INTRODUCTION

The establishment of the dorsoventral axis of the Drosophila embryo depends upon the production of a signal for ventral development (reviewed by Morisato and Anderson, 1995). This signal is generated extracellularly within the fluid-filled perivitelline space lying between the embryo plasma membrane and the vitelline membrane, the innermost layer of the eggshell. This signal acts as a ligand to activate the Toll transmembrane receptor, which in turn leads to nuclear translocation of a rel-like transcription factor, Dorsal, preferentially into ventral nuclei. Loss-of-function mutations in the dorsal gene or in any of ten upstream genes in the Toll signaling pathway (referred to collectively as the dorsal-group genes) cause all cells of the embryo to adopt a dorsal cell fate. The ligand that activates the Toll receptor appears to be a C-terminal proteolytic fragment of the Spätzle protein (Morisato and Anderson, 1994; Schneider et al., 1994). Proteolytic processing of Spätzle to generate the Toll ligand requires the activities of six additional dorsal-group genes (Morisato and Anderson, 1994). Three of these genes – gastrulation defective, snake and easter – encode proteins structurally related to zymogens of trypsin-like serine proteases that are activated by proteolytic cleavage (DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Konrad et al., 1998). These proteases appear to act sequentially, possibly in a cascade as seen for the proteases of vertebrate blood coagulation (Chasan et al., 1992; Smith and DeLotto, 1994).

Perivitelline fluid transfer experiments have shown that the Snake and Easter zymogens and the Spätzle precursor are freely diffusible in the perivitelline space (Stein and Nüsslein-Volhard, 1992), suggesting that local activation of the serine protease cascade is necessary to generate the Toll ligand only on the ventral side of the embryo (Chasan et al., 1992; Smith and DeLotto, 1994).

Local activation of this cascade depends upon stable positional information deposited in the future perivitelline space during oogenesis by somatic follicle cells that surround the developing oocyte (reviewed by Ray and Schüpbach, 1996). Because the follicle cells degenerate prior to the end of oogenesis, there is a significant time delay before this information is utilized in the embryo. Furthermore, transmission of this information must somehow be biochemically linked to local activation of the serine protease cascade that generates the Toll ligand. Deciphering the molecular nature of this signal transduction mechanism is a major unanswered question in understanding how embryonic dorsoventral polarity is established.

The follicle cells express three dorsal-group genes – windbeutel, pipe and nudel – that are likely to control the local production of the Toll ligand (Schüpbach et al., 1991; Stein et al., 1991; Hong and Hashimoto, 1995; Konsolaki and Schüpbach, 1998). Analysis of mutant clones within the follicle cell epithelium has demonstrated a ventral requirement for the windbeutel and pipe genes, but not for nudel, in the establishment of embryonic dorsoventral polarity (Nilson and...
Schüpbach, 1998). While cloning of the pipe gene has not been reported, the windbeutel gene appears to encode a resident ER protein, which may be required for the biogenesis of a secreted factor involved in the establishment of dorsoventral polarity (Konsolaki and Schüpbach, 1998). Such a factor might be required ventrally for the formation of a zymogen activation complex that triggers the serine protease cascade, as seen for the initiation of the coagulation cascade (Furie and Furie, 1988).

Our laboratory has focused on the nudel gene because genetic observations suggested that its product, while expressed during oogenesis, is required during early embryogenesis when it could have a direct role in activating the protease cascade (Hong and Hashimoto, 1995). The nudel gene encodes a large mosaic protein resembling extracellular matrix proteins (Hong and Hashimoto, 1995). Unusual among such proteins, Nudel has a centrally located domain with significant homology to the catalytic domains of trypsin-like serine proteases (Hartley, 1970). This homology includes the catalytic triad residues that are essential for proteolytic activity and a potential zymogen cleavage site, suggesting that Nudel could act as a protease in the establishment of dorsoventral polarity. However, the presence of extensive sequence C-terminal to the protease domain, unprecedented among trypsin-like serine proteases, makes it unclear whether this domain actually has catalytic activity and how this activity might be regulated. Nudel also has eleven copies of the LDL-receptor ligand-binding motif (Südhof et al., 1985), which has been implicated in several extracellular protein-protein interactions, in particular the binding of serum proteases and protease-inhibitor complexes (Willnow et al., 1994). These sequence features suggest that Nudel might function as the initiating protease in the serine protease cascade, perhaps in response to a positional cue generated by the pipe and windbeutel genes, and also as a scaffold to localize downstream proteases (Hong and Hashimoto, 1995).

