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37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
38. Cells were washed twice with culture medium, and control Fc or EphA3-Fc protein was added for 2 hours unless otherwise stated. These Fc proteins were preclustered with polyclonal antibody to human Fc (Jackson ImmunoResearch, West Grove, PA) for 30 min on ice. Midbrain neurons were cultured on laminin/poly-L-lysine–coated dishes for 3 days before the cleavage assay in Neurobasal/B27 medium (Gibco). After incubation, the cells were collected in SDS-PAGE sample buffer. The culture supernatant was spun at 14,000 rpm for 10 min at 4°C, then protein was precipitated with trichloroacetic acid and dissolved in SDS-PAGE sample buffer, adjusting the pH with 1M NaOH. For ephrin-A2 immunoblots, polyvinylidene difluoride membranes were probed with rabbit anti-ephrin-A2 (Santa Cruz Biotechnology) diluted 1:1500, and the secondary antibody was horseradish peroxidase (HRP)–conjugated sheep anti-rabbit immunoglobulin G (IgG) (Amersham Pharmacia Biotech) diluted 1:2000, with detection by an enhanced chemiluminescence kit. Epitope tags were detected with antibody to Myc 9E10 (100 ng/ml), antibody to Flag F2 (200 ng/ml), or antibody to HA 3F10 (50 ng/ml), with HRP-conjugated sheep anti-mouse IgG secondary antibody diluted 1:3500.
39. Videomicroscopy was as follows. Cultures were on 25-mm glass coverslips that were precoated overnight with mouse laminin (2 μg/ml). Ephrin-A2 plasmids were stably transfected into NIH-3T3 fibroblasts, and before the assay, these cells were plated at 0.5×10^5 to 2×10^5 cells/ml in Dulbecco's modified Eagle's medium with 10% calf serum. The medial half of the hippocampus was removed from E16 to E18 mice, dissociated in neurobasal/B27 medium, and plated between 10^4 and 10^6 cells/ml to obtain a good density for observation. After culturing for 16 to 40 hours, coverslips were viewed with a Zeiss Axiovert microscope and a charge-coupled device camera, with the stage warmed to 37°C, and 20 mM Hepes was added to the medium to maintain pH at ~7.4. Single-axon growth cones approaching a target cell were recorded every 30 s with phase-contrast optics. For GFP experiments, one to four images were recorded with fluorescein optics. All results were confirmed blind by an independent investigator.
40. We thank L. Ma, R. Davis, T. Inoue, T. Wei, D. Marks, D. Feldheim, Q. Lu, M. Kirschner, B. Freeman, D. Van Vactor, and K. Mikoshiba for help and discussions and D. Pan for mouse Kuz plasmids. This work was supported by a fellowship from the Society for Promotion of Arts and Science of Japan (M.H.) and by NIH grants HD29417 and EY11559 and a fellowship from the Klingenstein foundation (J.G.F.).

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Function of an Axonal Chemoattractant Modulated by Metalloprotease Activity

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The axonal chemoattractant netrin-1 guides spinal commissural axons by activating its receptor DCC (Deleted in Colorectal Cancer). We have found that chemical inhibitors of metalloproteases potentiate netrin-mediated axon outgrowth in vitro. We have also found that DCC is a substrate for metalloprotease-dependent ectodomain shedding, and that the inhibitors block proteolytic processing of DCC and cause an increase in DCC protein levels on axons within spinal cord explants. Thus, potentiation of netrin activity by inhibitors may result from stabilization of DCC on the axons, and proteolytic activity may regulate axon migration by controlling the number of functional extracellular axon guidance receptors.

There is evidence that proteolytic activity present on neuronal growth cones may regulate their migratory activity (*1*). This possibility is supported by the presence of axonal stalling defects in flies with mutations in the *kuzbanian* gene, which encodes a member of the ADAM class of metalloproteases (*2*). ADAM class metalloproteases are also implicated in other biological processes, including fertilization (*3*), lateral inhibition during neurogenesis (*4*), and protein ectodomain shedding of a variety of ligands and receptors [(*5*–*7*), reviewed in (*5*)].

