Neuronal and Non-Neuronal Collapsin-1 Binding Sites in Developing Chick Are Distinct from Other Semaphorin Binding Sites

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The collapsin and semaphorin family of extracellular proteins contributes to axonal path finding by repulsing axons and collapsing growth cones. To explore the mechanism of collapsin-1 action, we expressed and purified a truncated collapsin-1–alkaline phosphatase fusion protein (CAP-4). This protein retains biological activity as a DRG growth cone collapsing agent and saturably binds to DRG neurons with low nanomolar affinity. Specific CAP-4 binding sites are present on DRG neurons, sympathetic neurons, and motoneurons, but not on retinal, cortical, or brainstem neurons. Outside the nervous system, high levels of CAP-4 binding sites are present in the

mesenchyme surrounding major blood vessels and developing bone and in lung. These sites provide a substrate for the collapsin-1-dependent patterning of non-neuronal tissues perturbed in *sema III* (–/–) mice. The staining patterns for mouse semaphorin D/III and chick collapsin-1 fusion proteins are indistinguishable from one another but quite separate from that for semaphorin B and M-semaphorin F fusion proteins. These data imply that a family of high-affinity semaphorin binding sites similar in complexity to the semaphorin ligand family exists.

Key words: *collapsin; semaphorin; growth cone collapse; dorsal root ganglion; sympathetic neuron; alkaline phosphatase*

The extreme precision of axon pathfinding during neural development depends on both attractive and repulsive guidance signals (for review, see Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996). Chick collapsin-1 was isolated as a growth cone collapse factor for sensory neurons (Luo et al., 1993) and is capable of guiding developing axons away from certain territories by a repulsive mechanism. Insect semaphorin I (initially termed fasciclin IV) was identified as the antigen recognized by an axon tract-specific antibody (Kolodkin et al., 1992). Other members of the collapsin and semaphorin family have primarily been identified by nucleic acid sequence homology (Luo et al., 1995; Püschel et al., 1995; for review, see Dodd and Schuchardt, 1995; Kolodkin, 1996; Püschel, 1996). Whereas some semaphorins such as collapsin-1 and its murine homolog semaphorin D/III are secreted proteins, other semaphorins such as semaphorin B, C, M-F, and M-G/CD100 are associated with the cell surface via a transmembrane hydrophobic sequence near the C terminal (Inagaki et al., 1995; Püschel et al., 1995; Furuyama et al., 1996; Hall et al., 1996). Whether family members other than collapsin-1 and semaphorin D/III are repulsive for growing axons is not known.

Several studies have demonstrated that regulation of axonal extension by these proteins contributes to nervous system formation *in vivo*. Perturbation of grasshopper semaphorin I alters axon trajectory in the limb bud (Kolodkin et al., 1992). Motoneurons

are sensitive to semaphorin D/III *in vitro* (Varela-Echavarría et al., 1997), and ectopic expression of semaphorin II in selected *Drosophila* muscles alters motoneuron innervation (Matthes et al., 1995). The relative insensitivity of the neurotrophin-3 (NT-3)-dependent dorsal root ganglion (DRG) subpopulation to collapsin-1 or its murine homolog semaphorin D/III, coupled with the ventral spinal cord expression of the protein, has suggested that this semaphorin participates in the patterning of the central projections of some sensory neurons (Messersmith et al., 1995; Shepherd et al., 1997). Analysis of mice lacking semaphorin D/III has confirmed that certain DRG neurons project aberrantly in the spinal cord (Behar et al., 1996).

Semaphorins are expressed in a number of non-neuronal tissues (Püschel et al., 1995; Giger et al., 1996). Mice lacking semaphorin D/III develop an enlarged cardiac right ventricle and malformation of the skeleton (Behar et al., 1996). This may be attributable to loss of the normal effects of semaphorin D/III protein expression in the lung and in the mesenchyme surrounding developing bone (Giger et al., 1996). A role for semaphorins in immune function is suggested by the expression of M-semaphorin G (CD100) in T lymphocytes (Furuyama et al., 1996; Hall et al., 1996; Herold et al., 1996) and perhaps by the expression of semaphorin-like molecules by several viruses (Kolodkin et al., 1993). Several small cell lung cancer cell lines exhibit homozygous deletion in chromosome region 3p21.3 where semaphorin A and human semaphorin IV are located (Roche et al., 1996; Sekido et al., 1996; Xiang et al., 1996). This raises the possibility that some members of the semaphorin and collapsin family function as tumor suppressors.

The molecular mechanism of collapsin and semaphorin action remains poorly defined. We have provided evidence that a pertussis toxin-sensitive heterotrimeric GTP-binding protein mediates collapsin-1-induced growth cone collapse (Igarashi et al., 1993; Goshima et al., 1995; Jin and Strittmatter, 1997). The intracellular protein CRMP (TOAD and ULIP) seems to be

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required for collapsin-1 signaling, but its mechanism of action is unclear (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996). Downstream signaling events in collapsin-1 action include activation of *rac1* and depolymerization of growth cone actin filaments (Fan et al., 1993; Jin and Strittmatter, 1997).

Despite these hints about the intracellular cascades activated by collapsin-1, there is no information concerning the nature of a high-affinity cell surface binding site for any of the collapsins and semaphorins. In this study we have developed a collapsin-1-alkaline phosphatase fusion protein (CAP) to visualize cell surface collapsin-1 binding sites. Saturable, high-affinity binding is detected on selective neuronal populations and extraneural tissues. The localization of binding sites for different semaphorin family members is unique, implying a family of semaphorin receptors of complexity similar to that of the ligand family.