In the present work, we provide strong evidence by site-directed mutagenesis of its central serine protease domain that Nudel does function as a protease in establishing dorsoventral polarity. We have identified a putative active Nudel protease that is generated in early embryogenesis by apparent autoproteolytic zymogen cleavage. Nudel protease activation appears to occur at the embryo plasma membrane, raising the possibility that the serine protease cascade is assembled on this surface. Surprisingly, Nudel protease activation occurs independently of pipe and windbeutel function, and occurs uniformly around the embryo circumference. We propose that Nudel protease activation serves to trigger the activation of the Toll signaling pathway, while spatial regulation of the pathway occurs downstream, perhaps at a step involving a cofactor for the Nudel protease or a binding site for Nudel’s substrate.

**MATERIALS AND METHODS**

**Fly stocks**

The wild-type strain was Oregon R. The w^{118} strain was used for production of transgenic lines (Lindsley and Zimm, 1992). Mutant alleles (ndl^{14}, ndl^{15}, ndl^{133}) and deficiencies (Df(3)L)CH12, (2)3844) of nudel have been described previously (Hong and Hashimoto, 1995, 1996). Strongly dorsalizing allelic combinations of several dorsal-group genes were used: wind^{604}/wind^{605} (Konsolaki and Schüpbach, 1998), gdl^{+}gd^{+} (Konrad et al., 1988), pipe^{606}/pipe^{604}, snk^{073}/snk^{073}, ea^{+}eael^{02021} and Tl^{Df(3)R} (Schüpbach, 1998), (Anderson et al., 1985; Hashimoto et al., 1988; Chasan and Anderson, 1989).

**Generation and analysis of nudel transgenes**

The wild-type genomic nudel transgene contains the entire nudel coding sequence plus 1.5 kb and 0.5 kb, respectively, of 5’ and 3’ sequences in the pCaSpeR-hs vector (Pirrotta, 1988) in reverse orientation to the hsp70 promoter. A similar vector contains the nudel cDNA sequence fused to the 1.5 kb nudel promoter region. Site-directed mutagenesis of the catalytic serine residue (S133A) or the zymogen cleavage site P1 residue (R1144L) was performed using a two-step PCR strategy (Cormack, 1997) followed by DNA sequencing of PCR-derived regions.

Multiple independent lines for each transgene were generated by germline transformation (Spradling, 1986). Females were constructed that expressed one nudel transgene copy in a background null for expression from the endogenous nudel gene (either ndl^{14}/ndl^{133} or ndl^{14}/Df(3)CH12). Embryo collections (>200/line) were scored visually under oil (Wieschaus and Nüsslein-Volhard, 1986) and by examination of cuticle structures (Anderson et al., 1985). Hatching embryos were scored by the presence of empty eggshells.

**Generation and purification of Nudel antibodies**

N-terminal (aa 548-657), protease domain (aa 1145-1379) and C-terminal (aa 2518-2607) regions of Nudel (Hong and Hashimoto, 1995) were expressed as MBP (N-terminal) or trpE (protease domain, C-terminal) fusion proteins (Maina et al., 1988; Koerner et al., 1990), purified (Hashimoto et al., 1991) and injected into rabbits (N-terminal, C-terminal) or mice (protease domain) for polyclonal antibody production (Harlow and Lane, 1988). Immun serum were affinity-purified as described (Hashimoto et al., 1991). Except for protease domain antibody staining in Fig. 5D,E, antibodies used for immunostaining were further preabsorbed against fixed ovaries or embryos lacking Nudel protein.

