The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing

Ralf H.Adams¹, Marion Lohrum,
Andreas Klostermann, Heinrich Betz and
Andreas W.Püschel²

Abteilung Neurochemie, Max-Planck-Institut für Hirnforschung,
Deutschordenstr. 46, D-60528 Frankfurt/Main, Germany

¹Present address: EMBL, Meyerhofstr. 1, D-69117 Heidelberg,
Germany

²Corresponding author

R.H.Adams and M.Lohrum contributed equally to this work

The semaphorins are a large group of cell surface and secreted proteins implicated in axonal pathfinding. Here we show that the secreted mouse semaphorin D (SemD) is synthesized as an inactive precursor (proSemD) and becomes repulsive for sensory and sympathetic neurites upon proteolytic cleavage. ProSemD processing can be blocked completely by an inhibitor selective for furin-like endoproteases or mutagenesis of three conserved dibasic cleavage sites. Its C-terminal pro-peptide contains a processing signal that is essential for SemD to acquire its full repulsive activity. SemD processing is regulated during the embryonic development of the mouse and determines the magnitude of its repulsive activity. Similarly to SemD, the secreted semaphorins SemA and SemE display repulsive properties that are regulated by processing. Our data suggest that differential proteolytic processing determines the repulsive potency of secreted semaphorins and implicate proteolysis as an important regulatory mechanism in axonal pathfinding.

Keywords: axon guidance/collapsin/convertase/repulsion

Introduction

Most biologically active peptides, such as hormones, growth factors or neuropeptides are released from inactive precursors by endoproteolytic processing. Several different convertases have been identified that catalyse this maturation of pro-proteins and can be distinguished by the consensus sequences which they cleave (Barr, 1991; Steiner *et al.*, 1992; Loh, 1993). The best characterized class of convertases is a family of proteins with homology to the yeast Kex2 and the bacterial subtilisin serine proteases that cleave C-terminally to a dibasic motif (Barr, 1991; Hosaka *et al.*, 1991; Steiner *et al.*, 1992). This family includes endoproteases active both in the constitutive and the regulated secretory pathways such as furin and PC3, respectively.

Recently, a large family of proteins, the semaphorins (Kolodkin *et al.*, 1993; Luo *et al.*, 1993, 1995; Püschel *et al.*, 1995; Adams *et al.*, 1996; Püschel, 1996), has been

described which are thought to regulate axonal pathfinding in the developing nervous system of vertebrates and invertebrates (Kolodkin *et al.*, 1992; Luo *et al.*, 1993; Matthes *et al.*, 1995; Püschel *et al.*, 1996; Tessier-Lavigne and Goodman, 1996). This family is characterized by a highly conserved semaphorin domain of ~500 amino acids and contains both secreted and membrane-bound members. It can be subdivided into at least five different classes (Püschel *et al.*, 1995; Adams *et al.*, 1996; Püschel, 1996) based on the similarity of their semaphorin domain sequences and the presence of class-specific C-terminal domains (CTDs).

The best characterized representatives of this class are semaphorin D (referred to here as SemD) and its chick orthologue collapsin (Coll-1), which displays a strong repulsive activity on sympathetic, sensory and motor neurons (Luo *et al.*, 1993; Messersmith *et al.*, 1995; Püschel *et al.*, 1995, 1996; Shepherd *et al.*, 1996; Varela-Echavarría *et al.*, 1997). When applied to sensory growth cones, it induces a rapid and reversible collapse which is accompanied by a redistribution of F-actin (Fan *et al.*, 1993). The developmentally regulated differential sensitivity of distinct subtypes of sensory afferents to SemD is thought to be critical for patterning their termination in different laminae of the spinal cord (Messersmith *et al.*, 1995; Püschel *et al.*, 1996; Shepherd *et al.*, 1997). Inactivation of the mouse *semD/semAIII* gene results in defects in the sensory innervation of the spinal cord and abnormal cortical development (Behar *et al.*, 1996).

Here we show that SemD is synthesized as a pro-protein (proSemD) that requires proteolytic processing at clusters of basic residues to become repulsive for neurites. Two of these processing sequences are highly conserved in all known class III semaphorins, and function as cleavage sites for furin or related endoproteolytic pro-protein convertases. Cleavage of proSemD is essential, but not sufficient for the acquisition of high repulsive activity. Our data indicate that the C-terminal pro-peptide directs a reaction linked to proteolysis that is necessary to realize the full repulsive potency of SemD.

Results

Class III semaphorins are synthesized as pro-proteins

Three clusters of basic amino acid residues were identified in the secreted class III semaphorin SemD that contain the consensus recognition sequence RXK/RR (single letter code; Figure 1) characteristic for the dibasic endoprotease furin (Barr, 1991; Hosaka *et al.*, 1991; Steiner *et al.*, 1992). Two of these consensus sequences (PCS1, R551–R555 and PCS3, R758–R761) are conserved in all class III semaphorins published to date (Figure 1), while a third (PCS2, 731–733) is present only in SemD and its chicken

Fig. 1. Predicted processing consensus sites are conserved in class III semaphorins. A schematic representation of SemD is shown, with the positions of the signal sequence (black), semaphorin domain (hatched), Ig homology (grey) and putative processing sites (arrowheads) indicated. The sequences of three clusters of basic residues found in SemD (SD, accession No. X85993) are aligned with the corresponding sequences of SemA (SA, X85990), SemE (SE, X85994), collapsin-1 (C1, G410078), collapsin-2 (C2, U28240) and semaphorin IV (S4, U33920). All consensus sequences derived from this alignment contain recognition sites for the endoprotease furin (K/RXRR). The predicted cleavage sites after the Processing Consensus Sequences (PCS) are indicated by arrowheads, and the theoretical molecular weights of cleavage products are shown in kDa. The sequence of PCS1 and 3 is conserved in all class III semaphorins. No evidence was found for cleavage at PCS2 (open arrowhead), a site unique to SemD and Coll-1.

