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Plexin B1 inhibits integrin-dependent pp125^{FAK} and Rho activity in melanoma

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Summary

Semaphorins are secreted and membrane bound proteins that regulate axon guidance through receptors Plexins and neuropilins. Plexin B1, the Semaphorin 4D receptor, is a recently described tumor suppressor protein for melanoma. We recently showed that Plexin B1 abrogates activation of the oncogenic receptor, c-Met, by its ligand, hepatocyte growth factor (HGF), in melanoma. We have now investigated the effect of Plexin B1 on integrin-dependent pp125^{FAK} activation, and the small GTP-binding protein Rho, in melanoma. Integrin receptors and Rho play critical roles in melanoma progression, through regulation of migration, proliferation and apoptosis. We engineered two human melanoma cell lines expressing Plexin B1 and analyzed integrin-dependent migration, integrin-dependent pp125^{FAK} activation, and Rho activity. Results show that Plexin B1 abrogates integrin-dependent migration and activation of pp125^{FAK}. We also show that Rho activity is significantly reduced in cells expressing Plexin B1, and that Plexin B1 suppresses HGF-dependent Rho activation.

Keywords

melanoma; semaphorin; plexin; integrin; Rho

Introduction

Plexin receptors are a family of highly conserved transmembrane proteins, which alone or in cooperation with neuropilins, transduce effects of semaphorins (Castellani and Rougon, 2002). Semaphorins are secreted or membrane bound proteins originally described in the nervous system, but are also expressed in lung, kidney, bone and lymph tissue (Fujisawa, 2004; Moretti et al., 2006; Potiron et al., 2009; Takegahara et al., 2006). The best characterized effects of Plexin receptor signaling are on remodeling of the actin cytoskeleton (Bagnard, 2001; Korostylev et al., 2008; Nakamura et al., 2000). Plexin receptors from each of the four Plexin families regulate cell adhesion, cell migration, axon guidance, and neuronal pathfinding (Nakamura et al., 2000; Negishi et al., 2005). The downstream targets of Plexins include integrins, Rho GTP-binding proteins, cofilin and Ras, which control multiple facets of cell behavior, including cytoskeletal remodeling and cell migration.

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Loss or gain of Plexins and their cognate ligands, as well as mutations in Plexin receptors, are described in a variety of malignancies (Neufeld et al., 2005). Plexin B1, the receptor for semaphorin 4D, is mutated in prostate carcinoma (Wong et al., 2007), and its expression is down-regulated in renal cell carcinoma (Gomez Roman et al., 2008) and breast carcinoma (Rody et al., 2007). In breast carcinoma, a recent study has shown that loss of Plexin B1 is strongly correlated with poor prognosis (Rody et al., 2009). Our laboratory is interested in the role of Plexin receptors in melanocyte and melanoma biology. Melanocytes are derived from the neural crest, and migrate to the skin, where they reside in the basal epidermal layer. Although the genesis of melanoma is complex, melanomas are thought to arise from a stepwise progression of loss or gain of tumor suppressor and tumor promoter proteins, along with epigenetic changes, resulting in invasion, migration and metastasis. Metastatic spread of melanoma is the principle cause of morbidity and mortality associated with this disease. Our previous work showed that Plexin B1 expression is lost in melanoma in vivo, particularly in deeply invasive and metastatic tumors (Stevens et al., 2010). In combination with work showing that introduction of Plexin B1 into human melanoma cell lines abrogates metastasis in a mouse model (Argast et al., 2009), the data support a role for Plexin B1 as a tumor suppressor protein for melanoma. Although the mechanism by which loss of Plexin B1 promotes melanoma progression remains to be fully defined, we identified the c-Met receptor as a downstream target of Plexin B1 in melanoma (Stevens et al., 2010). Introduction of Plexin B1 into human melanoma cell lines significantly inhibited activation of the c-Met receptor by its ligand, hepatocyte growth factor (HGF). Because c-Met is frequently activated in metastatic melanomas, suppression of c-Met signaling is a potential mechanism by which Plexin B1 suppresses late stages of melanoma progression (Halaban et al., 1993).

