Netrin Stimulates Tyrosine Phosphorylation of the UNC-5 Family of Netrin Receptors and Induces Shp2 Binding to the RCM Cytodomain*

Received for publication, April 30, 2001, and in revised form, August 27, 2001
Published, JBC Papers in Press, August 30, 2001, DOI 10.1074/jbc.M103872200

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Caenorhabditis elegans UNC-5 and its mammalian homologues such as RCM are receptors for the secreted axon guidance cue UNC-6/netrin and are required to mediate the repulsive effects of UNC-6/netrin on growth cones. We find that C. elegans UNC-5 and mouse RCM are phosphorylated on tyrosine in vivo. C. elegans UNC-5 tyrosine phosphorylation is reduced in unc-6 null mutants, and RCM tyrosine phosphorylation is induced by netrin-1 in transfected HEK-293 cells, demonstrating that phosphorylation of UNC-5 proteins is enhanced by UNC-6/netrin stimulation in both worms and mammalian cells. An activated Src tyrosine kinase induces phosphorylation of RCM at multiple cytoplasmic tyrosine residues creating potential binding sites for cytoplasmic signaling proteins. Indeed, the NH2-terminal SH2 domain of the Shp2 tyrosine phosphatase bound specifically to a Tyr568 RCM phosphopeptide. Furthermore, Shp2 associated with RCM in a netrin-dependent manner in transfected cells, and co-immunoprecipitated with RCM from an embryonic mouse brain lysate. A Y568F mutant RCM receptor failed to bind Shp2 and was more highly phosphorylated on tyrosine than the wild type receptor. These results suggest that netrin-stimulated phosphorylation of RCM Tyr568 recruits Shp2 to the cell membrane where it can potentially modify RCM phosphorylation and function.

Neuronal growth cones in the developing nervous system are guided to their targets by attractive and repulsive guidance cues. Several proteins have been identified that play key roles in guiding axons along their stereotypical pathways, including members of the ephrin, Netrin, Semaphorin, and Slit protein families. Several classes of transmembrane receptors for these various guidance cues have been identified and characterized (see Refs. 1 and 2 for reviews). In the case of the netrins, there are two known classes of receptors, the deleted in colorectal cancer (DCC)† and UNC-5 families. The DCC family receptors (UNC-40, Frazzled, or DCC in Caenorhabditis elegans, Drosophila, and mammals, respectively) are required for the attraction of growth cones by netrins in vitro. Loss of function mutations in the corresponding genes result in misrouting of axons that are normally attracted to a netrin source in vitro (3–6). The UNC-5 family receptors, regarded as co-receptors of DCC, are required for the repulsive effect of the netrins. In C. elegans, the loss of UNC-5 function in neurons that normally project away from an UNC-6/netrin source results in misrouting similar to that observed in unc-6 mutants. Ectopic expression of UNC-5 in neurons that normally fail to respond to UNC-6 netrin causes their axons to be directed away from the source in an UNC-40 dependent manner (7–11). In Xenopus spinal cord neurons, DCC mediated attraction to netrin was converted to repulsion by expression of UNC-5 in these cells (12). However, some evidence suggests that the relationship of netrin mediated attraction and repulsion might be more complex. For example, unc-40 loss of function mutations in C. elegans impair neurite migrations away from the UNC-6/netrin source to a lesser extent than loss of function mutations in unc-5, indicating UNC-5 receptors may mediate their own UNC-40 independent repulsive response to UNC-6/netrin (9). Furthermore, Frazzled (the UNC-40/DCC Drosophila ortholog) is required for netrin mediated attraction, but not repulsion, suggesting the possible existence of additional netrin receptors in Drosophila (13).

All of the UNC-5 receptors, including the three mammalian subtypes rostral cerebellar malformation (RCM), UNC5H1, and UNC5H2 (14, 15) share a common structure. Their extracellular regions contain two immunoglobulin (Ig) and two thrombospondin type 1 domains, followed by a single transmembrane segment. Within their cytoplasmic regions they possess a juxtamembrane sequence, followed by a conserved ZZ-5 domain (homologous to a portion of zona occludens-1; (16)) and a C-terminal death domain (DD) (17). A DCC binding motif was recently located between the ZZ-5 and DD domain of UNC5H2 (12).

The cytoplasmic domains of guidance receptors are key effectors, directing either attractive or repulsive responses

* This work was supported in part by grants from the Canadian Institutes of Health Research (CIHR) (to T. P. and J. C.) and a Howard Hughes Medical Institute International Research Scholar Award (to T. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported by a CIHR postdoctoral fellowship.
‡ Supported by a CIHR grant.
§ Supported by the PVA Spinal Cord Research Foundation and the CNRP, administered by the ONF.
¶ Supported by a CIHR grant and a Distinguished Scientist of the CIHR.
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† The abbreviations used are: DCC, deleted in colorectal cancer, a netrin receptor; DD, death domain; RCM, rostral cerebellar malformation, a netrin receptor; Shp2, a Src homology 2 domain containing protein-tyrosine phosphatase type 2; Unc, uncoordinated movement in C. elegans; Unc-6, C. elegans netrin homolog; UNC-5, a C. elegans UNC-6 receptor, RCM homology; UNC-40, a C. elegans UNC-6 receptor, DCC homology; ZU-5, zona occludens-1 and Unc-5 homology domain; GST, glutathione S-transferase; PLC, phospholipase C.
Phosphorylation Regulates UNC-5/RCM Function

(12, 18). A chimeric receptor consisting of a DCC ectodomain and an UNC5 cytoplasmic domain (12) mediates a repulsive response to the netrin-1 gradient in a turning assay, indicating that the UNC5 cytoplasmic domain determines the response of growth cones to the netrin-1 signal.