orthologue Coll-1. Complete cleavage of SemD by furin (at position +1 C-terminally to the C-terminal arginine; Barr, 1991; Loh, 1993) would result in the generation of peptides with theoretical mol. wts of 60.5, 21.3, 3.1 and 1.3 kDa. In order to demonstrate the proteolytic processing of class III semaphorins, three variants of SemD were engineered that contained epitope tags introduced into different positions of the coding sequence to detect specifically the individual cleavage products (Figure 2A). The recombinant proteins were expressed in 293 cells and analysed by Western blotting. All three epitope-tagged proteins retained full biological activity, as shown by their repulsive effects on sensory neurites (Figure 5, and data not shown). A protein of ~65 kDa was detected in conditioned media from cells expressing the FlagSemD protein (Figure 2B, lane 1), whereas a theoretical mol. wt of 86.2 kDa would be expected for full-length SemD. A 33 kDa protein was secreted by cells transfected with an expression vector for SemDmyc (Figure 2B, lane 2). The coding sequence of the secreted alkaline phosphatase (AP; Flanagan and Leder, 1990) was fused to the C-terminus of SemD to allow detection of the postulated 1.3 kDa cleavage product. Expression of SemD-AP resulted in the production of a 68 kDa protein (Figure 2B, lanes 4, 6 and 8) corresponding to a fusion of the 1.3 kDa peptide with AP. Upon longer exposure, additional bands at 105 and 170 kDa became apparent which probably represent partially cleaved and uncleaved proteins (Figure 2B, lanes 4 and 6). The presence of the AP-tag in the 68 kDa protein was confirmed by Western blotting with an antibody specific for AP (Figure 2B, lanes 5 and 6). The apparent molecular weights of the SemD peptides detected in Western blots are considerably smaller than expected for full-length protein and are consistent with a proteolytic cleavage at the predicted processing sites PCS1 and 3. Similar results were obtained when the recombinant pro-

teins were expressed in a neuronal cell line (data not shown). Thus, SemD is synthesized as a precursor protein (proSemD) which is proteolytically processed to three peptides with mol. wts of 65, 33 and 1 kDa. No evidence was found for a cleavage of SemD at PCS2 in 293 cells (data not shown). This sequence may not be accessible in the mature protein or may require a protease not present in 293 cells.

To show that the proteolytic cleavage of SemD is characteristic for class III semaphorins, C-terminal fusion proteins with an AP tag were constructed for SemA (SemA-AP) and SemE (SemE-AP) and expressed in 293 cells. Western blot analyses of SemA-AP and SemE-AP detected proteins at 68 kDa corresponding to the short C-terminal fragment fused to AP as described for SemD-AP (Figure 2B, lanes 7 and 9). The identity of the utilized processing sites was confirmed further by mutational analysis (see below).

Proteolytic processing can be suppressed by an inhibitor selective for furin

In order to confirm that furin or a furin-like endoprotease is responsible for the proteolytic processing of SemD, N- or C-terminally tagged recombinant SemD proteins were expressed in 293 cells incubated with decanoyl-RVKR-chloromethylketone, a protease inhibitor that selectively blocks furin-like endoproteases (Vey, 1995). In the presence of inhibitor, bands corresponding to mol. wts of 95 and 170 kDa were detected in Western blots, which represent full-length FlagSemD and SemD-AP, respectively, whereas in its absence SemD was processed completely (Figure 3B). Thus, the inhibitor completely suppressed processing of SemD in 293 cells, demonstrating that furin or a related enzyme is responsible for the maturation of SemD in these cells.

Fig. 2. SemD is proteolytically cleaved into three fragments. (A) A schematic representation of SemD is shown, with putative processing sites indicated by black arrowheads. Epitope tags were introduced into the *semD* coding sequence at positions indicated by arrows. A Flag epitope (FlagSemD) was introduced between the signal peptide (black) and the semaphorin domain (light grey), and a myc tag (SemDmyc) between the semaphorin domain and the Ig homology domain (dark grey). In order to visualize the 1.3 kDa peptide, SemD was C-terminally fused to the coding sequence of a Flag-tagged secreted alkaline phosphatase (Flag-AP: SemD-AP; the theoretical molecular weight of AP is 55 kDa; Flanagan and Leder, 1990). The theoretical molecular weights of the cleavage products of SemD are indicated in kDa. (B) Epitope-tagged proteins (lane 1, FlagSemD; lane 2, SemDmyc; lanes 3 and 5, AP; lanes 4 and 6, SemD-AP) were expressed in 293 cells and analysed by Western blotting with anti-Flag M2 (lanes 1, 3, 4 and 7–9), anti-myc 9E10 (lane 2) or anti-AP (lanes 5 and 6) monoclonal antibodies. A 65 kDa protein was detected when SemD was N-terminally tagged, whereas a 33 kDa protein was observed when probing for the myc epitope. A major product of 68 kDa was revealed for the C-terminally tagged SemD, corresponding to a fusion of the last 11 amino acids to the Flag-tagged AP. The presence of the AP coding sequence in this peptide was confirmed by an antibody specific for AP (compare lanes 4 and 6). Note that lanes 3–6 were exposed ~5 times longer than lanes 1 and 2 to visualize partial cleavage products. 293 cells were transfected with expression vectors for SemA-AP (lane 7), SemD-AP (lane 8) or SemE-AP (lane 9) and concentrated conditioned media analysed after 48 h by Western blot using an anti-Flag antibody. In all cases, a 68 kDa protein was detected, indicating complete C-terminal processing. The sizes of molecular weight markers are indicated in kDa.

