Published in final edited form as:

Arch Biochem Biophys. 2008 July 15; 475(2): 169–174.

Mutagenesis of the cysteine-rich clip domain in the *Drosophila* patterning protease, Snake

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Abstract

A common motif found in invertebrate serine proteases involved in immunity and development is the clip domain, proposed to regulate catalytic activity or protein-protein interactions within proteolytic cascades. Snake functions in a cascade that patterns the *Drosophila* embryo, and provides an accessible model for exploring the structural requirements for clip domain function. We tested Snake zymogens bearing charged-to-alanine mutations in the clip domain for their ability to rescue embryos lacking endogenous Snake and for their interactions by S2 cell co-transfection with upstream Gastrulation Defective and downstream Easter in the protease cascade. Of 13 single and multiple substitutions, one double mutant in a predicted protruding region exhibited a severe defect in embryonic rescue but showed only minimal defects in the co-transfection assay. We discuss implications of these and other results for potential biological roles of the Snake clip domain and for use of the in vitro assay in predicting protease behavior.

Keywords

serine protease; charged-to-alanine mutagenesis; clip domain; *Drosophila*; dorsoventral polarity

Introduction

The clip domain is a cysteine-rich structural motif that has been found amino-terminal to the catalytic domain in many invertebrate serine proteases and serine protease homologs having regulatory function in immunity or development [1]; for example, 37 of 204 predicted serine protease-related genes in *Drosophila melanogaster* contain one or more clip domains [1,2]. Its name was derived from the observation that, in drawings showing its disulfide linkages, the domain somewhat resembled the shape of a paper clip [3]. Clip domains usually comprise 37–60 amino acid residues interlinked by three strictly conserved disulfide bonds, with the remaining residues showing relatively low similarity [1,2]. These domains have been proposed to play roles in protease localization, regulation of catalytic activity, or in promoting interactions with other components of proteolytic cascades [1,4,5], but relatively few functional studies have been performed to test these hypotheses.

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To date, structural information has been obtained for just three clip domains, including x-ray crystal structure of the clip domain from the catalytically inactive serine protease homolog prophenoloxidase-activating factor II (PPAF-II) from *Holotrichia diomphalia* [5], and NMR spectroscopy-derived structure of the two clip domains in the functional serine protease prophenoloxidase-activating proteinase-2 (PAP-2) from *Manduca sexta* [4]. These represent group 3 and group 2 of four sub-groups of clip domains defined by sequence homology, where groups 1a (including *Drosophila* Snake), 1b, and 2 (including *Drosophila* Easter) represent the clip domains associated with functional serine proteases, and group 3 represents those associated with serine protease homologs lacking catalytic activity [1,2]. Structural features that are likely to be shared by all clip domains include a three- to four-stranded, irregular β -sheet to which loops that form the outer rim and bottom of a prominent central cleft are attached by disulfide bonds. The outer rim of the cleft predominantly consists of hydrophilic residues derived from sequences lying between the third and fourth conserved cysteines (what we will call loop 3 residues), and the interior surface of the cleft consists of hydrophobic residues. In PPAF-II, the rim of the cleft is composed of loops and a short β -strand, while in the PAP-2 clip domains, this rim contains two antiparallel α -helices separated by a short connecting loop. The PPAF-II clip domain also is noted to have a protruding region or knob composed of residues lying between the first and second conserved cysteines (loop 1 residues).

Mutation of a strictly conserved valine at the bottom of the central cleft in PPAF-II abolished PPAF-II binding to partially processed prophenoloxidase, an event necessary for expression of phenoloxidase activity [5]. This result suggests that the central cleft, conserved in clip domains, serves as a binding site for other proteins such as substrates. Loss of the clip domain from a catalytically active Easter-type serine protease, PPAF-I, resulted in severely reduced activation of the truncated PPAF-I by plasma compared to activation of the intact PPAF-I zymogen [5]. These authors proposed that the clip domains could be important in binding to stereotypical molecular structures on pathogen surfaces, which could bring many molecules of the clip-domain containing proteases in a cascade in close contact with each other and allow signal amplification at that site. A similar role in pathogen binding has been proposed for the PAP-2 clip domains, possibly involving a positively charged patch on the β sheet involving residues lying within loop 1 or immediately carboxy-terminal to the second cysteine [4]. However, these putative roles in surface binding have not been tested experimentally.

