Cutting Edge: Bone Morphogenetic Protein Antagonists Drm/Gremlin and Dan Interact with Slits and Act as Negative Regulators of Monocyte Chemotaxis

Bo Chen, Donald G. Blair, Sergei Plisov, Gennady Vasiliev, Alan O. Perantoni, Qian Chen, Meropi Athanasiou, Jane Y. Wu, Joost J. Oppenheim and De Yang

J Immunol 2004; 173:5914-5917;
doi: 10.4049/jimmunol.173.10.5914
http://www.jimmunol.org/content/173/10/5914

References
This article cites 17 articles, 5 of which you can access for free at:
http://www.jimmunol.org/content/173/10/5914.full#ref-list-1

Subscription
Information about subscribing to J Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Bone Morphogenetic Protein Antagonists Drm/Gremlin and Dan Interact with Slits and Act as Negative Regulators of Monocyte Chemotaxis1,2

Bo Chen,* Donald G. Blair,* Sergei Plisov,† Gennady Vasiliev,† Alan O. Perantoni,† Qian Chen,‡ Meropi Athanasiou,§ Jane Y. Wu,** Joost J. Oppenheim,‡ and De Yang3§

Drm/Gremlin and Dan, two homologous secreted antagonists of bone morphogenetic proteins, have been shown to regulate early development, tumorigenesis, and renal pathophysiology. In this study, we report that Drm and Dan physically and functionally interact with Slit1 and Slit2 proteins. Drm binding to Slits depends on its glycosylation and is not interfered with by bone morphogenetic proteins. Importantly, Drm and Dan function as inhibitors for monocyte migration induced by stromal cell-derived factor 1α (SDF-1α) or fMLP. The inhibition of SDF-1α-induced monocyte chemotaxis by Dan is not due to blocking the binding of SDF-1α to its receptor. Thus, the results identify that Drm and Dan can interact with Slit proteins and act as inhibitors of monocyte chemotaxis, demonstrating a previously unidentified biological role for these proteins. The Journal of Immunology, 2004, 173: 5914–5917.

The Drm (also known as Gremlin), a 184-aa protein initially identified through differential screening as a transcriptional down-regulated gene in v-mos-transformed rat embryonic fibroblasts (1), belongs to the Dan family of secreted glycosylated proteins (2, 3), which contains a highly conserved cysteine knot domain shared by the TGFβ superfamily, PDGF, nerve growth factor, and other secreted proteins (4). Drm and Dan regulate early development (5–8), tumorigenesis (1, 9, 10), and renal pathophysiology (11). The action of Drm and Dan on development and possibly diabetic nephropathy is mediated by heterodimerizing with certain bone morphogenetic proteins (BMPs),4 in particular BMP2, 4, and 7 (2, 3, 11, 12) to subsequently block the ability of BMPs to bind their receptors (2, 12, 13). We have previously shown that the capacity of Drm to suppress transformation and tumorigenesis (1, 9, 10) is mediated by a mechanism that is independent of BMPs and involves both up-regulation of p21(Cip1) and down-regulation of p42/44 MAPK (9), suggesting additional target(s) for Drm and other Dan family members.

To identify additional target proteins for Drm and other Dan family members, we used a yeast two-hybrid screening approach with a Drm-GAL4 fusion construct as the bait to search for potential Drm-binding partners. This approach identified Slit as one type of the Drm-interacting proteins. We further characterized the interaction of Drm and Dan with Slit proteins and found that both Drm and Dan could inhibit monocyte migration induced by chemotactic factors. Thus, the data demonstrate for the first time that Drm and Dan physically and functionally interact with Slit proteins to act as a negative regulator for monocyte chemotaxis.

Materials and Methods

Yeast two-hybrid screening

The HybriZAP2.1 two-hybrid system (Stratagene, La Jolla, CA) was performed based on the protocol provided by the manufacturer. Briefly, yeast cells (YRG2) cotransfected with Drm-GAL4-BD bait plasmid and a 19-day postcoitus rat fetal kidney cDNA-GAL4-AD expression library were selected on HLT medium. Plasmids from surviving colonies expressing β-galactosidase were rescreened, sequenced, and BLAST-searched against GenBank.