The netrin-1 protein and its receptor, DCC, are required for commissural axon outgrowth in vitro and for the proper guidance of spinal commissural neurons in vivo (*8*–*12*). We previously characterized an unidentified proteinaceous activity, termed netrin-synergizing activity (NSA), that potentiates the axon outgrowth–promoting effects of netrin on rat E11 (embryonic day 11) dorsal spinal cord explants (*7, 13*). In a screen of several dozen known molecules (including many axon guidance molecules), none could potentiate netrin activity (*13*). Upon further screening, we found that netrin-1 activity was potentiated by IC-3, a specific hydroxamate inhibitor of metalloproteases (*6, 14*) (Fig. 1). In the absence of any factors, or in the presence of a low concentration of netrin-1 (~50 ng/ml), very little outgrowth was observed from these explants (Fig. 1, A and B). Robust outgrowth was observed from explants grown in the presence of both 40 μM IC-3 and netrin-1 (~50 ng/ml) (Fig. 1D), which was greater than that observed with a high concentration of netrin-1 (1 μg/ml) alone (Fig. 1E). In contrast, much less outgrowth was observed from explants grown in the presence of 40 μM IC-3 alone (Fig. 1C); the fact that any outgrowth was observed may reflect a potentiation of low levels of endogenous netrin-1 present in dorsal regions of the spinal cord (*9*). The responsive axons in these assays are commissural, as assessed by expression of the commissural axon markers TAG-1 and DCC (*15*). The degree of outgrowth observed in the presence of both IC-3 and netrin-1 (thick and long axon fascicles emerging primarily but not exclusively from the ventral cut edge of the explants) was similar to that observed with netrin-1 and NSA (*8, 13*). Quantification of axonal outgrowth (estimated as total axonal length) with IC-3 alone or IC-3 plus netrin-1 (Fig. 1F) revealed peak activity of IC-3 at 80 μM, and only slight activity at 20 μM.

The effect of IC-3 is likely to be due to its metalloprotease inhibitor activity because this compound has not been found to have any effect other than metalloprotease inhibition in a wide variety of cellular assays (including protein phosphorylation and cell viability) or in mice (*14*). To confirm this, we also compared the actions of a chemically distinct hydroxamate metalloprotease inhibitor, GM6001, and an inactive control isomer of this compound (*16*). As expected, GM6001 but not the control isomer showed a similar potentiation of netrin-1 activity to IC-3 (*15*), consistent with the potentiation of netrin-1 being due to inhibition of metalloprotease activity. Members of another class of inhibitors that function through chelation of the divalent cations required for metalloprotease function (including 1,10-phenanthroline and EDTA) could not be tested in these assays because they were toxic to the explants (*15*).

Dorsal spinal cord explants grown in the presence of IC-3 exhibited much brighter staining for DCC than did control axons grown in the absence of any factors (compare Fig. 2, C and D). This effect was specific for DCC, because explants grown with or without IC-3 and stained for TAG-1 exhibited no clear difference in staining intensity except at the ventral cut edge of the explant (Fig. 2, A and B). Quantification of fluorescence intensity across the dorsoventral axis of DCC-stained explants revealed an increase over almost the entire dorsoventral length of the explants (Fig. 2E).

Because IC-3 increased DCC staining levels within dorsal spinal cord explants, we tested whether DCC might be a substrate for

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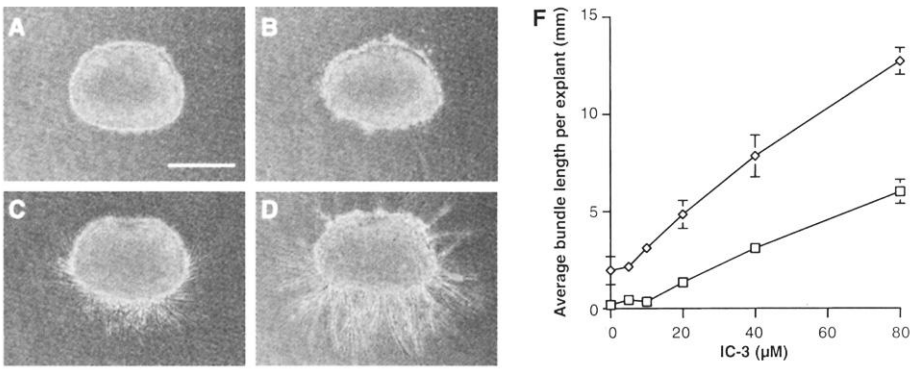