Studies of cytoplasmic signaling mechanisms have led to the notion that the response of a growth cone to a particular guidance cue depends on the internal state of the neuron, which, in turn, is under the influence of other coincident signals received by the neuron (19). So far, the immediate downstream signaling molecules for the DCC and UNC5 receptors are unknown. In C. elegans, suppressors of growth cone steering induced by ectopic UNC5 include four new genes as well as four genes previously known to be required for axon guidance, unc-6, unc-40, unc-44, and unc-34 (11). Interestingly, unc-44 encodes an ankryin-related protein, thus providing a possible physical link between UNC5 and the cytoskeleton. Homologs of UNC-34, Mena and ena, are profillin-binding proteins required for axon guidance in mice and Drosophila, respectively (20, 21).

In this study, we focus on the regulation of UNC5 receptor-mediated signaling. We demonstrate that UNC5 receptors are tyrosine phosphorylated in vivo and this tyrosine phosphorylation can be enhanced by netrin ligand. One phosphorylation site in the ZU-5 domain is identified and shown to bind with the Shp2 tyrosine phosphatase in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Constructs and Reagents—Full-length mouse RCM (14) was cloned into the mammalian expression vector pcDNA3 (Invitrogen) with an in-frame HA or FLAG tag in the carboxyl terminus. Mutagenesis of RCM was performed using the QuickChange system (Stratagene). For glutathione S-transferase (GST) fusion constructs, cDNA sequences corresponding to the mouse Grb2 SH2 domain (residues 79–160), bovine PLCγ SH2N domain (residues 537–658), and human Shp2 SH2 domain (residues 3–105) were cloned into pGEX vector (Amersham Pharmacia Biotech) and expressed as GST fusion proteins. The synthetic RCM ZU-5 peptide has the sequence Cys-Val-Tyr-Glu-Met-Tyr(P)-Val-Thr-Val-His-Arg-Lys (amide at the carboxyl terminus) and was confirmed by mass spectrometry.

Polyclonal RCM antisera was raised against a GST fusion protein expressing the region between the ZU-5 and the death domain of RCM (residues 160–244) (20). A mouse immunoglobulin G anti-DCC mouse monoclonal (AF5) was purchased from Oncogene Science, anti-PA mouse monoclonal (12CA5) was produced as ascites fluid (22), and other antibodies were purchased from Santa Cruz Biotechnology.

Generation of C. elegans Transgenic Lines and Tagged Proteins—General techniques for the handling of nematodes were described by Brenner (23). C. elegans strain N2 was used as the wild type. The alleles used were (LGI unc-5(e533), unc-40(e457), and unc-6(e400)) (9). The details of the construction of tagged pU5HA, pUNC40GFP, and the generation of C. elegans transgenic lines are described by Killeen et al.2

Cell Culture, DNA Transfection, and Tissue Lysis—Human embryonic kidney 293 (HEK-293) cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. DNA transformation was performed as described previously (24). 20 μg of plasmid DNA was used to transfected 2 × 10⁶ cells/100-mm plate. Control cells were treated in the same way, but with no DNA or with expression vector DNA only.

Brain tissue lysate was obtained by brief homogenizing brain tissues in PLC buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM NaF, 100 mM Na₃VO₄, and a mixture of protease inhibitors) (25, 26), incubating at 4 °C for 30 min, and centrifuging at top speed in a microcentrifuge at 4 °C for 10 min. The supernatants were used for immunoprecipitation and Western blotting.

All of the C. elegans transgenic strains used in this study contain the transgene of interest as a stable integration in the genome. Mixed stage populations of C. elegans were used to measure the phosphorylation levels of C. elegans proteins. The mixed stage populations contain a mixture of animals representing all of the different stages of development from embryo through the four larval stages and adult. Worm lysates were prepared from a mixed population of unstained animals grown on standard nematode growth media plates. The worms were washed off the plates and centrifuged at 3000 × g for 5 min. The worm pellet was resuspended in PLC buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0)), sonicated for 1 min, incubated at 4 °C for 1 h, and then centrifuged at top speed in a microcentrifuge at 4 °C for 10 min. The supernatants were used for immunoprecipitations and Western blotting. To examine protein phosphorylation levels at different developmental stages, aliquots from a worm population that was synchronized in development we were analyzed after hatched off from plates and assayed at 2-day intervals.