MATERIALS AND METHODS

Construction of expression vectors for collapsin-1-His₆ and collapsin-alkaline phosphatase fusion proteins. The C terminal of the chick collapsin-1 coding region was amplified by PCR using the 5'-*EcoRI* primer (CAATGGATGAATTCGTGTGAGCAGGT) and the 3'-(His)₆ primer (GGACTCTAGAGGTCATGCTGCATGATGATGATGATGATGGACACTCCGTG-GTGCCCT). pcDNA-collapsin-1 was used as the DNA template for the reaction (Luo et al., 1993; Goshima et al., 1995). The amplified fragment was digested with *EcoRI* and *XbaI* and cloned into the *NotI-XbaI* sites of pcDNA1 together with the *NotI-EcoRI* fragment (nucleotides 130–2160) of collapsin-1 cDNA to create pc-collapsin-1-His₆.

The entire alkaline phosphatase (AP) coding region of pSEAP (Clontech, Cambridge, UK) was amplified by PCR with the 5'-AP1 primer (CCAGTCTAGAATGCTGCTGCTGCTGCTGCTGCTGGGCTG-AGGCTACAG) and the 3'-AP-His₆ primer (CCAGAGATCTTCATGCTGCATGATGATGATGATGATGATGACCCGGGTGCGCGGCGTC-GGT), polished with *PfuI*, and ligated in the *EcoRV* of pcDNA1 site. This pcAP vector was used for (His)₆-tagged AP expression. In addition, pcDNA and AP vectors with *XbaI* or *EcoRI* cloning sites were generated. An AP3 fragment (52–1517) lacking the signal sequence was amplified with the 5'-AP3-*XbaI* primer (CCAGTCTAGAATCATCCAGTTGAGGAGGAGAACCCGGACTTCTGG) and the 3'-AP-His(6) primer. This fragment was polished with *PfuI* and blunt-end ligated to the *XbaI* site of pcDNA1. This pcAP3 plasmid was used for the construction of pcCAP-3, semaphorin B-alkaline phosphatase (sema B-AP), semaphorin D-alkaline phosphatase (sema D-AP), and M-semaphorin F-alkaline phosphatase (M-sema F-AP). These fusion protein vectors encode a Ser-Arg between the semaphorin and the alkaline phosphatase moieties. An AP4 fragment (52–1517) was amplified with the 5'-AP4-*EcoRI* primer (CCGAATTCATCATCCAGTTGAGGAGGAGAACCCGGACTTCTGG) and the 3'-AP-His₆ primer, polished with *PfuI*, and digested with *EcoRI*. This fragment was inserted at the *EcoRI-EcoRV* sites of pcDNA1 to create pcAP4. The CAP-4 construct created from pcAP4 contains a Glu-Phe linker between protein domains.

The collapsin-1 coding region was amplified from pcDNA-collapsin-1 with the T7 primer (TAATACGACTCACTATAGGG) and the 3'-*XbaI* primer (CCAGTCTAGAGACACTCCGTGGTGCCTCTC), digested with *NotI* and *XbaI*, and ligated into *NotI-XbaI* sites of pcAP3 to create pcCAP-3. The *EcoRI* fragment (–7–2160) of pcCAP-3 was inserted at the *EcoRI* site of pcAP4 to create pcCAP-4. The extracellular domain of semaphorin B (–7–2108) was amplified by PCR from pBluescript SK-semaphorin B (Püschel et al., 1995) with the 5'-T3 primer (AATTAACCTCACTAAAGGG) and the 3'-*XbaI* primer (GATTCTAGAGGGCCAGTAGGACCGCTGGGCATC), digested with *XbaI*, and ligated into the *XbaI* site of pcAP3 to create sema B-AP. The pBK-CMV-SemD (Püschel et al., 1995) clone lacks the initial 270 nucleotides of the coding region. The missing sequence was amplified by PCR with embryonic day 17 (E17) mouse embryo cDNA (Clontech) as template and ligated into the TA cloning vector (Invitrogen, San Diego, CA) to create TA-5'-semaphorin D. The 3' coding region from pBK-CMV-SemD was inserted into TA-5'-semaphorin D using the *EcoRV* site at position 325 to create the full-length clone TA-semaphorin D. The truncated form of semaphorin D lacking C-terminal basic rich residues (–8–2270) was amplified by PCR from TA-semaphorin D with the 5'-SP6 primer (ATTTAGGTGACACTATA) and the 3'-*XbaI* primer

Figure 1. Purification of a collapsin-1-alkaline phosphatase fusion protein. *A*, Schematic illustrating the structure of recombinant proteins. Because a full-length collapsin-1-alkaline phosphatase fusion protein (CAP-3) was degraded in the basic rich region at the C terminal of collapsin-1, a truncated form of collapsin-1 lacking the basic rich region at the C terminal was fused with alkaline phosphatase (CAP-4). *B*, SDS-PAGE of purified recombinant proteins. Fusion protein preparations (2 μ g) were analyzed by 10% SDS-PAGE. The CAP-3 preparation contains a significant fraction of degraded 66 kDa protein similar in molecular weight to alkaline phosphatase (AP) and a second degraded protein species of 110 kDa. Only a minor portion of the CAP-3 is full-length 165 kDa fusion protein. Although the CAP-4 preparation contains a minor amount of degraded 97 kDa protein, the majority of protein is full-length 160 kDa fusion protein. Collapsin-1-His₆ exhibits the expected full-length 95 kDa size.