Two clip domain-containing serine proteases, Snake and Easter, are downstream components of a four-member serine protease cascade that establishes the dorsoventral axis of the *Drosophila* embryo through ventral activation of a signaling ligand for the receptor Toll [6–8]. These proteases provide an attractive target for functional studies of clip domains because a sensitive assay is available to test the ability of engineered in vitro-transcribed mRNAs to rescue embryos lacking either of these proteases when injected in the first hours of embryonic development [7,9]. Previously, deletion of Snake's clip domain or mutation of any of its conserved cysteines was shown to abolish the ability of injected *snake* mRNA to rescue *snake*-mutant embryos, demonstrating the importance of the clip domain for Snake function [9]. Furthermore, transcripts encoding the N-terminal pro-domain of Snake or Easter, including the clip domain and other sequences, were reported to dorsalize wild-type embryos, suggesting the prodomain may compete for binding to other essential components of the cascade such as Gastrulation Defective (GD), the protease that activates Snake, Snake's substrate, Easter, or uncharacterized cofactors [9]. In the current work, we have taken advantage of the embryo injection rescue assay and an established cell culture co-expression model for dorsoventral protease interactions [10,11] to mutate the 10 charged residues in the Snake clip domain to alanine, individually and in groups, to further explore the structural requirements for the clip domain in the function of Snake.

Materials and methods

Site-directed mutagenesis of the snake cDNA

A full-length *snake* cDNA provided by Robert DeLotto [9] was subjected to mutagenesis using the QuikChange Site-Directed Mutagenesis Kit and PfuTurbo polymerase (Stratagene), and clones were sequenced prior to use in assays. The primers used for all mutagenesis reactions are listed in Supplemental Table 1. The sequence of Snake clip domain (from F92 through P139) is **FCRRSFDGRSGY**CILAYQCLHVIREYRVHG**TRIDICTHRNNVPVICCP**. The ten charged residues (bolded) were individually mutated to alanine to generate 10 single mutant constructs. Two double mutants, loop1 (R94A,D98A) and loop3R (R123A,D125A), and one triple mutant, loop3L (R115A,E116A,R118A), were generated by sequential mutagenesis reactions.

Functional assay by mRNA injection into embryos

The wild-type and mutant *snake* cDNAs, in the pBS-SK⁻ vector (Stratagene), were linearized with Bam HI cutting downstream of the *snake* 3'UTR and polyA tail, gel-purified, and used as template for in vitro production of 5'-capped, polyadenylated mRNA using the T7 mMessage mMachine kit according to manufacturer's instructions (Ambion). Phenol-chloroform extracted, isopropanol-precipitated mRNA was dissolved in RNase-free water and quantitated by UV spectrometry. The mRNAs were standardized to a concentration of 1.0 µg/µl and injected into 0–1 hour embryos derived from *snk*^{229/Df(3L)Exel8157} mothers in which any Snake protein produced would have an early truncation deleting the clip and catalytic domains [9]. By convention, these maternally mutant embryos are referred to as *snk*-null embryos. Following injection, embryos were maintained in a humid chamber at 18 °C as described [12,13]. Injected embryos were visually examined during early gastrulation and scored as no rescue, partial rescue, or full rescue as described ([12,14]; see Supplemental Fig. 1). Accuracy of this scoring was verified by performing cuticle preparations and Twist staining [15], although due to substantial losses of injected embryos during these procedures only gastrulation phenotype was routinely scored. The position of injection into dorsal, ventral or centrally localized cytoplasm had no effect on the rescue response, as previously described [9].

