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Cloning and Characterization of Neuropilin-1-Interacting Protein: A PSD-95/Dlg/ZO-1 Domain-Containing Protein That Interacts with the Cytoplasmic Domain of Neuropilin-1

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Neuropilin-1 (Npn-1), a receptor for semaphorin III, mediates the guidance of growth cones on extending neurites. The molecular mechanism of Npn-1 signaling remains unclear. We have used a yeast two-hybrid system to isolate a protein that interacts with the cytoplasmic domain of Npn-1. This Npn-1-interacting protein (NIP) contains a central PSD-95/Dlg/ZO-1 (PDZ) domain and a C-terminal acyl carrier protein domain. The physiological interaction of Npn-1 and NIP is supported by co-immunoprecipitation of these two proteins in extracts from a heterologous expression system and from a native tissue. The C-terminal three amino acids of Npn-1 (S-E-A-COOH), which is conserved from *Xenopus* to human, is responsible for interaction with the PDZ domain-containing C-terminal two-thirds of NIP. NIP as well as Npn-1 are broadly expressed in mice as assayed by Northern and Western analysis. Immunohistochemistry and *in situ* hybridization experiments revealed that NIP expression overlaps with that of Npn-1. NIP has been independently cloned as RGS–GAIP-interacting protein (GIPC), where it was identified by virtue of its interaction with the C terminus of RGS-GAIP and suggested to participate in clathrin-coated vesicular trafficking. We suggest that NIP and GIPC may participate in regulation of Npn-1-mediated signaling as a molecular adapter that couples Npn-1 to membrane trafficking machinery in the dynamic axon growth cone.

Key words: neuropilin-1; PDZ domain; axon guidance; yeast two-hybrid; neuron development; adapter protein; signal transduction

Neurons rely on particular combinations of guidance molecules to project their axons to target cells (Tessier-Lavigne and Goodman, 1996). The detection and transduction of these cues are mediated by receptors residing on growth cones. Guidance ligand–receptor pairs mediating attractive and repulsive responses have been identified (Luo et al., 1993; Keino-Masu et al., 1996; Kolodkin and Ginty, 1997; Leonardo et al., 1997). However, mechanisms responsible for converting these guidance signals into changes within the growth cone remain unclear. Because the cytoplasmic domains of the guidance receptors bear no close homology to known functional motifs, the identification of proteins associated with the cytoplasmic domains of these receptors will be important in elucidating the cellular mechanism underlying the response of neurons to guidance molecules.

The semaphorin family proteins share a 500-amino acid loosely conserved region called the “sema” domain (Kolodkin et al., 1993). Collapsin-1, the chicken homolog of semaphorin III, can induce the collapse of growth cones in cultured neurons, suggesting that this family of proteins serve an inhibitory or repulsive role in axon guidance (Luo et al., 1993). Neuropilin-1 (Npn-1), a semaphorin III receptor identified through expression cloning, binds semaphorin III with high affinity. Moreover, antibodies directed against the extracellular segment of Npn-1 blocked ligand-specific growth cone collapse (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Npn-1 was originally characterized as a cell surface molecule with a restricted expression pattern in the optic tectum of *Xenopus* (Takagi et al., 1991). The transmembrane and cytoplasmic domains of this receptor share >90% amino acid identity across species (Takagi et al., 1995; Kawakami et al., 1996). Consistent with the role of Npn-1 in axon guidance, overexpression of Npn-1 results in axonal defasciculation and sprouting in embryonic mice (Kitsukawa et al., 1995). Furthermore, growth cones of dorsal root ganglion (DRG) neurons derived from Npn-1 null mutant mice fail to collapse after application of semaphorin III (Kitsukawa et al., 1997). These data demonstrate that Npn-1 is a receptor for semaphorin III.

The semaphorins and neuropilins contribute to the projection of olfactory primary axons (Pasterkamp et al., 1998). Npn-1 is expressed in olfactory epithelium from early embryonic stages through adulthood (Kawakami et al., 1996), reflecting the capacity of this tissue to regenerate throughout the animal’s lifetime. Moreover, collapsin-1 can induce the collapse of the growth cones of olfactory neurons (Kobayashi et al., 1997). Using a yeast two-hybrid screen, we have identified and characterized a protein, Npn-1-interacting protein (NIP) from olfactory epithelium that interacts with the cytoplasmic domain of Npn-1. The interaction is also supported by co-immunoprecipitation of Npn-1 and NIP in extracts from a heterologous expression system and from a native tissue. NIP is expressed in a variety of mouse tissues. *In situ* hybridization and immunohistochemistry studies revealed that NIP and Npn-1 are co-localized in developing nervous system. The physical interaction and co-localization of NIP and Npn-1 suggest that NIP functionally interacts with Npn-1. The identification of NIP will allow further study of the Npn-1-mediated

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signaling events. Recently, NIP was independently cloned as RGS–GAIP-interacting protein (GIPC), which interacts with the C terminus of RGS–GAIP (a $G\alpha_{i3}$-associated protein located to the membrane of clathrin-coated vesicles) (De Vries et al., 1998). GIPC and RGS–GAIP may be involved in the regulation of vesicular trafficking by association with the G-protein-coupled signaling complex. This leads to the hypothesis that NIP and GIPC, as molecular adapters, may couple Npn-1 to the membrane trafficking machinery in the dynamic axon growth cones.

MATERIALS AND METHODS

Isolation of the Npn-1 cytoplasmic domain. The cytoplasmic domain of Npn-1 was obtained by RT-PCR amplification of RNA from adult mouse olfactory epithelium. Total RNA was purified using RNAzolB (Tel-Test, Friendswood, TX), reverse-transcribed into first-strand cDNA with random hexamers and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). The forward primer (HC89: ACGCGTCAGCGATCTCCAGGAAGCCAGGC) and reverse primer (HC94: ATAAGAATGCGGCCGCTCACGCCTCTGAGTAATTAC) were used to amplify the cytoplasmic domain of Npn-1. PCR products were digested with *Not*I and *Sal*I, subcloned into pPC97, and verified by DNA sequencing.

Yeast two-hybrid screen. A yeast two-hybrid screen was performed as described (Fields and Song, 1989) by using an oligo-dT-primed cDNA library from adult rat olfactory epithelium (Wang and Reed, 1993), (3.6×10^6 independent clones) fused to the GAL4 transactivator domain in the prey vector pPC86 (Chevray and Nathans, 1992). The fragment encoding the cytoplasmic domain of Npn-1 was subcloned into the *Sal*I–*Not*I sites of the bait vector pPC97. The plasmids were co-transformed into yeast strain HF7c (Feilotter et al., 1994), and positive clones were selected on triple-minus plates (Leu^-, Trp^-, His^-) and assayed for β-galactosidase activity. Plasmids rescued from His^+/β-gal^+ yeast were isolated and sequenced. For protein-protein interaction assays, NIP clones were co-transformed with either the cytoplasmic domain of Npn-1 or pPC97 empty vector into yeast. Yeast cells were assayed for β-galactosidase activity by immobilization on nitrocellulose filters, lysing the cells by immersion in liquid nitrogen for 10 sec, placing them onto 3MM papers presoaked in Z buffer (Miller, 1972), and incubation at 37°C for 30 min.

cDNA library screen and 5′ rapid amplification of cDNA ends. DNA fragments from clone Y91 were ^{32}P-labeled and used to screen an oligo-dT-primed cDNA library from adult mouse olfactory epithelium and an oligo-dT-primed λZAP II mouse postnatal day 2–3 eye cDNA library. The 5′ rapid amplification of cDNA ends (RACE)-PCR was performed according to the manufacturer's instructions (Marathon cDNA amplification kit; Clonetech, Palo Alto, CA).