(GATTCTAGAATCCATCGTGTTCAGGTTGGGGTG), digested with *HindIII* and *XbaI*, and inserted into *HindIII-XbaI* sites of pcAP3 to create sema D-AP. The extracellular domain of semaphorin F (–7–1986) was amplified by PCR from pBluescript SK-semaphorin F (Inagaki et al., 1995) with the 5'-*EcoRI* primer (GATGAATTCCTGCGATGGCCCCA-CCTGGGCTGTC) and the 3'-*XbaI* primer (GATTCTAGAGTTTTC-CAAGGGAGCCCGTGCCTC), digested with *EcoRI* and *XbaI*, and ligated into *EcoRI-XbaI* sites of pcAP3 to create M-sema F-AP. The ligation sites of all constructs were confirmed by DNA sequencing.

Expression and purification of recombinant proteins. Twenty to thirty micrograms of purified expression vector DNA were transfected into 1.5×10^7 HEK293T cells by the calcium-phosphate method. Conditioned medium was collected 3–5 d after transfection. Conditioned medium from cells transfected with pcAP-4 exhibited ~1 U of AP activity per ml, and medium from pcAP-transfected cells yielded 10 U of AP activity per ml. One unit of AP activity is defined as 1 μ mol of *p*-nitrophenyl phosphate hydrolyzed/min at 37°C (Flanagan and Leder, 1990).

Figure 2. Growth cone collapse activity of collapsin-1-His₆ and CAP-4. Growth cone collapse of chick E7 DRG explants (*A*), dissociated chick E7 sympathetic neurons (*B*), and chick E7 retinal explants (*C*) was determined with various concentrations of CAP-4 (closed circle) and collapsin-1-His₆ (Col-His; open circle) as indicated. The EC₅₀ values of collapsin-1-His₆ and CAP-4 are 50 pM and 5 nM, respectively, for both DRG and sympathetic neurons. The SEM from three to five determinations is shown.

To purify recombinant proteins, we supplemented 200 ml of medium to 50 mM imidazole-HCl, pH 7.3, and 0.5 M NaCl and then passed the medium through a 0.45 μ m filter (AP-His₆, CAP-3, CAP-4, sema B, sema D, and M-sema F) or centrifuged it at 100,000 $\times$ g for 90 min at 4°C (collapsin-His₆). The ultracentrifuge supernatant or the filtrate was applied to a 0.75 ml Ni-containing resin (Probond; Invitrogen). The column was washed with 5 ml of 50 mM imidazole, pH 7.3, and 0.5 M NaCl and then eluted with a stepwise gradient of imidazole buffer in 0.5 M NaCl. AP activity eluted primarily in the 200 mM imidazole fraction. The purified protein was frozen in liquid nitrogen and stored at -80°C. The small fraction of protein that aggregated during storage was removed by centrifugation at 15,000 rpm in a microfuge for 15 min at 4°C before the staining protocols.

Neuronal cell culture and growth cone collapse assay. The preparation of

DRG, sympathetic ganglion, and retinal cultures from E7 chick embryos has been described previously (Igarashi et al., 1993; Strittmatter et al., 1994). The growth cone collapse assay has been described in detail (Raper and Kapfhammer, 1990; Strittmatter et al., 1994; Goshima et al., 1995). For selective culture of DRG subpopulations, E10 chick dissociated DRG cells were maintained in medium containing either NT-3 or NGF at 40 ng/ml for 48 hr before CAP-4 staining.

Rat DRG, cortical plate, and brainstem neurons were obtained from E15 Sprague Dawley rat pups. After incubation in 0.05% trypsin EDTA for 15 min at 37°C, tissues were dissociated in DMEM containing 10% FCS and 0.1 mg/ml DNase using a 1 ml plastic micropipette tip. Dissociated cells were diluted in DMEM containing 10% FCS, 2 mM Gln, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The medium used for DRG cultures was supplemented with 10 ng/ml NGF and 10 ng/ml NT-3, and that for brainstem and cortical plate cells was supplemented with 0.5 ng/ml BDNF, 1 ng/ml NT-4/5, and 1 ng/ml NT-3. Recombinant human BDNF was generously provided by Cephalon (West Chester, PA) and NT-4/5 and NT-3 by Genentech (San Francisco, CA). Diluted cells were plated at a density of 125 cells/mm² on 14 mm round glass coverslips (Assistant), which had been precoated with 0.1 mg/ml poly-D-ornithine followed by 2 μ g/ml mouse laminin.

Motoneurons from embryonic day 15 rat pups were purified as described previously (Camu and Henderson, 1992). This procedure uses differential centrifugation and immunopanning with anti-p75 NGF receptor antibodies to obtain highly enriched motoneuron cultures. The cells were seeded at 25 cells/mm² onto poly-D-ornithine- and laminin-precoated glass coverslips in L-15 medium supplemented with 0.63 mg/ml sodium bicarbonate, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2% horse serum, 20 mM glucose, 5 μ g/ml insulin, 0.1 mM putrescine, 20 mM progesterone, 0.1 mg/ml conalbumin, 30 mM sodium selenite, 0.5 ng/ml BDNF, 1 ng/ml NT-4/5, and 1 ng/ml NT-3.

Staining of cell cultures. After 24–72 hr of incubation, cell cultures were washed once with HBH (Hank's balanced salt buffer with 0.5 mg/ml BSA and 20 mM HEPES, pH 7.0) and then blocked with DMEM containing 10% FCS and 100 IU/ml penicillin and streptomycin for 15 min at 23°C. The cultures were then incubated with various amounts of CAP-4 and collapsin-1-His₆ in DMEM/FBS for 2 hr at 10°C and washed with ice-cold HBH six times for 3 min each. Washed cells were fixed with 3.7% formaldehyde in 20 mM HEPES, pH 7.0, and 150 mM NaCl for 15 min, washed with HH (Hank's balanced salt buffer with 20 mM HEPES, pH 7.0) twice, and incubated in HH for 2 hr at 65°C to inactivate the heat-labile endogenous alkaline phosphatases. Bound heat-stable alkaline phosphatase was detected by incubation with *p*-nitrophenyl phosphate or with nitroblue tetrazolium (34 μ g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (18 μ g/ml) in AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂) for 24 hr at room temperature.