Fig. 1. The chemical metalloprotease inhibitor IC-3 potentiates netrin-mediated axon outgrowth from E11 rat dorsal spinal cord explants (*23*). (**A**) Explant cultured alone. (**B**) In the presence of netrin-1 (~50 ng/ml), there is little outgrowth, as in (A). (**C**) Some outgrowth is observed in the presence of 40 μM IC-3. (**D**) Robust outgrowth is observed in the presence of both 40 μM IC-3 and netrin-1 (~50 ng/ml). (**E**) A high concentration of netrin-1 (1 μg/ml) also elicits robust outgrowth. In all panels, dorsal is top and the ventral cut edge of the explant is at the bottom; scale bar, 200 μm. (**F**) E11 dorsal spinal cord explants were cultured for 36 to 40 hours in the presence (diamonds) or absence (squares) of netrin-1 (~50 ng/ml) and increasing concentrations of IC-3. The outgrowth under each condition was quantified by measuring the total length of all the axon fascicles emerging from each explant [a useful measure of outgrowth in response to netrin-1 (*8*)]. The data are from three independent experiments ± SEM. Where no error bars are visible, the symbol is larger than the error bars.

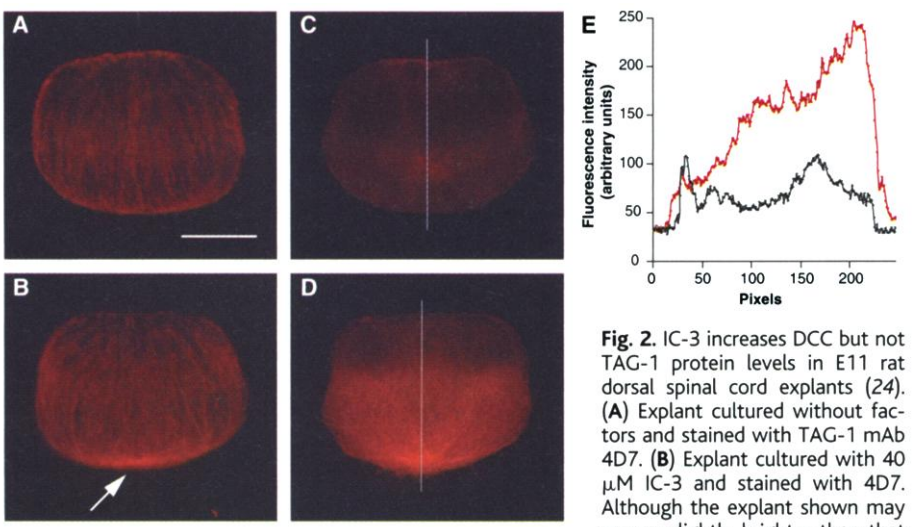

Fig. 2. IC-3 increases DCC but not TAG-1 protein levels in E11 rat dorsal spinal cord explants (*24*). (**A**) Explant cultured without factors and stained with TAG-1 mAb 4D7. (**B**) Explant cultured with 40 μM IC-3 and stained with 4D7. Although the explant shown may appear slightly brighter than that in (A), this was not observed consistently; the difference shown is no greater than that seen between multiple explants cultured under the same conditions. The only reproducibly noticeable difference in TAG-1–stained explants is the region of brighter staining at the ventral cut edge of explants grown in the presence of IC-3 [arrow in (B)]. This may reflect more complete and/or faster growth of commissural axons to the ventral cut edge within these explants. (**C**) Control explant stained with DCC mAb AF5. (**D**) Explant cultured with 40 μM IC-3 and stained with DCC mAb. Explants are oriented as in Fig. 1; scale bar, 200 μm. (**E**) Quantification of the fluorescence intensity of DCC mAb staining along the dorsoventral axis of a control explant [line along which intensity is measured is shown in (C)] and an explant cultured in the presence of 40 μM IC-3 [line along which intensity is measured is shown in (D)]. The explants shown here represent the average intensity difference within multiple pairs of observed explants.