To stimulate receptor cells, netrin-1 was purified as described by Serafini (27) or conditioned medium from netrin-1 expressing stable cells was applied to transfected cells for 20 min at 37 °C. Cells were incubated in medium containing 0.5% fetal bovine serum for 6–8 h prior to stimulation. Bovine serum albumin or conditioned medium from HEK-293 cells was used for control stimulation.

Immunoprecipitation and Western Blotting—Cells were rinsed once in ice-cold phosphate-buffered saline and routinely lysed in PLC lysis buffer as previous described (25, 26), except in Fig. 6, where buffer D (20 mM Hepes, pH 7.5, 0.5% Triton X-100, 10% glycerol, 20 mM NaF, 1 mg/ml bovine serum albumin, and 1 mM vanadate) (28) was used to demonstrate protein associations. Protein concentrations of cleared lysates were determined by a BCA assay (Bio-Rad). Immunoprecipitations were made as previously described (28) for 1–2 h at 4 °C and beads were routinely washed 3 times in HNTG buffer (20 mM Hepes, pH 7.5, 10% glycerol, 0.1% Triton X-100 and 150 mM NaCl) (25) except in Fig. 6, where buffer D was used. Proteins were separated on 7.5% SDS-polyacrylamide electrophoresis gels and transferred to an Immobilon-P membrane (Millipore). Membranes were blocked in TBST containing 5% bovine serum albumin for anti-phosphotyrosine blots or 5% skimmed milk for other blots, and immunoblotted as per standard protocols. Primary antibodies were detected with anti-mouse- or protein-A-horseradish peroxidase followed by treatment with Enhanced Chemiluminiscence (Pierce). To quantitatively measure the signals from Western blots, we used the Fluor-S™ Multimager (Bio-Rad), which uses an ultra-sensitive charge-coupled detector to capture signals and provide digital data. The relative levels of phosphorylated proteins were calculated as the ratio of the signal from an anti-phosphotyrosine antibody versus the signal obtained after stripping the blot and reprobing with an anti-HA antibody. The Tyr(P)/HA signal ratio from phosphorylated RCMHA was used as the control and indicated as 100%.

The peptide pull-down experiment involved coupling RCM 5-mer with anti-RCM antibody to 50 μg of GST fusion protein/ml lysate) or mouse brain whole cell lysates in PLC lysis buffer at 4 °C for 1 h and washed 3 times in HNTG buffer. The dephosphorylated peptide coupling gel was obtained by incubating phosphorylated peptide gel with potato acid phosphatase at 37 °C for 0.5 h. GST fusion proteins were produced in isoosproyl-1-thio-β-p-galactopyranoside-induced Esherichia coli which were then lysed in PLC buffer.

Nano-ESI/MS/MS/MS-based Phosphopeptide Mapping—The domains in the intracellular RCM region were made as GST fusion proteins. These GST fusion proteins were expressed in Epicurian coli cells, purified using glycinethione-Sepharose beads (Amerham Pharmacia Biotech), and trypsin digested to completion at 37 °C for 3 h using a 50:1 ratio of protein to protease. Phosphopeptides were desalted using ZipTip desalting columns (Millipore) eluted in 5% formic acid, washed with equilibration buffer, and eluted with 5% formic acid, 60% methanol. Tandem mass spectrometry (MS/MS) analysis was carried out on a QSTAR™ mass spectrometer (PE-Sciex) with a nano-electrospray ion (ESI) source (Protana A/S). Following the identification of tryptic ions of interest, product ion spectra were generated by collisionally induced dissociation. For product ion scans, collision energy was determined experimentally (29).

Statistical Methods—All data are expressed as mean ± S.D. An unpaired Student’s t test was used for statistical comparisons of the mean values between samples. A value of p < 0.05 was taken to indicate statistical significance.

RESULTS

Tyrosine Residues in UNC-5 Receptors Are Phosphorylated in Vivo—A number of receptors implicated in axon guidance ei-
**Phosphorylation Regulates UNC-5/RCM Function**

Fig. 1. *In vivo phosphorylation* of UNC-5 and UNC40 in *C. elegans* and RCM in mouse brain. A, anti-phosphotyrosine immunoblot of anti-HA immunoprecipitations from unc-5 (e53) animals transgenic for either functional wild-type unc-5 (pU5) or one of two functional unc-5::HA constructs (pU5::HAa or pU5::HAb). Worm lysates were immunoprecipitated with anti-HA antibodies and the precipitated proteins were analyzed by immunoblotting with the anti-phosphotyrosine monoclonal antibody 4G10 (left panel). The filters were then stripped and reprobed with anti-HA antibodies to show the position of UNC-5HA (right panel). B, anti-phosphotyrosine immunoblot of anti-RCM immunoprecipitations from embryonic day 14 (E14) and adult mouse brain lysates (upper panel). The same filters were reprobed with an anti-RCM antibody to show that RCM was indeed present in the anti-RCM immunoprecipitates (lane 2, lower panel). For size comparisons the first lane contains tyrosine-phosphorylated RCMHA immunoprecipitated by anti-HA antibody from HEK-293 cells that were co-transfected with RCM and v-Src. C, anti-phosphotyrosine immunoblot of anti-GFP immunoprecipitations from unc-5 unc-40 double null animals transgenic for a functional unc-5::HA construct and a functional wild-type unc-40::GFP construct (left panel). Anti-GFP reprobe, right panel. UNC5HA, RCM, and UNC40GF are indicated by the arrows. Molecular mass standards (in kilodaltons) are indicated to the left. These data are representative of the results of three independent experiments.

