Activation of a protease cascade involved in patterning the *Drosophila* embryo

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Dorsoventral patterning of the *Drosophila* embryo is initiated by a ventralizing signal. Production of this signal requires the serine proteases Gastrulation Defective (GD), Snake, and Easter, which genetic studies suggest act sequentially in a cascade that is activated locally in response to a ventral cue provided by the *pipe* gene. Here, we demonstrate biochemically that GD activates Snake, which in turn activates Easter. We also provide evidence that GD zymogen cleavage is important for triggering this cascade but is not spatially localized by *pipe*. Our results suggest that a broadly, rather than locally, activated protease cascade produces the ventralizing signal, so a distinct downstream step in this cascade must be spatially regulated to restrict signaling to the ventral side of the embryo.

serine protease | dorsoventral axis | transfection

Embryonic pattern formation depends on localized signals that inform cells of their relative positions within the developing embryo. An important model for studying the molecular mechanisms by which such signals are controlled in space and in time is the establishment of dorsoventral polarity in the *Drosophila* embryo. Here, cell fates along the dorsoventral axis are determined by an intracellular morphogen gradient, which in turn is dictated by an extracellular morphogen gradient of the ligand that activates the receptor Toll (1). The Toll ligand, which conveys a "ventralizing" signal necessary for ventral and lateral development, is activated by proteolytic processing of the Spätzle protein, a reaction that apparently occurs shortly after fertilization and only on the ventral side of the embryo. This crucial processing event requires four serine proteases—Nudel, Gastrulation Defective (GD), Snake, and Easter—and a ventrally restricted factor provided during oogenesis by the *pipe* gene (2). An important question has been, how do these components function to process Spätzle at the right time and place to establish embryonic dorsoventral polarity?

Genetic and molecular studies have suggested that the proteases in the Toll signaling pathway function sequentially in a proteolytic cascade, as seen for mammalian blood clotting. In such a cascade, the proteases exist as zymogens that become activated by cleavage at a defined site between a prodomain and the catalytic domain, and one protease activates the next downstream protease in the cascade (3). By genetic criteria, Nudel is the most upstream protease in the Toll signaling pathway, followed by GD, then Snake, and finally Easter, the protease that can process Spätzle to a biologically active form (4–7). The zymogen forms of Snake and Easter, as well as the Spätzle protein, appear to be freely diffusible in the extracellular perivitelline space surrounding the embryo (4, 8, 9). Thus, the activities of these proteases must be ventrally restricted to confine Toll ligand production to the ventral side of the embryo. Spatial control of the blood-clotting proteases, which are also diffusible as zymogens, is partly achieved through dependence of the first and all subsequent zymogen-activation steps on membrane-bound cofactors that are localized to the site of blood-vessel injury (10). By analogy, it has been proposed that the ventrally restricted factor provided by the *pipe* gene functions as a cofactor necessary for activation of the proteolytic cascade that produces the Toll ligand (2).

As the earliest acting protease in the Toll signaling pathway, Nudel would be expected to play an important role in triggering Toll ligand production. The Nudel protease is autoactivated without requiring the activities of the other proteases at the beginning of embryogenesis, which is consistent with its proposed role as the initiator of a protease cascade in which it activates the next downstream protease, presumably GD (11). However, Nudel protease activation does not seem to be ventrally restricted or regulated by *pipe*. We have recently shown that the Nudel protease is also required for modification of the extracellular matrix, raising the possibility that Nudel acts indirectly in dorsoventral patterning (12).

Our recent studies have suggested that GD, the next downstream protease from Nudel, plays a more clearly instructive role in triggering the Toll signaling pathway (6). Unusual structural features of GD have made it uncertain whether this protein functions as a protease (13). We obtained evidence that GD is a functional protease, which is probably activated by zymogen cleavage at an atypical site that has yet to be defined. We also showed that the level of GD determines the strength of the ventralizing signal, presumably by controlling the activation of the downstream proteases Snake and Easter. Finally, we showed that GD at high levels can ectopically induce the ventralizing signal in the absence of *nudel* or *pipe* function. This observation suggested that GD activity is normally restricted to the ventral side of the embryo by the action of these genes, which might control the proteolytic activation of the GD zymogen.

Here, we demonstrate by using a cultured cell expression system that the GD zymogen can directly trigger a proteolytic cascade in which it activates Snake, which in turn activates Easter, thereby providing evidence for biochemical interactions among these proteases. During activation of this cascade, the GD zymogen is cleaved to smaller forms that are also detected during early embryogenesis. We show that the GD zymogen is not prelocalized to a ventral site, and that GD zymogen cleavage, although requiring the Nudel protease, does not depend on *pipe*. Surprisingly, these results suggest that the protease cascade producing the ventralizing signal is initiated by a spatially uniform cleavage of the GD zymogen, with spatial regulation of a downstream step determining where signaling is to occur.