Coimmunoprecipitation and expression construct. The Npn-1 (ΔSEA) deletion construct was made by PCR amplification from full-length Npn-1 cDNA in pCI-neo vector with an N-terminal Myc tag. The full-length NIP cDNA was subcloned into pCIS with an N-terminal 10-amino acid hemagglutinin (HA) tag. HEK 293T cells were co-transfected with NIP and Npn-1 constructs by using calcium phosphate. After transfection (36–48 hr), cells were solubilized with 1% Triton X-100 in immunoprecipitation (IP) buffer (1× PBS, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, and 10 U/ml Trasylol). The lysate was centrifuged at 14,000 rpm for 20 min at 4°C, precleared by incubating with CL-4B Sepharose beads (Pharmacia, Piscataway, NJ), and incubated with anti-NIP antibodies or anti-myc antibodies at 4°C for 2 hr as indicated. The immunoprecipitates were bound to protein A beads and washed twice with IP buffer containing 1% Triton X-100, twice with IP buffer and 500 mM NaCl, and twice with IP buffer. Immunoprecipitated proteins were eluted with SDS sample buffer, separated using SDS-PAGE, transferred to polyvinylidene difluoride (PVDF), and subjected to immunoblot analysis with antibodies to Myc tag and NIP protein.

Brain membrane preparation and coimmunoprecipitation. Membrane preparations (P2) and solubilizations were performed according to published protocols (Luo et al., 1997), with modifications. Olfactory bulbs from postnatal day 2 CD-1 mice were homogenized twice using a glass–Teflon homogenizer in the presence of protease inhibitors. After determining the protein concentration of the P2 fraction by Bradford assay (Bio-Rad, Hercules, CA; 500-0006), aliquots of 300 μg of proteins were stored at −80°C until use. For coimmunoprecipitation, an aliquot of P2 was solubilized by 1% NP-40 and centrifuged for 10 min at 100,000 × *g*. The supernatant was then clarified by protein A-Sepharose beads. Affinity-purified antibodies (~10 μg) were preincubated with 40 μl of 1:1 protein A-Sepharose slurry for 1 hr, and the protein A–antibody complex was spun down at 2000 × *g* for 2 min and washed once with coimmunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, and protease inhibitors). The clarified P2 fractions were then added to the antibody-conjugated Sepharose beads, and incubated for 2–3 hr at 4°C. After incubation, the mixture was washed once with coimmunoprecipitation buffer, twice with Tris-buffered saline (TBS) buffer containing 300 mM NaCl, and three times with TBS buffer. The proteins were eluted by Laemmli sample buffer and subject to Western analysis with an anti-Npn-1 antibody (Kolodkin et al., 1997).

Antisera preparation. NIP anti-peptide and anti-fusion protein antibodies against NIP were generated in rabbits (Covance Research Products Inc., Denver, PA). A synthesized peptide corresponding to the C-terminal 12 residues of mouse NIP protein was coupled to bovine serum albumin using glutaraldehyde and used to make the anti-peptide antibody. The resulting antiserum was affinity-purified on an immobilized peptide column. To generate the NIP anti-fusion protein antiserum, a DNA fragment corresponding to the residues from 210 to 311 of NIP was subcloned into pTrcHisA (Invitrogen, San Diego, CA). The HIS_6 fusion protein purified by Ni-NTA (Qiagen, Hilden, Germany) chromatography was used as antigen. The resulting antiserum was purified on a fusion protein affinity column.

Western blot analysis of NIP protein. Whole tissue lysates were prepared from postnatal day 2 mice by homogenization in PBS buffer with 0.1 mM PMSF, 1% Triton X-100, and protease inhibitors. The lysates were sonicated for 1 min and centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatants were collected. Protein concentrations were measured by Bradford assay (Bio-Rad 500-0006). Proteins (50 μg/lane) were separated using SDS-PAGE, transferred to PVDF, and subjected to immunoblot analysis with anti-NIP antibodies.

Tissue preparation. CD-1 mouse embryos of embryonic days 12–16 (E12–E16) were immersion-fixed in freshly prepared 4% paraformaldehyde in 1× PBS overnight at 4°C. Fixed embryos were immersed in 30% sucrose in 1 ×PBS at 4°C overnight and embedded in OCT (Tissue-Tek, Torrance, CA). Cryostat sections (10–14 μm) were collected on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) glass slides. Adult mice or rats were anesthetized with pentobarbital and perfused with ice-cold PBS, followed by Bouin's solution (Sigma, St. Louis, MO). Tissues were harvested and post-fixed in the same fixative for 2 hr before immersion in 30% sucrose and 1× PBS at 4°C overnight. Adult tissue sections were prepared as described above.

In situ *hybridization. In situ* hybridization was performed on tissue sections with digoxigenin-labeled riboprobes as previously described (Vassar et al., 1993).

Immunohistochemistry. Immunohistochemistry was performed on embryonic mouse tissue sections as previously described (Davis and Reed, 1996). Tissue sections were blocked by 10% normal goat serum in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), reacted with antisera specific for NIP or Npn-1 (Kolodkin et al., 1997), incubated with biotinylated anti-rabbit IgG and avidin–biotin–horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized with diaminobenzidine.

RESULTS

Cloning and sequence analysis of a gene that interacts with the cytoplasmic domain of Npn-1

The C-terminal 39 residues of Npn-1, predicted to comprise the entire cytoplasmic domain, display >90% amino acid identity among *Xenopus*, chick, mouse, rat, and human (Fig. 1). This suggests an important role for this domain in Npn-1 function. A yeast two-hybrid screen was performed to identify proteins interacting with the cytoplasmic domain of Npn-1. The bait construct (pPC97-Nnp-1/C) contained a DNA fragment encoding the 39 amino acid Npn-1 cytoplasmic domain (Npn-1/C) fused to a GAL4 DNA binding domain in the yeast expression vector pPC97. A cDNA expression library from rat olfactory epithelium fused to the GAL4 transactivator domain in yeast expression