ther possess intrinsic tyrosine kinase activity, for example, the Eph receptors, or are substrates for tyrosine phosphorylation, such as Robo1 and the plexins (2, 30–32). Since phosphorylation is a rather general mechanism for regulating receptor function, and can control interactions with cytoplasmic targets, we examined whether *C. elegans* UNC-5 may become phosphorylated on tyrosine. To this end, we constructed several transgenic *C. elegans* lines, in an unc-5(e53) null mutant background, that contain a construct (pU5::HA) encoding a functional HA-tagged UNC-5 (UNC-5::HA). The unc-5 null mutant unc-5(e53), which displays severely uncoordinated (Unc) locomotion, has been described previously (9). The Unc phenotype is caused by defects in the migration of the motor neuron growth cones that migrate along the dorsoventral axis of the animal. Rescue of the Unc phenotype was complete in the transgenic lines containing an untagged UNC-5 protein and was nearly complete for the lines containing UNC-5::HA.3 To analyze UNC-5::HA tyrosine phosphorylation, lysates from two independent pU5::HA transgenic lines were immunoprecipitated using an anti-HA tag antibody and immunoblotted with an anti-phosphotyrosine antibody. This approach identified tyrosine phosphorylated UNC-5::HA in worms that express the epitope-tagged form of UNC-5 (Fig. 1A), but not in transgenic worms expressing wild type UNC-5 lacking the HA tag (negative control, Fig. 5A). The UNC-5 co-receptor, UNC-40 (GFP tagged), was also tyrosine phosphorylated in *C. elegans* (Fig. 1C).

To test whether mammalian UNC-5 homologs might also be tyrosine phosphorylated, endogenous RCM, a murine UNC5 family member, was immunoprecipitated from mouse brain lysates using anti-RCM antiserum raised against a fragment of the RCM cytoplasmic region between the ZU-5 and death domains. The specificity of the anti-RCM polyclonal antibodies was confirmed by immunoprecipitation, Western blotting, and immunostaining of HEK-293 cells transfected with the RCM cDNA (data not shown) and no band corresponding to the RCM protein was observed in immunoprecipitates using pre-immune serum (Fig. 1B). A low level of tyrosine phosphorylation was detected on RCM immunoprecipitated from an embryonic day 14 mouse brain lysate (Fig. 1B). RCM from adult mouse brain lysate was not tyrosine phosphorylated, even though the RCM protein was more abundant in the adult brain lysate (Fig. 1B). Changes in the level of UNC-5 phosphorylation also occurred during *C. elegans* development. The phosphorylation levels of UNC-5 appeared to increase from larval stage one through to the adult (data not shown). These results indicate that *C. elegans* UNC-5 and mouse RCM are tyrosine phosphorylated in *vivo*, and their phosphorylation status may change during development.

Netrin Stimulates the Phosphorylation of UNC-5 Receptors—or one—if the tyrosine phosphorylation of UNC-5 receptors is functionally relevant, one might expect to see changes in the levels of UNC-5 phosphorylation upon stimulation by its ligand. To pursue this point, we investigated the *in vivo* phosphorylation levels of *C. elegans* UNC-5 in the presence or absence of UNC-6/netrin. Immunoprecipitation experiments revealed that UNC-5::HA isolated from unc-6(e400) null and unc-40(ev457) null strains of *C. elegans* still contained phosphotyrosine, indicating that its phosphorylation was not entirely unc-6-dependent. However, compared with the level of
phosphorylation on UNC5HA in an unc-5 null strain, the relative levels of phosphorylated UNC5HA in unc-6 or unc-40 null mutant strains were 53 ± 27% (n = 3, p < 0.05) and 84 ± 35% (n = 3), respectively (Fig. 2A). This result indicates that phosphorylation of UNC-5 is stimulated by UNC-6 in vivo. There was no significant change in UNC5-5HA phosphorylation in an unc-40 background.

The ligand dependence of mammalian RCM phosphorylation was tested using FLAG-tagged RCM, which was co-transfected with DCC into HEK-293 cells. Netrin-1 stimulation induced a modest tyrosine phosphorylation of transfected RCM (Fig. 2B). To facilitate the further analysis of RCM phosphorylation, we co-transfected RCM into HEK-293 cells with a constitutively active tyrosine kinase, v-Src (33). v-Src induced a basal tyrosine phosphorylation of transfected RCM (Fig. 2B).