Materials and Methods

Fly Stocks. The wild-type strain was Oregon-R. Transgenic flies were generated in a w^{1118} background (14). The mutations and allelic combinations used here are pip^{386}/pip^{664}, snk^{073}/snk^{073}, $ndl^{111}/Df(3)CH12$ (11), and gd^{9}/gd^{9} (15).

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Abbreviations: GD, gastrulation defective; SNKS-A, catalytically inactive Snake; HA, hemagglutinin; EAS-A, catalytically inactive Easter substrate.

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Constructs. The EAΔN and SNKΔN constructs were made as described (4, 7). The GDΔN1, GDΔN2, and NDL-PD constructs were similarly made to link the Easter signal sequence to amino acid 212 or 254 of GD, or amino acid 1145 of Nudel (the C terminus of Nudel's protease domain was arbitrarily set at amino acid 1387). Full-length GD, Snake, and Easter constructs were generated by PCR of the cDNAs and were tagged at the C terminus with a 9-aa hemagglutinin (HA) epitope, an 83-aa fragment containing five copies of the Myc epitope and six His residues, and an 8-aa FLAG epitope, respectively (16). Constructs were expressed under the control of the metallothionein promoter in the pRmHa-3 vector (17) or the related pRmHa-3N vector containing a *Not*I site. The HA-tagged wild-type GD construct was also inserted into the pGerm8 transformation vector (18).

Transfections. Cultured *Drosophila* S2 cells (American Type Culture Collection) 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 vector plasmid 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 $CuSO_4$; cells and media were harvested 18–20 h later.

Immunomethods. Commercial antibodies included rabbit anti-Myc (Santa Cruz Biotechnology) and mouse monoclonal anti-HA (Covance, Princeton, NJ). The polyclonal Easter antibody was provided by D. Morisato (Harvard Medical School, Boston). Rabbit anti-GD was prepared by using a GD-trpE fusion protein (GD, amino acids 118–285), as described (19).

Western blots of proteins separated on SDS/13% PAGE gels and immunostaining were performed as described (11). Immunostaining of endogenous GD gave results similar to those shown for GDHA. For immunoprecipitations, ovaries or laid eggs were homogenized in lysis buffer (25 mM Tris, pH 7.5/0.15 M NaCl/0.3% Nonidet P-40/1 mM EDTA/1 mM EGTA/0.2 mM *N*-ethylmaleimide) containing protease inhibitors, and a soluble fraction was reacted with 1:100 monoclonal anti-HA and protein G-agarose (Sigma).

Results

GD Triggers a Proteolytic Cascade by Activating Snake That in Turn Activates Easter. To test whether GD, Snake, and Easter function in a proteolytic cascade, as suggested by genetic studies, we examined their biochemical activities by coexpression in *Drosophila* S2 cells. In many experiments, we used as substrates inactive forms of Easter and Snake in which the catalytic serine had been mutated to an alanine residue. This strategy was adopted because the active forms seemed to be unstable after zymogen cleavage and thus difficult to detect (see below).

To test first whether GD activates Snake, we examined by Western blotting the processing of catalytically inactive Snake (SNKS-A) after it was expressed with GD (Fig. 1*A*). Because our previous studies had suggested that GD activity depends on proteolytic processing, we initially tested the activities of two truncated GD forms. GDΔN1 lacks amino acids 1 through 211 after signal sequence cleavage, where Lys-211 is the nearest basic residue N-terminal to the conserved catalytic domain and thus might serve as a cleavage site for GD activation (6, 13). GDΔN2 lacks amino acids 1 through 253, corresponding to a potential cleavage site suggested by Konrad *et al.* (13) at the beginning of the conserved catalytic-domain sequence. Expression of SNKS-A with either of these GD forms resulted in the appearance of a 40-kDa C-terminal Snake polypeptide, approximately the size predicted for the Snake catalytic domain of 29 kDa plus a Myc-based C-terminal epitope tag of 9.5 kDa (Fig. 1*A*, lanes 3 and 4). In contrast, we found that Snake cleavage did not occur