Integrins are heterodimeric proteins composed of α and β chains that bind extracellular matrix proteins, such as fibronectin, collagen, laminin and vitronectin (Arnaout et al., 2007). Integrins link the cell membrane to the extracellular matrix through attachment of the cytoplasmic tail of the β chain to cytoskeletal associated proteins such as talin and α–actinin. Integrin activation results in pleiotropic effects including cell migration, invasion through basement membranes, and resistance to apoptosis (Reddig and Juliano, 2005). Integrin activation stimulates the phosphorylation and activation of focal adhesion kinase ($pp125^{FAK}$; Mitra et al., 2005) which controls several integrin-dependent processes, including cell migration, and cell survival (Tomar and Schlaepfer, 2009). The Rho family of small GTP-binding proteins includes Rho, Rac and Cdc42. The principle effect of Rho proteins is on the actin cytoskeleton, with each protein having a well defined role in the formation of stress fibers (Rho), lamellipodia (Rac) and filopodia (Cdc42) in virtually all cells studied (Hall et al., 1993; Heasman and Ridley, 2008). Rho proteins alternate between active (GTP-bound) and inactive (GDP-bound) forms. In addition to effects on cytoskeletal remodeling, signaling by Rho proteins impacts cell proliferation, gene expression, and apoptosis, through signaling to diverse downstream targets (Ridley, 2001). Integrins and Rho share multiple downstream targets and are cross-activating or inhibiting, depending upon the cell type (Barry et al., 1997; Hotchin and Hall, 1995).

Our prior investigation into signaling pathways of Plexin B1 in melanoma suggest that inhibition of c-Met signaling is an important mechanism by which Plexin B1 suppresses melanoma progression (Stevens et al., 2010). Constitutive signaling of c-Met stimulates $pp125^{FAK}$ and Rho activation, leading to tumor progression (To and Tsao, 1998). Further, increased expression and activation of $pp125^{FAK}$ and Rho are described in many malignancies, including melanoma, independent of c-Met receptor activation (Golubovskaya et al., 2009; Hao et al., 2009; Lydolph et al., 2009; Narumiya et al., 2009; Routhier et al., 2010; Timar et al., 1997). In this report we have studied two human metastatic melanoma cell lines (YUSIK and YUMAC), engineered to express human Plexin B1, to examine

effects of Plexin B1 on integrin-dependent pp125^{FAK} activation and Rho activity. YUSIK and YUMAC melanoma cells do not show constitutive activation of the c-Met receptor, which permits identification of downstream targets of Plexin B1, independent of its suppressive effects on c-Met. Our data suggest that Plexin B1 has multiple targets that contribute to its role as a tumor suppressor protein for melanoma.

Results

Plexin B1 suppress migration of YUSIK melanoma towards fibronectin, but not invasion through a basement membrane

Migration and invasion are key processes in melanoma metastasis. To assess the effect of Plexin B1 on migration of melanoma towards fibronectin, YUSIK cells were placed in basal medium for 18 h and then seeded in the upper well of a 96 well migration chamber. The bottom wells contained basal medium with fibronectin, or basal media alone. Cells were allowed to migrate for 24 h (Figure 1A). Cells expressing empty vector ("LacZ") and cells expressing Plexin B1 ("PBA") showed dose-dependent migration towards fibronectin. The rate of migration was significantly higher ($P < 0.001$) at a fibronectin concentration of 50 μg/ml. The addition of recombinant Sema4D did not affect migration of PBA cells (data not shown), consistent with prior reports that the receptor signals constitutively (Conrotto et al., 2004; Giordano et al., 2002; Stevens et al., 2010). To determine if Plexin B1 abrogates invasion through a basement membrane, cells were placed in the top well of a matrigel invasion chamber and allowed to invade for 48 h (Figure 1B). There was no significant difference in invasion of Plexin B1 expressing cells, compared with LacZ controls ($P = 0.16$). Similar results were obtained when invasion was carried out for 24 h (data not shown).

Plexin B1 abrogates integrin-dependent activation of pp125^{FAK}

pp125^{FAK} is a non-receptor tyrosine kinase localized to focal contacts, and was originally identified as a tyrosine phosphorylated protein in pp60$^{v\text{-}src}$ transformed cells (Kanner et al., 1990). Auto-phosphorylation of pp125^{FAK} occurs upon integrin activation, and is closely correlated with its' kinase activity (Hanks et al., 2003). We examined levels of active (phosphorylated) pp125^{FAK} in response to adhesion to fibronectin, an integrin-dependent process, in LacZ and PBA cells. Serum-starved cells were plated on poly-L-lysine, which does not activate integrins, or fibronectin, for 1 h. Unattached cells were rinsed off, and the remaining cells were lysed and blotted for active pp125^{FAK}. Figure 2A shows a representative Western blot from three individual experiments; Figure 2B shows densitometry analysis of active pp125^{FAK} normalized to total pp125^{FAK}. Activation of pp125^{FAK} in response to attachment to fibronectin was significantly higher ($P < 0.05$) in LacZ compared with PBA cells. In LacZ cells, active pp125^{FAK} increased an average of 2-fold when attached to fibronectin, compared with PBA cells, in which active pp125^{FAK} was increased by 1.4-fold. Similar experiments were performed in the second human meta-static melanoma cell line (YUMAC) engineered to express the Plexin B1 receptor. Because this cell line attached poorly to poly-L-lysine, activation of pp125^{FAK} was only tested in cells attached to fibronectin. Figure 2C shows Western blots from two experiments; Figure 2D show densitometry analysis in which active pp125^{FAK} is normalized to total pp125^{FAK}. Following attachment to fibronectin, YUMAC LacZ cells showed a 1.4 fold increase in active pp125^{FAK} compared with 1B2 cells, consistent with suppression of pp125^{FAK} activity by Plexin B1. Levels of β1-integrins were similar in LacZ and PBA cells, as analyzed by FACS analysis (data not shown). Because levels of pp125^{FAK} may be increased in melanoma, we determined levels of mRNA of pp125^{FAK} in YUMAC and YUSIK melanoma cells expressing Plexin B1, or empty vector, using comparative real time PCR (Figure 2E). The fold difference in pp125^{FAK} message between YUSIK LacZ and Plexin B1