Functional assay by co-transfection into *Drosophila* S2 cells

The wild-type and four mutant *snake* cDNAs (R95A, loop1, loop3L, loop3R) were cut from the pBS-SK⁻ vector with KpnI and NotI and subcloned into the pMT-V5/HisA vector (Invitrogen) for expression of untagged Snake in *Drosophila* S2 cell culture. The constructs encoding functional HA-tagged GD and FLAG-tagged Easter zymogens have been previously described [11]. All of these constructs were expressed under the control of the copper-inducible metallothionein promoter. Cultured *Drosophila* S2 cells (Invitrogen) were transiently transfected by using lipofectin (Life Technologies, Grand Island, NY). In parallel samples, 1.5 µg of each plasmid was introduced (up to 4.5 µg total), using empty vector to equalize the total amount of DNA. After transfection (20 h), cells were washed in serum-free media, and plasmid expression was induced with 0.7 mM CuSO₄. Harvesting of cells and media was performed 18–20 hours following induction. Settled cells were resuspended in the media by gentle pipetting, and a 100 µl aliquot of each cell suspension was spun to separate cells and media. The media samples were mixed with an equal volume (100 µl) of Laemmli sample buffer containing 100 mM DTT, while cell samples were resuspended in 200 µl of reducing sample buffer. These samples were boiled for 5 minutes prior to loading on gels.

Polyclonal antibodies and Western blotting

Anti-SNKCAT was generated against a trpE fusion protein containing 64 amino acids (aa 237–300) within the catalytic domain of Snake [9,16], by immunization into rabbits (Pocono Rabbit

Farm and Laboratory). The antibody was affinity-purified by positive selection against SNKCAT-trpE and negative selection against trpE, as previously described [17,18] and used at a working concentration of 1:500 for Western blots. The anti-GD antibody and an anti-Easter antibody provided by Donald Morisato have been previously described [11,19], and were used at concentrations of 1:1500 and 1:2000, respectively.

Western blots of proteins separated on 13% SDS-PAGE gels were probed with these rabbit polyclonal antibodies [20]. SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce) was used for all detection of anti-SNKCAT and for the detection of anti-Easter in Figure 5, while SuperSignal West Pico (Pierce) or Amersham ECL (GE Healthcare) were used for remaining experiments. Media samples are shown for blots of GD (though cell samples were also run), while cell samples are shown for blots of Snake and Easter because of the secretion defect of the mutant Snake forms and because the Easter cleavage product is only cell-associated [11].

Results

In vivo rescuing activity of wild-type and clip domain mutant forms of Snake

Using a charged-to-alanine mutagenesis approach [21,22], we tested the ability of ten singly mutated mRNAs, two doubly mutated mRNAs, and one triply mutated mRNA to rescue *snk*-null embryos. The positions of the mutated residues are illustrated in the two-dimensional diagram of the Snake clip domain (Fig. 1), where loop 3 residues lying between cysteines 3 and 4 are predicted to form the rim of a central hydrophobic cleft, and loop 1 residues lying between cysteines 1 and 2 may form a charged knob protruding perpendicular to the central cleft [4,5]. Rescue was scored as full, partial, or no rescue, corresponding to wild-type, partially dorsalized, and completely dorsalized gastrulation patterns respectively (Table 1; see Methods).

The major finding from this study was that only one of the thirteen mutants, loop1 (R94A, D98A), exhibited severely compromised function in the rescue assay with only 4% of the embryos being fully rescued and 86% remaining completely dorsalized. In contrast, most of the single charged-to-alanine changes rescued the *snk*-null embryos with efficiency approximately equal to wild type. Two modest deficiencies in rescuing activity were noted among the single mutants. First, the R95A mutant showed a decreased ability to provide full rescue (34%, compared to 61% for wild-type). Second, the R95A mutant, as well as the D98A and R118A mutants, showed an increase in the percentage of embryos that were not rescued and had a completely dorsalized phenotype (36%, 29%, and 35%, respectively, compared to 17% for wild-type *snake* mRNA). These results are consistent with delayed or reduced ability of Snake to reach a necessary threshold of activity within the limited time period when rescue can occur. A triple mutant, loop3L, that combines R118A with R115A and E116A, performed better than R118A, with a shift towards partial rescue (37% compared to 14% for R118A) and similar full rescue, suggesting that changes in charge density may impact clip domain function in subtle ways. The modest deficiencies seen for R95A and D98A, together with the severe deficiency in function of the loop1 (R94A, D98A) double mutant, suggest that the predicted protruding loop 1 region plays an important role in Snake function in vivo (Fig. 1).