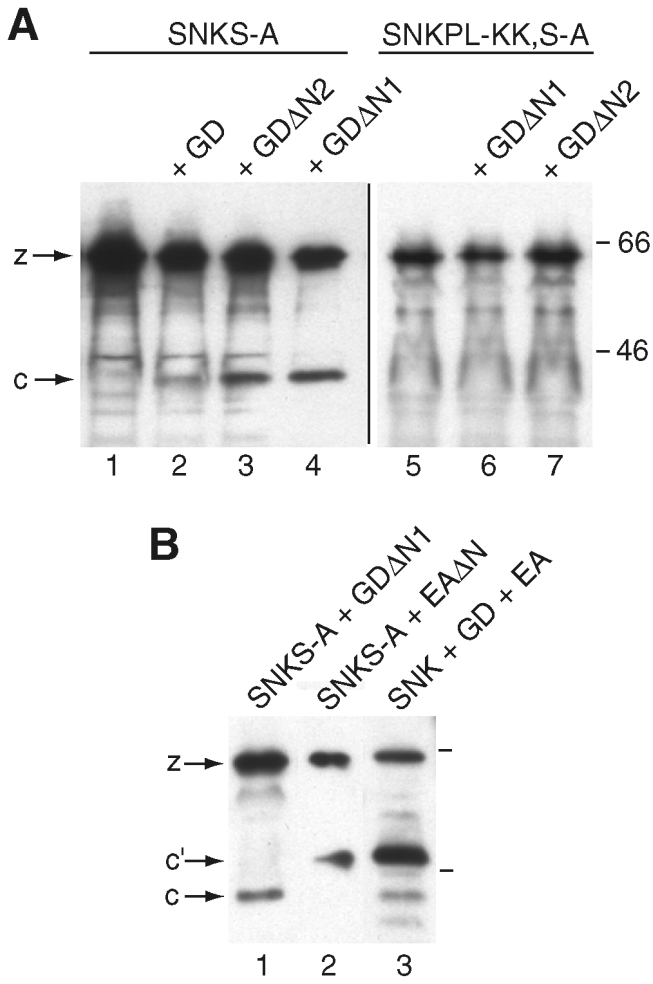

Fig. 1. Activation of Snake by GD. Western blots with anti-Myc antibody to detect Myc-tagged Snake in transfected S2 cells. (*A*) SNKS-A is cleaved from zymogen form (z) to 40-kDa C-terminal product (c) when expressed with preactivated GD (GDΔN1 and GDΔN2) or the GD zymogen (lanes 1–4). Double catalytic and zymogen-activation Snake mutant (SNKPL-KK, S-A) is not cleaved (lanes 5–7), suggesting that GD cleaves Snake at the zymogen-activation site. (*B*) Coexpression of SNKS-A and preactivated GDΔN1 (lane 1) produces the 40-kDa Snake product (c), whereas coexpression of SNKS-A and preactivated Easter, EAΔN (lane 2), produces the 50-kDa Snake product (c′) resulting from cleavage at a site other than the zymogen-activation site. Both cleaved forms are seen when the wild-type GD, SNK, and EA zymogens are coexpressed (lane 3).

when the Snake substrate contained a mutation in the zymogen-activation site (Fig. 1*A*, lanes 6 and 7; ref. 20). These results suggest that GD cleaves Snake at its zymogen-activation site. To determine whether this interaction between GD and Snake is specific, we examined the processing of SNKS-A by a preactivated form of Easter, EAΔN, containing only the functional catalytic domain after signal-sequence cleavage (4). EAΔN was not able to promote the processing of Snake at the zymogen-activation site (Fig. 1*B*, compare lanes 1 and 2), but rather induced cleavage at a distinct site to generate a larger Snake fragment, thereby demonstrating that the Snake zymogen is specifically activated by GD. This result also suggests that Snake is cleaved by Easter within the prodomain, which could either enhance or inhibit further Snake activation by GD as part of a positive or negative feedback loop (see *Discussion*).

Surprisingly, the GD zymogen was also able to induce the cleavage of SNKS-A to generate the 40-kDa Snake catalytic