expressing clones and YUMAC LacZ and Plexin B1 expressing clones was calculated using the Pfaffl equation (Pfaffl, 2001) with β-actin as a normalizing gene, and was 0.45 (±/−0.1 SD) and 0.22 (±/− 0.03 SD), respectively. Because a fold change of at least 1.3 is generally accepted to represent a significant change in gene expression (Dirisi et al., 1996; Huggins et al., 2008; Schena et al., 1996), we conclude that levels of $pp125^{FAK}$ are not regulated by Plexin B1 in these cell lines. These data suggest that Plexin B1 suppresses $pp125^{FAK}$ activation in melanoma, but does not regulate expression.

Plexin B1 suppresses Rho activity in melanoma

Rho is a critical signaling protein involved in regulation of the actin cytoskeleton, and expression and activation of Rho are increased in a variety of malignancies, including melanoma (Sanz-Moreno and Marshall, 2009). When Rho is active, cells displays actin stress fibers, which span the length of the cell and aid in cellular adhesion. Cells with prominent stress fibers are generally highly spread out and elongated. We analyzed stress fibers in YUSIK and YUMAC cells expressing empty vector and Plexin B1 by staining serum starved cells with fluor-594-conjugated *phalloidin* (Figure 3A). Immunofluorescence microscopy of stained cells showed that stress fibers are barely detectable in Plexin B1 expressing cells, compared with LacZ controls. The presence of stress fibers in LacZ cells, in the absence of growth factors, suggests that Rho is constitutively active in these cell lines.

We measured levels of active Rho in serum starved cells using a Rho G-LISA assay, which measures GTP bound Rho. Similar results were obtained in YUMAC and YUSIK cell lines (Figure 3B). YUSIK LacZ cells expressed 2.33 ng/ug total protein ($\times 10^2$) GTP-bound Rho compared with PBA cells, which expressed 1.0 ng/ug total protein ($\times 10^2$) GTP-bound Rho. The difference in GTP-Rho was significant ($P = 0.013$, $n = 4$ separate experiments). YUMAC LacZ cells expressed 2.54 ng/ug total protein ($\times 10^2$) GTP-bound Rho, compared with 1B2 cells, which expressed 1.3 ng/ug total protein ($\times 10^2$) GTP-bound Rho. The difference in GTP-Rho was significant between YUMAC LacZ and 1B2 cells ($P = 0.025$, $n = 3$ separate experiments).

Hepatocyte growth factor, also known as scatter factor, binds to and activates the c-Met receptor, resulting in activation of Rho (Takaishi et al., 1994). We have shown that Plexin B1 suppresses activation of the c-Met receptor, and migration in response to HGF (Stevens et al., 2010). To determine if Plexin B1 suppresses HGF-dependent activation of Rho, YUSIK cells were serum starved overnight and then treated with HGF (10 ng/ml) and GTP-bound Rho was measured (Figure 3C). Basal levels of GTP-Rho were 40% higher in LacZ compared with PBA cells. HGF stimulated a 16 and 40% increase in GTP-Rho in YUSIK LacZ cells at 10 and 30 min of treatment, respectively. In contrast, HGF had no effect on levels of GTP-bound Rho in cells expressing Plexin B1.

Discussion

The Plexin receptors belong to the c-Met family of scatter factor receptors, based on a conserved 500 amino acid “Sema domain” that is present in the extracellular region of the Plexin and c-Met receptors (Negishi et al., 2005). Plexin B1 is a non-tyrosine kinase receptor with a well-described function as a GTPase protein for R-Ras (Oinuma et al., 2004; Perrot et al., 2002; Saito et al., 2009). Previous reports show that Plexin B1 is a tumor suppressor protein for melanoma, and that Plexin B1 suppresses activation of the oncogenic c-Met receptor (Argast et al., 2009; Stevens et al., 2010). In this report we examined the effect of Plexin B1 on integrin-dependent $pp125^{FAK}$ activation, and Rho activity, in two human melanoma cell lines engineered to express the receptor. We chose human metastatic melanoma cell lines in which c-Met is not constitutively active, in order to identify targets of Plexin B1 that are independent of its suppressive effects on c-Met receptor signaling. Our

data show that Plexin B1 inhibits integrin-dependent pp125^{FAK} activity, and suppresses basal levels of active Rho in both cell lines. These results point to new downstream targets of Plexin B1 in melanoma that may contribute to its function as a tumor suppressor protein.

