The Chemorepulsive Activity of the Axonal Guidance Signal Semaphorin D Requires Dimerization*

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The axonal guidance signal semaphorin D is a member of a large family of proteins characterized by the presence of a highly conserved semaphorin domain of about 500 amino acids. The vertebrate semaphorins can be divided into four different classes that contain both secreted and membrane-bound proteins. Here we show that class III (SemD) and class IV semaphorins (SemB) form homodimers linked by intermolecular disulfide bridges. In addition to the 95-kDa form of SemD (SemD(95k)), proteolytic processing of SemD creates a 65-kDa isoform (SemD(65k)) that lacks the 33-kDa carboxy-terminal domain. Although SemD(95k) formed dimers, the removal of the carboxy-terminal domain resulted in the dissociation of SemD homodimers to monomeric SemD(65k). Mutation of cysteine 723, one of four conserved cysteine residues in the 33-kDa fragment, revealed its requirement both for the dimerization of SemD and its chemorepulsive activity. We suggest that dimerization is a general feature of semaphorins which depends on class-specific sequences and is important for their function.

The semaphorins are a large family of secreted and membrane-bound proteins that are involved in axonal navigation (1). To date, sequences of 15 vertebrate semaphorins have been published, and these can be divided into four classes (2–11) based on the similarity of their semaphorin domains and the presence of distinct sequence motifs in their COOH-terminal domains such as Ig homologies (classes III and IV), thrombospondin repeats (class V), and transmembrane segments (classes IV, V, and VI). The best studied vertebrate semaphorins are the murine SemD (2) and its chick ortholog collapsin-1 (3, 6, 12–15). When added to cultures of dorsal root ganglia, both induce a rapid and reversible collapse of sensory growth cones (3, 12, 15). Gradients of SemD originating from aggregates of cells transfected with an expression vector repel sensory and sympathetic axons in collag en gel co-cultures, demonstrating that semaphorins have the ability to exclude axons from regions expressing these proteins (6, 13, 15).

The secreted class III semaphorins are synthesized as preproteins that are processed proteolytically to 95- or 65-kDa isoforms (designated 95k and 65k, respectively) at several conserved dibasic cleavage sites (15). Semaphorins SemA, SemD, and SemE act as repellents for specific populations of axons, and the potency of this repulsion is regulated by proteolysis (15). Cleavage of pro-SemD at a COOH-terminal processing site generates the 95k isoform (SemD95k) and is required to activate its repulsive activity. Further cleavage of SemA, SemD, or SemE to a 65k form reduces their repulsive activities by at least 1 order of magnitude (15).

Semaphorins display specific and highly dynamic expression patterns in the developing nervous system as well as in non-neural tissues (2–6, 8, 9, 13, 14, 16–18). In vitro, specific subsets of spinal sensory afferents display a differential responsiveness to SemD which is regulated developmentally (5, 13, 14). It therefore has been proposed that SemD patterns spinal sensory innervation by dividing the spinal cord into dorso-ventrally organized subregions, one of which is accessible only to the prospective proprioceptive fibers and excludes thermo- and nociceptive axons. The phenotype of mice homozygous for an inactivated semD gene supports this hypothesis (19). In addition, it reveals functions of semD in the differentiation of other tissues such as heart and skeleton.

Although the biological effects of semaphorins have been studied in some detail, the structural requirements for their function have not been analyzed to a similar extent. Here we show that the class III and IV semaphorins form homodimers linked by intermolecular disulfide bridges. Proteolytic cleavage of dimeric SemD(95k) results in its dissociation to monomeric SemD(65k). Mutation of a single cysteine residue in SemD both prevents dimerization and abolishes its repulsive activity. We propose that dimerization is an essential step in the maturation of the chemorepulsive guidance signal SemD and may have a similar functional importance for other classes of semaphorins.