Some E10 DRG cultures were stained for trkC after CAP-4 staining. These samples were incubated with affinity-purified rabbit anti-trkC antibody (1 μ g/ml) directed against residues 798–812 of the porcine sequence (Santa Cruz Biotechnology, Tebu, France), and bound antibody was detected after incubation with fluorescein-labeled goat anti-rabbit IgG (10 μ g/ml; Sigma, St. Louis, MO).

Staining of tissue sections. E5, E7, E9, and E12 chick embryos were frozen on dry ice, and 20 μ m sections were cut on a cryostat and thaw-mounted onto Superfrost⁺ slides (Fisher Scientific, Houston, TX). Sections were fixed with 100% methanol at -80°C for 10 min. Fixed sections were rehydrated in PBS, equilibrated with HBH for 5 min, and then preblocked with HBH containing 20% fetal bovine serum for 1 hr. After preblocking, sections were incubated for 2 hr with AP fusion proteins diluted into HBH containing 20% fetal bovine serum. For competitive inhibition experiments, AP fusion protein and collapsin-1-His₆ were added. Sections were washed with HBH once for 5 min, with Tris-buffered saline (20 mM Tris-HCl and 135 mM NaCl, pH 7.5) three times for 5 min each, and with PBS for 5 min and then fixed with 3.7% formaldehyde in PBS for 15 min. The fixed sections were incubated in PBS at 65°C for 50 min to inactivate heat-sensitive endogenous alkaline phosphatases. Specimens were then washed with AP buffer three times for 5 min each and developed with nitroblue tetrazolium (34 μ g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (18 μ g/ml) in AP buffer for 24 hr at 23°C.

To section chick embryos without freezing, we fixed whole embryos with methanol for 3 hr, and 100–200 μ m sections were cut with a vibrating microtome. Sections were then air-dried onto slides and rehydrated in PBS. The staining procedure was as described for cryostat

Figure 3. Saturable staining of chick E7 DRG neurons by CAP-4. Dissociated cultures of chick E7 DRG neurons were stained with 10 nM CAP-4 (*top left*), with 10 nM CAP-4 in the presence of 50 nM collapsin-1-His₆ (*top right*), or with 10 nM human placental alkaline phosphatase (*bottom right*). CAP-4 stained a subpopulation of DRG neurons. The CAP-4 staining was abolished in the presence of excess collapsin-1-His₆. Human placental alkaline phosphatase did not stain DRG neurons. The growth cones of methanol-fixed DRG neurons were stained with 10 nM CAP-4 (*bottom left*). Scale bar: *bottom left*, 30 μ m; *right and top left*, 65 μ m.

sections, except that sections were pretreated with buffer containing 1 M NaCl and 0.1% Tween 20 to increase permeability.

RESULTS

Isolation of biologically active collapsin–alkaline phosphatase fusion protein

To visualize collapsin-1 receptors, we sought to develop a fusion protein consisting of collapsin-1 and secreted placental alkaline phosphatase. This method has been used extensively to visualize the distribution of the SEK/MEK family of receptors and their ligands by Flanagan and colleagues (Chen and Flanagan, 1994; Chen et al., 1995; Nakamoto et al., 1996). Initial constructs consisted of collapsin-1 followed by alkaline phosphatase and a His₆ tag (CAP-3). Purification of His₆-tagged proteins from transfected HEK293T cells by nickel affinity chromatography demonstrated that the majority of His₆-tagged fusion proteins were cleaved to a size nearly identical to that of alkaline phosphatase-His₆ (Fig. 1). There is a cluster of basic residues near the C terminal of the collapsin-1 sequence that may be a site for endoproteolysis. To avoid this degradation, we deleted the last 52 residues of collapsin-1 from the fusion protein. The truncated collapsin-1 fusion protein (CAP-4) could be purified from transfected HEK293T cells with an apparent molecular size of 160 kDa, as predicted for nonproteolyzed fusion protein (Fig. 1).

Purified recombinant collapsin-1-His₆, and to a lesser degree CAP-4, aggregated when frozen at concentrations exceeding 1 μ g/ml. Aggregation could be minimized by storing stock solutions with >0.5 M NaCl. The small fraction of aggregated protein in high salt could be removed by centrifugation of the protein.

The truncation in CAP-4 removes only a small portion of the collapsin-1 sequence that is not in the highly conserved sema domain, suggesting that the CAP-4 protein is likely to retain biological activity. Collapsin-1 induces growth cone collapse in DRG and sympathetic neurons but not in axons of retinal gan-

glion cells (Fig. 2). As expected, the alkaline phosphatase protein has no effect on growth cone morphology (data not shown). The CAP-4 protein induces growth cone collapse with a specificity indistinguishable from that of collapsin-1 and with an EC₅₀ of ~ 5 nM, ~ 100 -fold higher than that of collapsin-1-His₆ (Fig. 2). Thus, CAP-4 provides a biologically active, labeled and purified ligand for collapsin-1 binding sites.