In addition to studying the impact of clip domain mutations on activity of the full-length Snake protein, we sought to determine if prodomains containing these mutations would have reduced ability to dorsalize wild-type embryos as reported for the wild-type prodomain [9]. These authors had proposed that the prodomains of Snake and Easter could compete with the full-length proteins for interactions with other proteases or cofactors in the pathway. However, we were unable to reproduce the published finding of dominant negative activity for the wild-type Snake prodomain, using either Snake's own 5' and 3' UTRs or substituting *Xenopus* β -globin

5'UTR and *easter* 3'UTR (in an attempt to increase translation efficiency of the mRNA), and using the prodomain sequence either with or without cysteine 179 mutated to serine (since not clear which was used in the original study, and free cysteine 179 could result in abnormal disulfide bonding in the absence of the catalytic domain [23,24]) (data not shown). Therefore, we did not pursue these experiments with mutant prodomain constructs.

Interaction of mutant Snake proteins with other cascade components in cultured *Drosophila* S2 cells

The dorsoventral proteases GD, Snake, and Easter have been shown to function in a sequential cascade of zymogen activation when co-expressed in either *Drosophila* S2 cells or baculovirus-infected lepidopteran cells [10,11]. These cell culture-based assays are unlikely to faithfully mimic the interactions of the proteases in vivo, e.g., processing only appears to occur in the secretory pathway of co-expressing cells and not in conditioned media; however, they do demonstrate basic catalytic function of the proteases as well as a range of potential feedback cleavages among the proteases. The in vivo significance of these feedback cleavages seen in cell culture is not known.

The protease interactions detected in the S2 cell co-expression system [11] are briefly reviewed here (Fig. 2A). Co-expression of GD and Snake zymogens leads to cross-processing of both enzymes. Either low activity of the GD zymogen or small amounts of processed, active GD generated non-specifically within the secretory pathway appear to be capable of cleaving Snake at its zymogen cleavage site (reaction 1), and in turn activated Snake can process the GD zymogen (reaction 2), setting up a positive feedback loop. Activated Snake can also process other Snake molecules at an un-mapped site within the prodomain (reaction 3; [25]), so that a large C-terminal fragment of Snake is the predominant processed product in GD/Snake co-transfections (Fig. 2B, lanes 2 and 4). If the Easter zymogen is also present (triple transfection), it is activated by Snake (reaction 4), and in turn can process GD in the same region as does Snake (reaction 5), possibly leading to an additional positive feedback loop (or a negative one as suggested by [10]). Activated Easter also robustly cleaves Snake zymogen within the prodomain at an un-mapped site C-terminal to the Snake-dependent processing site (reaction 6; [11] and Fig. 2B, lanes 1 and 5). Similar events have been described in the baculovirus-infected lepidopteran cells, except that GD activation when co-expressed with Snake appeared to be autocatalytic, requiring the presence of Snake but not requiring that Snake be catalytically active [10]. The reason for this difference between the two co-expression systems has not been explored, however, it is possible that a distinct lepidopteran protease is capable of activating the GD zymogen when it is in complex with Snake.

To further explore the effects of Snake clip domain mutations on Snake function, we tested four of these mutants – R95A, loop1, loop3L, and loop3R – in the S2 cell expression system. These four were selected because the single R95A and double loop1 mutants showed modest to severe defects, respectively, in rescuing activity by embryo injection, so might show defects in specific interactions in the co-expression assays, while the triple loop3L and double loop3R mutants did not exhibit defects in the embryo injection assay and were expected to perform well in the co-expression studies. When expressed alone, all four mutants demonstrated a strong secretion defect compared to wild-type Snake but were present at normal levels without degradation products (Fig. 3A), suggesting a defect in trafficking the mutant Snake proteins through the secretory pathway [26]. Because the processing of co-expressed zymogens occurs within the secretory pathway and not in the media [11], we proceeded to test the mutants with the idea that relevant interactions could still occur intracellularly. For simplicity, the loop1 and R95A results in all co-transfections are presented prior to the loop3L and loop3R results.