**Western blot analysis**

Ovaries dissected in cold Ringer’s solution were frozen on dry ice. Embryos collected on apple juice plates were bleach-dechorionated, washed extensively with water and frozen. Samples were homogenized on ice in 2X SDS sample buffer containing 6 M urea and 100 mM DTT, boiled and soluble proteins loaded on 4-16% SDS-polyacrylamide gels. Typically, 10-20 egg chambers, 2 ovaries, or 25-40 embryos were loaded per gel lane. Addition of a protease inhibitor cocktail did not alter the band patterns observed. Protein transfer to nitrocellulose and western blotting were performed using standard methods (Harlow and Lane, 1988).

**Fixation and immunofluorescence staining**

Ovaries were fixed in 6% formaldehyde/heptane for 10 minutes (Verheyen and Cooley, 1994). Following 3X 15 minute washes in PBS/0.3% Triton X-100/0.5% BSA (PBT) and a 30 minute incubation in PBT/0.5 M sucrose, ovaries were transferred to OCT (Tissue-Tek) in foil molds and frozen in liquid nitrogen. Cryostat sections (8 μm) were transferred to gelatin-coated slides and stored at −20°C. Use of sectioned material was necessary to visualize the future perivitelline space after stage 10A and gave superior visualization of this compartment at all stages. Embryos were fixed, devitellinized and stored at −20°C in methanol as described (Patel, 1994), except that fixation was for 10 minutes. For staining with rhodamine-phalloidin, embryos were stained immediately after mechanical removal of vitelline membranes (Theurkauf, 1994).

Ovary sections were fixed to gelatin-coated slides by a 5 minute incubation in 4% paraformaldehyde in PBS, rinsed 3 times in PBS,
then blocked 15 minutes in PBT/2.5% normal goat serum. Primary and secondary antibody incubations were for 90 minutes and 30 minutes, respectively, in blocking solution, each followed by 3x5 minute washes in PBT. Slides were mounted in anti-fade solution (Verheyen and Cooley, 1994). Polyclonal anti-Sv23 vitelline membrane antibody (provided by G. Waring) and fluorescein- or Texas Red-conjugated secondary antibodies (Jackson Immunoresearch) were preabsorbed against fixed, devitellinized Oregon R embryos. Immunostaining of embryos was as described (Patel, 1994) except that our recipe for PBT was used. Rhodamine-phalloidin (Molecular Probes) was added to the secondary antibody incubation in some cases (Verheyen and Cooley, 1994). Nuclear staining by a 5 minute incubation in 4 μg/ml propidium iodide prior to the final washes allowed developmental staging of embryos. Specimens were examined using a BioRad MRC-600 confocal fluorescence microscope and images processed in Adobe Photoshop.

Ovaries and embryos derived from ndl215[l(3)3844] females lacking nudel mRNA expression showed no staining with Nudel antibodies. The internal vesicular staining with N- and C-terminal antibodies was confirmed to be genuine Nudel derived from follicle cell expression by the presence of Nudel polypeptides in immunoblots of postgastrulation embryos lacking surface Nudel staining, depletion of antibodies reacting with Nudel epitopes on ovary sections by preincubation of sera with these late embryos and absence of Nudel polypeptides in embryos derived from ndl215[l(3)3844] females that express high levels of a nonsecreted Nudel protein (E. L., unpublished data).