metalloprotease-dependent ectodomain shedding (*17*). Transfected CHO cells expressing DCC were metabolically labeled and then chased with medium that included or omitted IC-3. Cell extracts and supernatants were analyzed by immunoprecipitation with a monoclonal antibody to the extracellular domain of DCC (DCC mAb). A 160-kD protein was immunoprecipitated from extracts of DCC-transfected CHO cells but not from control cells (Fig. 3A). In addition to the presence of full-length DCC in cell extracts, a protein of ~130 kD, the predicted size of the entire DCC ectodomain, was also immunoprecipitated from the supernatant of DCC cells (Fig. 3B). This protein was absent from control transfected cells and was the same size as an immunoprecipitated protein from transfected cells expressing a soluble form of the complete DCC ectodomain (Fig. 3B). The metalloprotease inhibitors IC-3, GM6001, and 1,10-phenanthroline all abolished the presence of the ~130-kD protein from supernatants of cells transfected with full-length DCC, whereas the control isomer of GM6001 did not (Fig. 3B; only the IC-3 result is shown here) (*15*); these findings suggest that the protein resulted from a metalloprotease-dependent cleavage near the transmembrane region of DCC. We also investigated whether DCC could be shed from primary cultures of dissociated rat dorsal spinal cord (*18*). A protein corresponding to full-length DCC was visible in cell extracts that had been immunoprecipitated with DCC mAb, but not in extracts that were mock-immunoprecipitated with only the secondary antibody (Fig. 3C). A ~130-kD protein corresponding to the DCC ectodomain could be immunoprecipitated from the chase medium supernatant only if DCC mAb was included (Fig. 3D). The presence of IC-3 or GM6001 in the chase medium abolished the presence of this protein (1,10-phenanthroline could not be tested because it induced neural cell detachment). These results indicate that dissociated commissural neurons can shed, in a metalloprotease-dependent manner, the ectodomain of full-length DCC.

Although the axon outgrowth potentiation observed with metalloprotease inhibitors is similar to that observed with NSA (*8, 13*), it is not clear whether NSA acts by the same mechanism, because it failed to increase DCC expression within E11 dorsal spinal cord explants and failed to protect DCC from metalloprotease-dependent ectodomain shedding (*15*). It is possible that NSA acts in a mechanistically distinct manner that does not involve metalloprotease inhibition. Alternatively, it is possible that both NSA and IC-3 act as metalloprotease inhibitors, but that NSA (and perhaps also IC-3) inhibits degradation of some extracellular component other than DCC that is necessary for commissural axon guidance.

Our findings indicate that dorsal spinal cord explants display a metalloprotease activity that mediates (directly or indirectly) the proteolytic degradation of the netrin receptor DCC to a presumably nonfunctional form, and that the inhibition of this metalloprotease activity leads to enhanced responsiveness to soluble netrin-1. This functional effect may result from inhibition of DCC cleavage, although we cannot exclude the possibility that it may also result partly or entirely from inhibition of cleavage of other substrates. Nonetheless, our results imply that the balance of metalloprotease activity and metalloprotease inhibitory activity within the dorsal spinal cord or along the trajectory of commis-

