Collapsin-1 Covalently Dimerizes, and Dimerization Is Necessary for Collapsing Activity*

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Adam M. Koppel and Jonathan A. Raper†

From the Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Chick collapsin-1, the first identified vertebrate member of the semaphorin family of axon guidance proteins, repels specific growth cones. Like all family members, collapsin-1 contains within its sequence a semaphorin domain that is necessary for specifying activity. Two additional structural domains of collapsin-1, the immunoglobulin (Ig) domain and the basic tail, each potentiate collapsin-1 activity. We identify in this study another structural feature of collapsin-1 that is necessary for its function. Collapsin-1 covalently dimerizes, and dimerization is necessary for collapse activity. This dimerization is mediated through a cysteine at residue 723, between the Ig domain and basic tail. The semaphorin domain alone is not active since it cannot dimerize. The collapsing activity of the semaphorin domain can be reconstituted when made as a chimeric construct with an immunoglobulin Fc domain, which promotes dimerization.

Neuronal growth cones guide axons toward their appropriate targets in the developing nervous system by responding to molecular signals in their milieu. Members of the semaphorin family of proteins are an example of these guidance cues. In the nervous system, these molecules affect axon pathfinding, branching, and targeting (1–7). Collapsin-1, the founding member of the vertebrate semaphorin family, was identified as a repulsive, paralytic signal that causes the collapse of growth cones from cultured dorsal root ganglia (DRG)1 cells (8).

Collapsin-1 contains three structural domains: the family signature semaphorin domain at its amino terminus, a C-2 type immunoglobulin (Ig) domain, and a positively charged basic tail at its carboxyl terminus. We have shown previously that the semaphorin domain specifies the biological activity and the in situ binding pattern of collapsin family members (9, 10). The carboxyl end of the molecule does not specify either activity or binding but does potentiate the activity of collapsin-1.

In the course of an analysis to determine which structural elements are required for collapsin family activity, we generated a number of collapsin-1 deletion constructs and found that the semaphorin domain is necessary for activity (10). The semaphorin domain when produced by itself, however, had no biological activity. This was surprising since we had also shown in the deletion construct analysis that neither the Ig domain nor basic tail was required for activity. One simple explanation for these results was that an additional element in the carboxyl portion of the molecule is required for collapsin activity. The purpose of this study was to identify the additional structural element.

In this study, we show that collapsin-1 dimerizes and that dimerization is necessary for collapse activity. In addition, we show why the semaphorin domain of collapsin-1, although necessary for activity, is not sufficient. The cysteine at position 723, between the Ig domain and basic tail, is necessary for covalent disulfide bond dimerization. When fused to the Fc domain of human IgG, and thus produced as a dimer, the semaphorin domain alone is capable of collapsing growth cones.

EXPERIMENTAL PROCEDURES

Expression Plasmid—The expression plasmid used in this study, pAG-3, contains a pcDNA3 backbone (Invitrogen), a cytomegalovirus enhancer, a modified chicken β-actin promoter, and a rabbit β-globin splice acceptor sequence. The resulting β-actin/β-globin (AG) hybrid promoter provides enhanced protein expression (11). We modified the expression plasmid by adding either an amino-terminal signal sequence 2Mye-6His tag between HindIII and BamHI restriction sites making pAG-NT (amino-terminal tag) or adding a carboxyl-terminal 2Mye-6His tag between XhoI and NsiI restriction sites making pAG-CT (carboxyl-terminal tag). Collapsin-1 without signal sequence and with a stop codon was cloned into the BamHI and XhoI sites of pAG-NT to create Coll-1-pAG-NT, after PCR amplification with oligomers that placed the appropriate restriction sites on the 5’ and 3’ ends. Collap-1 with signal sequence and without stop codon was cloned into HindIII and XhoI sites of pAG-CT to create Coll-1-pAG-CT, following PCR amplification.