Activation of pp125^{FAK} by integrin engagement is an important step in migration, because pp125^{FAK} regulates the assembly of focal contact proteins at the leading edge of the migratory cell (Parsons et al., 2000). Constitutive p125^{FAK} activation, or increased expression, has been linked to melanoma progression, however, it is generally accepted that aberrant pp125^{FAK} activation, rather than increased expression, is more commonly seen in melanoma (Akasaka et al., 1995; Kahana et al., 2002; Scott and Liang, 1995; Vink et al., 1993). While we found slight variability in pp125^{FAK} expression between LacZ and Plexin B1 expressing cells at the protein level, we did not detect consistent differences in levels of pp125^{FAK}, and no evidence that pp125^{FAK} is regulated at the message level. We did observe significant inhibition of integrin-dependent pp125^{FAK} activation, however, as demonstrated by analysis of phosphorylated pp125^{FAK} in cells attached to fibronectin in melanoma cell lines engineered to express Plexin B1. The significance of this finding will require further study, as the effects of expression of Plexin B1 on migration towards fibronectin were quite modest. Because migration is a complex process, it is likely that the effects of Plexin B1 on integrin activation are not sufficient to robustly suppress migration. However, pp125^{FAK} activation suppresses apoptosis in cells detached from a cell matrix (“anoikis”; Gilmore, 2005), and promotes vasculogenic mimicry in melanoma (Hess et al., 2005). It is possible that the suppression of pp125^{FAK} activity by Plexin B1 suppresses aggressive behavior in melanoma through regulation of apoptosis or new vessel formation, in addition to its' effects on migration. Further studies will be needed to test this notion.

Melanoma and melanocyte migration towards fibronectin is integrin dependent (Scott et al., 1992; Tian et al., 2007). Plexin B1 inhibits integrin activity in other cell types (Oinuma et al., 2006). Interestingly, R-Ras activates integrins in melanoma (Gawecka et al., 2010). Because Plexin B1 is a GTPase activating protein for R-Ras, loss of Plexin B1 in melanoma may increase integrin affinity or avidity through enhanced R-Ras activation. While our data suggest that Plexin B1 suppresses integrin activation in melanoma, additional experiments will be needed to define the effect of Plexin B1 on integrin activation in melanoma.

Rho is a multifunctional protein that regulates assembly of the actin cytoskeleton, but also controls cell adhesion, motility, migration, and gene expression (Ridley, 1997). Plexin B1 receptor signaling regulates the activity of Rho, resulting in either activation or inhibition, depending upon the cell type (Barberis et al., 2005; Korostylev et al., 2008, Perrot et al., 2002). Because activation of Rho promotes melanoma progression (Ruth et al., 2006) our data suggest that Plexin B1 dependent suppression of Rho may contribute to the tumor suppressor effects of Plexin B1. Analysis of stress fibers, and measurement of GTP-Rho, showed that Plexin B1 suppresses basal levels of Rho activity in two melanoma cell lines by approximately 50%. Rho is a downstream target of c-Met activation (Takaishi et al., 1994). When cells were treated with HGF, LacZ cells showed the expected increase in Rho activation, whereas cells expressing Plexin B1 showed no detectable Rho activation. Whether the lack of Rho activation in response to HGF is due to suppressive effects of Plexin B1 on c-Met receptor activity, or to suppression of Rho through pathways independent of c-Met, remains to be determined. Experiments in which c-Met receptor is silenced, or activation is blocked with specific antagonists of receptor activation, will be required to answer this question. The functional significance of Rho suppression by Plexin B1 in melanoma remains to be defined. We attempted to determine if Rho contributes to migration or invasion of melanoma, however, inclusion of an inhibitor of the Rho downstream effector ROCK, did not detectably affect invasion of cells across a basement membrane, or migration of LacZ or PBA cells towards fibronectin (unpublished data). The

well documented role of Rho in the progression of cancer, as well as melanoma, suggests that suppression of Rho by Plexin B1 could be a potentially important mechanism by which Plexin B1 functions as a tumor suppressor protein for melanoma, however further studies will be needed to confirm this.