**Multiple Tyrosine Residues on UNC-5 Are Phosphorylated**—In an effort to localize the tyrosine phosphorylation sites in RCM, we introduced a series of deletions into the RCM cytoplasmic region (Fig. 3A). The deleted regions included the death domain (ΔDD), the death domain plus the DCC-binding domain (JM), or the juxtamembrane domain (ΔJM) (Fig. 3A, diagram). Upon co-transfection with v-Src into HEK-293 cells, all of the deletion mutants were phosphorylated on tyrosines, but appeared to have reduced levels of phosphorylation compared with full-length RCM (Fig. 3A). The relative levels of phosphorylation for full-length RCM (RCMHA, n = 6), ΔDD (n = 4), JMZU-5 (n = 4), JM (n = 4), and ΔJM (n = 4) were 100, 80 ± 25, 82 ± 30, 74 ± 28%, and 38 ± 24% (p < 0.05), respectively. These results suggest that RCM may be phosphorylated on multiple tyrosine sites in different regions of RCM. In this regard, it is of interest that an analysis of unc-5 deletion mutants expressed in C. elegans has indicated that UNC-5 is similarly phosphorylated on multiple tyrosine sites in vivo.  

Among potential RCM tyrosine phosphorylation sites, Tyr<sup>568</sup> in the ZU-5 domain is highly conserved among UNC-5 family members and is flanked by amino acids characteristic of tyrosine kinase substrate motifs and SH2 domain-binding sites (Fig. 3B). To test whether RCM Tyr<sup>568</sup> can be phosphorylated in vitro, we expressed the ZU-5 domain as a GST fusion protein in TKX bacteria (Stratagene), which also express a tyrosine kinase substrate motifs and SH2 domain-binding sites. Tyr<sup>568</sup> of the ZU-5 domain is highly conserved among UNC-5 family members and is flanked by amino acids characteristic of tyrosine kinase substrate motifs and SH2 domain-binding sites. 

Ion peaks corresponding to the Tyr<sup>568</sup> region peptide (Val-Tyr-Glu-Met-Tyr<sup>568</sup>-Val-Thr-Val-His-Arg) in the unphosphorylated and the singly phosphorylated states were identified from initial spectra (MS1) (Fig. 3C, a). Fragmentation of these parent ions produced secondary spectra (MS2) which allowed peptide sequencing and thus confirmed the peptide identity (Fig. 3C, b) and the specific phosphorylation of tyrosine 568 in RCM (Fig. 3C, c).
The phosphorylation of this residue in TKX bacteria implies that Tyr568 in the ZU-5 domain is a potential phosphorylation site in RCM.

Phosphorylation of Tyr568 in RCM Recruits Shp2—Individual substitution of tyrosines 449, 454, and 482 with phenylalanine caused a minor reduction in v-Src induced tyrosine phosphorylation of RCM in HEK-293 cells, with relative phosphorylation levels at 78 ± 28% (n = 4), 72 ± 25% (n = 4), and 74 ± 29% (n = 4) of full-length RCM, respectively. Surprisingly, in contrast to the other mutants, the Y568F mutation led to increased phosphorylation (152 ± 30% (n = 4), p < 0.05) relative to wild-type RCM. Phosphorylated Tyr568 can bind Shp2, a phosphopeptide corresponding to the RCM Tyr568 motif (Val-Tyr-Glu-Met-Tyr568-Val-Thr-Val-His-Arg-Lys) was synthesized and examined for its ability to bind to GST fusion proteins from bacterial lysates and polypeptides from mouse brain lysates. The phosphorylated ZU-5 peptide associated with GST fusion proteins containing the Shp2 NH2- or COOH-terminal SH2 domains, as well as the PLCγ1 NH2- or COOH-terminal SH2 domains, but not GST alone nor GST fusion proteins containing the RasGAP SH2 NH2-terminal domain, the Grb2 SH2 domain or the Src SH2 domain (Fig. 5A). Affinity isolation of the SH2-N domain of Shp2 depended on the phosphotyrosine residue, since treatment of the phosphorylated peptide with potato acid phosphatase abolished its binding to the Shp2 SH2-N domain (Fig. 5B). The phosphorylated peptide also pulled down PLCγ and Shp2, but not Grb2, RasGAP, or Nck, all of which contain SH2 do-