Deletion Constructs—Deletion constructs were cloned into either pAG-NT (Ig deletion, semaphorin domain, semaphorin deletion) or pAG-CT (basic tail deletion) following PCR using specific oligomers that restricted collapsin-1 sequence and placed BamHI on the 5’ end and either XhoI or NsiI on the 3’ end. The Ig deletion necessitated a two-step PCR protocol, since internal amino acids had to be removed. In the first step, the collapsin-1 sequence on either side of the Ig loop was amplified. The 5’ end of the internal reverse primer was complementary to the internal forward primer. Consequently, the two pieces of DNA amplified in the first round of PCR would act as the template for the second round of PCR after they had annealed together at low temperatures (45 °C for 5 min).

Point Mutation Construct—Complementary forward and reverse internal oligomers were synthesized to introduce a site directed point mutation at cysteine 723 (collapsin-1). Two base pairs were changed to introduce a cysteine to alanine mutation: TGT to GCT. The same two-step PCR procedure just described was then implemented using the same external primers that were utilized to clone collapsin-1 into pAG-NT. C723A was subsequently cloned into pAG-NT using BamHI and NsiI sites. Fe Fusion Plasmids—Human Fe in pSP73 (Promega) was a gift from Marc Tessier-Lavigne. PCR oligomers were synthesized to attach XhoI restriction site to the 5’ end of Fe and a hexahistidine tag and PstI restriction site to the 3’ end of Fe. This PCR product was cut with XhoI and PstI and cloned into XhoINsiI sites of Coll-1-pAG-CT to give Coll-1-Fc-6His in the pAG-3 vector. C723A was cloned into the Fe-6His-pAG-3 plasmid, replacing wild-type collapsin-1 by cutting both insert
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and vector with HindIII and StuI and ligating. The semaphorin domain alone was cloned into the Fc-6His-pAg-3 plasmid, replacing C723A by cutting both insert and vector with BamHI and XhoI and ligating.

Protein Expression—293T cells grown to ~70% confluence were transfected with 50–60 µg of plasmid DNA/150-mm dish using calcium phosphate precipitation with chloroquine for 4 h. Conditioned medium (serum free) or cell lysate was collected 18 h later, depending on whether the modified protein was readily secreted like collapsin-1. Baculovirus infection of insect cells (Hi-5) was used to produce a large amount of collapsin-1 and C723A protein for HPLC gel filtration. Baculovirus production and infection was carried out as described by the manufacturer (PharMingen; Baculovirus manual, 1992).

Protein Purification—All constructs used in this study contained either an amino- or carboxyl-terminal hexahistidine tag. Protein was purified and concentrated with nickel beads (200 µl slurry/150-mm plate) and elution with imidazole (200–500 mM in phosphate buffer; Qiagen manual). Western blots, using anti-Myc and anti-collapsin-1 antibodies, silver stains, and micro-Bradford assays were used to quantitate the amount of protein. Proteins were compared with a collapsin-1 standard that was similarly tagged. Calculations were based on the assumption that 30 µM pure, recombinant collapsin-1 causes approximately 50% collapse of DRG growth cones (8). Protein concentration estimates were judged to be accurate within a factor of 3.

Nickel-purified protein from the conditioned medium of baculovirus infected Hi-5 cells was separated under non-denaturing conditions by molecular mass using the Superox-12 gel filtration column from Amersham Pharmacia Biotech and analyzed on chromatogram using A280 readings. Aliquots (0.5 µl) were collected and tested using the collapse assay.