Surprisingly, in comparison to the embryo rescue assay, the loop1 mutant performed quite well in cleavages detectable in the S2 cell system. In double transfections of GD and Snake

zymogens (Fig. 3B), there was robust processing in the Snake prodomain known to be performed by Snake and not GD (reaction 3; Fig. 2A and B, lanes 2 and 3) and dependent on prior activation of Snake by GD (not seen in Snake single transfection, Fig. 3A; [11]). There was a variable decrease in Snake-dependent processing of GD in co-transfections with loop1 or R95A mutant Snake compared to wild type (reaction 2; Fig. 3B and data not shown), with the loop1 mutant more affected than R95A. However, in triple transfections with both GD and Easter zymogens, no differences were detected between wild-type and loop1 or R95A mutant Snake in cleavages of any of the proteases (Fig. 4). These results together suggest that loop 1 mutations do not show functionally significant impairments in Snake's catalytic activity or interactions with GD and Easter when assayed in the S2 cell system, and that Easter's ability to process GD may mask the modest defect seen for loop1 processing of GD in the double transfection.

It has previously been proposed that the clip domain of Snake blocks the catalytic site of the protease domain, preventing Snake cleavage of Easter except when Snake is present within a ventral activating complex [25]. If the loop1 mutant had lost this inhibitory role, the Snake protease might weakly but indiscriminately activate Easter throughout the extracellular space of the embryo, leading to weak lateralization or ventralization of the embryo. As one test of this possibility in vitro, we performed double transfections of Snake and Easter (Fig. 5) to see if increased processing of Easter could be detected in the loop1 mutant compared to wild-type. While previous studies had not detected any processing of Easter in such double transfections [11], using a more sensitive chemiluminescent reagent we did detect a small amount of processing of Easter that was dependent on the co-expression of Snake. The loop1 and R95A mutants showed similar or less processing compared to wild-type Snake, so we fail to find support for a role of these clip domain residues in blocking the catalytic site of Snake.

In contrast to the relatively robust performance of the loop1 and R95A mutants in the S2 cell system, the loop3L and loop3R mutants showed broad defects that were unexpected given their ability to rescue *snk*-null embryos. In the double transfections of Snake and GD (Fig. 3B), there was moderately reduced processing of GD and moderately to severely reduced processing of Snake, while in the double transfections of Snake and Easter (Fig. 5) there was no detectable processing of Easter by these Snake mutants. As seen for the loop1 mutant, however, when all three proteases were expressed together (Fig. 4), the presence of Easter appeared to compensate for the deficiencies of the loop 3 mutants, with apparently full restoration of GD processing and Easter-specific Snake processing, and somewhat reduced processing of Easter by the Snake loop3R and loop3L mutants.

Discussion

No single charged-to-alanine substitution within the clip domain, and only one of three multiple substitutions, resulted in a dramatic reduction in the ability of the Snake zymogen to rescue *snake*-null embryos. Similar findings that most single changes do not obviously impact function are common in charged-to-alanine mutagenesis studies [22,26], and for any individual residue could mean either that it is not important for Snake function or that multiple interactions occurring with a binding partner mask the effect of removing a single charged residue involved in these interactions. With the exception of the loop1 double mutant, the embryo rescue seen was relatively robust in view of the secretion defect seen for four representative mutants expressed in cell culture. Such combinations of secretion defect with relatively normal function of cell surface or purified proteins have been described previously for charged-to-alanine mutants [26]. In the case of Snake, it is possible that (a) the embryo's secretory system is more efficient at trafficking Snake to the cell surface than are the S2 cells, (b) a very small quantity of the mutant Snake protein in the extracellular space is sufficient for rescue of the cascade in

vivo, and/or (c) cofactor and/or feedback interactions within the cascade in vivo are able to compensate for decreased quantity or quality of Snake within the extracellular space.

Apparently contradictory findings were obtained in the embryo injection assay versus the S2 co-expression assay, wherein the loop1 mutant performed poorly in vivo yet well in vitro while the loop3L and loop3R mutants performed well in vivo yet poorly in vitro. Three possibilities for the loop1 impairment are suggested by the available data. The first and least interesting is that the loop1 mutant could have a particularly severe defect in secretion in embryos, and thus be unavailable to participate in the protease cascade within the perivitelline fluid. The second possibility is that the loop1 mutant has impaired interactions with GD, as hinted at by the modestly decreased processing of GD in the double transfections (Fig. 3B). Reduced interaction of GD and Snake in vivo could directly result in decreased Snake activation, reduce proposed feedback activation of GD [10,11], or directly impact Snake's activation of Easter if Snake and GD must remain bound together in a ventral complex for Easter activation to occur [25,27]. Finally, the S2 cell system involving overexpression of the proteases is already known to bypass an in vivo requirement for a ventral cofactor acting in Snake activation and/or Easter activation [11,19]. Thus, it is possible that the critical defect in the loop1 mutant is in interactions with this ventral cofactor, which may act to concentrate the protease zymogens on a surface. Such a role for clip domains has previously been proposed for binding to pathogen surfaces, thus concentrating components of the prophenoloxidase-activating cascade to allow localized signal amplification [4,5].