SemD processing is developmentally regulated

The purification of Coll-1 with different molecular weights from embryonic and adult chick brain membranes (Raper and Kapfhammer, 1990; Luo *et al.*, 1993) suggested that a processing similar to the one seen in 293 cells may also occur *in vivo*. In order to demonstrate processing of SemD during mouse embryogenesis, a polyclonal antiserum was raised against a bacterially expressed fragment from the CTD of SemD (Figure 3A). The affinity-purified antiserum was specific for SemD and recognized the 33 kDa CTD of recombinant SemD (Figure 3C, lane 1), but did not cross-react with SemA or SemE (data not shown). In Western blots of membrane preparations from different embryonic stages, the antibody detected two bands corresponding to mol. wts of 125 and 40 kDa, respectively (Figure 3C, lanes 2–4). The 125 kDa protein probably corresponds to proSemD or partially processed SemD. The higher molecular weight of native SemD as compared with the recombinant protein generated in 293 cells may reflect differences in post-translational modification such as glycosylation at conserved consensus sites (Püschel *et al.*, 1995), and corresponds in size to a protein detected

Fig. 3. SemD processing is suppressed by an inhibitor selective for furin-like proteases and is regulated during mouse embryogenesis. (A) A schematic representation of SemD is shown, with putative processing sites indicated by black arrowheads. The theoretical molecular weights of cleaved and uncleaved SemD fusion proteins as detectable with Flag or AP tags are indicated in kDa. Values in parenthesis correspond to the molecular weight of SemD cleavage products. The peptide used to generate an antiserum against the CTD (anti-CTD) is indicated by a black bar. (B) 293 cells were transfected with expression vectors for FlagSemD (lanes 1 and 2) or SemD-AP (lanes 3 and 4) and incubated for 48 h with vehicle alone (lanes 1 and 3) or decRVKRCmk (ful), a selective inhibitor of furin-like proteases (lanes 2 and 4). Concentrated conditioned media were analysed by Western blot using an anti-Flag antibody. (C) FlagSemD was expressed in 293 cells and analysed by Western blotting with the anti-CTD antiserum (lane 1) which revealed an immunoreactive protein of 33 kDa. In Western blots of membrane preparations from E12.5 (lane 2), E14.5 (lane 3) and E15.5 whole embryos (lane 4), the anti-CTD antiserum detected two proteins of 125 and 40 kDa. The sizes of molecular weight markers are indicated in kDa.

by an anti-Coll-1 antiserum in chick brain (Shepherd *et al.*, 1997). The 40 kDa peptide probably represents the fragment generated by cleavage of PCS1. Processing was developmentally regulated as the amounts of the 40 kDa peptide increased with development from very low levels at E12.5 to a strong signal at E15.5 (Figure 3C, lanes 2–4), a period of development when dramatic changes in the expression of *semD* also occur (Püschel *et al.*, 1996).

Mutational analysis of SemD processing sites

To confirm that processing of SemD occurs at the predicted sites, mutations in these sequences were introduced into N-terminally (for PCS1: FlagSemD) or C-terminally (PCS3: SemD-AP) epitope-tagged SemD (Figure 4A). Mutation of a single arginine in PCS1 (R555A: FlagSemDP1a) had no effect on the processing of SemD to the 65 kDa product (Figure 4B, and data not shown), which may reflect the presence of two independent and overlapping dibasic sites in the conserved KRRTRR motif. When the R555A substitution was combined with R551A (FlagSemDP1b) or R552A (FlagSemDP1c), cleavage of PCS1 was prevented completely, and a 95 kDa SemD protein was secreted from transfected cells (Figure 4B, lanes 3 and 4). Introduction of the corresponding mutation (R758,761A) into PCS3, however, had no effect on

Fig. 4. Mutation of the PCSs prevents processing. (A) A schematic representation of SemD is shown, with putative cleavage sites indicated by black arrowheads in the partial SemD sequences (SD: A549–D557 and K755–V772). Mutations were introduced at PCS1 into the N-terminally tagged FlagSemD (P1a–c) and at PCS3 (P3) into the C-terminally tagged SemD–AP. R→A substitutions in two C-terminal arginine residues revealed the presence of an additional cleavage site (PCS4: P4a, P4b, P34a and P34b). (B) 293 cells were transfected with expression vectors for FlagSemD (lane 1), mutated FlagSemD (lanes 2–4), SemD–AP (lane 5) or mutated SemD–AP (lanes 6–9) as indicated. Conditioned media were collected after 48 h, concentrated and analysed by Western blotting using the M2 anti-Flag antibody. Mutation of two (P1b and P1c), but not one (P1a), arginine residues inactivated PCS1. At the C-terminal end, all of SemD–AP (lane 5) and >95% of SemDP3–AP (lane 6) were processed. In contrast, mutation of PCS3 combined with substitutions in the motif RXXR (PCS4) prevented processing at the C-terminus (P34a and P34b). Note the slightly increased molecular weight of the P4a–AP fragment (lane 7) when PCS4 is no longer cleaved. Mutation P34b completely prevented processing, whereas P34a still allowed a small but detectable amount of cleavage (compare lanes 8 and 9). The sizes of molecular weight markers are indicated in kDa.

processing (data not shown). Replacement of all three arginines in PCS3 (SemDP3–AP: R758,760,761A; Figure 4A) resulted only in minor amounts of uncleaved SemD–AP fusion protein at 105 kDa (Figure 4B, lane 6). This could indicate that the C-terminal end of SemD is processed by a different type of protease at a site other than PCS3, or that additional processing sites are present in the C-terminus. Two additional substitutions (R767A and R770A) that were introduced downstream from PCS3 into an RXXR motif identified a fourth processing site (PCS4) in SemD (Figure 4A). Mutation of R767 in SemDP4a–AP resulted in a fusion protein of slightly increased molecular weight (Figure 4B, lane 7), probably consisting of the AP and the C-terminal 11 amino acids of SemD. C-terminal processing was almost completely suppressed when the mutations in PCS3 and PCS4 were combined (P34a or P34b; Figure 4B, lanes 8 and 9). Analysis of the combined mutations also revealed that cleavage of any single PCS did not influence processing at the other two sites to a significant extent (Figure 4B, and data not shown). Thus, the three sites used for proteolytic maturation can be cleaved independently.