Plasmids, Abs, reagents, and cell lines

pMex, pMex-Drm, pMex-HA-Drm, pMex-Drm*, and rabbit anti-Drm were previously described (2). Plasmids encoding Slit1-myc and its empty vector pcDNA2 were originally obtained from Dr. D. M. Ornitz. Anti-myc Ab and its aga-rose conjugate and anti-hemagglutinin Ab and its aga-rose conjugate were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse Dan, anti-Dan-neutralizing Ab, recombinant BMP 2 and 4 were obtained from R&D Systems (Minneapolis, MN). Recombinant human stromal cell-derived factor 1α (SDF-1α) was purchased from PeproTech (Rocky Hill, NJ). I125-SDF-1α with a specific radiactivity of 2000 Ci/mmol was obtained from Amersham Life Science (Cleveland, OH). HEK 293 and HT1080 cells, acquired from American Type Culture Collection (Manassas, VA), were maintained in DMEM supplemented with 10% FBS (Invitrogen Life Technologies, Carlsbad, CA). To generate cell lines stably expressing Drm and/or Slit, corresponding expression plasmids were transfected into HEK293 or HT1080 cells by using Effectene Transfection Reagent

1 This work was supported in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract NO1-CO-12400.
To purify Drm for chemotaxis assay, 10 ml of conditioned medium from Drm expression cells or its vector control cells was incubated with 1 ml of pre-washed heparin-acryl beads (Sigma-Aldrich, St. Louis, MO) for 2 h at 4°C. After washing three times with PBS, proteins were eluted by 10 mM Tris (pH 8.0)/1.5 M NaCl, desalted, and concentrated to 0.3 ml for both Drm and control.

**Immunoprecipitation and immunoblotting**

For cotransfection assay, indicated plasmids were transiently transfected into HEK 293 cells. Cells were harvested 48 h later and lysed in radioimmunoprecipitation assay buffer. For coculture assay, indicated stable cell lines were mixed (1:1) and the conditioned supernatants were harvested after 72 h of culture. For immunoprecipitation (IP), 10 µg of primary Ab agrose conjugate was added to lysates or conditioned supernatants and incubated at 4°C for 4 h. Beads were washed five times in radioimmunoprecipitation assay buffer. Immunoblotting (IB) was performed as previously described after SDS-PAGE and transfer (2).

**Chemotaxis assay**

Human PBMC were isolated by Ficoll density gradient centrifugation from leukopacks supplied by the Department of Transfusion Medicine (Clinical Center, National Cancer Institute (NCI), Bethesda, MD) with the approval of the NCI Human Research Committee. Monocytes were purified (>90%) from human PBMC by Percoll density gradient centrifugation (14) with all solutions prepared in endotoxin-free water. Monocyte chemotaxis was assessed using a 48-well microchemotaxis chamber assay (14) with minor modifications. Briefly, diluted SDF-1α and monocyte suspension (10^6 cells/ml) were, respectively, placed in the lower and upper wells of a chamber (NeuroProbe, Gaithersburg, MD), which were separated by a 5-µm polycarbonate filter (NeuroProbes). To test their effect on monocyte chemotaxis, Slit, Drm, Dan, and/or Ab were added to the lower or upper wells as indicated. Monocytes were suspended in and all factors were diluted with chemotaxis medium (RPMI 1640 containing 1% BSA, 2.5 mM HEPES, and 0.05% NaN3. After washing three times with PBS, proteins were eluted by 10 mM Tris (pH 8.0)/H11011. After measuring, the filters were removed and stained, and the cells migrating across the filter were counted. The results are presented as number of cells per high-power field.

**Binding assay**

Competitive binding was performed in triplicate by adding a constant amount of 125I-SDF-1α and increasing amounts of unlabeled SDF-1α or Dan to individual 1.5-ml microfuge tubes, each containing 10^6 monocytes suspended in RPMI 1640 containing 1% BSA, 2.5 mM HEPES, and 0.05% NaN3. After incubation at 37°C in humidified air with 5% CO2 for 1.5 h, the filters were removed and stained, and the cells migrating across the filter were counted. The results are presented as number of cells per high-power field.

**RNA extraction and RT-PCR**

Total RNA from purified human monocytes was extracted by using TRizol Reagent (Invitrogen Life Technologies). RT-PCR was performed according to the specification of a Qagen OneStep RT-PCR kit. Primer pairs for Robo (5'-TGGTC-3' and 5'-GCGAAGCTTATGAGCCGCACAGCCTAC-3'), Slit1 (5'-TGGTTCAGCTGTAGGAGAAC-3' and 5'-TTACGGGTCA GAACCTCCAG-3'), Slit2 (5'-TCAGTGGCAAGTTTCAACCA-3' and 5'-CTGTATGTTGCTTCAGGAGA-3'), Slit3 (5'-ACATGCGCCGTATCC TGGTCT-3' and 5'-CTCTGGCAGAATGGATGGAT-3'), and Drm (5'-GGAGGTGAGACCCACAGCCTAC-3' and 5'-ATTTGATACCTTTACCA-3').

**Statistical analysis**

Each experiment was performed at least three times with similar results. Statistical comparison between two groups was conducted by Mann-Whitney U analysis.