EXPERIMENTAL PROCEDURES

Expression Vectors and Transfection—To express recombinant semaphorins, cDNAs were cloned into the pBK-CMV expression vector (Stratagene). An epitope-tag (Flag: DYKDDDDK) was introduced between the signal peptide and semaphorin domain of SemD or fused to the carboxy terminus of SemB by polymerase chain reaction as described previously (15). Cysteine to alanine mutations (FlagSemDP1bC1: C567A and FlagSemDP1bC1: C723A) were introduced into the FlagSemDP1b sequence using oligonucleotides including the intended mutation (15) and verified by DNA sequencing. Amino acids Ser566–Val772 were deleted to generate FlagSemDＣ谭DCTD. To replace the COOH-terminal domain of SemD by the hinge-CH2-CH3 (Fc) region from the human G1 immunoglobulin, FlagSemDfC and FlagSemDP1bFc were constructed by replacing the sequences corresponding to amino acids Ser566–Val772 by oligonucleotides (5'-GGAACAGGTAAGTGGATCC-3') containing a splice donor. The resulting semD sequence was cloned into pBK-Fc (generously provided by S. Heller), which contains a genomic BamHI–NotI fragment encoding a splice acceptor and the Fc region (20). In SemD△CTDFlag, amino acids His657–Ala750 were replaced by DYKD-DDDKRS using a polymerase chain reaction-based strategy.
RESULTS

SemB and SemD Form Homodimers—Semaphorins are characterized by the presence of several conserved cysteine residues in both the semaphorin domain and the COOH-terminal domain which may form intra- or intermolecular disulfide bridges. To analyze this possibility we investigated the biochemical properties of two semaphorins. Recombinant SemB and SemD (Fig. 1) were expressed in HEK 293 cells, and cell lysates or concentrated conditioned media were analyzed by SDS-PAGE and Western blotting as described (15). Recombinant SemB contains a COOH-terminal domain which may form intra- or intermolecular disulfide bonds similar to other peptide sequences of this type (24). Also, mutation of Cys567 did not change the molecular mass of SemD (FlagSemD, FlagSemDP1b) and at the carboxyl terminus of SemB (SemBFlag, SemBTTCFlag). Mutation of PCS1 in FlagSemDP1b prevents processing at this position and allows the expression of SemD95k. The SemD fragment used to generate the anti-CTD (a-CTD) is indicated by a striped bar. The cytoplasmic domain and the transmembrane segment of SemB were deleted and replaced by a Flag-epitope in SemBTTTCFlag.

FIG. 1. Localization of epitopes in recombinant semaphorins. Schematic representations of SemD (panel A) and SemB (panel B) are shown with the positions of hydrophobic sequences (black: signal peptide and transmembrane segments), semaphorin domain (hatched), Ig homology domain (black), and the position of cysteine residues in the COOH-terminal domain indicated. The sites for proteolytic cleavage at the processed semaphorin sequences (PCS) are indicated by arrowheads, and the molecular masses of cleavage products as determined by SDS-PAGE are shown in kDa. A Flag-epitope (arrows) was introduced between the signal peptide and semaphorin domain of SemD (FlagSemD, FlagSemDP1b) and at the carboxyl terminus of SemB (SemBFlag, SemBTTCFlag). Mutation of PCS1 in FlagSemDP1b prevents processing at this position and allows the expression of SemD95k. The SemD fragment used to generate the anti-CTD (a-CTD) is indicated by a striped bar. The cytoplasmic domain and the transmembrane segment of SemB were deleted and replaced by a Flag-epitope in SemBTTTCFlag.

Displayed a molecular mass of 95 kDa (Fig. 2, A and B). Under reducing conditions SemBFlag, FlagSemD, and FlagSemDP1b were detected at the expected molecular masses of 94, 65, and 95 kDa, respectively (Fig. 2A, lanes 2 and 3; Fig. 2B, lanes 1 and 3). The apparent molecular mass of FlagSemD changed only slightly under non-reducing conditions (Fig. 2B, lane 2), but SemBFlag and FlagSemDP1b migrated at a molecular mass of about 190 kDa (Fig. 2A, lanes 5 and 6). This result indicates that both SemB and SemD form homodimers that are linked by one or several disulfide bonds. Because the 65-kDa isoform of SemD behaved as a monomer under non-reducing conditions, it appeared likely that the cysteine(s) responsible for dimerization of SemD reside within the COOH-terminal domain. This is supported by the observation that the COOH-terminal domain remained dimerized after proteolytic processing (Fig. 2C). Efficient dimerization of SemB depended on the presence of the transmembrane segment and/or the cytoplasmic domain, as the majority of SemBTTTCFlag migrated as a monomer of 94 kDa after deletion of its carboxyl-terminal end (amino acids His692–Ala760, Fig. 2, lanes 1 and 4).