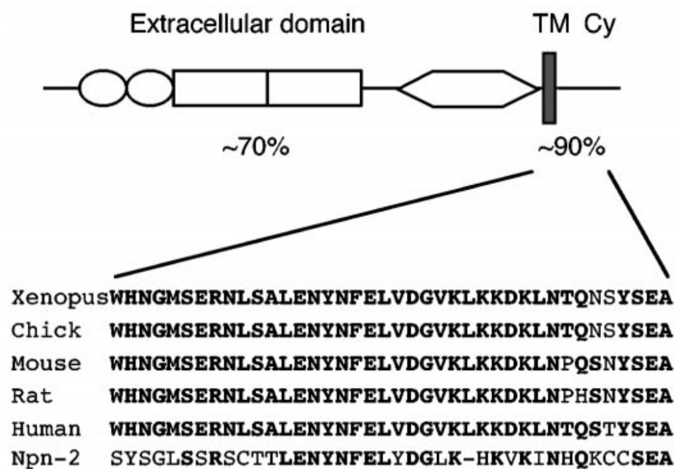

Figure 1. Domain structure of Npn-1 and sequence alignment of Npn-1 cytoplasmic domains. Npn-1s share ~70 and 90% amino acid identities in the extracellular and cytoplasmic domains, respectively. The amino acid sequences of cytoplasmic domains of Npn-1 (*Xenopus*, chick, mouse, rat, and human), and Npn-2a (rat) are aligned, and the identical amino acids are shown in bold. *TM*, Transmembrane domain, *Cy*, cytoplasmic domain.

vector pPC86 (Wang and Reed, 1993) was co-transformed with pPC97-Nnp-1/C into yeast strain HF7c. From $\sim 3 \times 10^6$ transformants, 28 clones that were both HIS^+ and β-galactosidase$^+$ were identified, rescued from yeast, and subjected to sequence analysis.

Sequence analysis revealed that the same gene was identified in 26 isolates representing eight independent overlapping cloning events. We named this gene NIP as Npn-1-interacting protein. The longest of these clones (Y16) encoded an open reading frame of 1645 bp fused in-frame to the GAL4 transactivator domain. Further analysis demonstrated that a protein motif resembling a PSD-95/Dlg/ZO-1 (PDZ) domain was shared by all of these 26 clones. A 1675 bp cDNA clone (mNIP9) was obtained by screening an oligo-dT-primed mouse olfactory epithelium cDNA library by using Y16 as probe. The PCR products obtained from 5′ RACE failed to yield sequences further upstream of mNIP9. Northern blot analysis of total RNA from adult mouse olfactory epithelium revealed a 1.7 kb band (data not shown). These results, together with Western blot analysis of endogenous proteins and HEK 293T cells transiently transfected with the isolated NIP cDNA (described below), suggest that mNIP9 contains the entire coding region of NIP protein.

The conceptually translated 333-amino acid protein encoded by mNIP9 predicts a cytoplasmic protein containing a single PDZ domain between amino acids 129 and 217. This domain includes residues that are conserved among other PDZ domain family members and contribute to the interaction with the C terminus of target proteins (Cabral et al., 1996; Doyle et al., 1996). Previous yeast two-hybrid screens with canonical PDZ binding sequences have resulted in the isolation of multiple PDZ domain-containing clones. NIP was among the proteins identified in one such screen (Rousset et al., 1998). Recently, an RGS-GAIP C terminus-interacting protein, GIPC, was identified by yeast two-hybrid system that is identical to NIP (De Vries et al., 1998). The NIP protein encodes an identified PDZ domain, several short consensus sequences for protein phosporylation, and an acyl carrier protein domain at the C terminus but no other extensive homologies to other proteins.

Defined regions of NIP and Npn-1 mediate the NIP–Npn-1 interaction

The C-terminal three residues of Npn-1, S-E-A-COOH, resemble the T/S-X-V-COOH motif that interacts with PDZ domains, with the exception of a less hydrophobic terminal residue (Ala). To test whether Npn-1 interacts with NIP through the C terminus, a truncated form of Npn-1, Npn-1/C (ΔSEA), lacking the three terminal residues, was fused to the GAL4 DNA binding domain in pPC97 for protein–protein interaction assays in yeast. The pPC97-Npn-1/C and pPC97-Npn-1/C (ΔSEA) plasmids were co-transformed with full-length NIP/GAL4 transactivator domain, and LEU^+/TRP^+ yeast colonies were identified. The positive clones were subjected to a β-galactosidase activity assay and HIS^+ selection. The deletion of the C-terminal three amino acids of Npn-1 abolished the interaction with NIP (Table 1).

The presence of the PDZ domain in all of the NIP clones isolated from the yeast two-hybrid screen implicated this domain in the interaction with the C-terminal tail of Npn-1. To examine whether the NIP–PDZ domain mediated the interaction with Npn-1, the PDZ domain [amino acids 120–248, ($NIP_{120-248}$)] was fused to the GAL4 transactivator domain. In parallel, other regions of the NIP protein (NIP_{1-148}, $NIP_{221-333}$, and $NIP_{120-333}$) were also subcloned into pPC86. These constructs were co-transformed with pPC97-Npn-1/C into yeast and assayed for β-galactosidase activity and HIS^+ selection (Table 1). $NIP_{120-248}$, the PDZ domain of NIP, displayed a weaker interaction with the Npn-1 C terminus than that of full-length NIP. The C-terminal one-third of NIP, $NIP_{221-333}$, itself showed a very weak interaction with Npn-1, whereas $NIP_{120-333}$, which contains both the PDZ domain and C terminus of NIP, displayed a binding affinity similar to that of the full-length NIP construct. The N-terminal one-third of NIP failed to interact with Npn-1. These observations suggest that the PDZ domain and C-terminal one-third of NIP may function together in the binding of the Npn-1 C-terminal tail.

Co-immunoprecipitation of Npn-1 and NIP from mammalian cell lines

The interaction between NIP and Npn-1 was examined by co-immunoprecipitation in a heterologous expression system. The full-length NIP, full-length Npn-1, or both were transiently expressed in HEK 293T cells. The HA-tagged NIP was specifically immunoprecipitated in the presence of the N-terminal Myc-tagged Npn-1 and the anti-Myc antibody (Fig. 2*A*). Similarly, the N-terminal Myc-tagged Npn-1 required the presence of NIP and the anti-NIP peptide antibody (described below) for specific immunoprecipitation (Fig. 2*B*). Deletion of the C-terminal three residues of Npn-1 abolished its interaction with NIP, although the expression level of truncated Npn-1 protein was comparable with the full-length construct (Fig. 2*B*). These co-immunoprecipitation assays further support the interaction between NIP and Npn-1.