In summary, we have identified two additional targets of Plexin B1 signaling in melanoma, independent of its effects on the c-Met receptor; integrin-dependent $pp125^{FAK}$ activation, and Rho activity, in two melanoma cell lines engineered to express the receptor. We found a modest, yet significant effect, of Plexin B1 on migration towards fibronectin, however, because of the myriad effects of $pp125^{FAK}$ activation on variety of cell processes, it is reasonable to propose that Plexin B1-dependent $pp125^{FAK}$ suppression may affect other cellular processes. In combination with the known effects of Plexin B1 on suppression of c-Met receptor activity, a complex picture emerges of the signaling pathway of Plexin B1 in melanoma. In cells driven by c-Met receptor activation, Plexin B1 is expected to suppress melanoma progression through inhibition of c-Met dependent downstream targets, including Rho and $pp125^{FAK}$. Because Plexin B1 suppresses $pp125^{FAK}$ and Rho activity, independent of its effects on the c-Met receptor, Plexin B1 is expected to be a powerful suppressor of $pp125^{FAK}$ and Rho in melanoma cells driven by constitutive c-Met activation. However, a subset of melanomas do not show aberrant activity of c-Met, and in those melanomas, the ability of Plexin B1 to inhibit integrin-dependent $pp125^{FAK}$ activation, and Rho activity, through pathways distinct from suppression of c-Met, suggests that Plexin B1 potentially functions as a tumor suppressor protein in a heterogeneous population of melanomas.

Methods

Reagents

Rabbit polyclonal antibodies to β-actin and mouse monoclonal antibodies against RhoA were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies, mouse IgG_2 antibodies, fibronectin from bovine plasma, hepatocyte growth factor (HGF) and soybean trypsin inhibitor were purchased from Sigma Co., (St Louis, MO, USA). Antibodies against focal adhesion kinase ($pp125^{FAK}$) phosphorylated on Tyr 397 were purchased from Millipore (Temecular, CA, USA). Antibodies against total $pp125^{FAK}$ were purchased from MBL (Nagoya, Japan). Fullrange rainbow molecular weight marker was purchased from Amersham Life Sciences (Arlington Heights, IL, USA). Fluoro-594-conjugated phalloidin was obtained from Molecular Probes Inc (Portland, OR, USA). Rho-GLISA assay was purchased from Cytoskeletal Inc. (Denver, CO, USA). Poly-L-Lysine was purchased from R&D systems (Minneapolis, MN, USA).

Cells and cell culture

The YUSIK and YUMAC human metastatic melanoma cell lines (Yale Skin Disease Research Core Center, New Haven, CT, USA) were engineered to express human full length Plexin B1 receptor using Lentiviral vectors as previously described (Stevens et al., 2010). YUSIK and YUMAC melanoma cells do not exhibit activation of c-Met receptor under basal conditions, but do respond to the c-Met ligand HGF by phosphorylation of the c-Met receptor (Stevens et al., 2010). The clone “PBA” (YUSIK; characterized in Stevens et al., 2010) and 1B2 (YUMAC; Figure S1) were used. Transductants were maintained in OptiMEM (Gibco, Carlsbad, CA, USA) with 5% fetal bovine serum (HyClone, Logan, UT, USA) and blasticidin (Invitrogen, Carlsbad, CA, USA) at 2 μg/ml.

Staining of cells for actin

10^5 cells were plated onto Lab-Tek Chamber Slides (Nalge Nunc International, Naperville IL, USA) and were allowed to attach for 24 h. Cells were placed in basal media 24 h prior to staining. Cells were rinsed with phosphate buffered saline (PBS) then fixed with 3.7% formalin in PBS for 10 min. Cells were permeabilized with 0.1% Triton-X/PBS for 3 min and then rinsed with PBS for 5 min. Slides were incubated with Fluoro-594-phalloidin as per manufacturer's instructions, and then rinsed with PBS and coverslipped.

Western blotting

Cells were lysed on ice using radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl) with protease inhibitors (Boehringer Mannheim GmbH, Mannheim, Germany) and phosphatase inhibitors (GBiosciences, St. Louis, MO, USA). Lysates were resolved on SDS-PAGE and transferred to nitrocellulose paper. Immunoreactive proteins were visualized using SuperSignal West Pico Substrate (Pierce Chemical, Rockford, IL, USA).

FACS analysis

For analysis of β_1-integrin surface expression, cells were trypsinized from the dish and the action of trypsin was stopped with soybean trypsin inhibitor. 10^6 cells were placed in PBS/0.1% bovine serum albumen and incubated with phycoerythrin-conjugated mouse monoclonal antibodies against β_1-integrins (clone 4B7R, Abcam, Cambridge, MA, USA). Negative controls consisted of cells stained with phycoerythrin-conjugated mouse IgG (Sigma Co.).