Processing regulates the repulsive activity of SemD

In order to address the functional significance of proteolytic processing, the repulsive activity of SemD proteins con-

taining mutations in one or several processing sites was quantified in a co-culture of aggregates of transfected 293 cells with sympathetic ganglia (SG) from chick embryos at 9 days of incubation (E9). Explants of dorsal root ganglia (DRG) from mouse embryos at 12.5 days of gestation (E12.5) gave similar results (data not shown). The activity of mutant proteins was expressed as relative repulsive activity (r.r.a.) in comparison with the activity of completely processed FlagSemD (r.r.a. = 100) determined in parallel (for details, see Materials and methods). Similar amounts of the various mutant proteins were expressed by transfected 293 cells as shown by Western blotting (Figure 5B).

Inactivation of single PCSs revealed considerable functional differences between the three sites. Whereas the repulsive effects of FlagSemDP3, FlagSemDP4a or FlagSemDP4b were indistinguishable from those of FlagSemD (data not shown), the mutation of PCS1 in SemDP1b resulted in a 6- to 10-fold increase of its r.r.a. (Figure 5A and C). Therefore, processing of the 95 kDa form of SemD at PCS1 appears to reduce its activity and may indicate the presence of an important functional determinant in the CTD. The consequences of combining two PCS mutations confirmed the non-equivalence of the individual sites. Aggregates expressing FlagSemDP1b3 with mutations in both PCS1 (P1b) and PCS3 (P3) displayed an increased repulsion relative to those producing FlagSemDP1b (Figure 5A and C). In contrast, when mutations in PCS1 (P1b) and PCS4 (P4b) were combined (FlagSemDP1b4b), the activity of the resulting protein was greatly reduced.

The dominant influence of PCS4 was clearly demonstrated by analysing FlagSemDP34a and FlagSemDP34b. Although still showing some weak repulsion (Figure 5A), their r.r.a. values were barely above those of unprocessed SemD (Figure 5C). The combination of mutations in FlagSemDP1b34a or FlagSemDP1b34b resulted in almost complete abolition of their repulsive activities (Figure 5B) to an r.r.a. of ~2 for both sensory and sympathetic neurites (Figure 5A and C; and data not shown). Western blotting confirmed that comparable amounts of the different mutant proteins were secreted by transfected 293 cells (Figure 5B).

To exclude an effect of the mutations on the formation of a SemD gradient in the collagen gel, 293 cells were transfected with expression vectors for SemD or selected mutants, and the growth cone-collapsing activity of the conditioned medium was measured (Figure 5D). This analysis confirmed the results obtained in the co-culture assays. FlagSemDP1b3 displayed a marked increase in collapsing activity compared with FlagSemD, whereas FlagSemDP1b34a showed a significant reduction in its activity to levels close to the background control of medium from mock-transfected cells. Both assays produced similar relative values for FlagSemDP1b3 and FlagSemDP1b34a, and demonstrated that a single R→A substitution in PCS4 decreased the protein's activity 500-fold. Thus, cleavage at PCS4 is required to generate a maximally active protein from unprocessed proSemD which has a very low repulsive and collapsing activity.

SemA and E are repulsive molecules regulated by proteolytic processing

The high conservation of the consensus sequences for furin-dependent processing in all class III semaphorins

Fig. 5. Processing regulates the repulsive activity of SemD. (A) 293 cells were transfected with expression vectors for FlagSemD (SemD), FlagSemDP1b (P1b), FlagSemDP1b3 (P1b3), FlagSemDP34a (P34a), FlagSemDP1b34a (P1b34a) and FlagSemDP1b34b (P1b34b). Sympathetic ganglia were explanted from E9 chick embryos and cultured in a collagen matrix with 5 ng/ml NGF at a distance of 500 μ m with aggregated cells. Micrographs were taken after 48 h of culture. (B) 293 cells were transfected with expression vectors for the indicated proteins and concentrated media analysed after 48 h by Western blot using an anti-Flag antibody. Similar amounts of the various proteins were expressed. (C) The relative repulsive activity (r.r.a.) displayed in co-culture assays was quantified as described in Materials and methods. For SemD mutants that displayed an activity higher than that of FlagSemD under standard conditions, transfections were adjusted to decrease the amount of secreted protein and r.r.a. values calculated based on the amount of protein as detected by Western blotting. An r.r.a. = 100 was assigned to FlagSemD. Means $\pm$ SEM of the r.r.a. values for the mutant SemD proteins are shown: FlagSemDP1b, 625 ± 116 ($n = 3$); FlagSemDP1b3, 1294 ± 235 ($n = 4$); FlagSemDP1b4b, 59 ± 9 ($n = 3$); FlagSemDP34a, 3.7 ± 1.5 ($n = 3$); FlagSemDP34b, 2.6 ± 1.5 ($n = 3$); FlagSemDP1b34a, 2.3 ± 0.3 ($n = 5$); and FlagSemDP1b34b, 1.8 ± 0.2 ($n = 3$). (D) E12.5 DRG were explanted from mouse embryos and cultured overnight in the presence of 5 ng/ml NGF to allow extension of neurites. The relative growth cone-collapsing activity (r.c.a.) of FlagSemD1b, FlagSemDP134a and medium from mock-transfected cells was determined by adding to these sensory growth cones conditioned media from 293 cells transfected with the respective expression vectors. An r.c.a. of 100 was assigned to FlagSemD. A total of 200–300 growth cones were evaluated per experiment. Means $\pm$ SEM of the r.c.a. values for the mutant SemD proteins are shown: FlagSemDP1b3, 1544 ± 225 ($n = 4$); FlagSemDP1b34a, 26 ± 4 ($n = 5$); and mock-transfected medium, 23 ± 3 ($n = 5$).

suggests a similar mechanism of regulation for all secreted semaphorins. In a search for potential targets of Sema and E, we observed a weak but reproducible repulsive effect of SemaA and a stronger one of SemaE on E7 SGs (Figure 6A and C). When the sequences corresponding to PCS1 were mutated in SemaA and SemaE, a significant increase in their repulsive activities was observed (Figure 6B and D). As also found for SemD, the mutations in PCS1 did not cause increased secretion of the mutant proteins (data not shown). Thus, as demonstrated for SemD, the repulsive activity of all secreted class III semaphorins tested here depends on the extent of their proteolytic processing.

