Role of the Spaetzle Pro-domain in the Generation of an Active Toll Receptor Ligand

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The cytokine Spaetzle is the ligand for Drosophila Toll, the prototype of an important family of membrane receptors that function in embryonic patterning and innate immunity. A dimeric precursor of Spaetzle is processed by an endoprotease to produce a form (C-106) that cross-links Toll receptor ectodomains and establishes signaling. Here we show that before processing the pro-domain of Spaetzle is required for correct biosynthesis and secretion. We mapped two loss-of-function mutations of Spaetzle to a discrete site in the pro-domain and showed that the phenotype arises because of a defect in biosynthesis rather than signaling. We also report that the pro-domain and C-106 remain associated after cleavage and that this processed complex signals with the same characteristics as the C-terminal fragment. These results suggest that before activation the determinants on C-106 that bind specifically to Toll are sequestered by the pro-domain and that pro-teolytic processing causes conformational rearrangements that expose these determinants and enables binding to Toll. Furthermore, we show that the pro-domain is released when the Toll extracellular domain binds to the complex, a finding that has implications for the generation of a signaling-competent Toll dimer.

Drosophila Spaetzle is the activating ligand of Toll, a class I transmembrane receptor required for embryonic dorsoventral patterning and innate immune responses to bacteria and fungi (1, 2). In vertebrates, a related family of receptors mediate responses to analogous microbial signals in the innate immune response, and these signaling events are a prerequisite for the development of adaptive immunity (3). It is an important objective of current research to understand the molecular mechanisms of pathway activation by Toll ligands, and such information should allow extrinsic regulation of the pathways in human autoimmune and infectious diseases (4).

Spaetzle protein is synthesized and secreted from the cell as an inactive, dimeric precursor (pro-protein) consisting of a pro-domain (25 kDa) and a C-terminal region that forms a cystine knot structure (C-106) (4) (14 kDa) (5). This structural motif is found in a number of vertebrate signaling molecules, and Spaetzle is most closely related to the neurotrophins, for example nerve growth factor (NGF) (6, 7). The precursor form of Spaetzle is secreted efficiently from expressing cells and is correctly folded, containing three intra-molecular disulfide bonds and a fourth that joins the two subunits together to form a homodimer. Previous studies have suggested that the pro-domain is a natively unstructured or “loopy” domain and that covalent association of the pro-domain and cystine knot is sufficient to completely suppress binding to the Toll receptor and signaling activity (8). In both dorsoventral patterning and innate immunity, specific stimuli activate a cascade of serine proteases, and the terminal member of the cascade cleaves the pro-protein at a specific site 106 amino acids from the C terminus (9, 10). In dorsoventral patterning an endoprotease with a trypsin-like specificity, Easter, is known to cleave Spaetzle (11, 12); and a related protease, Spaetzle-processing enzyme (SPE), has recently been shown to cleave Spaetzle in the immune response (13).

The precursor form of Spaetzle has no signaling activity, but C-106 binds and cross-links two molecules of the Toll ectodomain thereby activating the receptor pathway (8, 14). Thus the pro-domain of Spaetzle prevents C-106 from binding to Toll, and this suggests that unmasking of the N terminus of C-106 is necessary to form a stable complex between Toll and C-106.

In this study we report that the pro-domain is required for secretion, and after endoproteolytic cleavage, C-106 and the pro-domain remain tightly associated with each other. This noncovalent complex is active in signaling, and the pro-domain is displaced by binding to Toll. Taken together, these results suggest that proteolytic processing of Spaetzle causes structural rearrangements that unmask a binding surface on C-106 for interaction with Toll ectodomains.

MATERIALS AND METHODS

Spaetzle Isoforms—There are at least 15 splice isoforms of Spaetzle (15), and the exon structure of the gene is illustrated in...
supplemental Fig. S2. In this study the EST clone HL01462 was used throughout (accession number EF155533, see supplemental Fig. S1). The isoforms appear to be very similar functionally (15).

**Generation of Expression Constructs**—The generation of Toll and Spätzle pro-protein expression constructs is described previously (8). The constructs Mel-C106-FLAG and Toll-C106-FLAG were generated by two rounds of PCR. The Spätzle EST clone HL01462 (see supplemental Fig. S1) was purchased (Research Genetics Invitrogen) and used as a template in the first PCR. The following 5′ primers were used to fuse part of the Melittin or Toll signal peptide to the C106 sequence, respectively: atg tgc gta tac att tac tac atg gat gtt ggt ggc tca gacgacgca and atc atc cta cta cag tgg gga gaa gca tcc gtt ggt ggc tca gacgacgca (the asterisk denotes start of the C106 sequence). As a 3′ primer primer reaction 2). The resulting PCR product was purified using NucleoSpin PCR purification kit (Macherey-Naegel). DNA was sequenced on a 3100 Applied Biosystems sequencer.