Migration assay

Migration assays were performed using Chemicon QCM Chemotaxis 96-well Fluorometric Cell Migration Assay with 8 μm pore size (Millipore, Bedford, MA, USA). 10^5 cells were plated into the top well of the migration chamber in basal medium and incubated at 37°C for 24 h. The bottom wells contained basal media alone, or basal media with fibronectin in a range of doses. Cells were detached from the underside of the migration chamber plate with detachment solution and lysed and labeled with CyQuant GR Dye as described in the kit protocol. Fluorescence was measured at 480/520 nm with a Modulus Microplate Photometer (Turner Biosciences, Sunnyvale CA, USA). Background florescence (fluorescence of labeled media, in the absence of cells) was subtracted.

Invasion assay

Invasion assays were performed using CytoSelect 24-Well Cell Invasion Assay (basement membrane on 8 μ pore filters) from Cell Biolabs (San Diego, CA, USA). 5×10^4 cells were plated onto the top well of the invasion chamber in basal medium and incubated at 37°C for 24 h. Cells that invaded through the membrane were detached with dissociation buffer, lysed and quantified with CyQuant GR dye. Fluorescence was measured at 480/520 nm with a Modulus Microplate Photometer. Background florescence (fluorescence of labeled media, in the absence of cells) was subtracted.

Comparative real time PCR for $pp125^{FAK}$

Total RNA from *YUMAC and YUSIK* cells was isolated using the RNeasy Mini Kit (QIAgen, Valencia, CA, USA) according to manufacturer's instructions. Reverse transcription was performed using 0.75 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen). PCR was performed using iQ SYBR Green Supermix (BioRad Laboratories) on the Applied Biosystems ABI prism 7700 sequencedetection system (BioRad iCycler). Primers used for amplification of $pp125^{FAK}$ were: fwd: 5′-ATT GCT

GCC TCG GAA TGT TCT -3′; rvs: 5′-GCT GAG GTA AAA CGT CGA AAA-3′. The conditions were: 94°C, 3 min (1 cycle); 94°C 45 sec, 57.8°C, 30 sec, 72°C, 60 sec (40 cycles). Primers used for amplification of β-actin were: fwd: 5′-CAC-GCACGATTTCCCGCTCGG-3′; rvs: 5′-CAGGCTGTGCTATCCTGTAC-3′. The conditions were 95°C, 3 min (1 cycle); 95°C 1 5 sec, 54.5°C, 30 sec, 72°C, 40 sec (40 cycles). Samples were analyzed in triplicate, and each analysis was performed four times. Fold change in mRNA between samples was calculated using the Pfaffl equation (Pfaffl, 2001) using β-actin as a reference gene. Efficiency of β-actin amplification was assumed to be 94.3%; efficiency of $pp125^{FAK}$ amplification was assumed to be 93.6%.

Statistical analysis

Differences between means were analyzed by two-tailed Student's t-test. A p value <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Significance

Loss of the semaphorin receptor Plexin B1 promotes late stages of melanoma progression. pp125^{FAK} and Rho play a critical role in regulation of actin assembly, cell migration, and cell survival, and aberrant activation is associated with tumor progression. The present work is significant because it identifies pp125^{FAK} and Rho as downstream targets of Plexin B1 in melanoma. We propose that Plexin B1 suppresses progression of melanoma through inhibitory effects on multiple targets, including c-Met, pp125^{FAK} and Rho. Suppression of pp125^{FAK} and Rho activity by Plexin B1 in melanoma could result in suppression of cell migration, angioinvasion, and anchorage-independent growth.

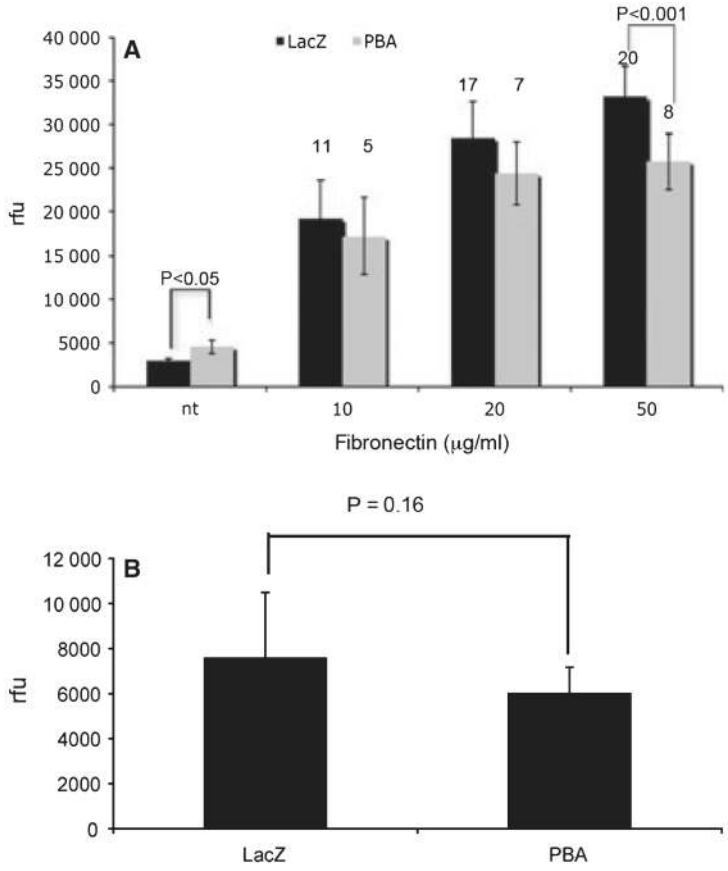

Figure 1.