The pro-peptide contains a signal required for SemD activation

Two mechanisms might explain the requirement for processing at the C-terminal end of SemD. The 11 C-terminal residues could exert an auto-inhibitory influence on the SemD protein. Alternatively, processing itself or a reaction coupled to it could enhance the repulsive activity of SemD. Precedents exist for both models (see Discussion). To distinguish between these possibilities, a recombinant protein (FlagSemDP1b Δ 11) was constructed that corresponds to the predicted product of processing FlagSemDP1b at PCS3 (Figure 7A). Deletion of the C-terminal 11 amino acids from FlagSemDP1b resulted in a protein that was

Fig. 6. Mutation of PCS1 increases the repulsive activity of SemA and SemE. 293 cells were transfected with expression vectors for SemA (A), SemAP1b (B), SemE (C) and SemEP1b (D). Sympathetic ganglia were explanted from E7 chick embryos and cultured in a collagen matrix with 5 ng/ml NGF with aggregated cells. Micrographs were taken after 48 h of culture ($n = 3$, 4–8 explants/experiment). Mutation of PCS1 in SemA (KRRFRR→KRAFRA, positions 549–553) and SemE (KRRFRR→KARSRA, positions 551–555) increased their repulsive activity.

as inactive as FlagSemDP1b34a (Figure 7C). In contrast, replacing the 11 amino acids by a Flag epitope (FlagSemDP1bΔ10F) restored some of the activity, without, however, reaching the level of FlagSemD (Figure 7A and C). Similarly, when the terminal 14 amino acids including PCS3 were replaced by an unrelated sequence containing a dibasic motif (Figure 7A and C: FlagSemDP1bΔ14R), the potency of the resulting mutant protein was partially restored. These results demonstrate that processing itself, possibly coupled to an additional reaction, and not removal of an auto-inhibitory peptide, is important. This activation of SemD appears to be dependent on the sequences surrounding the actual cleavage site since the artificial C-terminal ends used here did not fully support activation of proSemD.

In order to verify further the role of C-terminal cleavage in SemD activation, sequences containing PCS3 or PCS4 were added back to the C-terminal end of the inactive FlagSemDP1b34a or FlagSemDP1b34b constructs (Figure 7E). Introduction of PCS4 but not PCS3 could substantially rescue the repulsive activity of non-processed SemD mutants but did not allow a full recovery of repulsive potency (Figure 7D). These results confirm the essential function of PCS4 for SemD activity and demonstrate the presence of a signal in the pro-peptide which is responsible for the formation of its repulsive potency.

Discussion

SemD is processed by a furin-like endoprotease

Several lines of evidence demonstrate that the axonal guidance molecule SemD is synthesized as a pro-protein that undergoes proteolytic processing dependent on a furin-like endoprotease. Heterologous expression of SemD resulted in the secretion of three products whose sizes corresponded to those expected from cleavage at the conserved dibasic consensus sites. This processing was prevented by an inhibitor selective for furin-like serine

Fig. 7. Activation of the repulsive activity of SemD requires processing of PCS4. (A) C-terminal sequences introduced into FlagSemDP1b are shown. (B) Western blotting of conditioned media using the M2 antibody confirmed that comparable amounts of the various proteins were synthesized by 293 cells. (C) 293 cells were transfected with expression vectors for the indicated C-terminally mutated proteins and cell aggregates were cultured in a collagen matrix together with sympathetic ganglia explanted from E9 chick embryos in the presence of 5 ng/ml NGF. The r.r.a. of the mutant proteins were determined after 48 h (means of r.r.a. $\pm$ SEM): FlagSemDP1bΔ11, 2 ± 0.2 ($n = 4$); FlagSemDP1bΔ10F, 61 ± 9 ($n = 3$); and FlagSemDP1bΔ14R, 44 ± 4 ($n = 3$). The value for FlagSemDP1b34a is given as a comparison. FlagSemDP1bΔ11 corresponding to a protein processed at PCS3 is almost inactive. Activity can be partially restored by adding sequences unrelated to SemD that contain a dibasic motif. (D) Re-introduction of PCS4 but not of PCS3 partially restores repulsive activity to inactive unprocessed SemD. The sequence of FlagSemDP1b34a or FlagSemDP1b34b was C-terminally extended by adding the sequences displayed in (E) containing a Flag-epitope and 13 amino acids of SemD including PCS3 or 4. Aggregates expressing the indicated proteins were cultured in a collagen matrix together with explanted sympathetic ganglia from E9 chick embryos and the repulsive activities of the mutant proteins were determined after 48 h (means of r.r.a. $\pm$ SEM): FlagSemDP1b34a + P3, 6 ± 1 ($n = 3$); FlagSemDP1b34a + P4, 123 ± 6 ($n = 3$); FlagSemDP1b34b + P3, 6 ± 2 ($n = 4$); and FlagSemDP1b34b + P4, 267 ± 54 ($n = 3$).

proteases and by site-directed mutagenesis of consensus cleavage sequences.