**Site-directed Mutagenesis**—A QuikChange site-directed mutagenesis kit (Stratagene) was used to delete the consensus glycosylation sequences in the Spätzle pro-domain sequence, site 1 NQS (to NQA) and site 2 NDT (to NDA). The following primers were used according to the manufacturer's instructions: 5′-cag aag cag aat gat gac melittin Gct cgg ata cca gag acg aac c-3′ and its reverse complement for site 1; and 5′-cgg cct tgg agc gaa gat gac 149NQS (to NQA) and site 2 mutant (Y134N) into the Spätzle expression construct the following primer (and its reverse complement) was used according to the QuikChange instructions: 5′-cag ggc ctt gca ctt gga gag cac aat ccc tgc-3′. The primer 5′-gac tat cTag gac ctt cga ggc ctt cga gaa cag aag gac gac gac ggt ggt ggc tca gacgacgca and atc atc cta cta cag tgg gga gaa gca tcc gtt ggt ggc tca gacgacgca (the asterisk denotes start of the C106 sequence). As a 3′ primer primer reaction 2). The resulting PCR product was purified using Ni-NTA Superflow agarose (Qiagen) on an inertial flow filtration system (Pall).

**Generation of Toll ectodomain, Spätzle pro-protein, and generation of C106 were performed as described previously (8). The constructs Mel-C106-FLAG, Toll-C106-FLAG, and N-His-TEV-C106 were expressed by infection of Sf9 cells at 1.0 multiplicity of infection for 3 days. This working stock was used for protein expression. Expression and purification of the Toll ectodomain, Spätzle pro-protein, and generation of C106 was performed as described previously (8). The constructs Mel-C106-FLAG, Toll-C106-FLAG, and N-His-TEV-C106 were expressed by infection of Sf9 cells at 1.0 × 10^6 cells/ml and at a multiplicity of infection of 1.0 for 48 h. For large scale protein expression, 2–4 liters of Sf9 cultures were used. N-His-TEV-C106 culture supernatant was then concentrated and buffer exchanged to 150 mM NaCl, 20 mM Tris, pH 7.5, 10 mM imidazole binding buffer using a Centramate tangential flow filtration system ( Pall). Subsequently, the protein was purified using Ni-NTA Superflow agarose (Qiagen) on an AKTA FPLC system (GE Healthcare). The column was washed with 40 mM imidazole and eluted with 250 mM imidazole-containing buffer. Relevant fractions were pooled, and the protein was usually 95% pure. To obtain cleaved N-His-TEV-C106, purified protein was incubated with recombinant TEV protease (Invitrogen or produced according to Ref. 16; expression plasmid was a kind gift from D. Waugh, NCI, National Institutes of Health, Frederick, MD) as described by the manufacturer on a small scale (Fig. 4C) or at a ratio of 500 units of TEV per mg of protein for 6 h at 37 °C or 16 °C overnight in 50 mM NaCl, 50 mM Tris-HCl, pH 8.0, on a larger scale (Fig. 4D). Subsequently, TEV was removed by ion exchange or gel filtration on Superdex 200 (GE Healthcare). S2 cells were cultured and transfected as described previously (13).

**Spätzle Activation**—All expression constructs were cloned into pFastBac1 (Invitrogen) and recombinant baculoviruses generated according to the Bac-to-Bac procedure (Invitrogen). The initial viral supernatant was amplified by infecting SF9 cells grown serum-free in SF900 II medium at a multiplicity of infection of 0.1 for several days. This working stock was used for protein expression. Expression and purification of the Toll ectodomain, Spätzle pro-protein, and generation of C106 were performed as described previously (8). The constructs Mel-C106-FLAG, Toll-C106-FLAG, and N-His-TEV-C106 were expressed by infection of Sf9 cells at 1.0 × 10^6 cells/ml and at a multiplicity of infection of 1.0 for 48 h. For large scale protein expression, 2–4 liters of Sf9 cultures were used. N-His-TEV-C106 culture supernatant was then concentrated and buffer exchanged to 150 mM NaCl, 20 mM Tris, pH 7.5, 10 mM imidazole binding buffer using a Centramate tangential flow filtration system ( Pall). Subsequently, the protein was purified using Ni-NTA Superflow agarose (Qiagen) on an AKTA FPLC system (GE Healthcare). The column was washed with 40 mM imidazole and eluted with 250 mM imidazole-containing buffer. Relevant fractions were pooled, and the protein was usually 95% pure. To obtain cleaved N-His-TEV-C106, purified protein was incubated with recombinant TEV protease (Invitrogen or produced according to Ref. 16; expression plasmid was a kind gift from D. Waugh, NCI, National Institutes of Health, Frederick, MD) as described by the manufacturer on a small scale (Fig. 4C) or at a ratio of 500 units of TEV per mg of protein for 6 h at 37 °C or 16 °C overnight in 50 mM NaCl, 50 mM Tris-HCl, pH 8.0, on a larger scale (Fig. 4D). Subsequently, TEV was removed by ion exchange or gel filtration on Superdex 200 (GE Healthcare). S2 cells were cultured and transfected as described previously (13).
Spätzle Activation