RESULTS

Nudel’s protease activity is required for the establishment of embryonic dorsoventral polarity

To examine the function of the central serine protease domain of Nudel, we introduced separately into a genomic nudel transgene two point mutations having predictable effects on protease function (see Methods and Fig. 1A). One mutation (S1332A) altered the essential catalytic serine residue, which eliminates enzymatic activity of serine proteases (Jin and Anderson, 1990; Smith et al., 1994), while the second mutation (R1144L) altered the P1 residue in the predictedzymogen cleavage site, which is predicted to alter the cleavage specificity required for zymogen activation without adversely affecting catalytic activity of the protease (Tate et al., 1987; Smith et al., 1995). These transgenic nudel constructs were assayed for their ability to rescue embryos derived from female flies lacking expression from the endogenous nudel gene (Table 1 and Methods). Such embryos arrest early in development, apparently due to an additional requirement for the Nudel protein in the structural integrity of the embryo (Hong and Hashimoto, 1996). A single copy of the wild-type nudel transgene fully rescued nudel activity to give hatching larvae. In contrast, both site-directed mutants were able to rescue the early arrest phenotype but were inactive in the establishment of dorsoventral polarity, yielding completely dorsalmost embryos. These results strongly suggest that, despite its unusual structure, Nudel is an authentic serine protease regulated by zymogen cleavage and that Nudel’s protease activity is essential for the establishment of embryonic dorsoventral polarity.

Partial processing of Nudel occurs during oogenesis

To explore the temporal regulation of Nudel’s protease activity, we sought to identify potentially active forms of Nudel protease in ovary and egg extracts by Western blotting. We reasoned that an active Nudel protease resulting from cleavage at the zymogen cleavage site would be absent in extracts containing only the zymogen cleavage mutant (R1144L). Using affinity-purified polyclonal antisera recognizing the central protease domain and unique N- and C-terminal regions of Nudel (Fig. 1A), we uncovered extensive processing of Nudel occurring in a temporally defined manner across oogenesis and early embryogenesis (partially outlined in Fig. 1B). Although processing during oogenesis is not altered in the zymogen cleavage mutant (R1144L; data not shown), indicating that Nudel’s protease activity is required for the establishment of dorsoventral polarity.

Fig. 1. Analysis of Nudel processing that generates Nudel protease. (A) Tools used in this analysis. Top Nudel diagram shows signal sequence (light gray), 11 LDL-receptor ligand-binding motifs (dotted), serine protease domain (black), partial protease-like domain (white dots on black), serine/thrreonine-rich regions that may be O-glycosylated (cross-hatched), potential glycosaminoglycan attachment sites (black triangles) and a potential RGD integrin recognition sequence (open triangle). Not shown are 23 potential N-glycosylation sites. Below this are bars indicating the nudel transgenic constructs. Bottom bar shows regions of Nudel recognized by N-terminal, protease domain and C-terminal polyclonal antibodies. (B) Schematic diagram of Nudel processing defined by Western blots. During oogenesis, Nudel comprises a 210 kDa N-terminal fragment and a 250 kDa C-terminal fragment containing the protease domain (Fig. 2), generated by proteolytic cleavage independent of Nudel’s protease activity. In embryogenesis, further Nudel-independent processing separates the protease domain from the C-terminal polypeptide and yields intermediate forms of the C-terminal polypeptide (Fig. 3B,C, lanes 1-2). In the presence of Nudel protease function, processing at the zymogen cleavage site yields a putative active 33 kDa Nudel protease and further processing of the remainder of Nudel occurs (Fig. 3B,C, lanes 4). Nudel fragments marked with solid lines are documented by western blots, while those outlined by dashed lines are inferred from sizes of observed bands. Comparison of fragments to the Nudel diagram in A is approximate, as cleavage sites have not been mapped.
Nudel exists as a zymogen during oogenesis, we will briefly describe the major Nudel polypeptides present during oogenesis for comparison to those found during embryogenesis.