Interaction of Npn-1 and NIP *in vivo*

The interaction of Npn-1 with NIP was examined *in vivo*. NIP was solubilized from postnatal day 2 mouse olfactory bulb membrane extract with 1% NP-40 and immunoprecipitated with the affinity-purified NIP peptide antibody (described below). The resulting complexes were then examined by Western blotting for the presence of Npn-1. As shown in Figure 2*C*, Npn-1 co-immunoprecipitates with NIP from solubilized olfactory bulb membrane extract. In control experiments, preimmune serum did

Table 1. Interaction of NIP with Npn-1 determined by filter assay of β-galactosidase activities and His^+ selection

	Npn-1/C		Npn-1/C (ΔSEA)		Vector	
	β-galactosidase activity	His^+	β-galactosidase activity	His^+	β-galactosidase activity	His^+
NIP	+++++	+	−	−	−	−
$NIP_{1–148}$	−	−				
$NIP_{120–333}$	++++	+	−	−	−	−
$NIP_{120–248}$	+++	+				
$NIP_{221–333}$	+	+				
Vector	−	−				

Npn-1/C, The cytoplasmic domain of Npn-1; Npn-1/C (ΔSEA), the cytoplasmic domain of Npn-1 with the deletion of the last three amino acids; +, positive reaction; −, negative reaction.

not precipitate Npn-1. These experiments indicate that NIP interact with Npn-1 *in vivo* and further suggest that NIP may mediate the regulation of Npn-1 signaling by directly interacting with the cytoplasmic domain of Npn-1.

NIP expression in mouse tissues

To analyze the expression pattern of NIP, two antibodies were generated against two nonoverlapping portions of NIP protein. An anti-peptide antibody recognizing amino acids 321–333 of NIP and an anti-fusion protein antibody against residues 171–320 of NIP were generated, and each recognized a single 40 kDa protein in NIP-transfected HEK 293T cells (data not shown) and in mouse olfactory bulb cell lysate (Fig. 3*A*). Using the anti-peptide antibody, the tissue distribution of NIP protein in post-natal day 2 mice was examined (Fig. 3*B*). In agreement with the tissue distribution of Npn-1 (Soker et al., 1998), NIP was present in multiple neuronal and non-neuronal tissues. The higher molecular weight bands seen in liver and lung were also observed with the anti-fusion protein antibody and may reflect a splice variant. The intense, low molecular weight protein detected in the eye was only detected with the anti-peptide antibody and likely reflects cross-reaction with lens crystalin protein that contains an amino acid sequence similar to the peptide sequence used to generate the anti-NIP antibody. The broad tissue distribution of NIP and Npn-1 is consistent with the observations suggesting that Npn-1 may play additional physiological roles outside of the nervous system (Kitsukawa et al., 1995; Soker et al., 1998).

NIP expression in developing CNS and PNS

Npn-1 is a receptor for semaphorin III, which mediates a repulsive effect on growth cones of neurons from DRG, sympathetic ganglion, olfactory epithelium, and additional cranial ganglions (Kawakami et al., 1996). If NIP functionally interacts with Npn-1, one might expect NIP and Npn-1 to colocalize in the same neurons and within the same subcellular compartments. To determine the expression pattern of NIP, *in situ* hybridization with digoxigenin-labeled NIP RNA antisense probes and immunohistochemistry with anti-NIP antibodies were performed on neuronal tissue sections derived from E14 mouse embryos. *In situ* hybridization experiments revealed NIP mRNA enrichment in both DRG and spinal cord neurons (Fig. 4*A*). Within the spinal cord, NIP-reactive neurons were more numerous in the dorsal horn than in the ventral horn. No signal was detected from the adjacent section with the NIP sense probe (Fig. 4*B*).

NIP protein is also detected in multiple areas in the developing brain and sympathetic system. From horizontal sections of E14 mouse embryo, NIP immunoreactivity was observed in the optic nerve (Fig. 5*E*) and in the olfactory nerve bundles that terminate on the surface of the olfactory bulb (Fig. 5*H*). Staining with antibody against Npn-1 in the adjacent sections revealed a similar expression pattern (Fig. 5*D,G*). In sagittal sections of E14 mouse embryo, Npn-1 and NIP immunoreactivity also co-localized in superior cervical ganglion (Fig. 5*A,B*) and other brain regions, including clusters of neurons in the brainstem, trigeminal ganglion, and Rathke's pouch (data not shown). Sections incubated with preimmune serum failed to stain (Fig. 5*C,F,I*). The co-localization of NIP and Npn-1 protein in developing nervous system further supports their functional interaction and suggests a role for NIP in axon guidance.

NIP expression in primary olfactory axon bundles and their terminals of adult animals

Olfactory neurons undergo constant replacement throughout embryonic and adult life. Olfactory neuronal differentiation and axon outgrowth in the adult mimic many aspects of the processes observed during embryonic development. Npn-1 mRNA (Kawakami et al., 1996) and protein (Fig. 6*A,C*) persist into adulthood in the olfactory system in contrast to its downregulation in most other adult neuronal tissues. Consistent with its initial isolation from adult olfactory epithelium, NIP is expressed in the mature olfactory epithelium. The NIP immunoreactivity was observed almost exclusively in the axon bundles that underlie the olfactory epithelium (Fig. 6*B*) and in their terminal processes forming the glomeruli of the olfactory bulb (Fig. 6*D*). Furthermore, the immunostaining of primary olfactory axon bundles and their terminals is not homogeneous with anti-NIP antibody (Fig. 6*B,D*) and with anti-Npn-1 antibody (Fig. 6*A,C*). The co-localization of Npn-1 and NIP proteins in the same subset of glomeruli revealed by immunostaining of two adjacent olfactory bulb sections suggests these two proteins may play important roles in the specific projection of primary olfactory axons to the olfactory bulb (Fig. 6*C,D*).

Both NIP and Npn-1 immunoreactivities were also detected in the adult vomeronasal organ, and their axons terminated at the accessory olfactory bulb (data not shown). In all cases, no staining was observed from the adjacent section incubated with preimmune serum.

DISCUSSION

The potential role of NIP in Npn-1-mediated cellular responses

Npn-1 has a short, but conserved cytoplasmic domain, which shares no homology with known protein motifs. How does Npn-1