Protein Biochemistry—Proteins were generally analyzed by SDS-PAGE on 10–12% acrylamide gels (Hoefer system) and bands stained with Simply Blue Stain (Invitrogen). For immunoblots, gels were transferred onto Hybond-P polyvinylidene difluoride membrane using transfer buffer with 15% methanol for which computed extinction coefficients applied. These extinction coefficients were determined by comparison with the A_{280} extinction coefficient of 6 M guanidine hydrochloride for which computed ε_{280} extinction coefficients applied. These were obtained using Prot Param based on the primary amino acid sequence of the proteins.

Analysis of Toll–Spätzle Complexes—Proteins were dialyzed into 25 mM Tris-HCl, pH 7.5, 25 mM NaCl. Subsequently, 600 pmol of cleaved or uncleaved N-His-TEV-C106 or C106 were mixed with 600 pmol of His-tagged Toll or buffer in 300 μl and incubated for 1 h at room temperature. Subsequently, the samples were analyzed by ion exchange chromatography (not shown) or native PAGE followed by anti-penta-His (Qiagen) immunoblot. After mixing with 2× native PAGE loading dye (Invitrogen), the samples were separated on 3–8% Tris acetate gel. The gel was soaked in 0.1% SDS buffer for 30 min and then washed in water two times for 5 min before the proteins were transferred to polyvinylidene difluoride membrane (GE Healthcare).

Gel Filtration Analysis—For experiments shown in Fig. 4D, a GE Healthcare Superdex 75 HR 10/30 column was used with 150 mM NaCl, 25 mM Tris-HCl, pH 7.5, as running buffer. The flow rate was 0.25 ml/min and 100 μl of Ni-NTA eluate (after TEV cleavage of N-His-TEV-C106) was loaded. GE Healthcare low molecular weight standards were used to calibrate the columns, and Unicorn software (GE Healthcare) was used to integrate peaks and determine precise elution volumes. For the experiment in Fig. 5, a mixture of cleaved Spätzle and Toll ectodomain with a slight molar excess (1.2-fold) of Spätzle over Toll was analyzed. In detail, 200 μg of TEV-cleaved Spz (3.2 nmol) were mixed with 300 μg of Toll ectodomain (2.7 nmol) in phosphate-buffered saline; the mixture was left for 2 h at room temperature and then loaded on a GE Healthcare Superdex 200 HR 10/300 column with 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, as a running buffer. The flow rate was 0.5 ml/min.

Surface Plasmon Resonance (Biacore)—All experiments were performed using a Biacore 2000 instrument with research grade CM4 sensor chips using 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20 as a running buffer (flow 10 μl/min). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide were used to activate the chip surface for amine coupling (Biacore). Sensorgrams were recorded with high sensitivity and corrected by subtraction of a control signal (empty flow cell). For experiments shown in Fig. 5A, cleaved and uncleaved N-His-TEV-C106 pro-proteins were immobilized on the chip (2000 and 500 response units, respectively; the difference in immobilization was taken into account), and 2-fold dilutions of Toll were injected starting with 19.4 μM using the settings described above. Sensorgrams were analyzed using BiaEval 3.1 software, and K_{D} estimates were determined by fitting selected curves using the simultaneous k_{a}/k_{D} function with a 1:1 binding model.