Dimerization of SemD Requires Cysteine 723—The different behavior of FlagSemD and FlagSemDP1b upon non-reducing SDS-PAGE indicates that the COOH-terminal domain of SemD is important for dimerization. To identify the residues that form intermolecular disulfide bonds we substituted four cysteine residues in the 33-kDa SemD COOH-terminal domain fragment which are conserved among all class III semaphorins by alanine. Three of these residues lie in the COOH-terminal domain and one at the carboxyl terminus of the semaphorin domain. Mutation of cysteines 598 and 650 had no effect on dimerization (data not shown), and their location in the Ig homology domain suggests that they might form an intramolecular disulfide bond similar to other peptide sequences of this type (24). Allophanation of Cys687 did not change the molecular mass of SemD determined by SDS-PAGE under non-reducing conditions (Fig. 3, compare lanes 3 and 6). In contrast, muta-
analyzed by Western blotting using an anti-Flag antibody and lane 2. FlagSemDP1b (lanes 1 and 5), and FlagSemDP1bC2 (lanes 3 and 6) were expressed in HEK 293 cells. Concentrated conditioned media (lanes 1, 3, 4, and 6) or cell lysates (lanes 2 and 5) were separated by SDS-PAGE under reducing (+βME: 5% (v/v) β-mercaptoethanol) and non-reducing (−βME) conditions and analyzed by Western blotting using an anti-Flag antibody. Panel B, FlagSemD (lanes 1 and 2) and FlagSemDP1b (lanes 3 and 4) were expressed in HEK 293 cells. Concentrated conditioned media were separated by SDS-PAGE under reducing (+) and non-reducing (−) conditions and analyzed by Western blotting using an anti-Flag (lanes 1 and 2) or an anti-CTD antibody (lanes 3 and 4). Panel C, FlagSemD was expressed in HEK 293 cells. Concentrated conditioned media were separated by SDS-PAGE under reducing (+) and non-reducing (−) conditions and analyzed by Western blotting using an anti-CTD antibody. Molecular mass markers are indicated in kDa.

FIG. 3. Cys723 of SemD is essential for dimerization. FlagSemD (lane 1), FlagSemDP1b (lanes 2 and 5), FlagSemDP1bC1 (lanes 3 and 6), and FlagSemDP1bC2 (lanes 4 and 7) were expressed in HEK 293 cells. Concentrated conditioned media were separated by SDS-PAGE under reducing (+βME: 5% β-mercaptoethanol) and non-reducing (−βME) conditions and analyzed by Western blotting using an anti-Flag antibody. The positions of processed monomer (65 kDa), unprocessed monomer (95 kDa), and dimers (190 kDa) are indicated by arrows.

FIG. 4. Cross-linking of SemD by sulphydryl oxidizing agents. FlagSemD, FlagSemDCTD, FlagSemDP1b, and FlagSemDP1bC2 were expressed in HEK 293 cells. Concentrated conditioned media (lane 1) were incubated with 100 µM iodoacetamide (lane 2) and subsequently with 100 µM dithiothreitol (lane 3). Proteins treated in this way were incubated with increasing amounts of (o-phenanthroline)2-Cu2+ (CP; in µmol: lane 4, 0; lane 5, 1; lane 6, 5; lane 7, 10; lane 8, 50). Samples were separated by SDS-PAGE under non-reducing conditions and analyzed by Western blotting using an anti-Flag antibody. Positions of the processed monomer (65 kDa), unprocessed monomer (95 kDa), and dimers (190 kDa) are indicated by arrows.

The size of the different recombinant proteins was determined by gel filtration chromatography, to verify the molecular mass observed by SDS-PAGE under non-reducing conditions (Fig. 5). The fractions were analyzed by SDS-PAGE and Western blotting using an anti-Flag antibody, and the protein concentration of the peak fractions was measured (Fig. 5A and data not shown). FlagSemD was processed to the 65-kDa form and migrated with an apparent molecular mass of 70 kDa, consistent with the expected size for an SemD monomer. The same fractions were analyzed for the presence of the COOH-terminal domain by using the anti-CTD antiserum raised against a fragment of the SemD COOH-terminal domain. In Western blots this antibody revealed a protein that migrated at a molecular mass of approximately 50 kDa, which probably corresponds to a dimer of the 33-kDa COOH-terminal domain (Fig. 5B). SDS-PAGE under non-reducing conditions confirmed that the COOH-terminal domain existed as a disulfide bond-linked dimer (data not shown). When the semaphorin domain was expressed without the COOH-terminal domain...
(FlagSemD) it migrated at a size similar to that of FlagSemD.