High-affinity binding of CAP-4 to cultured neurons

Neuronal cultures were incubated with CAP-4 to visualize collapsin-1 binding sites, and bound protein was visualized by alkaline phosphatase reaction (Fig. 3). Dense staining was observed in chick E7 DRG soma, neurites, and growth cones (Fig. 3). Although the intensity of staining varied between different DRG neurons in the culture, sympathetic neurons were uniformly and intensely stained (Fig. 3; see below). Non-neuronal cells were not stained in the mixed cultures. To demonstrate further the specificity of CAP-4 staining, we analyzed several control conditions. Alkaline phosphatase yielded no staining (Fig. 3), indicating that the staining was dependent on the collapsin moiety of the fusion protein. Heating of CAP-4 at 65°C destroys growth cone collapse activity and abolishes staining of DRG neurons (data not shown). Most selectively, CAP-4 staining was prevented by the addition of excess collapsin-1-His₆ (Fig. 3).

Using an alkaline phosphatase substrate that produces a soluble product, we could quantitate the amount of CAP-4 bound to cultured neurons (Fig. 4). Specific CAP-4 binding to sympathetic neurons is saturable with a K_D of 3.3 nM. Collapsin-1-His₆ inhibits CAP-4 binding with an apparent K_i of 250 pM. The K_D of CAP-4 and the K_i of collapsin-1-His₆ in this binding assay are very close to the potency of these proteins in the growth cone collapse assay (Fig. 2), indicating that the CAP-4 staining is likely to reflect a biologically relevant site leading to growth cone collapse. The slightly higher K_i for collapsin-1-His₆ in the binding assay as

Figure 4. Quantitation of CAP-4 binding to chick sympathetic neurons. *A*, Saturation of CAP-4 binding to sympathetic neurons. Dissociated cultures of chick E7 sympathetic neurons were stained with various amount of CAP-4 (*Total*; open circle) or CAP-4 plus 100 nM collapsin-1-His₆ (*Nonspecific*; closed circle). Bound alkaline phosphatase activity was quantitated after the incubation with *p*-nitrophenyl phosphate by measuring the optical density at 415 nm. Specific CAP-4 binding was calculated by subtracting nonspecific from total binding (*Specific*; square). *B*, Scatchard analysis of specific CAP-4 binding to sympathetic neurons. Scatchard analysis of specific CAP-4 binding from *A* is shown. The calculated K_D is 3.3 nM, and the B_{max} is 1 nmol of CAP-4 bound per well. *C*, Inhibition of CAP-4 binding by collapsin-1. The binding of 4 nM CAP-4 to chick sympathetic neurons in the presence of the indicated concentrations of collapsin-1-His₆ was quantitated as described in Materials and Methods. The IC_{50} is 500 pM, which corresponds to an apparent K_i of 250 pM assuming competitive inhibition and a K_D for CAP-4 of 3.3 nM. Error bars indicate SEM for three to five determinations in *A–C*.

opposed to its EC_{50} in the collapse assay may be because of the effects of longer incubation time and lower incubation temperature in the binding assay or because of the possibility that partial receptor occupancy induces growth cone collapse.

Selectivity of CAP neuronal staining

Only certain neuronal populations respond morphologically to collapsin-1 (Luo et al., 1993; Fig. 2). Neuronal populations known to be sensitive to the collapsing effects of collapsin-1 and semaphorin D/III include DRG neurons, sympathetic neurons, and motoneurons (Fig. 2; Luo et al., 1993; Shepherd et al., 1996). In contrast, retinal ganglion cell growth cones and cortical neurons are insensitive to collapsin-1 (Luo et al., 1993; Shepherd et al., 1996). Furthermore, collapsin-1 has no effect on the shape of kidney COS cells. CAP-4 stains sympathetic neurons and motoneurons specifically but not retinal neurons, brainstem neurons, cortical neurons, or COS cells (Fig. 5; data not shown). Sympathetic neurons are strongly and uniformly stained by CAP-4. The motoneuron-enriched culture prepared by differential centrifugation and anti-p75 panning contains two populations of cells: a group with large cell bodies that are islet-1-positive, p75-positive, and L14-positive and a second set with smaller cell bodies that are islet-1-negative, p75-positive, and L14-positive (Henderson et al., 1993). Although the larger cells are clearly motoneurons, the identity of the smaller cells in these cultures is less clear. CAP-4 stains the majority of the large cells but few if any of the smaller cells. CAP-4 staining of these cells is most prominent within the soma region (Fig. 5), although at high magnification, staining of axons is also detectable. Overall, the selectivity of staining matches the morphological sensitivity of growth cones.

Two populations of DRG neurons with different collapsin-1 sensitivities have been documented (Messersmith et al., 1995; Shepherd et al., 1997). The NGF-dependent, trkC-negative, collapsin-1-sensitive pool projects dorsally in the spinal cord. In contrast, the NT-3-dependent, trkC-positive, collapsin-1-insensitive pool of primary muscle afferents extends from the dorsal cord into the collapsin-1-expressing ventral spinal cord. If CAP-4 binds to physiologically relevant collapsin-1 receptor sites, then CAP-4 staining should be selective for the first population. To examine this hypothesis, we cultured chick E10 neurons in NGF or in NT-3 to enrich for different subpopulations of neurons (Messersmith et al., 1995; Shepherd et al., 1997). CAP-4 staining is robust in the NGF-treated cultures and nearly absent in the NT-3-treated cultures (Fig. 6*A*). To confirm this selectivity, we also cultured E10 DRG cells with a mixture of NT-3 and NGF and then stained cells for both CAP-4 binding and immunoreactive trkC. A majority of CAP-4-binding-positive neurons are trkC-negative, whereas a majority of CAP-4-binding-negative neurons are trkC-positive (Fig. 6*B*). These data extend the coincidence of CAP-4 staining and physiological responsiveness to collapsin-1.

