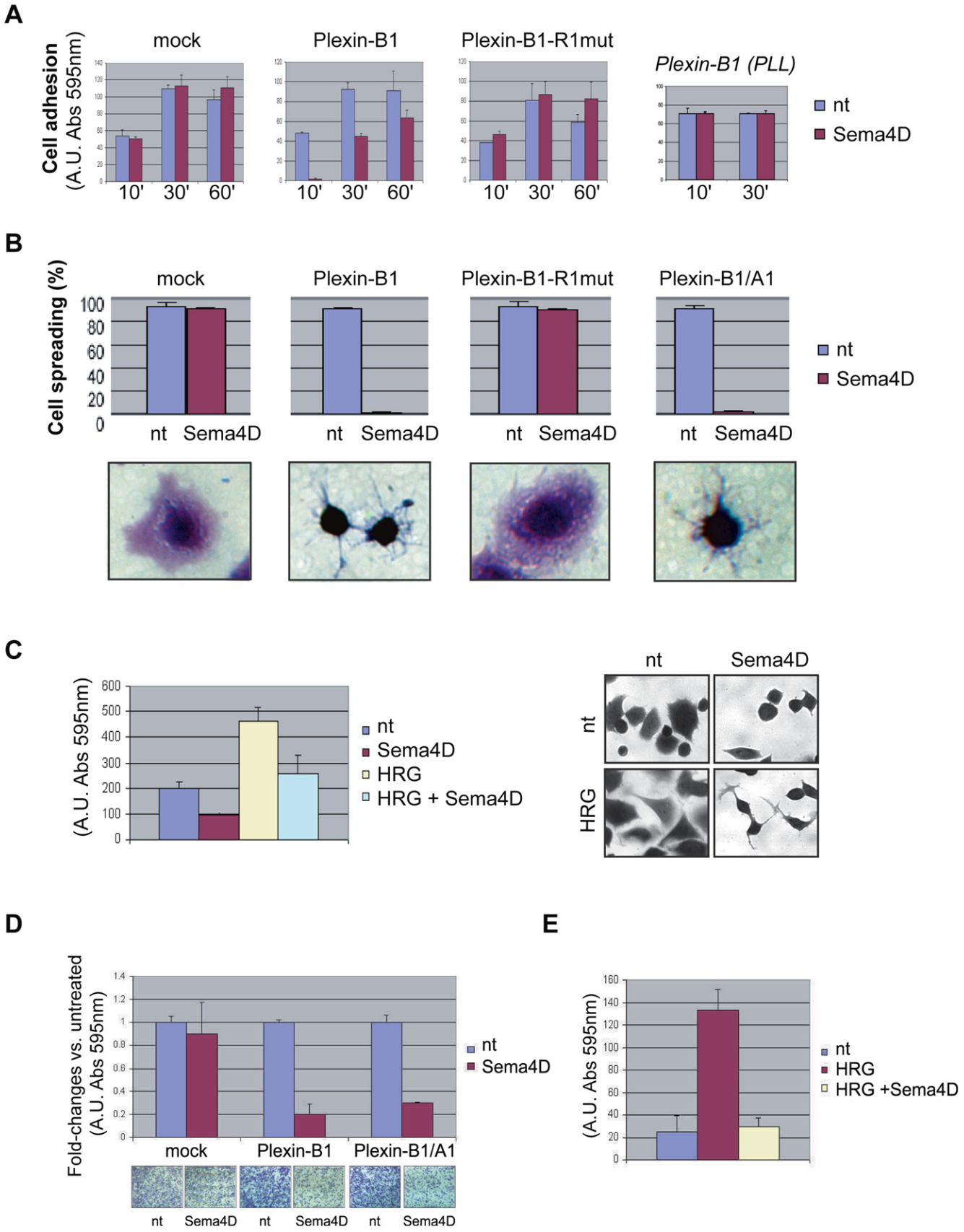

Figure 4. Plexin activation inhibits integrin-dependent cell adhesion, cell spreading on extracellular matrix and cell migration. ***A–B***) NIH-3T3 cells (mock transfected, or expressing Plexin-B1, the chimeric receptor Plexin-B1/A1, or the nonfunctional receptor Plexin-B1-R1mut) were detached with 1 mM EDTA. Cell were seeded in microtiter wells coated

with 10 μg/ml of fibronectin or alternatively coated with 1 μg/ml poly-L-lysine (PLL) where indicated. After indicated times in the presence or absence of 5 nM Sema4D, the adherent cells were fixed, stained with crystal violet, and photographed. Cell adhesion was eventually measured by eluting dye and reading absorbance at 595 nm (***A***). Before that, percentage of cell spreading at 1 h was determined by evaluating over 100 cells in 2 distinct wells (***B***); micrographs at the bottom depict representative fields of cells stimulated with Sema4D. Results are representative of at least 3 experiments performed in duplicate, with consistent results. Intracellular signaling of Plexin-B1 and Plexin-A1 hampers cell-substrate adhesion and completely blocks the extension of lamellipodia and cell spreading. A.U. = arbitrary units. ***C***) Adhesion and spreading on fibronectin of SKBR3 cells, expressing endogenous Sema4D-receptor, were tested as above. Cell-substrate adhesion was quantified after 3 h incubation in the presence or absence of Sema4D and/or 0.2 nM Heregulin-β1 (HRG), a known motogenic factor for these cells. Micrographs on right (taken at 3 h) show that cell spreading was inhibited by Sema4D, both basally and in the presence of the motogenic factor Heregulin-β1. ***D***) Haptotactic migration of NIH-3T3 cells (mock transfected or expressing the indicated plexins) on a gradient of fibronectin, in absence of calf serum and in presence or absence of 5 nM Sema4D (see Methods for details). After 4 h, migrated cells were fixed and stained with crystal violet (see pictures at bottom). Cell migration was quantified by A_{595nm}, as above. Results are representative of at least 3 experiments performed in duplicate with consistent results. ***E***) Sema4D inhibits chemotactic migration of SKBR3 cells in response to 0.2 nM Heregulin-β1. Cell migration, after 16 h, was measured as above.