Furin is an integral membrane protein which is located mainly in the trans-Golgi network but can also be detected at the cell surface (Molloy *et al.*, 1994; Vey *et al.*, 1994). While furin is expressed ubiquitously as a component of the constitutive secretory pathway, another related protease, PC3, is found in the regulated secretory pathway

Fig. 8. Differential processing of proSemD generates several isoforms with distinct properties. SemD is synthesized as an inactive precursor, proSemD. Differential processing by furin or a related protease at three processing sites (PCS1, 3 or 4) during intracellular maturation results in the formation of several SemD isoforms [proSemD, SemD(65k) and SemD(95k)] which differ in their repulsive activity (indicated by a red bar on the left) over a range of three orders of magnitude. The individual PCSs have different functional properties. Processing at PCS3/4 coupled to a post-cleavage modification activates SemD. Secreted proSemD has the lowest, SemD(65k) (cleaved at PCS1/3/4) and SemD(95k) (cleaved at PCS3/4) the highest activity. After secretion, proSemD could be activated by cleavage at PCS3 or 4. Proteolysis of SemD(95k) at PCS1, in contrast, results in the reduction of its potency.

of neuroendocrine cells (Barr, 1991; Steiner *et al.*, 1992). All members of this enzyme family recognize and cleave dibasic peptide motif but differ with respect to their specific sequence requirements (Barr, 1991; Steiner *et al.*, 1992; Loh, 1993). Whereas furin prefers the extended consensus sequence RXK/RR, R/KR or RXXR are sufficient for cleavage by other enzymes from this group (Barr, 1991; Nakayama *et al.*, 1992; Steiner *et al.*, 1992; Watanabe *et al.*, 1992, 1993; Loh, 1993). These consensus sequences are also present in clusters of basic amino acid residues (PCS1 and 3) that are conserved in all known class III semaphorins but absent from other semaphorins, including the secreted D-SemaII.

Processing of proSemD activates its repulsive properties and generates functionally different isoforms

Proteolytic processing is a prerequisite for the generation of active SemD. Our mutational analysis uncovered a distinct contribution of each PCS to the functional properties of processed SemD (Figure 8). ProSemD is almost completely inactive. Processing at one or several PCSs results in an increase in repulsive activity by a factor of 15–500, depending on the combination of cleaved processing sites. PCS4 makes the largest contribution to this increase in repulsive potency, probably due to an additional secondary reaction. In contrast, cleavage of the activated SemD(95k) at PCS1 has an opposite effect and considerably reduces its activity (Figure 8).

Up until now, SemD and its orthologue Coll-1 were unique among vertebrate semaphorins as the only molecules displaying repulsive properties. Here we demonstrated repulsion of sympathetic fibres by two different class III semaphorins. This allowed us to show that the importance of proteolytic processing is not restricted to SemD, as the activity of SemA and E was increased considerably by inactivating the processing sequences corresponding to PCS1.

Processing of SemD also occurs *in vivo*. An antibody raised against a fragment from the CTD specifically recognized a 40 kDa peptide in membrane preparations from mouse embryos of various stages, in addition to a 125 kDa protein. The abundance of the 40 kDa peptide increased during embryogenesis, further supporting a developmental regulation of processing. The 125 kDa protein probably corresponds to proSemD or partially processed SemD. A protein of similar size was detected with an antibody raised against Coll-1 (Shepherd *et al.*, 1997). The larger size of endogenous murine SemD in comparison with the recombinant protein probably results from differences in post-translational modification in 293 cells. Although the precise reasons for the differences in the composition of SemD forms are not resolved, all presently available data are consistent with a regulated processing of SemD in the developing embryo. Given the abundance and ubiquitous expression of furin and related proteases (Barr, 1991; Steiner *et al.*, 1992; Loh, 1993; Zheng *et al.*, 1994, 1997), a specific mechanism is likely to exist that prevents complete processing of the 95 kDa Coll-1/SemD during its intracellular maturation.

SemD processing is coupled to an additional step for activation

The requirement for proteolytic processing to generate active SemD could reflect a necessity to remove an auto-inhibitory domain and thus release the active protein. Alternatively, processing might provide the substrate for a coupled secondary reaction that modifies SemD to increase its activity (Barr, 1991; Steiner *et al.*, 1992; Loh, 1993; and references therein). Processing at dibasic sites often is accompanied by subsequent removal of the C-terminal basic residues by carboxypeptidases and covalent modification (Skidgel, 1988; Loh, 1993). Amidation of the terminal residue has been shown to be important for the biological activity of several peptides (Loh, 1993), whereas the pro-domain of the blood coagulation factor IX directs γ -carboxylation of the mature protein (Jorgensen *et al.*, 1991; Bristol *et al.*, 1994). Our analysis of C-terminal mutations indicates that PCS4 might direct a similar activation of SemD that is dependent on prior proteolytic cleavage. Further experiments will have to clarify this observation.

Differential processing of SemD suggests novel roles for proteases in axonal pathfinding

The distinct contributions of individual processing sites to SemD activation underscore the importance of proteolytic activity in the nervous system. Differential expression and regulation of protease activity would allow cells to regulate the repulsive activity finely by changing the relative abundance of the different SemD isoforms (Figure 8). In addition, proteolytic cleavage might also serve to localize

and modulate precisely the effects of SemD. Secreted proSemD could be activated locally by proteases synthesized by cells lining axonal pathways or by migrating growth cones (Figure 8). Local extracellular proteolysis could be achieved by a protease such as furin, a related molecule or a different class of proteases such as the matrix metalloproteases (Matrisian, 1992). Such a protease could even be produced by the growth cone itself. In agreement with the involvement of proteases in chemorepulsion is the identification of a growth cone-collapsing activity from adrenal tissue which is inactivated by preincubation with protease inhibitors and was suggested to be itself a protease (Baird and Raper, 1995). Cleavage at PCS1, in contrast, would reduce repulsion, and cells equipped with appropriate enzymes might thereby be able to traverse repulsive regions along their trajectory. Thus, changes in the response to SemD, as seen for sensory neurites (Püschel *et al.*, 1996), might involve not only a down-regulation of putative receptors but also an up-regulation of proteases.