Collapse Assay—Serial dilutions of purified protein were assayed for growth cone collapse on explanted chick embryonic day 7 DRG and embryonic day 78 sympathetic chain ganglia as described earlier (8). Briefly, explants were dissected from chick embryos and incubated at 37 °C overnight on a glass coverslip in 500 µl of F-12 medium supplemented with glucose, glycine, penicillin, streptomycin, bovine pituitary extract, insulin, selenium, transferrin, and nerve growth factor. The following day, nickel-purified protein was added to the explanted culture. After 1 h of incubation at 37 °C, the explants were fixed in 4% paraformaldehyde sucrose/PBS solution for 1 h. Glass coverslips were transferred to Petriperm dishes and visualized under magnification (>40). Growth cones were scored as either being spread or collapsed. The percentage of collapsed growth cones were then plotted against the concentration of purified protein added to the cultured explant. Collapse curves were constructed from a representative experiment. All constructs were tested at least three times, and the reported concentrations causing 50% collapse represent an average of the multiple experiments.

RESULTS

Collapsin-1 Is Produced as a Dimer—We engineered constructs missing particular domains to determine structural elements within collapsin-1 necessary for its function. The Ig domain deletion was missing amino acids 601–664, the basic tail deletion was missing amino acids 736–781, the semaphorin domain deletion was missing amino acids 30–566, and the semaphorin domain construct was missing amino acids 601–781 (for numbering of sequence, see Ref. 9). Collapsin-1, the basic deletion of collapsin-1, and the Ig deletion of collapsin-1 are all produced as covalent dimers as seen when run on a non-reducing, denaturing gel (Fig. 1, A and B). These dimers dissociate under reducing conditions, suggesting that cysteine-cysteine disulfide bonds are responsible for their dimerization. All three of these constructs collapse DRG growth cones. The semaphorin domain is not produced as a dimer when made by itself, and it does not collapse DRG growth cones (Fig. 1, A and B).

The correlation suggested that a cysteine in the carboxyl portion of collapsin-1 is responsible for covalent intermolecular disulfide bonding between pairs of collapsin monomers.

A factor complicating the analysis of the deletion constructs is that semaphorin family members are proteolytically cleaved at specific sites. Three basic regions within the molecule are cleaved by furin-dependent proteolytic processing; amino acid 565 (KRRTRR at the carboxyl terminus of the semaphorin domain), amino acid 735 (RDRKQRR at the amino terminus of the basic tail), and amino acid 761 (KKGRNRR within the basic tail) (12). This processing generates multiple bands on Western blots. When the epitope tag for purification and immunoblot identification is placed at the amino terminus of the molecule, two bands are detected: reducing gels (collapsin-1 at 100 kDa and N terminus fragment at 60 kDa) and four bands detected on non-reducing gels (collapsin-1 dimer at 200 kDa, collapsin-1 covalently attached to COOH-terminal fragment at 130 kDa, collapsin-1 monomer at 100 kDa, and NH₂-terminal fragment at 60 kDa; Fig. 1, B and C). The primary cleavage site affecting this analysis is the one at amino acid 565, at the carboxyl

FIG. 1. Dimerization of collapsin-1 and deletion constructs. A, structural schematics of the deletion constructs and the concentrations of each that induced 50% collapse (EC₅₀ pM). B, collapsin-1 and collapsin-1 deletion proteins were run on a 7.5% SDS-polyacrylamide gel under both reducing and non-reducing conditions and visualized on Western blots with a monoclonal antibody against a Myc tag incorporated into the amino terminus. The expected bands are indicated with an arrow. The other bands represent proteolytic fragments. C, putative protease cleavage site at KRRTRR just after semaphorin domain and potential proteolyzed products resulting from cleavage. Vertical line between two collapse molecules represents covalent bond. Myc tag was used for identification purposes; 6xHis tag was used for nickel column purification. An additional protease cleavage site exists just before the basic domain (small arrow).
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**DISCUSSION**

The direct comparison of collapsin-1 behavior on reducing and non-reducing SDS-PAGE indicates that collapsin-1 is produced as a covalent dimer. Mutated collapsin-1 in which the cysteine at residue 723 is changed to an alanine (C723A), a dimer should be produced. We also fused Fc to C723A, a dimer should be produced. We also fused Fc to C723A, a dimer should be produced. We also fused Fc to unmutated collapsin-1, to assure that the Fc addition does not perturb collapsing activity. Collapsin-1-Fc dimerized at the appropriate molecular weight and had an activity profile similar to collapsin-1. Unlike C723A, C723A-Fc migrated as a dimer under non-reducing conditions (Fig. 4B). C723A-Fc is at least 10 times more active than C723A and almost as potent as collapsin-1 or collapsin-1-Fc (Fig. 4C).