The nudel mRNA is expressed by somatic follicle cells surrounding the oocyte in mid-oogenesis (stages 7-10A; Hong and Hashimoto, 1995). We detected three major Nudel polypeptides in extracts of stage 10 egg chambers (Fig. 2, lanes 1-3). The least abundant 350 kDa form is of sufficient mass to contain the predicted ~290 kDa full-length core Nudel polypeptide and was recognized by all three domain antibodies. However, the predominant Nudel species in stage 10 egg chambers are a 210 kDa N-terminal polypeptide and a 250 kDa C-terminal polypeptide containing the protease domain. Because all of these polypeptides were produced in ovaries expressing only a nudel cDNA transgene (not shown), we suggest that the N-terminal and C-terminal polypeptides are likely to be derived by proteolytic cleavage of the 350 kDa precursor followed by further modifications, such as addition of carbohydrates, rather than by an alternative mRNA splicing mechanism. Consistent with this interpretation, the 350 kDa polypeptide is present only in egg chambers expressing the nudel mRNA, while the 210 kDa and 250 kDa polypeptides can be detected at high levels in mature stage 14 egg chambers (Fig. 2, lanes 4-6). Additional smaller N-terminal polypeptides, apparently derived by processing of the 210 kDa form (Fig. 2, lane 4), are first detected in stage 11 egg chambers no longer expressing the nudel mRNA.

**Nudel protease appears to be activated during early embryogenesis**

While the Nudel protease domain was detectable only in the 250 kDa polypeptide in mature ovarian egg chambers (Fig. 2, lane 5), four additional Nudel protease forms were found in extracts of laid fertilized eggs (Fig. 3A; such extracts contain all contents of the embryo, perivitelline space and vitelline membrane that are solubilized by boiling in sample buffer). The smallest and most abundant of these is a 33 kDa form similar in size to the Nudel serine protease domain generated by in vitro translation (32 kDa; not shown), suggesting that it might contain the protease domain with very little surrounding sequence. New bands at 38 kDa and 75-80 kDa were also seen, while the 250 kDa polypeptide appeared to be reduced in abundance compared to ovaries. These smaller Nudel polypeptides are transiently present during embryogenesis, disappearing by 2 hours of development at 22°C (Fig. 3A, lane 3). Based upon the previously defined temperature-sensitive period for nudel function, extending 1½-2 hours into embryogenesis (Hong and Hashimoto, 1995), one or more of these embryonic Nudel protease forms is likely to be the catalytically active Nudel protease required for the establishment of dorsoventral polarity.

### Table 1. Rescue of nudel function by transgenic constructs

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<thead>
<tr>
<th>Transgene</th>
<th>% Early arrest</th>
<th>% Dorsalized</th>
<th>% Hatch</th>
<th>No. of lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Wild-type</td>
<td>n.d.</td>
<td>n.d.</td>
<td>88</td>
<td>3</td>
</tr>
<tr>
<td>Catalytic serine mutant</td>
<td>6</td>
<td>94</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>(S1332A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zymogen cleavage mutant</td>
<td>6</td>
<td>94</td>
<td>0</td>
<td>4</td>
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<tr>
<td>(R1144L)</td>
<td></td>
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<td>n.d., not determined.</td>
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The pattern of these smaller Nudel protease forms is altered by mutations affecting Nudel protease function (Fig. 3B). In eggs containing only the zymogen cleavage mutant (R1144L), the 33 kDa form was absent and the 38 kDa form was present at high levels (Fig. 3B, cf. lanes 1 and 4). The simplest interpretation of this result is that the major 33 kDa form in wild-type embryos is derived from a transient 38 kDa intermediate by proteolysis of the zymogen cleavage site and thus likely represents the fully active Nudel protease. Eggs containing the catalytic serine mutant (S1332A) as the only Nudel protein showed the identical pattern as seen in the zymogen cleavage mutant, indicating that Nudel’s own proteolytic activity is required for generation of the 33 kDa Nudel protease form (Fig. 3B, cf. lanes 2 and 4).