Plexin B1 inhibits migration of melanoma towards fibronectin. (A) Shown is quantitative analysis (relative fluorescence units, rfu) of PBA and LacZ migration towards fibronectin over a 24-h time period. LacZ and PBA cells migrated towards fibronectin in a dose-dependent manner, however, migration of LacZ cells was higher at all concentrations of fibronectin, compared with PBA cells. The numbers above the bars indicate the fold change in migration at each fibronectin concentration, compared with migration towards basal media without added fibronectin ("nt"; no treatment). The difference in migration between LacZ and PBA cells was statistically significant at a fibronectin concentration of 50 μg/ml ($P < 0.001$). Each column represents the averaged results of nine separate wells, ±SD. Results are representative of two experiments. (B) Shown is quantitative analysis (relative fluorescence units, rfu) of PBA and LacZ cells that invaded through a basement membrane substrate over a 48-h time period. No significant difference in invasion was detected between LacZ and PBA cells ($P = 0.16$). Each bar represents the average fluorescence intensity from 3 separate experiments, from duplicate wells ±SD.

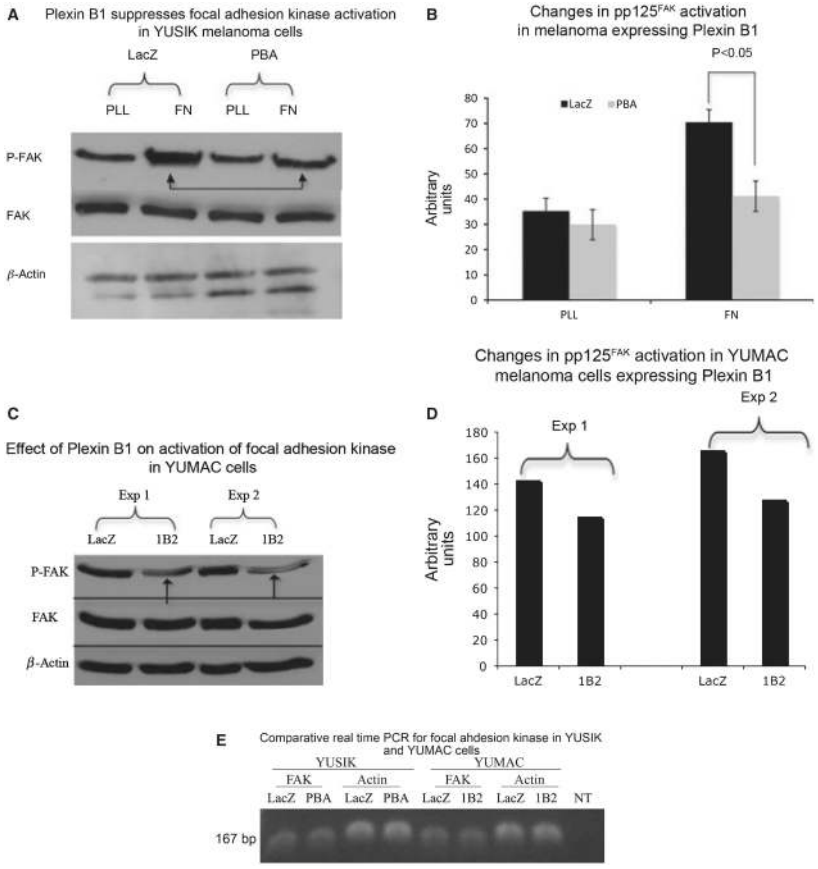

Figure 2.