To localize high-affinity collapsin-1 binding sites *in situ*, we stained methanol-fixed cryostat and vibratome sections of chick embryos. In the E7 chick, transverse sections of the spinal cord demonstrate staining of motoneurons and DRG (Fig. 7). The dorsal half of the cord is relatively unstained. Motoneurons from cervical, thoracic, and lumbar spinal cord are stained with equal intensity. In the E4 and E5 chick, staining is less prominent, but the same general pattern is apparent (data not shown). By E12, CAP-4 staining of motoneurons is minimal, but axonal tracts in the ventral midline that are not labeled at E7 are prominently stained. This location corresponds to descending reticulospinal

Figure 5. CAP staining of different neuronal populations. Chick E7 sympathetic neurons were stained with 10 nM CAP-4 (*Ch Sym*, *CAP-4*), and this staining was abolished in the presence of 50 nM collapsin-1-His₆ (*Ch Sym*, *CAP-4* + *Col*). Rat E15 DRG neurons were also stained with 10 nM CAP-4 (*Rat DRG*, *CAP-4*), and labeling was abolished by the addition of 50 nM collapsin-1-His₆ (*Rat DRG*, *CAP-4* + *Col*). Although large diameter cells in rat E15 motoneuron-enriched cultures were stained with 10 nM CAP-4 (*Rat MN*, *CAP-4*), rat cortical neurons (*Rat Cortex*, *CAP-4*) and rat brainstem neurons (*Rat BS*, *CAP-4*) were not stained. Scale bar, 50 μ m.

fibers, which also develop between E7 and E12 (Okado and Oppenheim, 1985). As seen for the staining of the primary neuronal cultures, all CAP-4 labeling in these sections is competitively inhibited by excess collapsin-1-His₆. There is no detectable staining of retina or cerebral cortex at any of these ages (see Fig. 9B; data not shown).

Localization of extraneuronal CAP-4 binding sites in developing chick

CAP-4 staining is not found exclusively in neurons. Several extraneuronal tissues are prominently labeled in the E7 chick embryo. The highest level of staining is found in developing mesenchyme, especially surrounding bone and major blood vessels such as the aorta (Fig. 8). The density of staining in these areas exceeds that in motoneurons and DRG neurons. Specific CAP-4 staining of the aorta is found in the mesenchyme surrounding the aorta,

but there is no labeling of the aorta (Fig. 8). High levels of staining are also present throughout the developing lung (Fig. 8). The mesenchyme surrounding a subset of developing bones is stained intensely (Fig. 8). The mesenchymal tissue immediately adjacent to the vertebral bodies between bone and dura exhibits the densest reaction product of any site in the developing chick (Fig. 7). Mesenchyme surrounding bone is also labeled by CAP-4 in the proximal segments of the wing bud (Fig. 8). Visceral organs including the heart, the liver, the kidney, and the intestine are essentially unstained (data not shown). The skin does not exhibit significant labeling.

Staining by other semaphorin–alkaline phosphatase fusion proteins

Collapsin-1 belongs to a large family of semaphorin proteins. Mouse semaphorin D/III is homologous to chick collapsin-1. The

Figure 6. CAP-4 staining of E10 DRG neuronal subpopulations. *A*, Dissociated chick E10 DRG neurons were cultured in either NGF or NT-3 for 48 hr and then stained with 3 nM CAP-4 as described in Materials and Methods. Note the prominent staining of the NGF-dependent neurons and the absence of staining of NT-3-dependent cells. Scale bar, 50 μ m. *B*, Dissociated chick E10 DRG neurons were cultured in both NGF and NT-3 for 48 hr and then stained with CAP-4 and anti-trkC antibody as described in Materials and Methods. The percentage of CAP-4-binding-positive neurons also exhibiting trkC immunoreactivity is low compared with the fraction of CAP-4-negative neurons that are trkC immunoreactive. The percentage of E10 DRG neurons stained by CAP-4 was $50 \pm 2\%$. The SEM from three separate experiments is shown.

amino acid sequence identity within the sema domain is 94%. We considered whether binding sites for semaphorins are selective for certain members of this family. To test this, we expressed semaphorin B-, D-, and M-F-alkaline phosphatase fusion proteins with a truncated structure analogous to CAP-4. Murine semaphorin B is more closely related to chick collapsin-4 (amino acid identity of sema domain, 45%) than to chick collapsin-1, -2, -3, or -5 (31–34%). Murine M-semaphorin F is also more closely related to chick collapsin-4 (amino acid identity of sema domain, 51%) than to other known chick collapsins (38–41%). It is unclear whether murine semaphorin B and M-F are homologs of collapsin-4 or homologs of as yet unidentified chick collapsins. Each of the semaphorin-AP fusion proteins could be highly purified from transfected-cell conditioned medium (Fig. 9*A*). Although semaphorin B and M-F are transmembrane proteins (Inagaki et al., 1995; Püschel et al., 1995), the semaphorin B and M-F fusion proteins lack the transmembrane segment and are secreted into the culture medium.

The staining pattern exhibited by these semaphorin fusion proteins was compared with that of CAP-4. Sema D-AP staining is indistinguishable from CAP-4 labeling and can be blocked by excess collapsin-1-His₆ (Fig. 9*B*; data not shown). Thus, ligands and receptors of the semaphorin family can react across the chick-mouse species barrier. In contrast, sema B-AP and M-sema F-AP fusion proteins label different cell populations, and staining is not competitively inhibited by excess collapsin-1-His₆. Sema B-AP and M-sema F-AP both stain retinal ganglion cells and the medial longitudinal fasciculus in the midline of the dorsal midbrain. These structures are not labeled with CAP-4 or sema D-AP. Neither sema B-AP nor M-sema F-AP bind to the periaortic mesenchyme that is strongly labeled by CAP-4 and sema D-AP (Fig. 9*B*).