Inhibitors and analogues of proteases might regulate the activity of repulsive signals

Many proteases and protease inhibitors (Gloor *et al.*, 1986; Mansuy *et al.*, 1993; Zheng *et al.*, 1994, 1997; Osterwalder *et al.*, 1996) expressed in neural tissue have been implicated in developmental or functional plasticity (Monard, 1988; Sumi *et al.*, 1992; Seeds *et al.*, 1995, 1996; Fambrough *et al.*, 1996). The most common hypothesis to account for their effects on neuronal development and activity has been a role in the penetration of the extracellular matrix (Monard, 1988; McGuire and Seeds, 1990; Nordstrom *et al.*, 1995). Our demonstration that the activity of a repulsive molecule involved in axonal pathfinding is modulated by proteolytic processing suggests additional roles. A set of specific proteases and their inhibitors could serve locally to activate or modulate repulsive signals and thereby contribute to their specificity.

There may also exist other mechanisms for modulating proteolytic processes. The *Drosophila masquerade* (*mas*) gene encodes a protein that displays sequence similarity to the catalytic domains of proteases but has no apparent enzymatic activity. Its mutation results in disturbances of axonal pathfinding (Murugasu-Oei *et al.*, 1995, 1996). It has been speculated that this protein might act as a competitive inhibitor of serine proteases (Murugasu-Oei *et al.*, 1996). By binding to protease cleavage sites of repulsive molecules similar to SemD, the Mas protein might mask these sites and prevent their processing or cleavage. Consistent with this idea, the mutant phenotype has properties suggestive of an increase in repulsive activity in the embryo (Murugasu-Oei *et al.*, 1996). Similar effects should be observed if the PCS1 of SemD is protected against cleavage. Thus, our results suggest that proteases may play an essential role in determining the strength of repulsive cues and indicate a novel role for proteolysis in developmental and functional plasticity of the nervous system. The balance of intra- and extracellular proteolytic activities, specific protease inhibitors, as well as accessibility of protease recognition sites could determine locally not only the strength of adhesive interactions but also of signals repulsive for neurites along specific axonal pathways.

Materials and methods

Site-directed mutagenesis

Epitope tags and mutations were introduced into the *semD* coding sequence by PCR following standard protocols (detailed protocols and primer sequences are available on request). To generate FlagSemD, the coding sequence for DYKDDDD was introduced between the codons for G25 and K26 (Püschel *et al.*, 1995). The myc epitope EQKLISEEDL (Evan *et al.*, 1985) was synthesized as a double-stranded oligonucleotide and ligated into an *ApaI* site corresponding to the codons for G579 and P580. The SemD-coding sequence was extended by adding a Flag epitope to the C-terminal end and fused in-frame to the secreted AP from pAP-Tag (Flanagan and Leder, 1990) via a *BglII* site. All constructs were cloned into the pBK-CMV vector (Stratagene). Mutations (Table I) were introduced into the PCS sequences using oligonucleotides with the mutations shown in Figures 4 and 7. Amplified PCR fragments were cloned into the FlagSemD or SemD-AP expression vectors and verified by DNA sequencing.

Generation of antibodies

To generate a polyclonal antiserum against the CTD, a fragment of SemD corresponding to amino acids V653–E753 was expressed in M15 bacteria using the pQE11 vector (Quiagen). The His₆-tagged protein was purified on an Ni-NTA-agarose column (Quiagen) as specified by the manufacturer. New Zealand white rabbits were immunized with recombinant protein by a commercial service (Eurogentec). Antisera were affinity purified by covalently coupling recombinant protein in 6 M guanidinium-HCl and 9% (w/v) polyethylene glycol (PEG) 20 000 to a MiniLeak matrix (KemEnTec) according to the manufacturer's specifications. Serum was diluted 1:5 in phosphate-buffered saline (PBS), bound to the column and eluted with 0.1 M glycine (pH 2.5).

Transfection and Western blot

To express recombinant semaphorin proteins, human embryonic kidney 293 cells (ATCC CRL 1573) grown in 90 mm dishes (Falcon) were transfected with 12 µg of expression plasmid DNA by calcium phosphate co-precipitation (Chen and Okayama, 1987), and the transfection medium was replaced by serum-free medium after 5 h of incubation. Conditioned media were harvested after 3 days of culture and concentrated 75-fold using Centrplus concentrators (Amicon). To test the effect of decanoyl-RVKR-chloromethylketone (decRVKRCmk; Vey, 1995), a specific inhibitor of furin-like proteases, 293 cells grown in 30 mm dishes were transfected with 2 µg of expression vector and incubated for 48 h with serum-free medium containing 100 µM decRVKRCmk. Aliquots of concentrated media were separated by SDS-PAGE, blotted onto nitrocellulose membranes, incubated for 1 h in blocking buffer [3% (w/v) bovine serum albumin in PBS], followed by anti-Flag M2 (Kodak), anti-myc 9E10 (Boehringer, Mannheim) or anti-AP (Zymogen) antibodies in blocking buffer for 1 h as specified by the manufacturer. After incubation with horseradish peroxidase (HRP)-coupled anti-mouse antibody (Dianova) in blocking buffer for 45 min, the blot was developed by enhanced chemiluminescence (ECL, Amersham). Membranes were prepared from whole embryos following the method of Raper and Kapfhammer (1990). Then 50 µg of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Western blots were developed as described above with the following modification: the affinity-purified anti-CTD antiserum was diluted 1:10 000 in 1% (w/v) non-fat milk powder, 1% (v/v) goat serum and 0.1% (w/v) Tween-20 (Sigma) in blocking buffer and detected with anti-rabbit-HRP as secondary antibody (Dianova).