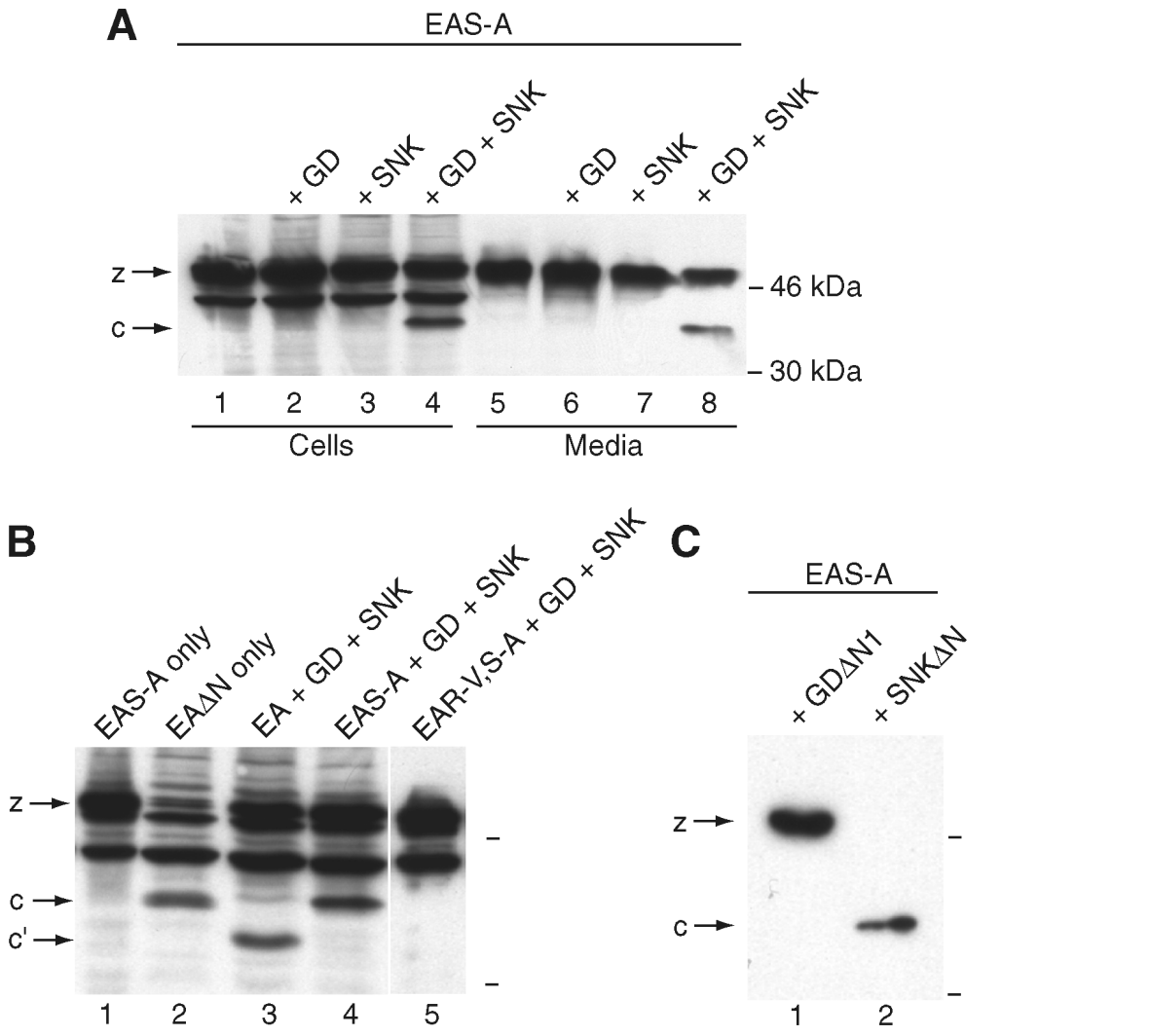

Fig. 2. Activation of Easter by Snake when all three zymogens are coexpressed. Western blots with anti-Easter antibody to detect Easter in cell pellet (*A* and *B*) and culture medium (*A* and *C*) after transfection of S2 cells. (*A*) EAS-A is cleaved from zymogen form (z) to 35-kDa C-terminal product (c) when expressed with GD + SNK zymogens (lane 4 or 8), but not when expressed with only GD (lane 2 or 6) or SNK (lane 3 or 7). (*B*) When expressed with GD + SNK zymogens, EAS-A is cleaved to generate a 35-kDa product (c, lane 4) that migrates similarly to EAΔN, the functional Easter catalytic domain (lane 2); wild-type EA is cleaved to generate predominantly a smaller product (c′, lane 3). Double catalytic and zymogen-activation mutant EAR-V,S-A is not cleaved (lane 5). (*C*) EAS-A is cleaved when expressed with preactivated SNK (SNKΔN; lane 2) but not when expressed with preactivated GD (GDΔN1; lane 1).

domain, although at lower levels than those seen with the truncated GD forms (Fig. 1*A*). This observation led us to test whether coexpressing the zymogen forms of all three proteases could result in the activation of Easter. We found that a catalytically inactive Easter substrate (EAS-A) was cleaved when expressed with both GD and Snake but not when expressed with either GD or Snake alone (Fig. 2*A*, lanes 1–4 and 5–8, respectively). EAS-A was cleaved to a 35-kDa form representing a C-terminal fragment, as judged by its reactivity with an antibody against a C-terminal FLAG-epitope tag (not shown). This C-terminal fragment migrated similarly to EAΔN (Fig. 2*B*, compare lanes 2 and 4; ref. 4). In addition, mutation of the zymogen-activation site in EAS-A eliminated production of the 35-kDa polypeptide when the mutant protein was expressed with GD and Snake (Fig. 2*B*, lane 5). These results suggest that, when expressed with GD and Snake, Easter can be cleaved at its zymogen-activation site and therefore activated.