**Results and Discussion**

**Identification and characterization of Drm-Slit interaction**

Screening ~1.5 × 10^6 of cDNA using the yeast two-hybrid system yielded 25 clones that survived on HLT medium and had β-galactosidase expression. Rescreening revealed 21 plasmids containing authentic Drm-interacting components. Sequencing and BLAST search identified both Slit1 and Slit2 as Drm-interacting proteins (data not shown). To further confirm Drm-Slit interaction, we showed that Drm and Slits could be coimmunoprecipitated from the supernatants of mixed cultures of cells expressing Drm and myc-tagged Slit1 (Fig. 1a, lane 2) or from a lysate of coexpressing cells (Fig. 1a, lanes 4). As controls, the supernatant (Fig. 1a, lane 1) or lysates (Fig. 1a, lanes 3 and 5) of cells expressing either Drm or Slit1 alone showed no evidence of association. Similarly, Drm could be precipitated from supernatant of cocultures of cells expressing both myc-tagged Slit2 and Drm (Fig. 1b, lane 4) by anti-myc tag antiserum, but not from the supernatant of vector-transfected cells (Fig. 1b, lane 1) or from singly expressing cells (Fig. 1b, lanes 2 and 3).

To analyze whether glycosylation of Drm might affect the interaction between Drm and Slit, we mutated Drm’s glycosylation site (42ND545E to 42IEA45E) (2) and investigated whether the mutation affected the interaction between Drm and Slit1. Mutated Drm could not be coprecipitated with myc-tagged Slit1 (Fig. 1c, lane 4), suggesting that either glycosylation of Drm is necessary or Slit binding involves a site at or near the site of glycosylation. Since nonglycosylated Drm resulting from the same mutation is still able to bind BMPs and to interfere with BMP-mediated biological activity (2), it is unlikely that Drm’s structure is drastically altered by the mutation per se or by the lack of glycosylation.

Drm has been shown to bind BMP2, 4, and 7 and to subsequently block BMPs’ ability to bind their receptors (2, 3, 12, 13). To determine whether the binding of BMP to Slits could affect the Drm-Slit interaction, we added exogenous BMP-4 or BMP-2 to conditioned medium containing Drm and Slit1 or Slit2 before immunoprecipitation. Exposure to 10–100 nM of BMP-4 (Fig. 1d, left panel) or for up to 48 h (Fig. 1d, right panel) failed to interfere with the immunoprecipitation of Drm and Slit1 (Fig. 1d) or Slit2 (data not shown). BMP-2 also did not interfere with Drm-Slit interaction (data not shown). These results provide further support for the notion that the region of Drm responsible for BMP binding is distinct from that interacting with Slits.

**FIGURE 1.** Drm interacts with Slit1 and Slit2 proteins. a, Drm binds Slit1. Coculture assay (lanes 1 and 2) and cotransfection assay (lanes 3–5) were performed to determine the interaction between Drm and Slit1. Culture supernatants or lysates were IP and IB with Abs as indicated. b, Drm binds Slit2. Coculture assays were performed as described in a. c, Glycosylation of Drm is critical for the interaction. Drm⁺ is the construct with a mutated glycosylation site. HT1080 cells were transfected with the indicated plasmids; after 48 h, conditioned medium was harvested and then processed for IP and IB with Abs as indicated. d, BMP-4 does not block Drm-Slit interaction. HT1080 cells stably expressing Drm and Slit1 were cocultured. Left panel, Increasing amounts of BMP-4 were added. Right panel, One hundred nanomolar BMP-4 were added and cultured with increasing time. IP and IB assays were then performed as indicated above.
**Drm as negative regulator of monocyte chemotaxis**

It has recently been reported that members of the Slit family of secreted guidance proteins, which regulate neuronal migration and axon growth (15, 16) via interaction with their cellular roundabout receptor (Robo), can also block leukocyte chemotaxis (17). We investigated whether Drm could modulate the capacity of Slit2 to inhibit the chemotaxis of human monocytes in response to the chemokine SDF-1α, using serum-free conditioned supernatants of Slit2-expressing HEK293 cells and Drm-expressing HT1080 cells as the sources of Slit2 and Drm, respectively. The chemotaxis of human monocytes in response to SDF-1α showed a bell-shaped dose-response curve, with an optimal concentration of SDF-1α at 100 ng/ml (Fig. 2a). As previously reported (17), Slit2 inhibited ~50% of the chemotaxis of monocytes in response to the optimal concentration of SDF-1α (Fig. 2b, third bar). When added in combination with Slit2, Drm (Fig. 2b, fifth bar), but not the control supernatant (Fig. 2b, fourth bar), completely abrogated the chemotactic migration of monocytes induced by SDF-1α. Similar to inhibiting SDF-1α-induced monocyte chemotaxis, Drm also inhibited monocyte chemotaxis when fMLP was used as a chemotactic factor (Fig. 2c), suggesting that Drm acted as an inhibitor of monocyte chemotaxis independent of the chemotactic factors used.