**FIG. 3.** Phosphorylation of RCM deletion mutants (A) in v-Src co-transfected HEK-293 cells, and the identification of Tyr568 as one of the tyrosine phosphorylation sites in RCM by mass spectrometry (C). A, anti-phosphotyrosine immunoblot of anti-HA immunoprecipitations from HEK-293 cells transfected with RCMHA or deleted forms of RCMHA as indicated at the top of the blot (upper panel). The filter was then stripped and reprobed with an anti-HA antibody to show that wild type RCMHA and the deletion mutants, marked by asterisks, were present in each lane (lower panel). The data are representative of the results of four independent experiments. B, amino acid sequence alignment of different UNC-5 receptors and the human ZO-1 protein in the ZU-5 region around RCM Y568. Conserved tyrosine residues are shown in bold. C, RCM Y568 can be phosphorylated in vitro. a, a nano-ESI-MS (MSI) spectrum identifying triply charged ion peaks corresponding to the unphosphorylated (432.9) and phosphorylated (459.3) forms of the tryptic peptide YVEMY568VTVHR (1296.6 atomic mass units), obtained from a GST-ZU-5 fusion protein. b and c, MS2 spectra of the phosphorylated and unphosphorylated ions illustrating the 80 atomic mass units (one phosphate) increase in mass of single charged peptide in the 459.3-derived spectrum (b) versus the 432.9-derived spectrum (c).
tyrosine kinases, can directly bind specific SH2 domain proteins and can also phosphorylate the p62 dok-1 scaffolding protein, which in turn binds SH2 proteins such as p120-Ras-GAP, Nck, and Shp2 (26, 40, 41). Such data have strongly implicated tyrosine phosphorylation as playing a significant role in directed cell movements and axon guidance.

In addition to receptors with intrinsic tyrosine kinase activity, a large number of receptors lack covalently linked catalytic domains, but nonetheless associate with tyrosine kinases and require tyrosine phosphorylation to engage SH2 domain proteins and transmit intracellular signals. Cytokine receptors, for example, interact with cytoplasmic Jak tyrosine kinases, which phosphorylate the activated receptor, thereby creating binding sites for the SH2 domains of Stat transcription factors (42). Recent data indicate that a number of guidance receptors, although lacking any intrinsic kinase activity, may nonetheless serve as substrates for tyrosine kinases and be regulated by tyrosine phosphorylation. The Drosophila Roundabout (Robo) receptor, which mediates the repulsive effects of the Slit ligand on axonal crossing of the mid-line, can be phosphorylated on multiple tyrosine residues by the AbI tyrosine kinase (18). Substitution of one of the conserved tyrosine phosphorylation sites (in the CC1 Robo motif) creates a hyperactive Robo receptor (18), suggesting that one role of tyrosine phosphorylation is to attenuate the strength of Robo signaling. In a similar vein, the Drosophila Dacm receptor is tyrosine phosphorylated and binds the SH2 domain of the Nck (Dock) SH2/SH3 adaptor in a fashion that appears important for the pathfinding of Bolwig’s nerve (43). In addition, plexins, receptors which mediate the effects of the semaphorin guidance cues, become tyrosine phosphorylated in cells (32). Although the semaphorins contain an extended and conserved “SP” cytoplasmic signaling domain, they lack intrinsic tyrosine kinase activity, suggesting that their phosphorylation results from an associated tyrosine kinase.

The importance of tyrosine phosphorylation in axon guidance in vertebrates is underscored by the finding that receptor-like tyrosine phosphatases are important in several aspects of neuronal development, ranging from cell fate determination to axon guidance and fasciculation (31, 44, 45). Thus, genetic analysis in Drosophila has revealed that two receptor-like tyrosine phosphatases (DPTP10d and DPTP69D) are positive regulators of Slit/Robo repulsive signaling (46). Our results show that members of the UNC-5 family of guidance receptors are also targets for tyrosine phosphorylation in vivo.

Phosphorylation Regulates UNC-5/RCM Function

Tyrosine kinases were originally identified through their ability to affect cell growth and differentiation, but more recent data have also implicated phosphotyrosine signaling in the control of guided cell movements and thus in the formation of complex tissues such as the nervous system (2, 31, 35). Notably, Eph receptors, which form the largest family of vertebrate receptor tyrosine kinases, control events such as axon guidance and topographic map formation in the mammalian central nervous system, as well as guiding the movements of neural crest cells and the interactions of cells that form the cardiovascular system (36, 37). Furthermore, the C. elegans Eph receptor VAB-1 regulates morphogenetic cell movements (38, 39). Eph receptor signaling re-organizes the actin cytoskeleton, and can result in the collapse of axonal growth cones, and thus in the repulsion of Eph receptor-expressing cells from cells that display relevant ligands (ephrins) on their surface. Although the physiological cytoplasmic targets of Eph receptors remain uncertain, activated Eph receptors, in common with other receptor tyrosine kinases, can directly bind specific SH2 domain proteins and can also phosphorylate the p62 dok-1 scaffolding protein, which in turn binds SH2 proteins such as p120-Ras-GAP, Nck, and Shp2 (26, 40, 41). Such data have strongly implicated tyrosine phosphorylation as playing a significant role in directed cell movements and axon guidance.