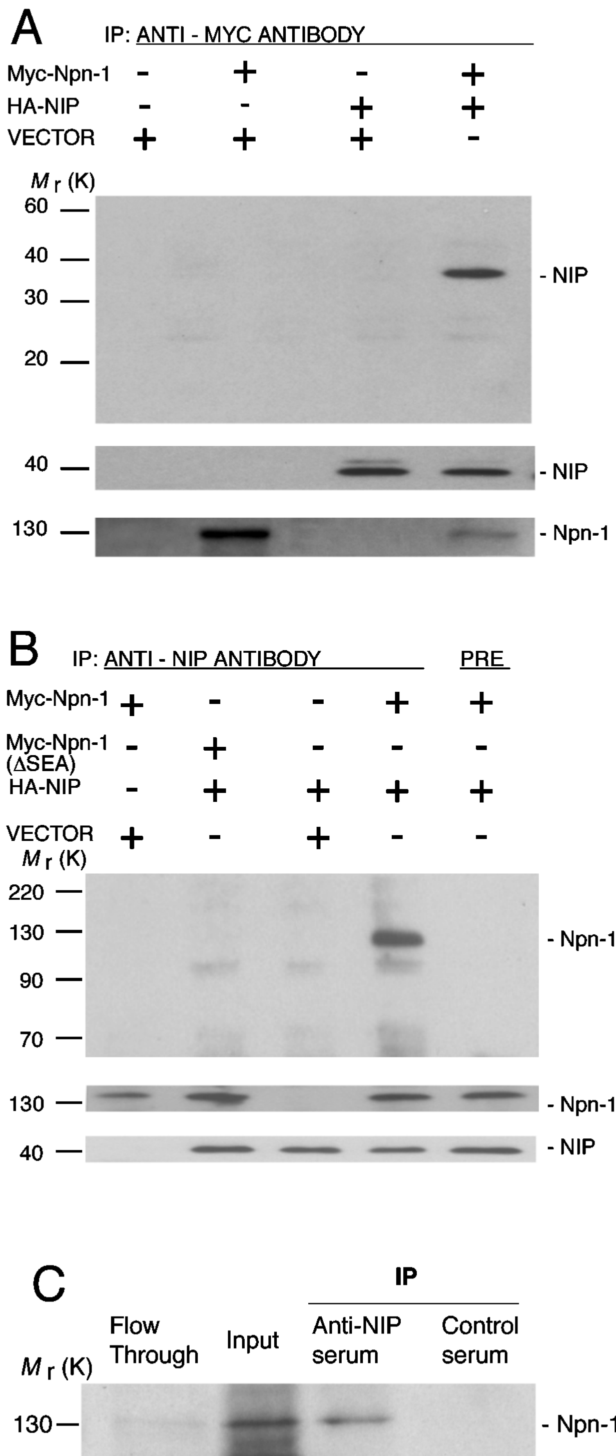

Figure 2. Co-immunoprecipitation of NIP and Npn-1. *A*, The full-length N-terminal Myc-tagged Npn-1 with or without HA-tagged NIP were expressed in HEK 293T cells and immunoprecipitated with an anti-Myc antibody. The anti-NIP antibody was used to detect the NIP protein. In the *bottom two panels*, aliquots of whole-cell extract were immunoblotted with anti-Myc or anti-HA antibodies. Molecular size markers are indicated in kilodaltons. *B*, HA-tagged NIP was co-expressed with Myc-tagged full-length Npn-1 or mutated Npn-1 lacking the C-terminal three residues in HEK 293T cells. Extracts were subjected to immunoprecipitation with the anti-NIP peptide antibody or preimmune serum. The resulting immunoprecipitates were probed with anti-Myc antibody to detect Npn-1 and its mutant form. In the *bottom two panels*, aliquots of total cell extracts were immunoblotted with anti-Myc or anti-HA antibodies to confirm the similar expression levels of the constructs. *C*, NIP was immunoprecipitated from solubilized olfactory bulb membrane extract (input) by using the NIP peptide antibody and the resulting immunoprecipitates were probed with a Npn-1 antibody. Npn-1 was co-immunoprecipitated with NIP. Preimmune serum failed to immunoprecipitated Npn-1.

transduce an extracellular signal into the changes within the cells? Npn-1 must be coupled with intracellular signaling molecules directly or through an adapter protein. The extraordinary conservation of the cytoplamic domain of the Npn-1 protein across species may suggest an important role for this domain in terms of the Npn-1 functions. One isoform of Npn-2 (a_0) also shares the last three amino acids with Npn-1 (Fig. 1). Considering Npn-1 and Npn-2 both mediate the same process of axon growth cone collapse, it is attractive to think that the C-terminal tails of these two proteins are involved in the same intracellular signal transduction events. Alternatively, Npn-1 may couple to intracellular signaling events through a co-receptor, like KDR/Kit-1 (Soker et al., 1998). For example, a recent study has shown that the ectodomains of Npn-1 linked by GPI to the cell surface retains a functional response to semaphorin III in chicken retinal ganglion cells (Nakamura et al., 1998). One key to distinguishing between these two hypotheses is to identify and characterize the proteins interacting with the cytoplasmic domain of Npn-1.

The interaction of NIP with the cytoplasmic domain of Npn-1, identified through a yeast two-hybrid screen and confirmed by biochemical interaction assays, makes this protein an attractive candidate for serving an adapter function. One of the important roles of PDZ domain-containing proteins is to act as molecular adapters that target proteins to proper subcellular compartments or assemble signal transduction components into closely associated protein complexes. In transfected HEK 293T cells, neither the C-terminal three residues of Npn-1 nor exogenous NIP are required for plasma membrane localization of Npn-1 (data not shown). However, other models for the participation of NIP in the regulation of Npn-1 signaling can be envisioned. For example, NIP may function to block the interaction of Npn-1 with other signal molecules through the C-terminal tail. A detailed understanding of the dynamics of NIP interaction with Npn-1 and other proteins should provide further knowledge about the molecular mechanism of Npn-1-mediated physiological responses.

Collapsin-1- and semaphorin III-induced growth cone collapse is also mediated by the heterotrimeric G-protein-coupled signal transduction pathway (Goshima et al., 1995). Pertussis toxin-mediated ADP ribosylation of the α subunit of G_o or G_i classes of G-protein blocks collapsin-1-induced growth cone collapse in DRG neurons. The collapsin-1 response mediator protein CRMP-62 (Goshima et al., 1995) is an intracellular component of this G-protein-coupled signaling cascade and shares homology with the UNC33 gene that is required for directed axon extension in *Caenorhabditis elegans*. Introduction of CRMP-62 antibody into DRG neurons can block the collapsin-1-induced growth cone collapse, although a collapsin-1-specific G-protein-coupled receptor has not been identified. *Rac1*, a *ras*-related monomeric GTP-binding protein, is also implicated in the collapsin-1-induced growth cone collapse (Jin and Strittmatter, 1997). *Rac1* appears to participate in pathways that modulate membrane ruffling and lamellipodia (Ridley et al., 1992), in agreement with the essential rearrangement of actin fibers during growth cone collapse. Recently, NIP was independently cloned as GIPC through its inter-