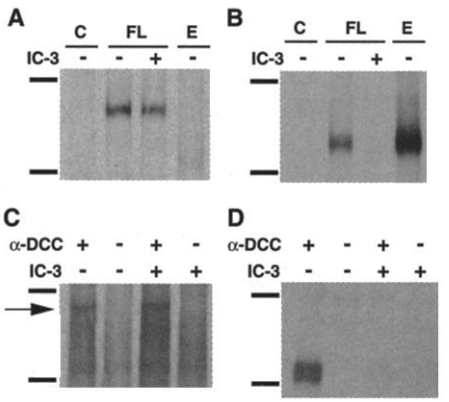

Fig. 3. DCC is a substrate for metalloprotease-dependent ectodomain shedding from transfected CHO cells and dissociated dorsal spinal cord neurons (*17, 18*). (**A**) DCC immunoprecipitations from cell extracts of metabolically labeled CHO cells chased in the presence or absence of IC-3 (indicated above each lane) and previously transfected with one of three plasmids: control vector (C), an expression plasmid encoding full-length DCC (FL), or an expression vector encoding a DCC mutant molecule that lacks the intracellular and transmembrane domains and produces the entire DCC ectodomain as a secreted protein (E). Control CHO cell extracts contained no DCC-immunoprecipitable proteins, whereas extracts from FL transfected cells contained a protein of 160 kD, the predicted size of full-length DCC. The secreted extracellular domain can be seen as a faint band within cell extracts at ~130 kD. (Molecular mass markers are indicated to the left of each autoradiogram; the upper marker in each case is 200 kD and the lower marker is 116 kD.) (**B**) DCC immunoprecipitations from supernatants of the metabolically labeled CHO cells whose cell extracts are shown in (A). Control supernatants contained no DCC-immunoprecipitable protein, whereas FL transfected CHO cells contained a protein of ~130 kD that was the same size as the constitutively expressed DCC extracellular domain. The presence of the extracellular DCC fragment in the supernatant was abolished by inclusion of IC-3 in the chase medium. (**C**) DCC or mock immunoprecipitation from cell extracts of dissociated E13 commissural neurons chased in the presence or absence of IC-3. A faint band corresponding to full-length DCC (arrow) can be seen in the presence of DCC mAb (α-DCC). The band above the 116-kD marker is nonspecific and is present in all immunoprecipitations from extracts of dissociated dorsal spinal cord cells. (**D**) DCC or mock immunoprecipitations from supernatants of the dissociated E13 commissural neurons chased in the presence or absence of IC-3 in (C). The ~130-kD protein observed in the presence of DCC mAb and absence of IC-3 corresponds to an ectodomain fragment of DCC. Close examination of this band on this and other gels reveals it to be a doublet, suggesting that there is more than one cleavage of DCC from these neurons. Chase media containing IC-3 and/or mock immunoprecipitations do not contain a DCC-immunoprecipitable protein. Note that the percentage of DCC shed from CHO cells during the 1-hour chase was far less than that shed from neurons, because an increase in full-length DCC in extracts of cells exposed to IC-3 was only occasionally detected in the former (A) but was readily detected in the latter (C).

sural neurons may be an important regulator of commissural axon guidance. Interestingly, it was recently shown in *Caenorhabditis elegans* that the MIG-17 metalloprotease, a member of the ADAM family, is required for proper migration of distal tip cells (DTCs), and a possible role for MIG-17 in regulating the function of the netrin UNC-6 was suggested by interactions between *mig-17* and *unc-6* mutant alleles in DTC migration (*19*). Thus, metalloprotease regulation of netrin and/or netrin receptor function may be phylogenetically conserved and involved in both axon guidance and cell migration.

The ability of axons to alter their responses to guidance cues as they progress through the environment is known to be essential for their progression from one intermediate target to the next (*20, 21*), and there is evidence that altered axonal responsiveness may result from increased or decreased insertion of receptor protein in the axonal membrane (*21*) or by alteration of downstream signaling pathways through activation of modulatory second messenger systems (*22*). The regulated shedding of axon guidance receptor ectodomains provides an additional mechanism through which axonal responsiveness to guidance cues can be regulated to help shape the precise trajectories of axons that are essential to the proper wiring of the nervous system.