The molecular mass of native FlagSemDP1b as determined by gel filtration chromatography was 190 kDa, consistent with the expected size for homodimeric SemD(95k). Little immunoreactivity was detected at higher molecular masses (data not shown), indicating that no additional oligomeric complexes were formed and that the SemD(95k) homodimer represents the majority of recombinant protein secreted by HEK 293 cells. Introduction of the C723A mutation into FlagSemDP1b (FlagSemDP1bC2) prevented dimerization as indicated by a reduction of the molecular mass to approximately 100 kDa. These experiments confirm that Cys723 in the COOH-terminal domain is essential for dimerization of SemD and that processing at PCS1 results not only in the removal of the COOH-terminal domain but also in the conversion of SemD to a monomeric form. These results also revealed that SemD(65k) and the dimerized COOH-terminal domain do not appear to associate as independent entities and elute at a molecular mass of 70 and 50 kDa, respectively.

Thus, Cys723 appears to be essential for the repulsive activity of SemD.

The COOH-terminal domain of class III semaphorins might function exclusively by promoting the formation of SemD dimers, or it may have additional functions. To distinguish between these possibilities, the COOH-terminal domain was replaced by the constant part of the human \( \gamma \)-immunoglobulin, and the activity of the resulting hybrid protein was analyzed in a co-culture assay. These chimeric proteins displayed almost no detectable repulsive activity irrespective of processing at PCS1 (Fig. 6C). Similarly, FlagSemD(CTD) did not show any repulsive effects despite a significantly higher expression compared with that of the other tested SemD variants (Fig. 6B; note that five times less medium was loaded in lane 4). Western blot analysis confirmed that with the exception of FlagSemD(CTD) all three proteins were expressed at comparable levels (Fig. 6B).

DISCUSSION

The function of semaphorins as chemorepulsive axonal guidance signals has been analyzed in some detail (1, 3, 5–7, 12–15, 19, 25). However, the structural requirements for the activities of these proteins are still poorly understood. Here, we present evidence that two distinct semaphorins, the membrane protein SemB and the secreted SemD, form homodimers linked by intermolecular disulfide bonds when expressed in HEK 293 cells. Upon SDS-PAGE under non-reducing conditions, SemD(65k) and SemD(95k) differ in their dimerization behavior. FlagSemD, FlagSemD(CTD), FlagSemDP1b, and FlagSemDP1bC2 were expressed in HEK 293 cells and subjected to gel filtration chromatography. The collected fractions were analyzed by Western blotting and the protein concentration determined by spectrophotometry at 280 nm. The results were plotted as percent protein concentration of the peak fraction. The molecular masses corresponding to the peaks of concentration are indicated by arrowheads. Panel B, fractions from the gel filtration chromatography of FlagSemD were separated by SDS-PAGE under reducing conditions and analyzed by Western blotting using an anti-Flag antibody (\( \alpha \)-Flag) or an anti-CTD antibody (\( \alpha \)-CTD; Ref. 15). The peaks of immunoreactivity are indicated by black arrowheads, and the corresponding molecular masses are in kDa. White arrowheads show the molecular masses of FlagSemDP1b and FlagSemDP1bC2 for comparison. The proteolytic fragments of SemD processing, semaphorin domain and the COOH-terminal domain behave as independent entities and elute at a molecular mass of 70 and 50 kDa, respectively.
Fig. 6. Cys723 is essential for the repulsive activity of SemD. Panel A, HEK 293 cells were transfected with expression vectors for FlagSemD (SemD), FlagSemDP1bC1 (P1bC1), and FlagSemDP1bC2 (P1bC2). Sympathetic ganglia were explanted from chick embryos at 9 days of incubation and cultured in a collagen matrix with 5 ng/ml nerve growth factor at a distance of 500 μm with aggregated cells and analyzed after 48 h of culture. The relative repulsive activity (rra) displayed in co-culture assays was quantified as described previously (15). An rra = 100 was assigned to FlagSemD. Means ± S.E. of the rra values for the mutant SemD proteins are shown: FlagSemDP1bC1, 14.6 ± 5.4 (n = 8); FlagSemDP1bC2, 1.2 ± 0.2 (n = 8). Western blot analysis confirmed that similar amounts of the different proteins were expressed after transfection of HEK 293 cells (Fig. 3 and data not shown). Panel B, FlagSemD (lane 1), FlagSemDFc (lane 2), FlagSemDP1bFc (lane 3), and FlagSemDCTD (lane 4; five times less medium was loaded than in lanes 1–3) were expressed in HEK 293 cells. Concentrated conditioned media were separated by SDS-PAGE and analyzed by Western blotting using an anti-Flag antibody. Similar amounts of all four proteins were detected with the exception of FlagSemDCTD. Panel C, HEK 293 cells were transfected with expression vectors for FlagSemD (SemD), FlagSemDFc (SemDFc), FlagSemDP1bFc (SemDP1bFc), and FlagSemDCTD (SemDCTD). Aggregated cells were cultured in a collagen matrix with sympathetic ganglia explanted from chick embryos at 9 days of incubation. Micrographs were taken after 48 h of culture (n = 6).