To determine whether Snake is responsible for the cleavage of EAS-A in this experiment, we expressed EAS-A with either preactivated Snake (SNKΔN; ref. 7), or preactivated GD (GDΔN1). SNKΔN promoted efficient conversion of EAS-A to the 35-kDa C-terminal form, whereas GDΔN1 was unable to promote cleavage of this substrate (Fig. 2*C*, compare lanes 1 and 2), supporting the conclusion that Snake is the protease that activates Easter.

In the experiments described above, we could not directly demonstrate that Easter had become an active protease because the catalytically inactive EAS-A substrate was used. However, similar experiments using wild-type Easter provided indirect evidence that a proteolytically active Easter is generated. When the wild-type Easter zymogen was expressed with both GD and Snake, but not either alone, a fragment of 32 kDa lacking the C-terminal FLAG tag was the most abundantly cleaved form of Easter detected, although the 35-kDa form could still be detected, albeit weakly (Fig. 2*B*, lane 3, and data not shown). These results suggest that the active Easter protease is unstable and may undergo further processing in a reaction that requires its own proteolytic activity. In further support of the conclusion that both Snake and Easter become activated when expressed with the GD zymogen, we found that both the 40-kDa Snake catalytic-

domain fragment generated by GD and the 50-kDa Snake product generated by Easter could be detected in cells expressing all three wild-type zymogens (Fig. 1*B*, lane 3).

GD Is Cleaved When It Triggers the Activation of Snake and Easter. The results described above suggested that the GD zymogen has some activity against Snake, but that the processing of GD gives rise to more active forms. To determine whether the GD zymogen undergoes proteolytic processing when it promotes the activation of Snake and Easter, we analyzed the processing of GD by Western blotting (Fig. 3*A*). When expressed alone, GD exists predominantly as the full-length zymogen, although minor processed forms can be detected (lane 1). However, when expressed with Snake or both Snake and Easter, two cleavage products at 46 kDa and 50 kDa are seen prominently (lanes 3 and 4), whereas little cleavage is seen if GD is expressed with Easter (lane 2). Both processed forms react with an antibody to a C-terminal HA tag present in the protein, indicating that they represent C-terminal fragments of GD, and the 46-kDa cleavage product is similar in size to GDΔN1 (not shown).

The cleavage of GD in these reactions depends on GD's own catalytic activity as well as that of Snake (Fig. 3*B*, lanes 1–3). This result suggests that GD can promote its own activation, perhaps through self-cleavage or positive feedback regulation involving the downstream proteases. To test whether GD is cleaved by itself, Snake, or Easter, we coexpressed the catalytically inactive GD substrate (GDS-A) with preactivated GDΔN2, SNKΔN, or EAΔN. We also examined whether GD is cleaved by Nudel's protease domain, NDL-PD. All of the proteases appeared capable of cleaving GD (Fig. 3*C*, lanes 1–5). However, SNKΔN generated both 46- and 50-kDa forms of cleaved GD, previously seen when the GD, Snake, and Easter zymogens are coexpressed (Fig. 3*A*), whereas NDL-PD and GDΔN2 primarily produced the 46-kDa form and EAΔN primarily produced the 50-kDa form.

Regulated Cleavage of GD *in Vivo* Depends on *nudel* but Not *pipe*. Our earlier studies suggested that GD activity is restricted to the ventral side of the embryo by the action of *pipe* as well as *nudel* (6). To test whether proteolytic activation of GD is regulated by these genes, we examined processing of GD in wild-type and mutant backgrounds.

Western blot analysis of GD present in wild-type ovary extracts identified a polypeptide of 64 kDa, the size predicted for the GD zymogen (Fig. 4*A*, lane 1). This polypeptide was absent in ovary extracts derived from gd^9 mutant females and was replaced by a smaller polypeptide (Fig. 4*A*, lane 2), whereas in several other *gd* mutants, no specific GD polypeptide could be detected (not shown). In no case did we detect 27- through 34-kDa GD species in ovary extracts, as described earlier by Konrad *et al.* (13). Together, our findings suggest that GD is present principally as a full-length zymogen during oogenesis.

To avoid strong background bands in the 46-kDa region of gels that complicated detection of endogenous GD, we used a transgenic fly strain expressing the HA-tagged GD used in our *in vitro* experiments, which rescued wild-type *gd* function in *gd* mutant embryos (not shown). As in the case with endogenous GD, we detected a single 64-kDa band corresponding to the GDHA zymogen by immunoprecipitation from ovary extracts (Fig. 4*B*, lane 2).