Luciferase Assays—The stably transfected cell line 648–1B6, derived from S2 cells (8), was used to monitor induction of a drosomycin-luciferase reporter gene. Cells were grown at 25 °C in Schneider’s medium (Biowest) supplemented with 10% fetal calf serum, 10^{5} units/liter penicillin, 100 mg/liter streptomycin, and 1 μg/ml puromycin. To monitor induction of the Toll pathway, cells were seeded at a density of 10^{6} cells/ml in 24-well plates and stimulated overnight by addition of recombinant Spätzle to the culture medium. Cells were then lysed in reporter lysis buffer, and luciferase activity was measured in a luminometer (BCL Book, Promega) immediately after adding the substrate (luciferin; Promega).

RESULTS

The Spätzle Pro-domain Is Required for Folding and Secretion of the Active Fragment—Previous studies (8) revealed that the pro-domain of Spätzle is natively unstructured and appears to suppress binding of the Spätzle active fragment to the Toll receptor prior to proteolytic processing. To determine whether active C106 could be synthesized and secreted without being linked to its pro-domain, we produced a construct in which C106 is linked directly to the secretory signal sequence from honeybee melittin (17) or from the Toll receptor. As shown in Fig. 1, removal of the pro-domain drastically reduced the ability of C106 to be secreted into the culture medium with most of the synthesized protein being present in the cellular fraction (compare respective lanes 2–5 in Fig. 1, A and B). By contrast, intact full-length Spätzle secretes efficiently (Fig. 1A, lanes 6 and 7). Samples were also analyzed by reducing and nonreducing SDS-PAGE, and this reveals that the characteristic intermolecular disulfide bond that normally connects two polypeptide chains in the Spätzle dimer (5, 8) is absent in the synthesized C106 protein (Fig. 1C). Instead the protein appears to form multiple disulfide-linked aggregates.

Description and Structural Analysis of Spätzle Cystine Knot Mutant Alleles—The importance of Spätzle in Drosophila was initially established by the phenotypic characterization of mutant embryos (18). In an attempt to define discrete sites in Spätzle that abolish function, we sequenced the spätzle (spz) gene isolated from three loss of function mutants generated in this mutagenesis screen (spz^{2,3,4}) (18), and a fourth allele, spz^{Efs}, which was isolated in a genetic screen for mutants on the third chromosome affected in their antifungal response.5 Two of these are point mutations (spz^{2} and spz^{Efs}), mapped to the pro-domain, providing genetic evidence that it plays an important biological role (see below and Fig. 2A). The two other alleles

5 V. Leclerc and J.-M. Reichhart, personal communication.
contain point mutations in the C106 region, Q207R (spz\(^3\)) and C232Q (spz\(^4\); Fig. 2A, see also supplemental Fig. S1). On the basis of an existing model (7), they would be expected to severely compromise the cystine knot structure because of changes in its interior packing or absence of one canonical cystine bond, respectively. Importantly, these two residues are conserved in NGF and other neurotrophins (Fig. 2B), thus highlighting the functional relevance of the structural relationships between Spätzle and neurotrophins. Comparing the NGF structure with the C106 model (7), we found that many of the conserved residues are located on the molecular surfaces of the NGF structure (Fig. 2C). In NGF, residues that mediate binding to the p75 receptor are located in two distinct binding areas (19) (Fig. 2D). It is interesting to note that, according to our analysis, equivalent residues in C106 are either conserved or charge reversals (Fig. 2E; see also “Discussion”). The Gln residue affected in the spz\(^3\) allele falls within a cluster of conserved residues involved in receptor interaction in NGF (Fig. 2B). Rather than rendering the protein dysfunctional because of defects in interior packing, as mentioned earlier, it is possible that this mutation could cause conformational changes in the surface residues surrounding the Gln residue, thereby abrogating their interactions with the Toll receptor. Further mutagenesis studies will help to ascertain if the C106 N terminus is in fact a site of interaction with Toll, analogous to that in NGF for its receptor p75.