The Snake zymogens containing multiple charged-to-alanine mutations in loop 3, likely corresponding structurally to the exposed rim of a central cleft in the clip domain, are among the mutants performing with wild-type function in vivo, yet they show severe impairment of Snake-dependent processing reactions in double transfections with either GD or Easter (Fig. 3B, Fig. 5). This impairment suggests that the Snake catalytic domain has very low activity under these experimental conditions. This could result from impaired cleavage of the Snake zymogen or from a reduced ability to adopt the open and active conformation of the Snake catalytic domain following zymogen cleavage, e.g., if a proposed blockage of the catalytic site by the Snake clip domain [25] is not relieved when the loop 3 residues are mutated. Interestingly, these defects are masked in the triple transfections containing Easter in addition to GD and Snake (Fig. 4). The cleavage of Easter in the triple transfections is strictly dependent on Snake's catalytic activity [11], arguing that the Snake catalytic domain is correctly folded and can function in the loop3L and loop3R mutants. The production of a small amount of active Easter may itself be sufficient to allow the robust Easter-dependent cleavages of GD and Snake seen in the triple transfection, especially if the latter cleavage within the Snake prodomain results in relief of Snake catalytic domain inhibition by the mutant clip domains to allow more normal levels of Easter activation. An alternative possibility suggested by these results is that GD, Snake and Easter function best in a mutually activating complex, and that multiple intermolecular interactions among these proteases overcome the loop 3 defects both in the S2 cell triple transfections and in the embryo injection assays.

Our results demonstrate the importance of loop 1 charged residues (R94, R95, D98) in the clip domain in Snake function in vivo, and suggest that loop 3 residues may regulate Snake catalytic function under some experimental conditions. The contrasting results obtained in the embryo injection assay and S2 co-expression assay highlight the importance of using multiple assays and the need for caution in interpreting data from any single assay of protease function. Determining whether the Snake clip domain functions as a protein-protein interaction module for binding to either GD or to a ventral cofactor, or if it regulates Snake catalytic function, will require development of both in vitro assays using purified proteases and better tools for studying protease activation and localization in vivo. Questions accessible using the current assays include mapping sites within Snake that are processed by Snake and Easter and

determining their significance to the protease cascade, and performing charged-to-alanine mutagenesis of the Easter clip domain to see if mutations in loop 1 and loop 3 show similar behavior to that seen for Snake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Maggie Elalayli and Jacklyn Hall for assistance in generating the anti-SNKCAT antibody; Robert DeLotto, David Stein, Donald Morisato, Siegfried Roth, the Bloomington Stock Center, and Exelixis for reagents and fly stocks; and anonymous reviewers for their suggestions. This work was supported by NIH RO1 GM067738 to E.K.L.

Abbreviations used

PPAF-I or -II

prophenoloxidase-activating factor I or II

PAP-2

prophenoloxidase-activating proteinase-2

GD

Gastrulation Defective

SNK

Snake

EA

Easter

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Fig. 1.

Diagram of the Snake clip domain showing mutated residues. Shown is the clip domain sequence from F92 to P139 (see Methods text for linear view) with the characteristic disulfide linkages (C₁-C₅; C₂-C₄; C₃-C₆) and arrangement of loops 1 and 3 indicated. The charged residues that were mutated individually and in groups to alanine are indicated by black circles. A single asterisk identifies R95, that when mutated resulted in a modest defect in embryo rescuing activity, while double asterisks identify the R94 and D98 residues that were both mutated in the loop1 mutant resulting in a severe defect in rescuing activity.

Fig. 2.