Figure 7. *In situ* localization of CAP-4 binding sites in developing chick nervous system. Transverse thoracic vibratome sections (dorsal surface at top) of chick E7 and E12 embryos were stained with 10 nM CAP-4. DRG and the motoneurons (MN) of the ventral horn of the spinal cord were stained in E7 embryos (top panel). In E12 embryos, staining of the ventral horn was minimal, but the ventral midline of the spinal cord was stained (bottom panel; arrow). This ventral midline staining was not detectable in E7 specimens (middle panel; arrow). Staining of mesenchyme at the surface of the vertebral bodies was prominent in E7 preparations (middle panel). Scale bar: top and middle panels, 60 μ m; bottom panel, 120 μ m.

DISCUSSION

Labeling of a candidate collapsin-1 receptor

We have localized a CAP-4 binding site in developing chick tissues. There are three conceivable explanations for this CAP-4 binding site: (1) an artifact of the *in vitro* binding assay, (2) an “accessory” binding site that serves to localize soluble collapsin-1 by limiting its diffusion, and (3) a physiologically relevant recep-

Figure 8. Localization of CAP-4 binding sites in extraneuronal sites. Transverse thoracic sections (dorsal surface at top) of the chick embryo were stained with 10 nM CAP-4. Low magnification images of sections from E9 embryos reveal intense mesenchymal staining (CAP-4) that is blocked by the addition of 50 nM collapsin-1-His₆ (CAP-4 + Col-His). The positions of the spinal cord (sc), the aorta (*), the limb (l), and the heart (h) are indicated. The mesenchyme around the aorta was stained with 10 nM CAP-4 (Aorta, CAP-4) in the E9 chick embryo. This staining was blocked in the presence of 50 nM collapsin-1-His₆ (Aorta, CAP-4 + Col-His). The lung parenchyma was stained with 10 nM CAP-4 (Lung, CAP-4). The histological border of lung tissue is indicated by the asterisks. In the limb, the mesenchyme surrounding bone was stained with 10 nM CAP-4 (Limb, CAP-4). Scale bar: two top panels, 800 μ m; two bottom left panels, 100 μ m; two bottom right panels, 200 μ m.

tor molecule. Several lines of evidence support the third view that CAP-4 labels a collapsin-1 binding site that mediates its inhibitory effects on growth cone motility. CAP-4 binding sites are saturable and of high affinity. The affinity of CAP-4 in the binding assay (3 nM) is essentially identical to its EC₅₀ in the growth cone collapse assay (5 nM). Likewise, the affinity of collapsin-1 in the CAP-4 binding assay (250 pM) is within an order of magnitude of its EC₅₀ in the growth cone collapse assay (50 pM). Furthermore, CAP-4 stains the same neurons that are sensitive to collapsin-1-induced axon repulsion. It is unlikely that the C-terminal truncation in CAP-4 unmasks a binding specificity distinct from that of intact collapsin-1 because the CAP-3 preparation, containing full-length collapsin-1-AP and proteolytic N-terminal truncations, exhibits a staining pattern for cultured neurons and for tissue sections indistinguishable from that of CAP-4 (data not shown). Overall, the data favor the hypothesis that CAP-4 labels a physiologically relevant collapsin-1 receptor.

Neuronal localization of collapsin-1 receptors

CAP-4 binding sites are visualized on those neurons the axons of which are repulsed by collapsin-1 and not on neurons insensitive to collapsin-1-mediated inhibition. Thus, the distribution of the binding sites can account for the selectivity of collapsin-1 effects. Binding sites in growth cones are positioned to mediate acute effects on growth cone structure. Binding sites in other segments of the neuron may reflect receptor protein undergoing synthesis

and transport to the growth cone. Alternatively, the sites along the axon shaft may mediate the stimulatory effects of collapsin-1 on axonal transport (Goshima et al., 1997).

Cultured DRG neurons display heterogeneous CAP-4 binding. It has been suggested that those NT-3-dependent primary muscle afferent fibers that project to the semaphorin III-expressing ventral cord of the mouse are insensitive to semaphorin III and that this allows their projection to the correct location (Messersmith et al., 1995). In the chick, those DRG neurons expressing the high-affinity receptor for NT-3 (trkC) are insensitive to collapsin-1 at E10 (Shepherd et al., 1997). CAP-4 staining exhibits the same pattern of selectivity among E10 DRG neurons as does collapsin-1 sensitivity, with little labeling of the NT-3-dependent, trkC-positive subpopulation. Although DRG axon and growth cone staining is easily observed in cultured cells, DRG axonal projections to the dorsal spinal cord and periphery are below the detection limit in our tissue sections.

At all levels of the neuraxis, developing motoneurons are repulsed by semaphorin D/III (Varela-Echavarría et al., 1997). In tissue culture, purified rat E15 motoneurons are stained with CAP-4. CAP-4 binding to developing chick ventral spinal cord is easily visualized in tissue sections and corresponds to the location of motoneuron cell bodies.