In addition to receptors with intrinsic tyrosine kinase activity, a large number of receptors lack covalently linked catalytic domains, but nonetheless associate with tyrosine kinases and require tyrosine phosphorylation to engage SH2 domain proteins and transmit intracellular signals. Cytokine receptors, for example, interact with cytoplasmic Jak tyrosine kinases, which phosphorylate the activated receptor, thereby creating binding sites for the SH2 domains of Stat transcription factors (42). Recent data indicate that a number of guidance receptors, although lacking any intrinsic kinase activity, may nonetheless serve as substrates for tyrosine kinases and be regulated by tyrosine phosphorylation. The Drosophila Roundabout (Robo) receptor, which mediates the repulsive effects of the Slit ligand on axonal crossing of the mid-line, can be phosphorylated on multiple tyrosine residues by the AbI tyrosine kinase (18). Substitution of one of the conserved tyrosine phosphorylation sites (in the CC1 Robo motif) creates a hyperactive Robo receptor (18), suggesting that one role of tyrosine phosphorylation is to attenuate the strength of Robo signaling. In a similar vein, the Drosophila Dacm receptor is tyrosine phosphorylated and binds the SH2 domain of the Nck (Dock) SH2/SH3 adaptor in a fashion that appears important for the pathfinding of Bolwig’s nerve (43). In addition, plexins, receptors which mediate the effects of the semaphorin guidance cues, become tyrosine phosphorylated in cells (32). Although the semaphorins contain an extended and conserved “SP” cytoplasmic signaling domain, they lack intrinsic tyrosine kinase activity, suggesting that their phosphorylation results from an associated tyrosine kinase.

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Tyrosine Phosphorylation of UNC-5/RCM in Vivo and in Vitro—Although they have been intensively studied at the genetic level, little is known about the biochemical signaling pathways activated by the netrin receptors UNC-40/DCC and UNC-5, nor is there any information concerning their phosphorylation beyond the fact that they lack obvious kinase domains. We find that the netrin-1 receptors, UNC-5 and UNC-40, are both phosphorylated on tyrosine in C. elegans and that the tyrosine phosphorylation of UNC-5 in vivo is partially dependent on UNC-6/netrin. Intriguingly, UNC-5 appears to have multiple tyrosine phosphorylation sites and it is therefore possible that the partial tyrosine phosphorylation of UNC-5 observed in an unc-6 null mutant of C. elegans (Fig. 2A) is due to different phosphorylation sites having a variable dependence on netrin stimulation. Although, as far as is known, all the functions of UNC-5 depend on UNC-6, it is also possible that other ligands exist for UNC-5 that regulate its ability to respond to UNC-6. A possible candidate for another UNC-5 ligand is UNC-129, (47), a family member of TGF-β that is also required to guide pioneer motor axons along the dorsoventral axis of C. elegans. Mutations in unc-129 suppress ectopic unc-5-induced growth cone guidance (11). UNC-129 does not appear to act through a conventional transforming growth factor-β
receptor (47) and is therefore a potential ligand for UNC-5. As such, UNC-129 could modulate UNC-5 phosphorylation, possibly by altering its sensitivity to UNC-6. These ideas are now being examined experimentally.

Of relevance to the function of UNC-5 tyrosine phosphorylation, work by Killeen et al. (48) indicates that deletion mutants with decreased tyrosine phosphorylation show a corresponding reduced ability to rescue unc-5 null mutants of *C. elegans*. These results suggest that tyrosine phosphorylation is a hallmark of fully active UNC-5 and raise the possibility that tyrosine phosphorylation is important for UNC-5 function in vivo. Another indication that UNC-5 tyrosine phosphorylation may be physiologically relevant is the finding that mammalian RCM is tyrosine phosphorylated in the embryonic mouse brain, suggesting that this UNC-5 modification is conserved in evolution. In HEK-293 transfected cells, RCM is weakly phosphorylated after netrin stimulation (Fig. 2B), although both the basal and netrin-stimulated tyrosine phosphorylation is greatly enhanced by the co-expression of an activated Src tyrosine kinase. There are several possible explanations for the rather modest RCM tyrosine phosphorylation in HEK-293 cells prior to v-Src expression. Netrin function may be regulated in vivo by a co-activator (48) such as netrin synergizing activity (49) or even an UNC-129 homologue, which may not be present in our cell culture experiments or RCM phosphorylation may normally be regulated by a tyrosine kinase which is not fully expressed or activated in HEK-293 cells. The identity of the tyrosine kinase that phosphorylates UNC-5 in vivo remains to be established. Regardless, the finding that UNC-5 and RCM contain phosphotyrosine in *C. elegans* and the mouse embryo, respectively, shows that their phosphorylation is a physiological event.

To identify specific UNC-5 phosphorylation sites, we screened several tyrosine point mutations and carried out phosphopeptide mapping of bacterially expressed proteins using nano-ESI-MS/MS. These approaches revealed that a conserved tyrosine within the ZU-5 domain (Tyr568) is one of the potential tyrosine phosphorylation sites in RCM.