Two Loss of Function Mutations in Spätzle Map to a Discrete Site in the Pro-domain and Are Defective in Biosynthesis and Secretion—The mutant allele, spz\(^2\), which also leads to a strongly dorsalized phenotype in the embryo similar to spz\(^3\) and spz\(^4\) (20), is a tyrosine to asparagine amino acid exchange in residue 134. This residue is located in the pro-domain 31 amino acids prior to the proteolytic processing site and is included in all known splice isoforms of Spätzle (Fig. 2A and supplemental Fig. S2). Remarkably, the fourth loss of function allele of spz, spz\(_{\text{L55}}\), contains a change in the adjacent residue proline 135 to leucine (supplemental Fig. S1). Thus two out of four sequenced mutant alleles of spz map to the pro-domain, establishing genetically its importance. To study the functional basis of the defect in Spz\(^2\), we expressed the protein in the Drosophila cell line SL2. As shown in Fig. 3, wild-type Spätzle is efficiently secreted from SL2 cells as a dimer, as indicated by the presence of a band of an apparent mass of 65 kDa (compare lane 4 with lane 2 in Fig. 3A). By contrast, Spz\(^2\) cannot be detected as dimer or monomer in supernatants (lane 6 in Fig. 3A). However, some Spz\(^2\) protein can be detected in reducing conditions (compare lanes 6 and 2 in Fig. 3B), indicating that a low level of protein can be secreted in multimeric form. Spz\(^2\) is also present in monomeric or multimeric form in cell lysates (compare lane 5 with lane 1 in Fig. 3, A and B). This result strongly suggests that the loss of function associated with the spz\(^2\) allele is caused by a defect in biosynthesis and secretion and not in signaling. We then carried out a similar analysis with Spz\(_{\text{US}}\) protein. We could not detect the presence of protein in supernatants (compare lane 3 with lane 2 and lane 6 with lane 5 in Fig. 3C), but we did observe the protein in cell lysates (Fig. 3C, lane 9). Taken together, these results identify a sequence region within the pro-domain, including residues 134 and 135, that is essential for the efficient folding and secretion of dimeric Spätzle pro-protein.

Glycosylation of the Pro-domain Does Not Affect Secretion of Spätzle—One hallmark of the primary sequence of the pro-domain is the presence of two potential N-linked glycosylation sites (Asn-49 and Asn-108; see Figs. 2A and supplemental Fig. S1), the latter being in exon H close to the site of the spz\(^2\) mutation (see Figs. S1 and supplemental Fig. S2). We next asked whether the pro-domain was modified by N-linked glycosylation during biosynthesis. Furthermore, as N-glycosylation has been shown to promote protein folding (reviewed in Ref. 21), we wanted to address whether this modification was also required for biosynthesis and secretion or whether the protein backbone by itself was sufficient to bring about this effect. To address these questions we mutated the respective serine or threonine residue in either the first or both of the acceptor sites to alanine to produce Spz\(_{\text{NGly1}}\) and Spz\(_{\text{NGly1},1+2}\). These constructs were then expressed in SL2 cells, and protein secretion was analyzed as for Spz\(^2\) and Spz\(_{\text{US}}\). As shown in Fig. 3, D and E, the protein produced by the Spz\(_{\text{NGly1}}\) construct has the same mobility in SDS-PAGE as wild-type Spätzle but Spz\(_{\text{NGly1},1+2}\) migrates faster (compare lane 4 to lanes 2 and 3 in Fig. 3, D and E). This shows that Asn-108 is a site for the addition of an N-linked glycan. Treatment of SL2 cells with the glycosylation inhibitor tunicamycin increases electrophoretic mobility of Spz similarly to the Spz\(_{\text{NGly1},1+2}\) mutation, confirming

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**FIGURE 1.** The Spätzle Pro-domain is required for efficient secretion and folding of its active fragment. A and B, FLAG peptide-tagged Spätzle constructs in which the pro-domain was deleted and a honeybee Melittin or Drosophila Toll signal peptide fusing to the C106 sequence were expressed in Sf9 cells using recombinant baculoviruses. A FLAG-tagged full-length Spätzle (Pro-Spz) was also expressed as a control. Supernatant (A) and cell pellet (B) fraction samples were taken 48 h postinfection and analyzed by immunoblot with anti-FLAG antibody. Lane 1, uninfected control; lanes 2 and 3, C106 fused to the melittin signal sequence (Mel-C106); lanes 4 and 5, C106 fused to the Toll signal sequence (Toll-C106); lanes 6 and 7, full-length Spätzle (Pro-Spz). C, analysis of the cell lysates of insect cells infected with the Mel-C106-FLAG baculovirus on reducing (lane 1) and nonreducing (lane 2) SDS-PAGE reveals protein aggregation because of incorrect disulfide bond formation, * nonspecific band cross-reacting with the anti-FLAG antibody.
that the precursor form of Spz is N-glycosylated (compare lanes 6–8 to lanes 2–4 in Fig. 3E). Fig. 3, D and E, also shows that the glycosylation of Asn-108 does not have a significant effect on the secretion of Spätzle or its assembly into a dimer.