In contrast to the undetectable level of GD processing during oogenesis, a dramatically different picture was seen in embryogenesis. In extracts of laid eggs containing GDHA, we detected a major species of 46 kDa, similar in size to the smaller of the two cleaved GD forms seen *in vitro*, in addition to the GD zymogen (Fig. 4*B*, lanes 4–7). The 46-kDa form was strongly detected in the first hour of embryogenesis and persisted for the first 3 h of embryonic development.

We next examined the processing of GDHA in *nudel* and *pipe* mutant backgrounds. The 46-kDa form could not be detected in *ndl* mutant eggs (Fig. 4*C*, lane 1); this finding is consistent with genetic data suggesting that the Nudel protease is required for the activity of GD and the other proteases. In contrast, the 46-kDa cleaved GD product was detected in *pip* mutant eggs

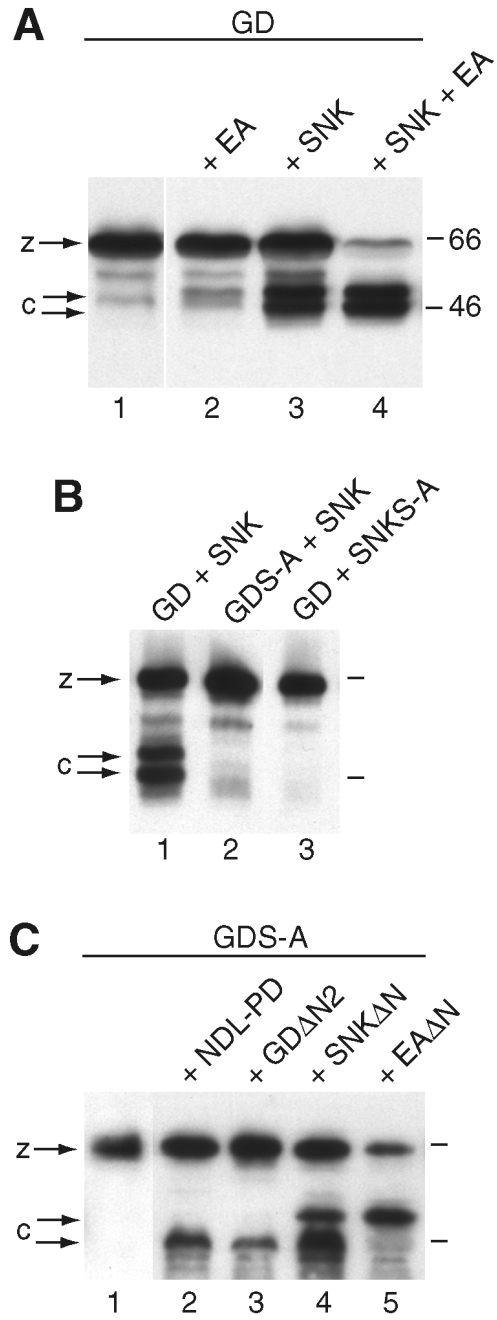

Fig. 3. Cleavage of GD *in vitro*. Western blots with anti-GD antibody to detect HA-tagged GD in culture medium of transfected S2 cells. (*A*) GD zymogen (z) is efficiently cleaved to generate two C-terminal forms (c) at 46 kDa and 50 kDa, when expressed with the Snake zymogen (lane 3) or both Snake and Easter zymogens (lane 4) but not when expressed with just the Easter zymogen (lane 2). These cleaved forms are also recognized by an anti-HA antibody (not shown). (*B*) Cleavage of GD (lane 1) requires the catalytic activity of GD itself (lane 2) and Snake (lane 3). (*C*) GDS-A is cleaved primarily to the 46-kDa form when expressed with Nudel's protease domain NDL-PD (lane 2) or GDΔN2 (lane 3); to both the 46- and 50-kDa forms when expressed with SNKΔN (lane 4); or to primarily the 50-kDa form when expressed with EAΔN (lane 5).