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17. The shedding assay was similar to that of J. Arribas *et al.* [*J. Biol. Chem.* **271**, 11376 (1996)]. Briefly, CHO cells were cultured in DME-H21 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% glutamine, and proline (20 μg/ml) and seeded in six-well culture plates 12 to 18 hours before transfection [using Fugene (Boehringer-Mannheim)] with expression constructs for full-length DCC or the DCC ectodomain (as a control) in pcDNA (Invitrogen). After 48 hours, cells were washed, then starved for 1 hour with DME-H21 medium lacking cysteine and methionine but supplemented with penicillin/streptomycin and dialyzed FBS, labeled for 4 hours with starvation medium with added glutamine, proline, and [^{35}S]cysteine/methionine labeling mix (150 μCi/ml, Amersham), washed, and chased for 1 hour with complete medium that contained either no additives or 20 μM IC-3. DCC was immunoprecipitated from equivalent amounts (based on radioactive counts incorporated into protein) of each supernatant and cell extract with DCC mAb AF5 (Oncogene). Immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and prepared for autoradiography.
18. The dorsal portions of 10 E13 spinal cords were dissected in L15 medium, washed twice with 1 ml of Ca^{2+}/Mg^{2+}–free phosphate-buffered saline (PBS), digested for 20 min at 37°C with 1 ml of 0.025% saline trypsin versene in Ca^{2+}/Mg^{2+}–free PBS, washed twice with serum-containing medium, and dissociated by trituration. Cells (1.5×10^6) were plated and cultured overnight in 2 ml of complete medium [DME-H21 supplemented with 40 mM glucose, 5% FBS, and N3 additives; H. Romijn, A. Habets, M. Mud, P. Walters, *Brain Res.* **254**, 583 (1981)] onto six-well tissue culture dishes precoated with polyornithine (5 μg/ml) and laminin (3 μg/ml) (Gibco-BRL). Cultures were metabolically labeled and immunoprecipitated as above for the CHO cells, except that the cells were washed three times with PBS before direct addition of labeling media containing N3 additives, supplemental glucose, and 5% dialyzed FBS. Cells were then washed once with complete medium before adding chase medium with or without 20 μM IC-3. Immunoprecipitations, SDS-PAGE, and autoradiography were performed as in (*17*).
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23. E11 rat dorsal spinal cord explants were dissected and cultured as described [M. Tessier-Lavigne, M. Placzek, A. G. Lumsden, J. Dodd, T. M. Jessell, *Nature* **336**, 775 (1988)] (*8, 13*). In some cases, explant medium was supplemented with a concentrated high-salt extract of stably transfected netrin-expressing cells [R. Shirasaki, C. Mirzayan, M. Tessier-Lavigne, F. Murakami, *Neuron* **17**, 1079 (1996)] that was diluted 1:133 into the culture medium to give a final netrin-1 concentration of ~50 ng/ml. Control explants with no netrin-1 were cultured with an equivalent dilution of 1.0 M NaCl and 20 mM Na_2HPO_4, pH 7.0. The metalloprotease inhibitor IC-3 (10 or 20 mM stock in water) was tested at final concentrations of 5 to 80 μM. For the quantification shown in Fig. 1E, axon fascicles emerging from each explant were measured and added to determine the total length of all fascicles per explant (isolated axons, which were rare, were not counted). Measurements were made using Scion Image 1.62a (which allows analysis in only one focal plane; the plane with the most visible fascicles was therefore chosen) and analyzed using Cricket Graph III 1.5.1.
24. Whole-mount immunohistochemistry was performed as described (*10*) using TAG-1 mAb 4D7 [M. Yamamoto, A. M. Boyer, J. E. Crandall, M. Edwards, H. Tanaka, *J. Neurosci.* **6**, 3576 (1986)] or DCC mAb AF5. Fluorescence intensity was quantified using NIH Image software.

25. We thank R. Black at Immunex for IC-3 metalloprotease inhibitor; Z. Werb, C. Bargmann, and S. Strickland for thoughtful contributions; and S. Faynboym for netrin-containing cell extracts. Supported by predoctoral fellowships from NSF and the California affiliate of the American Heart Association (M.J.G.). M.T.-L. is an investigator of the Howard Hughes Medical Institute.

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