The Pro-domain and C106 Remain Associated after Endoproteolysis—In a previous study we showed that C106 could be generated by partial proteolysis with trypsin, a treatment that completely degrades the pro-domain (8).
intention of studying the activation process in a purified system more similar to that found in vivo, we engineered an expression construct that could be processed by a specific endoprotease. We used overlapping PCR to generate a Spätzle construct in which a 7-residue recognition sequence for TEV protease (22) was inserted between the Spätzle pro-domain and C106 (see Fig. 4A). This protein should be cleavable by TEV protease in a way that mimics activation by Easter or SPE. Additionally, a hexahistidine tag was inserted between the pro-domain and TEV protease recognition site to facilitate purification and detection of the pro-domain. When expressed in insect cells using a baculovirus expression system, secretion of this protein construct (termed Spätzle N-His-TEV-C106) was directed into the supernatant where the protein was detected by anti-His antibodies and contained peptide fragments corresponding to the Spätzle pro-domain as analyzed by mass spectrometry, which confirms that this protein fragment was the Spätzle pro-domain (data summarized in Fig. 4C). An additional band (D) visible only under nonreducing conditions is likely to correspond to N-His-TEV-C106 cleaved at only one of the two sites in the dimer (Fig. 4E).

Thus, TEV cleaves N-His-TEVC106 protein, generating pro-domain and C106 fragment, which can be distinguished from each other on denaturing SDS-PAGE. After repeating the cleavage reaction with larger quantities of purified N-His-TEV-C106 (Fig. 4D, lane 1), we attempted to separate the two products under native conditions using immobilized metal affinity chromatography, on the basis that only the His-tagged pro-domain should interact strongly with the resin. After loading the column, buffers of low (10 mM) or intermediate (40 mM) imidazole concentrations were used to wash out any nonspecifically bound C106 from the column. However, C106 was not detected in the flow-through and wash fractions (not shown) but instead eluted with the His-tagged pro-domain in the 250 mM elution buffer (Fig. 4D, lane 2). This suggested a noncovalent interaction between the protein domains. The eluate was then analyzed by calibrated gel filtration, and as shown in Fig. 4D, the protein eluted as a single peak (P1) corresponding to a molecular mass of ~70 kDa. This finding indicates that C106 and the pro-domain remained noncovalently associated after backbone cleavage under native conditions. Treatment with high salt (0.5 M NaCl) or mild chaotropes (1 M MgCl2) did not dissociate the complex and C106, and the pro-domain remained associated when fractionated by ion-exchange chromatography (not shown). Furthermore, the association was specific as demonstrated by the fact that when fused to heterologous proteins (for example human MD-2) proteolysis releases the pro-domain (result not shown). These results are surprising as it had previously been assumed that Spätzle processing would lead to the spontaneous release of pro-domain from the active C106 fragment (23). Our data
rather indicate that pro-domain and C106 remain associated after backbone cleavage.

**Activated Spätzle Pro-domain-C106 Complex Binds to Toll and Causes Release of the Pro-domain**—To address whether backbone-cleaved N-His-TEV-C106 is able to bind to the Toll receptor, we used surface plasmon resonance (see "Materials and Methods"). Purified, uncleaved, and TEV-cleaved N-His-TEV-C106 were immobilized in different flow cells of a CM4 biosensor chip. As shown in Fig. 5A, purified Toll ectodomains did not bind to immobilized uncleaved N-His-TEV-C106 but bound to the cleaved TEV-C106 form. This experiment was repeated at several different concentrations of Toll ectodomain, and these data indicate a $K_D$ value of 60–100 nM for the Toll/TEV-C106 interaction, consistent with values previously found for isolated C106 (80 nM (8)). Specific binding was confirmed by band shift analysis in native PAGE (Fig. 5B). Mixtures of His-tagged Toll with purified, uncleaved, and TEV-cleaved N-His-TEV-C106 were separated by native PAGE and detected by anti-His immuno blotting. The mixture containing Toll and uncleaved N-His-TEV-C106 showed only one high molecular mass band (Fig. 5B, lane 5) that corresponded in size to the band observed for a sample consisting of Toll only (lane 1). Conversely, a mixture consisting of Toll and TEV-cleaved N-His-TEV-C106 (Fig. 5B, lane 3) showed an additional band migrating more slowly than that of Toll, suggesting that the Toll ectodomain was able to form stable higher mass complexes with the cleaved N-His-TEV-C106, in a way similar to C106 (Fig. 5B, lane 7) (8).