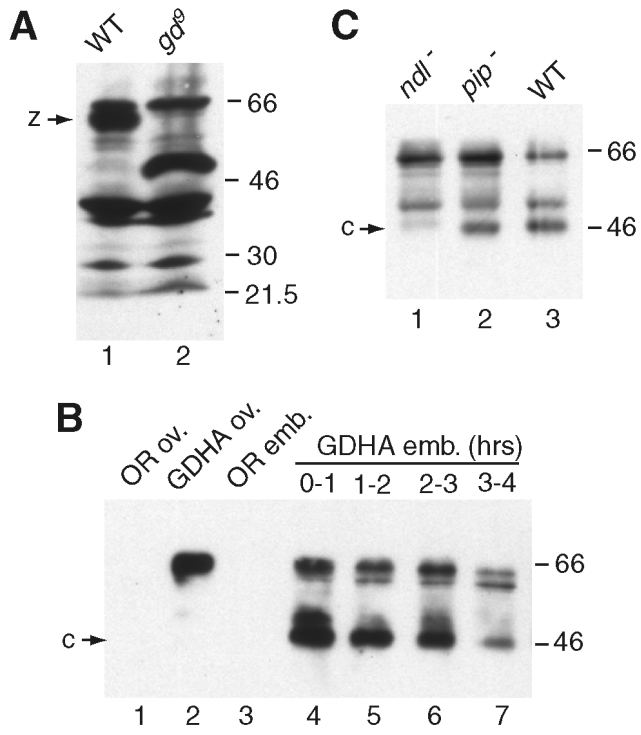

Fig. 4. Cleavage of GD *in vivo*. (*A*) Anti-GD Western blot of ovary extracts from wild-type (WT; lane 1) and gd^9 (lane 2) mutant females. GD zymogen (z) is absent and replaced by a smaller polypeptide in gd^9. (*B*) Time course of GDHA cleavage. Shown are immunoprecipitations with anti-HA antibody of extracts from ovaries (lanes 1 and 2) or laid eggs (lanes 3–7) at indicated times of embryonic development (lane 3 = 0–4 h), which have been probed on Western blots with anti-GD. The same band pattern is seen when the antibodies are used in reverse order (not shown), indicating that the 46-kDa band (c) represents a C-terminal GD fragment. (*C*) The 46-kDa form of GDHA (c) is seen in wild-type (WT; lane 3) and pip^- (lane 2) but not in ndl^- (lane 1) background. Immunoprecipitations of egg extracts and blots were performed as in *B*.

(Fig. 4*C*, lane 2). Surprisingly, this result suggests that GD processing *in vivo* occurs independently of the spatial cue generated by the action of *pipe*.

GD Is Localized Uniformly on the Oocyte Surface. We also examined the localization of GD during oogenesis to test the possibility that prelocalization of GD to a ventral site is important for determining the spatial distribution of the ventralizing signal. We found that after its secretion in late oogenesis, GD is present uniformly within the perivitelline space surrounding the oocyte (Fig. 5*A*). The distribution of GD was indistinguishable from that of Nudel (Fig. 5*B*), suggesting that GD is localized at the oocyte surface. In biochemical fractionation experiments (not shown), GD was found in a 16,000 × *g* pellet fraction from which it could be released only by strong denaturing agents or high pH. These findings suggest that the GD zymogen, apparently like the Snake and Easter zymogens, is uniformly distributed in the perivitelline space and, like Nudel, is not freely diffusible within this compartment (4, 8, 11).

Discussion

GD Activates a Proteolytic Cascade. Prevailing models based largely on genetic data have suggested that GD, Snake, and Easter function in a cascade of sequential zymogen activation, like the mammalian proteases involved in blood clotting (3, 21). The *in vitro* experiments described here provide biochemical evidence in support of this idea. Although our data strongly imply that GD

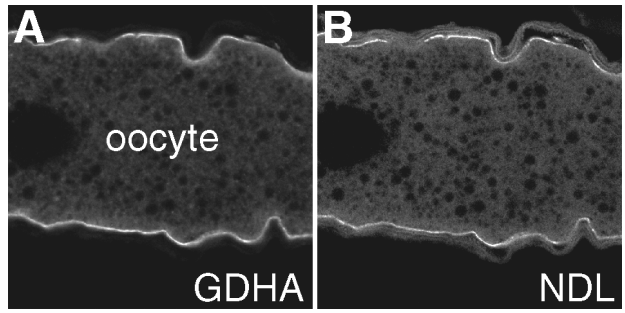

Fig. 5. GD is uniformly localized on the oocyte surface. Immunolocalization of GDHA in a sectioned stage 14 egg chamber representing a mature egg within the ovary. Anti-GD antibody detects GD (*A*) on oocyte surface in a pattern overlapping that of Nudel (*B*), which was visualized with an antibody to its protease domain (11).

directly activates Snake and that Snake directly activates Easter, experiments with purified proteins will be necessary to obtain proof of an enzyme–substrate relationship between these proteases.

A surprising finding is that the cascade of GD, Snake, and Easter is activated when all three proteases are coexpressed as zymogens (Fig. 2). One possible explanation is that high concentrations of these proteases generated by overexpression in S2 cells promote protein–protein interactions that are important for cascade activation *in vivo*. For example, the proteases may normally be activated after being brought together in zymogen-activation complexes, as is the case for the proteases involved in blood clotting (10).