Sympathetic neurons are also sensitive to collapsin-1 and stain with CAP-4. Presumably this contributes to the restriction of

Figure 9. Differential localization of semaphorin binding sites. **A**, Purification of semaphorin B, D, and M-F fusion proteins. Fusion proteins (2 μ g) were analyzed by 10% SDS-PAGE. Semaphorin B extracellular domain-alkaline phosphatase fusion protein (*Sema B*) is purified in its full-length form of 150 kDa. The truncated semaphorin D-alkaline phosphatase fusion protein preparation (*Sema D*) contains the predicted protein of 165 kDa and a degraded protein of 97 kDa. The M-semaphorin F extracellular domain-alkaline phosphatase fusion protein (*M-Sema F*) preparation exhibits protein staining at the predicted size of 150 kDa and a minor fraction of a degraded protein at 66 kDa. **B**, Localization of semaphorin-alkaline phosphatase binding sites. Cryostat sections of E9 chick embryo were stained with *CAP-4*, *Sema B-AP*, *Sema D-AP* and *M-Sema F-AP* (10 nM). Although *CAP-4* and *Sema D-AP* stained the mesenchyme around the aorta in transverse thoracic sections, *Sema B-AP* and *M-Sema F-AP* did not. Coronal sections containing the midline of the dorsal midbrain and transverse sections of the nasal retina were stained by *Sema B-AP* and *M-Sema F-AP* but not by *CAP-4* or *Sema D-AP*. The dorsal surface is at the top of each section. Scale bar, 100 μ m.

sympathetic innervation to certain regions of the embryo. The density of CAP-4 binding sites in sympathetic neurons is greater than that in DRG neurons or motoneurons, emphasizing the

potential importance of semaphorin signaling in autonomic nervous system formation.

At E12, CAP-4 labels axonal tracts in the ventral midline. The

spatial and temporal development of CAP-4 binding sites in this location is similar to that of the reticulospinal tract (Okado and Oppenheim, 1985). This suggests that collapsin-1 signaling may contribute to the establishment of some descending pathways in the spinal cord.

Non-neuronal functions for collapsin-1

Several extraneuronal sites are intensely and specifically stained with CAP-4. This finding implies that collapsin-1 might participate in pattern formation for tissues other than the nervous system. The high level expression of collapsin-1 and semaphorin D/III mRNA in the mesenchyme surrounding bone (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1997) is matched with high level CAP-4 staining in this location. For both collapsin-1 and CAP-4 binding sites, this mesenchymal tissue exhibits the highest expression levels. Thus, a local semaphorin ligand–receptor interaction may contribute to bone patterning. These CAP-4 binding sites provide a molecular substrate for the rib duplication and other abnormalities of bone development that occur in *sema III* (–/–) mice (Behar et al., 1996). This hypothesis contrasts with the view that high mesenchymal collapsin-1 expression in mesenchymal structures functions primarily to exclude extending axons from certain areas of the embryo (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1997). It is possible that both functions are served by mesenchymal collapsin-1 expression during development.

The lung also coexpresses CAP-4 binding sites (Fig. 8) and semaphorin D/III mRNA (Giger et al., 1996). Thus, collapsin-1 signaling may contribute to the formation of lung architecture, and disruption of collapsin-1 signaling in the lungs of *sema III* (–/–) mice may cause the hypertrophy of the right ventricle of the heart (Behar et al., 1996). The high levels of CAP-4 staining in the mesenchyme surrounding the aorta is diffuse and does not seem to be caused by neuronal fiber ingrowth. Staining in this area does not colocalize with the neuronal markers such as neurofilament (T. Takahashi and S. M. Strittmatter, unpublished observations). It is possible that collapsin-1 and semaphorin D/III may participate in the development of the vascular architecture. If so, this might provide an alternative explanation for the right ventricle hypertrophy observed in *sema III* (–/–) mice.

How might collapsin-1 binding sites function in these developing non-neuronal tissues? Collapsin-1 can regulate the motility of the growth cone via the actin-modulating small GTP binding protein rac1 (Jin and Strittmatter, 1997). Conceivably, mesenchymal development might be determined in part by collapsin-1 regulation of the same rac1–actin pathway in non-neuronal cells. Although CRMP seems to mediate collapsin-1-induced growth cone collapse (Goshima et al., 1995), its expression is neural-specific (Wang and Strittmatter, 1996), unlike rac1. Therefore, any collapsin-1 effects in non-neural tissues must use a distinct CRMP-independent signaling pathway.

Some regions of the embryo such as the retina and the skin exhibit collapsin-1 and semaphorin D/III mRNA expression (Wright et al., 1995; Giger et al., 1996; Shepherd et al., 1996) but no CAP-4 binding. The function of collapsin-1 in these locations is unclear. It is possible that collapsin-1 functions to exclude certain growing axons or migrating cells from these regions. Several organs including the liver, kidney, and heart express undetectable levels of both CAP-4 binding sites and semaphorin D/III mRNA. These organs are unlikely to use collapsin-1 directly during developmental morphogenesis.

A family of semaphorin receptors

Although semaphorin D/III and collapsin-1 seem to be homologous proteins binding to identical sites in the chick embryo, semaphorin B and M-F interact with a different set of binding sites in the developing chick. Furthermore, staining by sema B–AP and M-sema F–AP is not competitively inhibited by collapsin-1. The distribution of these binding sites may reflect the localization of a receptor for collapsin-4 or for as yet unidentified chick collapsins. Regardless of the natural ligand for these sites, the data provide further proof of CAP-4 specificity for collapsin-1 receptors. The findings also imply that there is a family of semaphorin receptors, with a diversity equal to that of the semaphorin ligand family. The preliminary data suggest that sema B and M-sema F might contribute to axonal guidance in the medial longitudinal fasciculus and in the projection of retinal ganglion cells.

The demonstration of CAP-4 as a sensitive, high-affinity ligand for collapsin-1 binding sites is likely to be of great value in the ongoing isolation and characterization of the semaphorin receptor family.

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