Co-culture assay

293 cells were transfected as described above, and cell aggregates formed as described previously (Püschel *et al.*, 1995). Dorsal root ganglia from E12.5 mouse embryos or sympathetic ganglia from E9 chick embryos and cell aggregates were placed in a 10:1 mixture of collagen (Boehringer Mannheim) and Matrigel (Collaborative Research) ~500 µm apart and cultured in the presence of 5 ng/ml murine nerve growth factor (NGF; generously provided by H.Rohrer) in 30 mm polyornithine-coated plastic dishes (Greiner). After polymerization of the matrix for 60 min at 37°C, primary culture medium was added. Only cultures that extended neurites within the first 12 h of incubation were used for quantification.

Quantification of co-culture assays

To compare the activity of the different SemD proteins, their repulsive potency was determined relative to that of SemD. The r.r.a. indicates

Table I. Summary of SemD mutants

Expression vector	Mutations
SemDP3-AP	PCS3: R758A, R760A, R761A
SemDP4a-AP	PCS4: R767A
SemDP34a-AP	PCS3: R758A, R760A, R761A PCS4: R767A
SemDP34b-AP	PCS3: R758A, R760A, R761A PCS4: R770A
FlagSemDP1a	PCS1: R555A
FlagSemDP1b	PCS1: R551A, R555A
FlagSemDP1c	PCS1: R552A, R554A
FlagSemDP3	PCS3: R758A, R760A, R761A
FlagSemDP4a	PCS4: R767A
FlagSemDP4b	PCS4: R770A
FlagSemDP34a	PCS3: R758A, R760A, R761A PCS4: R767A
FlagSemDP34b	PCS3: R758A, R760A, R761A PCS4: R770A
FlagSemDP1b34a	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R767A
FlagSemDP1b34b	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R770A
FlagSemDP1b4a	PCS1: R551A, R555A PCS4: R767A
FlagSemDP1b4b	PCS1: R551A, R555A PCS4: R770A
FlagSemDP1b3	PCS1: R551A, R555A PCS3: R758A, R760A, R761A
FlagSemDP1bΔ11	PCS1: R551A, R555A deletion of T762-V772
FlagSemDP1bΔ10F	PCS1: R551A, R555A T762-V772 replaced by TDYKDDDDKRS
FlagSemDP1bΔ14R	PCS1: R551A, R555A N759-V772 replaced by KQEDRRQLQGR
FlagSemDP1b34a + P3	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R767A C-terminal addition of RSDYKDDDDKSRGGESKKGRNRRTH
FlagSemDP1b34b + P3	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R770A C-terminal addition of RSDYKDDDDKSRGGESKKGRNRRTH
FlagSemDP1b34a + P4	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R767A C-terminal addition of RSDYKDDDDKSRGGTHEFERAPRSV
FlagSemDP1b34a + P4	PCS1: R551A, R555A PCS3: R758A, R760A, R761A PCS4: R770A C-terminal addition of RSDYKDDDDKSRGGTHEFERAPRSV

the amount of SemD that would cause the same degree of repulsion as measured by the radius of inhibition for neurite extension (r_i). The r_i obtained in a parallel culture with cells transfected with 10 μ g of the FlagSemD expression vector served as a reference with a defined r.r.a. = 100 (100 arbitrary units) in all experiments. The r_i measured for a mutant protein was expressed as a fraction of that determined for FlagSemD in the reference transfection. Thus, a value of r.r.a. = 2 indicates that the amount of mutant protein produced by a cell aggregate after transfection of 10 μ g of the respective expression vector results in a repulsion equal to that caused by 2 units of FlagSemD, i.e. the respective protein is 50-fold less active than SemD. The relationship between r_i and the relative amount of SemD produced ($P_{rel}[\text{SemD}]$) was determined experimentally as $r_i = \log(P_{rel}[\text{SemD}])$, as expected for an exponential decrease in the concentration of a secreted substance over

distance from its source, assuming a diffusion-based gradient. To obtain this calibration curve, the repulsive effects of 293 cells transfected with different amounts of the FlagSemD expression vector were measured as the radius of inhibition for neurite extension (r_i) in μ m. The total amount of transfected DNA was kept constant by adding the expression vector pBK-CMV to a total of 10 μ g of DNA per transfection. The relative amounts of SemD protein (P_{rel}) produced were determined by Western blot analysis of the secreted FlagSemD and SemD-AP and, in addition, by a chemiluminescence assay (Tropix) for SemD-AP ($n = 4$). Both methods gave the same results. In parallel, r_i values were determined in a co-culture of cell aggregates and chick E9 SG. The r.r.a. of a mutant was obtained by calculating the amount of $P_{rel}[\text{SemD}]$ that causes an r_i equal to that of the mutant based on the calibration curve described above. The activity of mutants with an r.r.a. >100 was measured under conditions where their r_i was smaller than that of the reference transfection by reducing the amount of transfected expression vector as described above for the calibration experiment.

Collapse assay

DRG were explanted from E12.5 mouse embryos onto poly-L-lysine (Sigma) and laminin- (Gibco) coated 4-well plastic dishes (Greiner) and cultured overnight in primary culture medium (see above) in the presence of 5 ng/ml NGF (5% CO₂, 37°C). Recombinant proteins were expressed by transient transfection of 293 cells as described above, and conditioned media concentrated with Centrifuil concentrators (Amicon). Collapse assays were performed after 18 h of culture by adding different concentrations of concentrated media diluted in 2 ml of primary culture medium, and the amount of protein that causes a half-maximal effect was determined. The amounts of recombinant protein were quantified by Western blot analysis prior to the collapse assays. The cultures were fixed 30 min after addition of recombinant proteins in 4% (w/v) paraformaldehyde, 10% (w/v) sucrose in PBS. After fixation, the cultures were washed with 10% sucrose in PBS, and the number of collapsed growth cones determined (Raper and Kapfhammer, 1990). The relative collapsing activity (r.c.a.) of a mutant indicates the amount of SemD that would cause an equal degree of collapse as the mutant at the half-maximal effective concentration. The r.c.a. of FlagSemD served as a reference with an r.c.a. = 100.

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