GD is cleaved when it triggers the activation of Snake and Easter *in vitro*. We think that cleavage is important for activation of GD's proteolytic activity. This conclusion is supported by the findings that a cleaved form of GD, but not the zymogen, reacts with active-site inhibitors specific for active serine proteases (6), and that two truncated forms of GD are more active than the zymogen in processing Snake (Fig. 1*A*). Because GD lacks a typical zymogen-activation site, as in Snake or Easter, it is unclear where GD is cleaved (13). The ability to generate more active forms of GD by truncation at two different sites may indicate that GD does not normally require cleavage at a single specific site to become activated.

Although present as a zymogen during oogenesis, GD apparently is cleaved during early embryogenesis to a smaller polypeptide similar in size to the 46-kDa form generated during the activation of Snake and Easter *in vitro* (Figs. 3 and 4). This cleavage must be significant for GD function *in vivo*, as it relies on the activity of the Nudel protease that acts genetically upstream of GD in the Toll signaling pathway. Additionally, the cleaved form of GD is detectable throughout the first 3 h of embryonic development when the Toll ligand is produced (9), which is consistent with it being the functional GD form that activates the downstream proteases. Although further studies are required to definitively demonstrate enzymatic activity, these findings strongly suggest that the smaller form of GD present during early embryogenesis represents activation of the GD zymogen.

How is the GD zymogen normally cleaved? In using the coexpression assay, we observed that the Nudel protease and preactivated forms of GD and Snake are capable of inducing the cleavage of GD to the 46-kDa form (Fig. 3*C*). However, only the Nudel protease seems to be essential for this cleavage *in vivo* (Fig. 4*C*), because mutagenesis of GD's catalytic serine or a mutation in the *snake* gene did not block the appearance of the 46-kDa GD polypeptide in the early embryo (data not shown). A model consistent with all of our data is one in which the Nudel

protease, detectable during the first 2 h of embryogenesis (11), directly cleaves the GD zymogen. Once activated, GD and perhaps even the downstream proteases could promote further GD-zymogen cleavage through a positive feedback loop. In the coexpression experiments carried out in the absence of the Nudel protease, a protease normally present in the S2 cells may have cleaved a small amount of GD sufficient to trigger the subsequent activation of Snake and Easter. This explanation is compatible with the idea that GD can be activated by the cleavage of an unusually labile region rather than at a specific site in its primary structure.

Regulation of the Dorsoventral Protease Cascade. We have previously shown that GD activity seems to be restricted to the ventral side of the embryo (6). One possible explanation is that GD is prelocalized to a ventral site (11, 22). We have now shown to the contrary that the GD zymogen is uniformly distributed at the oocyte surface, similarly to Nudel (Fig. 5). Alternatively, GD could be proteolytically activated only on the ventral side of the embryo (11). Our current results argue against this possibility, because GD-zymogen cleavage is not regulated by *pipe*, the key gene that ventrally restricts Toll ligand production (Fig. 4*C*). Activation of the Nudel protease, required for GD cleavage, is also not regulated by *pipe* (11).

If cleavage of Nudel and GD, necessary to trigger the proteolytic cascade leading to the Toll ligand, is not ventrally localized, then how could Toll ligand production be restricted to the ventral side of the embryo? The last step in the cascade, processing of Spätzle to the active Toll ligand, is controlled by *pipe* (9). Thus, spatial regulation could occur at an intermediate step, perhaps involving the proteolytic activation of Snake or Easter. For example, the GD protease, although broadly activated, may activate Snake only when its substrate is associated with the ventral factor provided by *pipe*.

The mammalian blood-clotting cascade normally generates a blood clot only at the site of tissue injury and thus is a signaling pathway that transmits spatial information. Our studies suggest that, in contrast, the protease cascade involved in dorsoventral patterning initially transmits not spatial but temporal information, which is, perhaps, a cue that embryogenesis has begun (21). Spatial information in the form of the ventral cue is then integrated at a distinct downstream step in the cascade. As a consequence, the ventralizing signal is generated at the right time and place to pattern the embryo.

Feedback control also seems to be important for defining the temporal and spatial dimensions of signaling by the dorsoventral cascade, as has been shown for other signaling pathways involved in creating complex developmental patterns (23). Earlier studies suggested that a negative feedback loop involving the most downstream component in the Toll signaling pathway inhibits activation of the Easter protease (24). Our data also raise the possibility that both positive and negative feedback loops modulate the activation of proteases upstream of Easter. Such feedback could amplify a subtle asymmetry in protease activation level not detectable by our current methods. The integration of positive and negative feedback loops, and of temporal and spatial cues as outlined above, likely provides the precise control of signaling necessary to establish embryonic dorsoventral polarity.

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