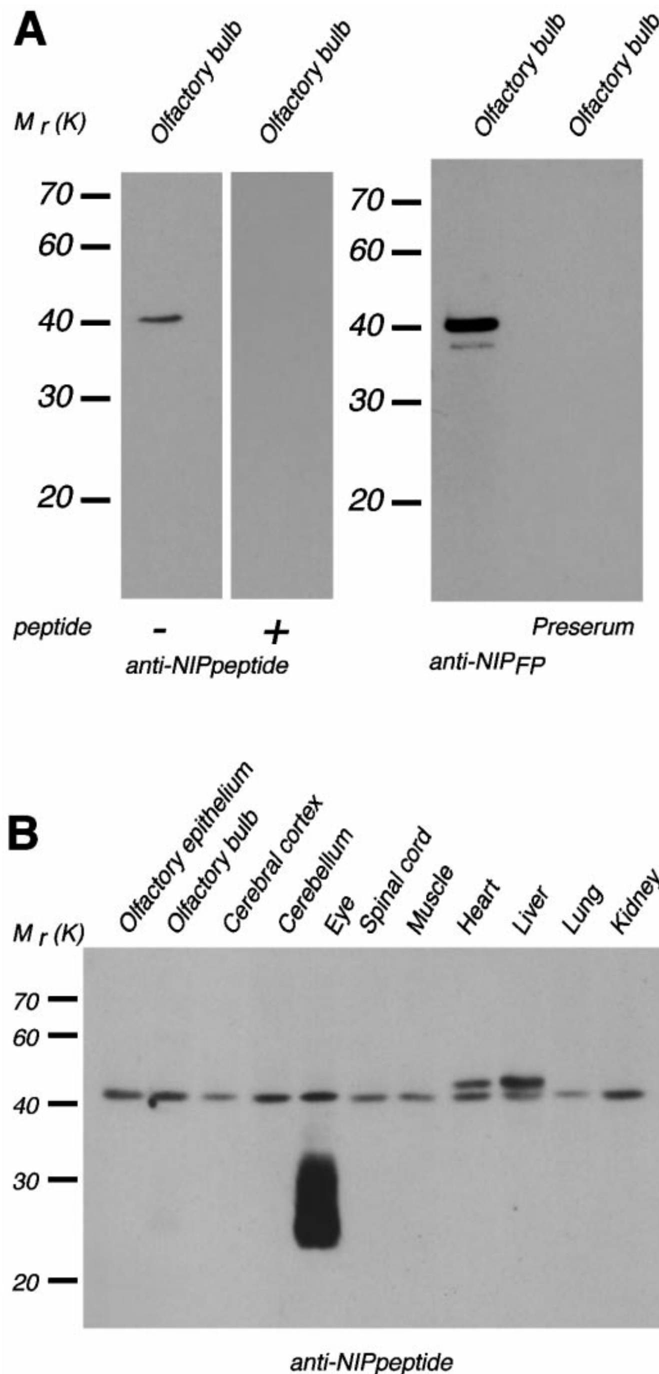

Figure 3. Distribution of NIP protein in postnatal day 2 mouse tissues. *A*, The NIP anti-fusion protein and the anti-peptide antibodies recognize a 40 kDa protein in the olfactory bulb whole-cell lysate. Detection of the NIP protein by the anti-peptide antibody was blocked by preincubation with specific peptide. Preimmune serum failed to detect the NIP protein. *B*, Immunoblot of 11 tissues revealed a broad tissue distribution of NIP protein. Equal amounts of whole tissue lysates (50 μg of proteins) were separated by gel electorphoresis and probed with the anti-peptide antibody.

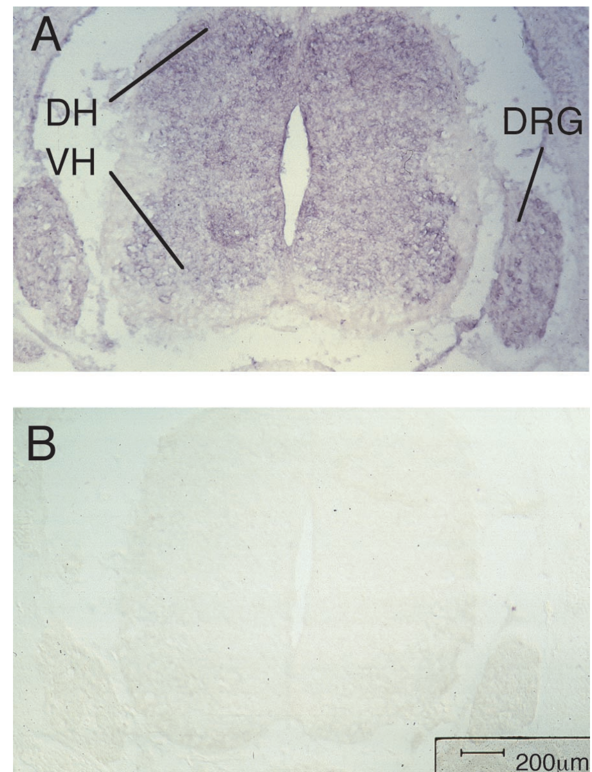

Figure 4. Expression of NIP mRNA in neurons of the embryonic mouse spinal cord and DRG. NIP mRNA is expressed in neurons of the dorsal and ventral spinal cord as well as DRG revealed by *in situ* hybridization on a transverse spinal cord section of an E14 embryo using digoxigenin-labeled NIP riboprobe (*A*). The sense probe failed to detect any signals in the adjacent section (*B*). *DH*, Dorsal horn of spinal cord; *VH*, ventral horn of spinal cord.

action with RGS-GAIP (De Vries et al., 1998). This study suggested that GIPC functions as a carrier for palmitoyl moieties for the palmitoylation of RGS–GAIP and Gα subunits and may be involved in the regulation of G-protein-mediated vesicle trafficking. It is attractive to speculate that NIP links Npn-1 to Gα and other components in the G-protein-coupled signal transduction pathway participating in vesicle trafficking and mediates membrane reconstruction during the dynamic rearrangement of axon growth cones encountering repulsive cues.

The PDZ domain containing C-terminal two-thirds of NIP is responsible for the interaction with the cytoplasmic domain of Npn-1

PDZ domains participate in many important signaling pathways (Kornau et al., 1997). Typically, PDZ domains recognize a short, T/S-X-V-COOH, consensus sequence (Songyang et al., 1997). PDZ domain-containing molecules serve to anchor proteins at specific subcellular locations and organize intracelluar signal transduction components. PSD-95, a component of postsynaptic densities, clusters glutamate receptors and K^+ channels at synapses through the specific interaction between individual PDZ domains and the C termini of these two proteins (Sheng and Wyszynski, 1997; Craven and Bredt, 1998). InaD, a scaffolding protein with five PDZ domains, assembles the *Drosophila* phototransduction machinery (Chevesich et al., 1997; Tsunoda et al., 1997). Mutation in a single PDZ domain of InaD prevents the recruitment of particular components into the signaling transduction complex and results in a corresponding physiological defect.

The PDZ domain of NIP interacts with the last three amino acids of Npn-1. However, in the β-galactosidase filter assay, the NIP-PDZ domain alone displayed a weaker interaction with Npn-1 than that of the C-terminal two-thirds of NIP, which contains both the PDZ domain and the remaining C-terminal of