**Phosphorylation of RCM Tyr568 Creates an SH2 Domain Docking Site**—Both PLCγ1 and Shp2 SH2 domains bind a phosphorylated Tyr568 RCM peptide in vitro. Subsequent analysis of PLCγ1 has not identified a netrin-dependent interaction with intact RCM, raising doubt as to whether this interaction is relevant to RCM signaling. In contrast, the results of *in vitro* peptide pull downs, receptor immunoprecipitations, and point mutation experiments all indicate that Shp2 is recruited to the phosphorylated RCM receptor after netrin stimulation, in a fashion that requires binding of the Shp2 SH2 domains to the Tyr568 phosphorylated motif. Since a T568F substitution increased RCM tyrosine phosphorylation under some circumstances, it is possible that RCM is itself a direct substrate for Shp2 and that one function of Shp2-binding to RCM is to modulate the phosphorylation of other tyrosine sites on RCM. It is also possible that Shp2 may directly contribute to signaling downstream of RCM. For example, Shp2 can regulate the activity of the RhoA GTPase (50) and Shp2 is known to be important for cell migration and the normal organization of focal adhesions during mammalian embryogenesis and in cultured murine cells (51, 52). Consistent with these results, Rho family GTPases can be activated by chemorepellants or chemotactants leading to either localized actin-based protrusion or retraction of the growth cone in different cell types (53). Increasing Shp2 activity can potentially reduce RhoA activity and focal adhesions and thereby increase cell motility through an effect on cell adhesion. By modulating RhoA activity, Shp2 could also have a localized effect on actin dynamics within the growth cone and thus contribute to polarized movements in response to netrin. The recruitment of Shp2 to RCM may therefore have a direct influence on the control of cell movement in response to netrin stimulation.
The functional relevance of Tyr^{568} phosphorylation in vivo is presently being tested in C. elegans. For this purpose, the equivalent of the RCM Y568F mutation was introduced into C. elegans UNC-5 (Y538F). UNC-5 Y538F has a slightly reduced ability to rescue the uncoordination phenotype of unc-5 null animals relative to rescue experiments using wild type UNC-5; however, visual examination of dorsal/ventral axons revealed no significant difference in axonal pathfinding between the animals containing UNC-5 Y538F and those containing wild type UNC-5. This result indicates that the loss of one signaling pathway may not result in a strong defect in vivo. This is not uncommon in receptor signal transduction. For example, mice lacking either the β-platelet-derived growth factor receptor or platelet-derived growth factor-B exhibit pericyte defects, which result in perinatal lethality (54–57). However, mice homozygous for β-platelet-derived growth factor receptor mutants that no longer activate phosphatidylinositol 3-kinase or PLCγ or both, were phenotypically normal (58), and defects were only identified upon analysis of mutant cells in culture or experimental manipulation of mutant animals. These results support the idea that receptor signals may be redundant or complementary in vivo, such that the loss of multiple signaling pathways may be required for observable defects. We are in the process of introducing multiple tyrosine to phenylalanine mutations into UNC-5 to test the ability of UNC-5 lacking multiple phosphorylation sites to rescue the unc-5 null phenotype. Preliminary results show that, relative to the single-site mutants, the multiple-site mutants have a severely reduced ability to rescue the uncoordination of an unc-5 null mutant.

In summary, our results show that the UNC-5 netrin receptor and its mammalian homologue RCM are tyrosine phosphorylated in vivo in a fashion that is partially dependent on netrin stimulation. Phosphorylation of a specific tyrosine motif in RCM creates a binding site for the SH2 domains of the Shp2 tyrosine phosphatase. These data indicate that tyrosine phosphorylation is involved in UNC-5 signaling and the control of cell movement.

Acknowledgments—We thank Drs. Nicki Broughton and Berivan Baskin for early work on UNC-5/RCM; Dr. Eric R. Fearon for human netrin-1 and DCC constructs; Drs. Lorene M. Lanier, Frank Gertler, and Marc Tessier-Lavigne for a chicken netrin-1 stable cell line; Dr. Jerry Gish for technical help; Paul P. Taylor and Alex Zhongman for help with mass-spectrometry; and Dr. Elke Stein for technical information.

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FIG. 6. Shp2, but not PLCγ, co-immunoprecipitates with RCM from intact cells after netrin-1 stimulation and from mouse brain. A, anti-DCC, FLAG, PLCγ, and Shp2 immunoblots of anti-FLAG immunoprecipitates from RCM-FLAG and DCC transfected HEK-293 cells with or without netrin-1 stimulation for 20 min. Since the molecular weights of DCC, RCM, and Shp2 are different and can be separated by SDS-polyacrylamide gel electrophoresis, the blot was cut into 3 pieces, which were immunoblotted with different antibodies to reveal the respective proteins. B, anti-DCC, HA and Shp2 immunoblots of anti-HA immunoprecipitates from HEK-293 cells co-transfected with DCC and wild type or Y568F mutant RCMHA. C, anti-PLCγ (upper left panel) and anti-Shp2 (lower panel) immunoblots of anti-RCM and its preimmune serum immunoprecipitations from mouse embryonic day 14 brain lysates. The anti-PLCγ filter was then stripped and reprobed with anti-RCM antibody to show that RCM was present in anti-RCM antibody immunoprecipitates (lane 2; upper right panel). These data are representative of the results of three independent experiments.
Phosphorylation Regulates UNC-5/RCM Function

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J. Biol. Chem. 2001, 276:40917-40925.
doi: 10.1074/jbc.M103872200 originally published online August 30, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103872200

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