We next sought to determine whether the cleaved pro-domain-C106 complex is active in signaling using an SL2 cell line derivative containing a luciferase reporter gene under the control of the drosomycin promoter (8). This cell line is responsive to the addition of purified C106 from which the pro-domain had been removed by tryptic digestion. The addition of TEV-treated pro-domain-C106 complex (N-His-TEV-C106) to the cell culture medium resulted in a dose-dependent activation of the reporter, with an ED$_{50}$ value similar to that of isolated C106. On the other hand, like the unprocessed full-length Spätzle pro-protein, untreated N-His-TEV-C106 was inactive (Fig. 5C). Consequently, we conclude that backbone cleavage renders the Spätzle active and that noncovalent association between pro-domain and C106 does not suppress signaling.

These observations prompted us to investigate whether binding of Toll to cleaved pro-domain/C106 displaced the pro-domain from the complex or leads to the formation of a complex consisting of both Spätzle domains and the Toll ectodomain. Purified Toll ectodomain was mixed with a molar excess of cleaved pro-domain-C106 complex, and the products were separated by gel filtration. As shown in Fig. 5D, the mixture elutes from the column in two discrete peaks; the first peak (high $M_r$; lanes 1–5) contained only Toll ectodomain and C106. The second peak (lower $M_r$; lanes 7–9) contained pro-domain and excess C106. This suggested that Toll binding to the activated pro-domain-C106 complex released the pro-domain (shown schematically in Fig. 5E). This was in agreement with the earlier observation that the higher molecular weight complex formed in Toll+cleaved N-His-TEV-C106 mixtures (Fig. 5B, lane 3) showed a similar electrophoretic mobility compared with that in mixtures containing only Toll and the C106 portion (Fig. 5B, lane 7). We conclude from these experiments that binding of the cleaved Spätzle complex to Toll ectodomain does not lead to the formation of a ternary complex but causes release of the pro-domain, leaving C106 alone bound to Toll.

**DISCUSSION**

**Functions of the Spätzle Pro-domain in Biosynthesis and Secretion**—In this study we have shown that the pro-domain of Spätzle is required for efficient folding and secretion. Like Spätzle, other related cystine knot proteins such as vertebrate neurotrophins are produced as precursors and processed after secretion by proteolysis into an active form. Sequences within the precursors are known to be important for secretion and biosynthesis (24) as the NGF pro-protein but not the active

![Diagram](image-url)
fragment can be refolded into a functional protein when produced in an insoluble form by *Escherichia coli* cells (25). Taken together these results suggest that the pro-domain of this family of proteins function as folding enhancers for their cognate active domains during the process of biosynthesis and secretion. Our finding that Spätzle C106 is not efficiently secreted when fused to only an endoplasmic reticulum signal sequence provides an explanation for the puzzling observation that an mRNA with a similar arrangement of signal sequence and C106 caused lateralization (because of activation-independent, and therefore spatially unrestricted, signaling at very low level) when injected into Spätzle mutant embryos, whereas the expectation is that it should cause a strong ventralization (spatially unrestricted, high Toll activation) if it were to be secreted (26). Our data also provide an explanation for the phenotype of flies carrying the *spz* or *spz*mutation, and map a region of importance for C106 folding to two residues within exon H of the *spätzle* transcription unit (see supplemental Figs. S1 and 2). It is interesting to note that exon H encodes part of the cystine knot sequence and is included in all *spätzle* splice isoforms, which may reflect its functional requirement.

**Implications of an Activated Pro-domain-C106 Complex**—It was previously assumed that Easter or SPE cleavage at a specific site rendered the Spätzle protein active and led to the simultaneous release of its pro-domain, which was then free to diffuse away from the site of activation and exert the function of an inhibitor (23) (reviewed in Ref. 27). The notion that endoproteolytic backbone cleavage at a defined site is sufficient to activate Spätzle is supported by our experiments using a Spätzle construct with an “Easter-like” (TEV) cleavage site. Contrary to expectations we found that the pro-domain and C106 remain stably associated with each other after proteolytic activation of purified Spätzle pro-protein *in vitro*. This association was also shown in embryo extracts from transgenic flies, which expressed a Spätzle form in which pro-domain and C106 were individually tagged.