A combination of Drm and Slit2 almost completely inhibited the monocyte migration induced by either SDF-1α (Fig. 2d, fifth bar) or fMLP (Fig. 2c, fifth bar) suggested that either Drm or Slit2 enhanced the suppressive effect of Slit2 or Drm by itself exhibited a suppressive effect on monocyte chemotaxis. To distinguish between the two possibilities, we examined whether pure recombinant Drm could inhibit monocyte chemotaxis (Fig. 2d). Drm dose-dependently inhibited the SDF-1α-induced monocyte migration when present together with monocytes in the upper wells (Fig. 2d, fourth to seventh bars), but not when present together with SDF-1α in the lower wells (Fig. 2d, eighth bar). Of note, Drm was not toxic for monocytes since coincubation of monocytes with Drm at even the highest concentration used for 1.5 h did not result in a loss of monocyte viability (data not shown), and Drm did not affect the background migration of monocytes (Fig. 2d, second bar).

**Dan interacts with Slits and acts as an inhibitor of monocyte chemotaxis**

The Dan family contains at least seven members including Drm and Dan (2–4, 12). To address whether acting as an inhibitor of monocyte chemotaxis is unique to Drm, we investigated whether Dan could also bind to Slits and inhibit SDF-1α-induced chemotaxis of monocytes. Similar to Drm, Dan could interact with both Slit1 (lane 2) and Slit2 (lane 3) as demonstrated by coimmunoprecipitation and Western blotting (Fig. 3a). Dan, when added simultaneously with monocytes to the upper wells of a chemotaxis chamber, also suppressed the chemotaxis of monocytes in a dose-dependent manner in response to an optimal concentration of SDF-1α with ~95% suppression at 2000 ng/ml (Fig. 3b). The suppressive effect of Dan at 500 ng/ml on SDF-1α-induced monocyte chemotaxis was dose-dependently neutralized by anti-Dan Ab (Fig. 3c). The neutralization was ~90% and 100% at 2 and 10 μg/ml anti-Dan Ab, while isotype-matched control Ab used at 10 μg/ml had no effect (Fig. 3c). Therefore, not only Drm but also Dan acts as an inhibitor of monocyte chemotaxis.

How does Dan or Drm inhibit monocyte chemotaxis? SDF-1α induces chemotaxis by binding to its receptor CXCR4, followed by the activation of a heterotrimeric G protein in target cells. Therefore, one possible mechanism by which Dan or Drm inhibits the chemotaxis of monocytes might simply be by abolishing the chemotactic activity of SDF-1α. This appears unlikely because neither Drm (Fig. 2d) nor Dan (Fig. 3d) inhibited monocyte chemotaxis when added simultaneously with SDF-1α into the lower wells of the chemotaxis chamber. Another possibility might be by hindering the binding of SDF-1α to CXCR4 on target monocytes. However, as shown in Fig. 3r, Dan did not inhibit the binding of 125I-SDF-1α to human monocytes, whereas unlabeled SDF-1α expectedly did so, suggesting that Dan’s suppressive effect on SDF-1α-induced monocyte chemotaxis is not due to competitive binding to the CXCR4 receptor. Since Slits can inhibit...
monocyte chemotaxis by signaling through their receptor Robo (17) and both Drm and Dan can bind to Slit1 and Slit2 (Figs. 1 and 3a), we hypothesized that the mechanism of Drm- or Dan-mediated inhibition of monocyte chemotaxis might be by promoting the suppressive effect of endogenous Slits. To search for support of this possibility, we determined whether monocytes express Slits and their receptor Robo by RT-PCR. The data demonstrated that monocytes express endogenous Slit1, Slit2, and Robo mRNA (Fig. 3f).

Chemotaxis of leukocytes, including monocytes, is critical for the development of inflammation and immunity, yet past research has predominantly focused on chemoattraction and positive regulation of this process. To our knowledge, only Slit protein has previously been reported to act as negative regulator of chemotaxis (17). The identification of Drm and Dan as Slit-interacting proteins and negative regulators of monocyte chemotaxis not only reveals a novel function beyond their roles as BMP antagonists but also broadens the search for chemotaxis inhibitors. Further elucidation of the signaling mechanism(s) responsible for Drm- and/or Dan-mediated inhibition of monocyte chemotaxis may provide useful clues for the design of potential therapeutic inhibitor(s) that attenuate leukocyte migration for the treatment of autoimmunity, inflammation, HIV infection, cancer, and other diseases.

Acknowledgments

We thank Dr. David M. Ornitz (Washington University, St. Louis, MO) for proving plasmid encoding Slit1-myc and Dr. Allan M. Weissman (NCI) for helpful discussion.

References