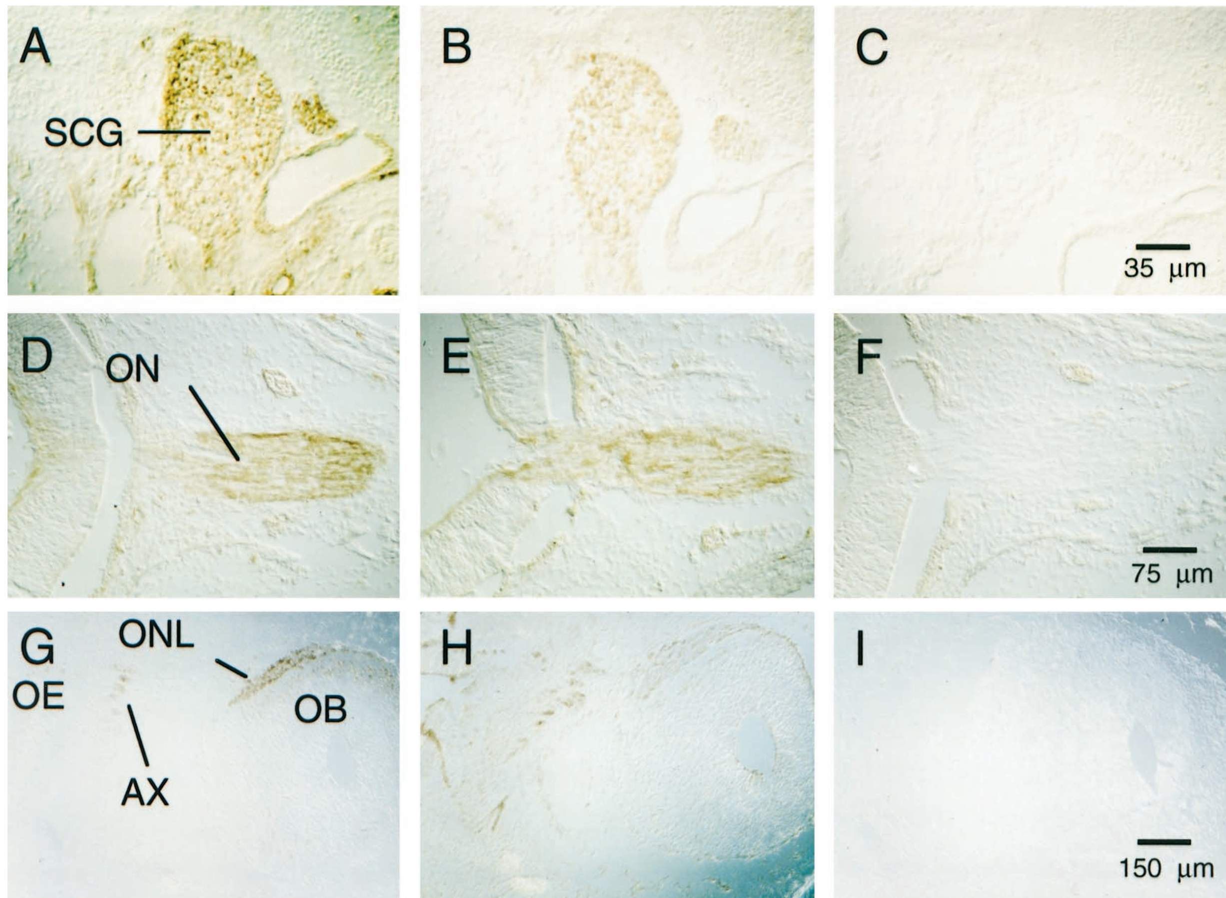

Figure 5. Expression of Npn-1 and NIP protein in the embryonic mouse CNS and PNS. Both Npn-1 and NIP proteins are expressed in E14 superior cervical ganglion neurons (SCG) (*A, B*), the optic nerve (*ON*) (*D, E*), the olfactory axon (*AX*) underneath the olfactory epithelium (*OE*), and the olfactory nerve layer (*ONL*) terminating at the surface of olfactory bulb (*OB*) (*G, H*). The sections were stained with anti-Npn-1 (*A, D, G*) and anti-NIP (*B, E, H*) antibodies. Preimmune serum failed to detect signals (*C, F, I*).

NIP. This could result from instability or improper folding of the NIP-PDZ domain fusion protein in the yeast or from the less hydrophobic alanine at the C-terminal tip of Npn-1. Mutation of the canonical PDZ recognition site by introduction of a terminal alanine has been reported to result in disruption of the interaction with PDZ domains (Songyang et al., 1997; Rousset et al., 1998). The C-terminal one-third of NIP also displayed a very weak binding to Npn-1, consistent with a second binding site in NIP that recognized the cytoplasmic domain of Npn-1. Based on these observations, the interaction of NIP with the cytoplasmic domain of Npn-1 is mediated by the entire C-terminal two-thirds of the NIP protein.

The NIP-mediated interaction described here occurs with the isolated cytoplasmic domain of the Npn-1 receptor and is therefore semaphorin III-independent. NIP could function positively to link Npn-1 with signaling molecules or the cytoskeleton. Alternatively, NIP could act as an inhibitory protein to mask critical regions of Npn-1 for the interacting with other signaling molecules in the absence of semaphorins. The modulation of NIP and Npn-1 interaction by semaphorin *in vivo* may provide clues regarding the physiological function of NIP in Npn-1-mediated responses.

The expression of NIP overlaps with that of Npn-1

During development, the expression of NIP in CNS and PNS neurons coincides with their time of axonal outgrowth. In the CNS of E14 mice, NIP is expressed in the olfactory and optic nerves, spinal cord, several neuron clusters in the brain stem (data not shown), and the intermediate zone of cortex (data not shown), a pattern nearly identical to that of Npn-1 (Kawakami et al., 1996). Interestingly, in E14 mouse spinal cord, Npn-1 is restricted to the dorsal horn, whereas Npn-2 is expressed in the ventral half of spinal cord (Chen et al., 1997). In light of the homology shared by the C-terminal tail of Npn-1 and Npn-2 (a_0), particularly the identity of the last three residues (S-E-A-COOH) critical for NIP/Npn-1 interaction, we predict that NIP could also interact with cytoplasmic tail of Npn-2 (a_0). The presence of NIP throughout the spinal cord at this stage revealed by both *in situ* hybridization (Fig. 4*A*) and immunostaining (data not shown) may suggest that this protein may interact with both of these receptor molecules.

Npn-1 displays a temporally restricted pattern of expression in the optic nerve (Kawakami et al., 1996). Npn-1 and NIP are present in the retina of E14 mouse embryos (Fig. 5*D,E*), but this expression disappears in the adult optic nerve (data not shown). In contrast, NIP and Npn-1 are localized to axon bundles in adult olfactory tissue. Olfactory neurons undergo constant replacement throughout adult life and project new axons to specific target glomeruli in the olfactory bulb. The ability of semaphorin III and collapsin I to disrupt growth cones of olfactory axons (Kobayashi et al., 1997) and especially the colocalization of NIP and Npn-1