The Semaphorin Domain Is Active when Dimerized—The loss of all collapsing activity that results from the deletion of the semaphorin domain raised the possibility that this domain is the primary locus of collapsin-1 activity. When made by itself, however, it is produced as a monomer and is not active. Since dimerization is a requirement for activity, we tested the possibility that the semaphorin domain would be active if fused to Fc (Fig. 5A). Semaphorin domain-Fc migrates as a dimer under non-reducing conditions and collapses DRG growth cones (Fig. 5, B and C). We therefore conclude that the semaphorin domain is necessary for collapsing function. Further, it has significant collapsing activity if it is able to dimerize using the normal carboxyl-terminal portion of collapsin-1, or any other protein motif that promotes dimerization.

**A Single Point Mutation Significantly Reduces Collapsin Dimerization and Activity**—The cysteine involved in covalent disulfide binding (i) resides in the carboxyl portion of collapsin-1 and (ii) cannot be in the Ig domain or basic tail since deleting either of these two regions does not prevent covalent dimerization. There is a stretch of 71 amino acids between the Ig domain and the basic tail. This stretch was not deleted in either the Ig domain deletion or the basic tail deletion, but it was removed in the semaphorin domain construct. This stretch of amino acids (665–735) contains only one cysteine residue, located at position 723. Its conversion to an alanine (C723A) generates a product with a molecular mass identical to the 100-kDa mass of collapsin-1 under reducing conditions. How-ever, when analyzed under non-reducing conditions, the C723A mutant is produced in a monomeric instead of the normal dimeric form (Fig. 2, A and B). After comparing the activity of collapsin-1 and C723A, we determined that C723A is about 50-fold less active than collapsin-1 just after purification (Fig. 2C), and substantially less active 1 day later (data not shown).

**The Active Form of Collapsin-1 Is a Dimer**—To confirm that it is the dimeric form of collapsin-1 that is active, collapsin-1 or the C723A mutant protein were run under non-denaturing conditions on a HPLC gel filtration column. The resulting fractions were tested for activity. Both collapsin-1 and C723A eluted in fractions corresponding to dimerized and monomeric forms. Collapsin-1 primarily eluted as a dimer while only a small fraction of C723A eluted as a dimer (Fig. 3). As expected, collapsin-1 had significantly more total activity than C723A. The HPLC fractions of collapsin-1 with the most potent activity are those that contain the highest amount of dimer. The same is true for the active fractions recovered when C723A was run on the column (Fig. 3). From these observations, we conclude that most if not all of the collapsing activity from both native collapsin-1 and C723A can be attributed to their dimerized forms. It appears that a small amount of non-covalent dimerization can occur in the absence of the cysteine at residue 723.

**Substantial Activity Can Be Rescued from C723A by Reconstituting Its Dimerization**—Using PCR, we covalently fused the Fc portion of human IgG onto the carboxyl terminus of C723A. We did this to determine whether reintroducing covalent disulfide-linked dimerization could rescue C723A activity (Fig. 4A) (13). The Fc region of immunoglobulins forms homodimers as a result of two covalent disulfide bonds. Thus, with Fc fused to C723A, a dimer should be produced. We also fused Fc to unmutated collapsin-1, to assure that the Fc addition does not perturb collapsing activity. Collapsin-1-Fc dimerized at the appropriate molecular weight and had an activity profile similar to collapsin-1. Unlike C723A, C723A-Fc migrated as a dimer under non-reducing conditions (Fig. 4B). C723A-Fc is at least 10 times more active than C723A and almost as potent as collapsin-1 or collapsin-1-Fc (Fig. 4C).