190 kDa. Although semaphorins SemD and SemB both form dimers, the specific residues involved appear to be located at different positions. Dimerization of SemD depends on its COOH-terminal domain and is abolished by mutagenesis of Cys723. Cysteine residues are found at a similar position in other class III semaphorins, and it is likely that these will also form dimers. In contrast, SemB does not contain a cysteine residue at a corresponding position. Deletion of the putative membrane-spanning segment and cytoplasmic domain reduced the amount of SemB dimerization dramatically. Therefore, intermolecular interactions dependent on these sequences might precede the formation of disulfide bonds. Thus, although dimerization may be characteristic for semaphorins, the sequences responsible for it could be class-specific.

Mutational analysis of SemD revealed an important role of Cys723 not only in dimerization but also in its chemorepulsive activity. When analyzed in a co-culture assay, mutation C723A (FlagSemDP1bC2) almost completely abolished the repulsion of sympathetic axons. The inactivity of this mutant therefore is likely caused by its inability to form dimers as loss of both activities coincides. Thus, dimerization appears to be a prerequisite for SemD to display its repulsive activity. The function of Cys567 is less clear. Its mutation also resulted in a reduction in repulsive activity, and this residue may therefore be important for proper folding of SemD.

Previously, we have reported that proteolytic processing of SemD at PCS1 to the 65-kDa form reduces its repulsive activity (15). Here we show that cleavage not only removes the COOH-terminal domain but also results in dissociation of SemD dimers. Determination of the molecular mass of SemD by gel filtration chromatography showed that SemD(65k) behaves as a monomer and does not remain associated with the dimerized COOH-terminal domain. Thus, dissociation of SemD homodimers might explain the reduced activity of SemD(65k) compared with SemD(95k). However, monomeric SemD(65k) is still significantly more active than FlagSemDCTD which is equivalent in sequence to the 65-kDa fragment of FlagSemD. The COOH-terminal domain may be required as a co-factor in addition to the semaphorin domain to activate putative SemD receptors or, alternatively, to promote the adoption of an active conformation. Artificial dimerization of the semaphorin domain by replacing its COOH-terminal domain with the constant part of human IgG1 did not result in active SemD and thus cannot substitute for the effects mediated by the COOH-terminal domain. In this respect semaphorins display a behavior different from that of another group of axonal guidance molecules, the ephrins, which are activated by fusion to an Fc fragment (27, 28). In contrast, replacement of the SemD COOH-terminal domain by that of other class III semaphorins allows the synthesis of at least partially active SemD.2 Our results show that dimerization mediated by the COOH-terminal domain is necessary but not sufficient for the formation of active SemD (15). In addition, processing of SemD at the carboxyl-terminal PCS3 and PCS4 is essential for producing active SemD (15). Because FlagSemDP1bC2 is as inactive as unprocessed SemD it appears possible that dimerization may be required for correct processing of pro-SemD to its active form. For example, dimerization could be required for recognition of PCS3/4 by the processing enzyme(s).

In summary, dimerization of SemD appears to be an essential part in its maturation process. Formation of dimeric molecules is not restricted to the class III semaphorins but can be found in at least one other class of these proteins. Although it may be a general characteristic of this family, different classes of semaphorins probably depend on different molecular mechanisms to accomplish it.

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