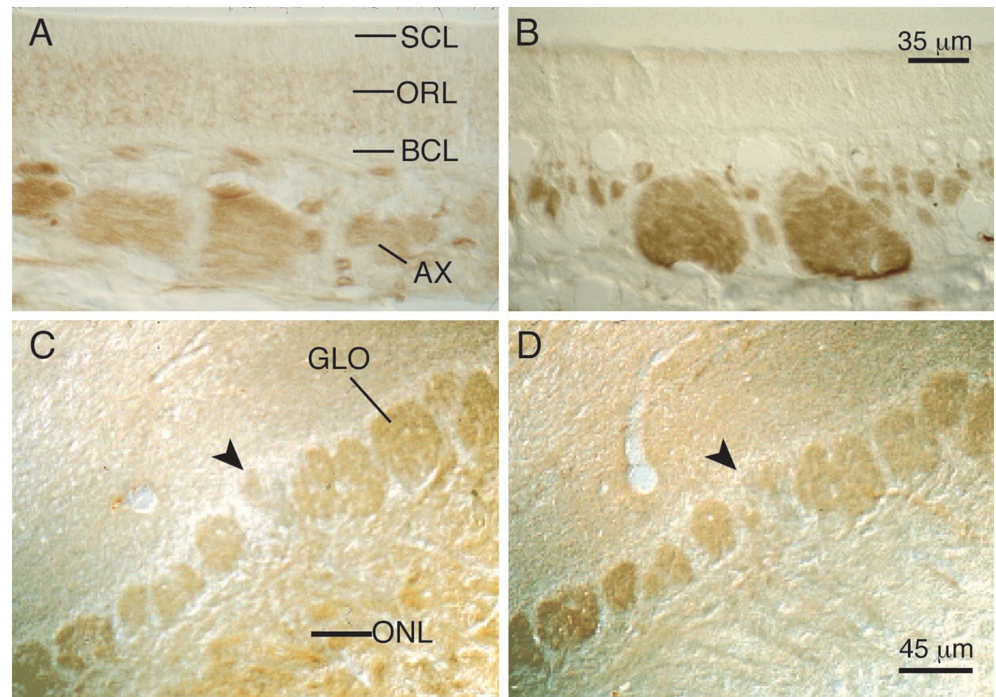

Figure 6. Heterogeneous expression of NIP and Npn-1 proteins in the adult rat olfactory nerve bundles and terminals. Transverse sections of adult rat olfactory epithelium and bulb were immunostained with the anti-NIP and anti-Npn-1 antibodies. Npn-1 staining was detected predominantly in the olfactory axon bundles (*AX*) underneath the olfactory epithelium (*A*) and also in the olfactory neuron layers (*ORL*) and glomeruli (*GLO*) (*C*), but not in the sustentacular cell layer (*SCL*) and basal cell layer (*BCL*). NIP staining was detected exclusively in the olfactory axon bundles underneath the olfactory epithelium (*B*) and at the surface of the olfactory bulb covered by olfactory nerve layer and glomeruli (*D*). Both Npn-1 and NIP immunoreactivities were detected in olfactory nerve terminals within the same subset of glomeruli on two adjacent olfactory bulb sections (*C, D*). *Arrowheads* point to the glomeruli that show weaker staining with both anti-Npn-1 and anti-NIP antibodies (*C, D*).

in subset of these axons strongly suggests a functional role for this complex in olfactory axon guidance. Similarly, in the PNS, both NIP and Npn-1 are expressed by neurons in the developing DRG and superior cervical ganglion. These neurons are also targets of semaphorin III- and collapsin-1-mediated axon growth cone collapse.

The extensive overlapping of NIP and Npn-1 expression patterns, their co-localization to primary olfactory axon terminals, and their biochemical interaction both in the mammalian cell line and *in vivo* provide evidence that NIP acts as an immediate downstream component in Npn-1-mediated functions.

REFERENCES

Cabral JH, Petosa C, Sutcliffe MJ, Raza S, Byron O, Poy F, Marfatia SM, Chishti AH, Liddington RC (1996) Crystal structure of a PDZ domain. Nature 382:649–652.

Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M (1997) Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19:547–559.

Chevesich J, Kreuz AJ, Montell C (1997) Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. Neuron 18:95–105.

Chevray PM, Nathans D (1992) Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci USA 89:5789–5793.

Craven SE, Bredt DS (1998) PDZ proteins organize synaptic signaling pathways. Cell 93:495–498.

Davis JA, Reed RR (1996) Role of Olf-1 and Pax-6 transcription factors in neurodevelopment. J Neurosci 16:5082–5094.

De Vries L, Lou X, Zhao G, Zheng B, Farquhar MG (1998) GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. Proc Natl Acad Sci USA 95:12340–12345.

Doyle DA, Lee A, Lewis J, Kim E, Sheng M, MacKinnon R (1996) Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell 85:1067–1076.

Feilotter HE, Hannon GJ, Ruddell CJ, Beach D (1994) Construction of an improved host strain for two hybrid screening. Nucleic Acids Res 22:1502–1503.

Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340:245–246.

Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM (1995) Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376:509–514.

He Z, Tessier-Lavigne M (1997) Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90:739–751.

Jin Z, Strittmatter SM (1997) Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17:6256–6263.

Kawakami A, Kitsukawa T, Takagi S, Fujisawa H (1996) Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. J Neurobiol 29:1–17.

Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, Culotti JG, Tessier-Lavigne M (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell 87:175–185.

Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 78:425–435.

Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H (1995) Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development 121:4309–4318.

Kitsukawa T, Shimizu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T, Fujisawa H (1997) Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron 19:995–1005.

Kobayashi H, Koppel AM, Luo Y, Raper JA (1997) A role for collapsin-1 in olfactory and cranial sensory axon guidance. J Neurosci 17:8339–8352.

Kolodkin AL, Ginty DD (1997) Steering clear of semaphorins: neuropilins sound the retreat. Neuron 19:1159–1162.

Kolodkin AL, Matthes DJ, Goodman CS (1993) The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75:1389–1399.

Kolodkin AL, Levengood DV, Rowe EG, Tai YT, Giger RJ, Ginty DD (1997) Neuropilin is a semaphorin III receptor. Cell 90:753–762.

Kornau HC, Seeburg PH, Kennedy MB (1997) Interaction of ion channels and receptors with PDZ domain proteins. Curr Opin Neurobiol 7:368–373.

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M (1997) Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors. Nature 386:833–838.

Luo Y, Raible D, Raper JA (1993) Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217–227.

Luo J, Wang Y, Yasuda RP, Dunah AW, Wolfe BB (1997) The majority

of N-methyl-D-aspartate receptor complexes in adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). Mol Pharmacol 51:79–86.

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM (1998) Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron 21:1093–1100.

Pasterkamp RJ, De Winter F, Holtmaat A, Verhaagen J (1998) Evidence for a role of the chemorepellent semaphorin III and its receptor neuropilin-1 in the regeneration of primary olfactory axons. J Neurosci 18:9962–9976.

Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410.

Rousset R, Fabre S, Desbois C, Bantignies F, Jalinot P (1998) The C-terminus of the HTLV-1 Tax oncoprotein mediates interaction with the PDZ domain of cellular proteins. Oncogene 16:643–654.

Sheng M, Wyszynski M (1997) Ion channel targeting in neurons. Bioessays 19:847–853.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform--specific receptor for vascular endothelial growth factor. Cell 92:735–745.

Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, Chishti AH, Crompton A, Chan AC, Anderson JM, Cantley LC (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science 275:73–77.

Takagi S, Hirata T, Agata K, Mochii M, Eguchi G, Fujisawa H (1991) The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron 7:295–307.

Takagi S, Kasuya Y, Shimizu M, Matsuura T, Tsuboi M, Kawakami A, Fujisawa H (1995) Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. Dev Biol 170:207–222.

Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274:1123–1133.

Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, Becker A, Socolich M, Zuker CS (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388:243–249.

Vassar R, Ngai J, Axel R (1993) Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell 74:309–318.

Wang MM, Reed RR (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364:121–126.