![Figure 2](https://www.jbc.org/content/jbc/early/2018/07/26/jbc.2018025286/Fig2.large.jpg)

**Fig. 2.** C723A, a collapsin-1 point mutant, does not dimerize and has significantly reduced activity. A, structural schematics of collapsin-1 and the point mutation C723A. B, collapsin-1 and C723A were run on a 7.5% SDS-polyacrylamide gel under both reducing and non-reducing conditions and visualized on a Western blot with a monoclonal antibody against collapsin-1.C, dose-response curves comparing collapsin-1 and C723A activity. The percentage of collapsed DRG growth cones is plotted against the concentration of purified recombinant protein displaying on a logarithmic scale. EC_{50} (pM), the concentration of protein causing 50% collapse.
modest activity of the C723A mutant protein is associated exclusively with the non-covalently dimerized form. We infer then that the residual activity associated with C723A is a result of some non-covalent dimerization.

These findings indicate that collapsin-1 is produced as a covalent dimer whose biological activity is almost entirely lost by preventing disulfide bond formation at C723A. Because the addition of an Fc fragment to the carboxyl terminus of the mutant C723A form of collapsin-1 reconstitutes dimerization and rescues biological activity, we infer that the cysteine at residue 723 has no additional structural role beyond that of promoting dimerization.

The semaphorin domain of collapsin-1 is necessary for biological activity but not biologically active when made by itself. A likely explanation is that dimerization is necessary for activity and the key residue responsible for dimerization is located outside the semaphorin domain. This is confirmed by the observation that the semaphorin domain-Fc chimera is produced as a dimer and has collapsing activity. The semaphorin domain-Fc construct is about thirty fold less active than full length collapsin-1. This lesser potency can be attributed to the absence of the Ig and basic domains, each of which contribute 3-fold and 20-fold, respectively, to the potency of collapsin-1 (10). Their absence predicts a 60-fold reduction in potency for the semaphorin domain-Fc construct that is very close to the forty fold that is observed. The Ig and basic domains of collapsin-1 bind strongly to neuropilin, a recently identified transmembrane semaphorin family member that plays a role in lymphocyte aggregation and activation, has also been found to be produced as a homodimer (14, 15). We therefore suggest that all semaphorins are produced and function as dimers.

Since semaphorin family members homodimerize and dimerization is necessary for functional activity, the interesting possibility arises that family members may form heterodimers that have distinct biological activities. Functionally unique heterodimers would add a significant degree of complexity to the potential signaling role of the semaphorin family. Current estimates suggest that any given higher vertebrate has at least 10 unique semaphorin family members. A family of 10 molecules that can form both homo- and heterodimers could make as many as 55 unique combinations with potentially as many biological activities. A necessary minimal condition required for the formation of heterodimers is that their mRNAs have to be co-expressed in the same cell. Collapsins-1, -2, and -3 have unique but overlapping mRNA expression patterns in the chick and mouse (6, 16). It is therefore plausible that in particular cells heterodimers could form in vivo.

A precedent for the heterodimerization of signaling molecules generating a biological activity distinct from that of homodimers is provided by members of the transforming growth factor-β signaling family: inhibin and activin. Each is composed of two inhibin subunits covalently associated by disulfide bonds: inhibin α and inhibin β. Inhibin proper consists of a heterodimer between inhibin α and one of two inhibin β subunits. Activin consists of a homodimer between two inhibin β subunits. Interestingly, inhibins and activins have different biological effects on certain systems in which they play a role. In the female reproductive system, for example, inhibin suppresses secretion of follicle-stimulating hormone, whereas activin enhances follicle-stimulating hormone secretion (17–19). This is a striking example of how the dimerization of different
combinations of related subunits can generate signaling molecules with unique biological activities.