Overview of cleavages detected among the dorsoventral proteases in the S2 cell co-expression assay system. (A) Schematic diagram of cleavages occurring upon co-expression of GD and Snake (reactions 1–3) or upon co-expression of GD, Snake, and Easter (reactions 1–6) [11]. See Results text for description of these reactions. (B) Processing of a catalytic serine-mutated Snake substrate (SNKS-A) by pre-activated (ΔN) forms of Easter, Snake, and GD results in distinct C-terminal products (lanes 1–3). GD-dependent processing has been mapped to the zymogen cleavage site (product marked with arrow) while Easter- and Snake-dependent processing sites are at unmapped positions within the Snake prodomain. In double transfections of GD and functional Snake, the Snake-dependent cleavage predominates (lane 4), while in triple transfections of these proteases plus Easter, the Easter-dependent cleavage product predominates (lane 5). Lanes 1, 3, and 5 have been presented previously in Figure 1B of [11] and are included here for comparison with lanes 2 and 4 documenting Snake-dependent Snake cleavage from same gel. In contrast to Figures 3–5 in this paper that use an untagged Snake recognized by a Snake catalytic domain antibody, the SNKS-A substrate includes an ~10 kDa C-terminal 5-myc-6-His tag and was detected using an anti-myc antibody. Approximate molecular weights of the Snake-related bands are indicated at left, and molecular weight markers are indicated at right in this and future blots.

Fig. 3.

Secretion defect of Snake mutants and processing defects seen in co-transfections with wild-type GD zymogen. (A) All tested mutants (loop3R, loop1, R95A, and loop3L) have a secretion defect compared to wild-type (WT) Snake in S2 cells. M and C indicate the media and cells, respectively. (B) Co-transfection of GD with empty pMT vector or with wild-type or mutant Snake. IB, immunoblotting antibody. Top panel, the loop1 and R95A Snake mutants show variable and modestly reduced processing of the GD zymogen (GDz) to its C-terminal cleavage products (GDc, 46–50 kDa doublet) compared to wild-type Snake, while loop3R and loop3L show greater reduction in GD processing. Bottom panel, R95A and loop1 mutants show normal Snake-dependent processing of the Snake zymogen (SNKz) to a 43 kDa C-terminal product (SNKcs, where s indicates Snake dependence of this form), while loop3R and loop3L show moderately and strongly reduced processing, respectively.

Fig. 4. Processing defects are largely masked in the triple transfections with GD, Easter, and mutant Snake zymogens. Top panel, GD. Middle panel, Snake, with Easter-dependent processing product SNKze (32 kDa) indicated. Bottom panel, Easter, showing slightly decreased processing by the loop3L mutant and moderately reduced processing by the loop3R mutant.

Fig. 5. There is no increased processing of Easter in double transfections with the mutant Snake zymogens. Snake/Easter double transfections are compared to GD/Snake/Easter triple transfections showing full processing of Easter zymogen and to Easter/pMT double transfection showing no processing. Top panel, minimal processing of Snake occurs in these double transfections of two zymogens. The significance of slightly different processed products in some of the mutants compared to wild-type Snake is not known. Bottom panel, a small amount of Easter is converted to the activated form (EAc) in a Snake-dependent manner by wild-type, R95A and loop1, but not by loop3R or loop3L mutants.

Table 1
Phenotypic rescue by wild type and clip domain mutants of Snake

construct	number	full rescue (%)	partial rescue (%)	no rescue (%)
WT	272	165(61)	61(22)	46(17)
SnkR94A	64	45(70)	11(17)	8(13)
SnkR95A	430	146(34)	129(30)	155(36)
SnkD98A	62	33(53)	11(18)	18(29)
SnkR100A	97	67(69)	19(20)	11(11)
SnkR115A	94	56(60)	24(25)	14(15)
SnkE116A	64	48(75)	9(14)	7(11)
SnkR118A	74	38(51)	10(14)	26(35)
SnkR123A	36	21(58)	10(28)	5(14)
SnkD125A	47	25(53)	14(30)	8(17)
SnkR130A	44	30(68)	6(14)	8(18)
loop1	485	20(4)	50(10)	415(86)
loop3L	49	23(47)	18(37)	8(16)
loop3R	140	88(62)	26(19)	26(19)