Plexin B1 inhibits integrin-dependent pp125^{FAK} activation in melanoma. (A) One hour after attachment to fibronectin (100 μg/ml), robust activation of pp125^{FAK} was detected in LacZ cells. In contrast, PBA cells showed less activation of pp125^{FAK} following attachment to fibronectin, compared with LacZ cells. Both cell types showed constitutive activity of pp125^{FAK}, as shown by active pp125^{FAK} in cells plated on poly-L-lysine. Shown is a representative Western blot from three separate experiments in which total cell lysates were resolved on 10% SDS-PAGE. (B) Densitometry analysis of Western blots from three separate experiments are shown. Phosphorylated pp125^{FAK} was normalized to total pp125^{FAK}. Columns represent the averaged density of bands (arbitrary units) from three experiments, ±SD. Active pp125^{FAK} was significantly higher ($P < 0.05$) in LacZ cells compared with PBA cells when cells were attached to fibronectin. (C) Results from two experiments are shown in which YUMAC LacZ and 1B2 Plexin B1 expressing clones were allowed to attach to fibronectin (100 μg/ml) for 1 h. Robust activation of pp125^{FAK} was detected in LacZ cells. In contrast, 1B2 cells showed less activation of pp125^{FAK} following attachment to fibronectin, compared with LacZ cells. Shown are 10% SDS-PAGE in which total cell lysates were resolved. (D) Densitometry analysis of each experiment is shown. Phosphorylated pp125^{FAK} was normalized to total pp125^{FAK}. Columns represent the average density of each band (arbitrary units). (E) PCR products from one of 4 separate experiments for pp125^{FAK} and β-actin were resolved on 1% agarose gel. A band corresponding to the expected molecular weight of pp125^{FAK} was observed in YUSIK and YUMAC melanoma cells. No difference in levels of pp125^{FAK} PCR product were observed between LacZ and Plexin B1-expressing clones, which was confirmed by comparative real time PCR.

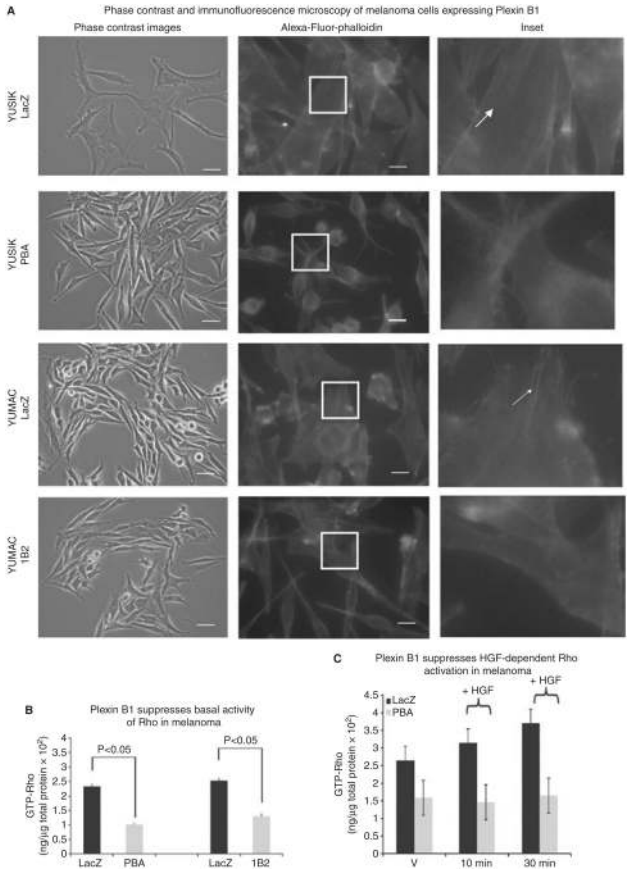

Figure 3.

Plexin B1 inhibits the activity of Rho in melanoma. (A) Shown are representative images of PBA, LacZ (YUSIK) and 1B2, LacZ (YUMAC) cells under phase contrast microscopy (bar = 10 μ), grown in complete media, and immunofluorescence microscopy of cells that were plated on multiwall dishes, and placed in basal media overnight, followed by stained for actin using phalloidin conjugated with Alexa-fluoro-594 (bar = 50 μ). Actin stress fibers (arrow) are visualized in LacZ cells, whereas cells expressing Plexin B1 contain few stress fibers. (B) GTP-bound Rho was measured in LacZ and cells expressing Plexin B1 in YUSIK and YUMAC cells. Shown is the amount of GTP-bound Rho as ng/μg/total cellular protein ($\times 10^2$). Melanoma cells expressing Plexin B1 showed significantly lower levels (approximately 50%, $P < 0.05$) active Rho, compared with LacZ cells. Each column represents the averaged results of 4 experiments (LacZ and PBA) or three experiments (LacZ and 1B2) in which samples were measured in duplicate, ±SD. (C) GTP-bound Rho was measured in YUSIK LacZ and PBA cells in basal medium following treatment with hepatocyte growth factor (HGF) (10 ng/ml). Shown is the amount of GTP-bound Rho as ng/μg/total cellular protein ($\times 10^2$). Levels of GTP-Rho are higher in LacZ compared with PBA cells (2.64 ± 0.12 vs. 1.59 ± 0.10 ± SD respectively) in the absence of HGF. Treatment with HGF stimulated an increase in GTP-Rho at 10 and 30 min in LacZ cells. In contrast, HGF had no effect on levels of GTP-Rho at either time points in PBA cells. Each column represents the average of triplicate wells ±SD. The results are representative of two separate experiments.