Given that the pro-domain does not remain associated when fused to a heterologous protein, it is likely that this interaction is specific. This result is contrary to a previous finding that “polarizing” activity (C106) purified from embryos was not bound to N-terminal sequences (18). However, the purification process used involved boiling embryonic extracts at an acidic pH, a treatment that is likely to cause dissociation of the non-covalent complex we have characterized. Several conclusions can be drawn from our observations. First, the pro-domain does not interfere with the activation of Toll. Studies using Western blot analysis showed that pro-domain remains stable after cleavage by Easter and also presented evidence that it can act as a negative regulator of Toll signaling, either by directly antagonizing the receptor or by modulating the activity of the protease cascade (23, 28). As isolated C106 and the pro-domain/C106 activate Toll with very similar characteristics, it appears that
the pro-domain does not interfere with signaling at the level of the receptor. Indeed we did not observe any antagonist activity when SL2 cells were stimulated with 10 nM C106 in the presence of increasing concentrations of purified pro-domain (data not shown). Studies are under way in our laboratory to ascertain its effect on Easter activation or the Spa¨tzle processing reaction. The idea of a diffusible inhibitor fits the theoretical proposals of pattern formation involving lateral inhibition (see for example Ref. 29) and can be reconciled with our finding that the pro-domain is released from C106 after binding to the Toll ectodomain. Once released, pro-domain would be free to diffuse in the perivitelline space and cause inhibition at lateral positions in the embryo. This is an attractive idea as released pro-domain would be about a quarter the mass of the pro-domain-C106 dimer complex and therefore able to diffuse more rapidly, a key requirement of a lateral inhibition model. On the other hand, the release of a negative regulator (Spa¨tzle pro-domain) at all positions of Spa¨tzle activation would not be favorable, as this could preclude maximum pathway activation at ventral positions. Our data suggest that pro-domain would not be released until after receptor binding, ensuring a high initial ventral signal followed by subsequent lateral inhibition. Assuming that cleavage of Spa¨tzle pro-protein leads to the generation of an activated pro-domain-C106 complex (as opposed to free C106 as previously assumed) the active species would be a slowly diffusing, 80-kDa protein complex, not a highly diffusible 24-kDa C106 fragment. This could have a significant effect on the availability of Spa¨tzle ligand in more dorsal positions and the properties of the dorsal gradient generated in the embryo as even small changes in the shape or slope of the dorsal gradient have profound effects on development of the dorsoventral axis (23). The existence of a pro-domain-C106 complex prior to receptor activation may further enforce maximum Toll activation at ventral positions by limiting the diffusion of the Toll activator before engagement to the receptor. Based on these new findings, we propose an extended model of Spa¨tzle activation in Drosophila dorsoventral patterning (Fig. 6).

Functional and Structural Similarities of Spa¨tzle to Other Cystine Knot Growth Factors—In addition to Spa¨tzle, two other cystine knot proteins are known to require the pro-domain for folding (reviewed in Refs. 30 and 31). Our previous data have suggested that the pro-domain of Spa¨tzle is relatively unstructured or disordered, being devoid of secondary structure elements or globular domains (8), properties that were recently reported for the NGF pro-peptide also (32).

Our results suggest that like protease zymogens such as chymotrypsinogen (33), proteolytic processing of Spa¨tzle pro-protein induces a conformational rearrangement that exposes the binding determinants of C106 and makes them available to bind the Toll ectodomain. In the structure of NGF bound to the neurotrophin receptor p75 (19), 23 residues of the NGF monomer contribute to binding, and these are localized in two discrete regions of the molecules, including the N terminus (Fig. 2D). Interestingly, a number of residues in both sites (but not the N-terminal residue) are conserved or are charge reversals compared with Spa¨tzle C106 (Fig. 2E). It is therefore plausible that binding of C106 to the Toll ectodomain is similar to that of NGF to p75, involving the burial of hydrophobic surfaces together with electrostatic complementarity provided by surrounding salt bridges and hydrogen bonds (Fig. 2, D and E). Furthermore, work just published shows that pro-peptide of NGF is also stably associated with the cystine knot. The site of interaction was mapped to a conserved, surface-exposed hydrophobic residue (Trp-21; see Fig. 2E) (34). We plan to map residues involved in the binding of C106 to the receptor using site-directed mutagenesis and probe the conformational rearrangements that may occur during Spa¨tzle processing using mass spectrometry. The fact that the Spa¨tzle active fragment associates noncovalently with its pro-domain is also reminiscent to the post-proteolytic association of transforming growth factor-β with its pro-peptide (31). Also termed latency-associated peptide, the pro-peptide is bound noncovalently and is released upon acid treatment, further proteolysis or conformational changes induced by molecular binding (31). One may speculate whether the properties of (i) their pro-peptides with regard to folding and regulation of activation and (ii) their active fragments regarding receptor binding and activation may be common features among certain vertebrate and invertebrate cystine knot growth factors. Our findings provide further
examples of these interesting structural and functional similarities within this group of growth factors.

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