Semaphorins are signaling molecules that are active at picomolar concentrations (8). They most likely function by binding to receptors expressed on growth cones. Some of our previous data suggest that the semaphorin receptor is a complex, consisting of neuropilin, a component common to all family members, and another unknown component that mediates a particular response (9). Growth cone repulsion in response to collapsin-1 results from the reorganization of F-actin away from the filopodia containing activated receptor (20). A plausible scenario, therefore, is that collapsin-1 activates its receptor, activated receptor initiates intracellular signal transduction, and intracellular signals induce cytoskeletal rearrangement. The data from this study suggest that collapsin-1 needs to dimerize in order to bind and activate its receptor complex.

Receptor dimerization triggers signal transduction in most high affinity receptor families such as the receptor tyrosine kinases (21). What varies, however, is how ligands interact with their receptors to induce dimerization. Is a particular ligand itself a dimer or does it bind receptor as a monomer and somewhere change receptor confirmation to induce dimerization? Certain growth factors like vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and platelet-derived growth factor (PDGF) must covalently dimerize in order to bind and activate their receptors (22, 23). Others like epidermal growth factor bind and activate their receptors as a monomer (24, 25). From the data presented in this paper, it seems that collapsin-1 acts in a similar fashion to VPF/VEGF and PDGF; ligand dimerization is necessary for receptor activation. As in this study with collapsin-1 (C723A), VPF/VEGF also dimerizes to a small degree and has some residual activity when a crucial intermolecular disulfide bond cysteine is mutated (23).

One question not addressed in this study is whether collapsin-1 needs to be a dimer in order to bind its receptor. If collapsin-1 does not need to be a dimer to bind its receptor, then monomer (such as C723A) would be expected to act as a competitive inhibitor of functional dimer. In contrast, if collapsin-1 does need to dimerize to bind receptor, then excess monomer would have no effect on the functional dimer activity. In this latter case, dimerization probably helps to form the appropriate structural binding motif. Our preliminary data suggest that collapsin-1 monomer (C723A) does not compete with functional monomer.

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![Figure 4: Fusion of an Fc domain rescues C723A dimerization and activity](image)

A, structural schematics of the fusion proteins tested. The lines connecting two molecules represent covalent disulfide bonds. Note that C723A is missing this intermolecular covalent bond. B, collapsin-1, collapsin-1-Fc, C723A, and C723A-Fc were run on a 7.5% SDS-polyacrylamide gel under both reducing and non-reducing conditions and visualized on Western blots with a monoclonal antibody against collapsin-1. Arrows indicate the expected band in each lane. The lower bands represent the products of proteolytic cleavage. The higher bands in the non-reducing Fc lanes may represent higher order protein aggregate. C, collapse activity curves comparing collapsin-1, collapsin-1-Fc, C723A, and C723A-Fc. The percentage of collapsed DRG growth cones is plotted against the concentration of purified recombinant protein displayed on a logarithmic scale. EC50 (pM), the concentration of protein causing 50% collapse.

![Figure 5: Fusion of an Fc domain rescues semaphorin domain dimerization and activity](image)

A, structural schematics of the proteins tested. B, collapsin-1, semaphorin domain, and semaphorin-Fc constructs were run on a 7.5% SDS-polyacrylamide gel under both reducing and non-reducing conditions and visualized on Western blots with a monoclonal antibody against Myc. Arrows indicate the expected band in each lane. C, dose-response curves comparing the activities of collapsin-1, semaphorin (Sema), and semaphorin-Fc (Sema-Fc) constructs. The percentage of collapsed DRG growth cones is plotted against the concentration of purified recombinant protein displayed on a logarithmic scale. EC50 (pM), the concentration of protein causing 50% collapse.
dimer. Consequently, it seems that, like VEGF and PDGF (23), secreted collapsin family members bind and activate their receptors as dimers. It may be possible, then, to develop a dominant negative by engineering and overexpressing defective collapsin-1 dimerizing partner that would prevent receptor binding and activation.

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