The pattern of several other embryonic Nudel polypeptides is also dependent upon Nudel protease function. The 75-80 kDa doublet recognized by the protease domain antibody was absent in eggs containing the site-directed mutants (Fig. 3B, cf. lanes 1 or 2 and 4); these forms could be derived either by an alternative cleavage of the 250 kDa C-terminal polypeptide or by SDS-stable association of the 33 kDa Nudel protease with another molecule such as an inhibitor (Misra et al., 1998). A 170 kDa N-terminal polypeptide disappeared in wild-type eggs but persisted in eggs containing the site-directed mutants (not shown). The 250 kDa C-terminal polypeptide appeared to undergo further processing in wild-type eggs to yield two major C-terminal 50-60 kDa forms, while intermediate C-terminal forms accumulated in the absence of functional Nudel protease (Fig. 3C, cf. lanes 1 or 2 and 4). Similarly to the apparent step-wise generation of the 33 kDa Nudel protease, this result demonstrates that production of other Nudel polypeptides during embryogenesis requires at least two proteases, one independent of Nudel protease activity and a second either directly or indirectly dependent on Nudel.
protease activity. The processing steps involved in generating the 33 kDa Nudel protease and C-terminal cleavage products are schematically outlined in Fig. 1B. The occurrence of Nudel-dependent processing in the first hour of embryogenesis suggests that Nudel protease is activated at this time.

**Other dorsal-group genes are not required for Nudel protease activation**

We were interested to know if other components of the Toll signaling pathway are required for Nudel protease activation and processing in the embryo. For example, the pipe or windbeutel gene products might be required to activate Nudel protease only on the ventral side of the embryo. Alternatively, other known proteases in the pathway might be responsible for proteolytic cleavages required to activate the Nudel protease, as might occur in a positive feedback loop, or for processing the remainder of Nudel. Western blot examination of Nudel in extracts of fertilized eggs derived from females mutant for the pipe, windbeutel, gastrulation defective, snake, easter or Toll gene showed normal processing of Nudel protease, as well as C-terminal and N-terminal Nudel polypeptides (data not shown). Thus, Nudel protease activation appears to proceed independently of known components of the Toll signaling pathway that act outside the embryo.

**Uniform distribution of secreted Nudel on the oocyte surface**

To determine if Nudel is deposited within the future perivitelline space surrounding the oocyte where it could interact directly with the serine proteases required for dorsoventral polarity, we examined Nudel distribution in sectioned egg chambers by immunostaining. All of the Nudel domain antibodies showed an identical immunostaining pattern (illustrated with C-terminal antibody, Fig. 4A-C). Staining within follicle cells overlying the oocyte, representing Nudel synthesis, was detected in mid-oogenesis (stages 7-10; Fig. 4A). Strong staining was also detected within the extracellular space lying between the oocyte and the follicle cell epithelium (Fig. 4A), presumably representing secreted Nudel, which persisted throughout the remainder of oogenesis (Fig. 4B). No dorsoventral asymmetry of Nudel staining was detected either within the follicle cells or within the extracellular space (Fig. 4A).

Secreted Nudel lies in a layer distinct from the nascent vitelline membrane, visualized by double-labeling with Nudel protease domain antibody and antibody to the S623 vitelline membrane protein (Fig. 4D; Pascucci et al., 1996). Counterstaining with rhodamine-phalloidin to label the cortical actin cytoskeleton of the oocyte showed that secreted Nudel is always closely apposed to the oocyte surface (Fig. 4C). Nudel is likely to be fixed at this surface, as Nudel polypeptides are largely insoluble in ovary extracts without the addition of denaturing agents (e.g., 1% SDS or 6 M urea; E. L., unpublished data).

**Spatially uniform activation of Nudel protease**

Because Nudel was localized at the oocyte surface, we expected it to be localized on the embryo surface. Therefore, to look at its distribution during embryogenesis, we used the whole-mount immunostaining method in which the vitelline membrane is removed (Patel, 1994). We were surprised to find that, in contrast to the case with the oocyte, the protease domain antibodies showed different staining patterns than seen with N- and C-terminal antibodies, which gave similar staining patterns.

The C-terminal antibody brightly stained the entire surface of wild-type embryos undergoing the first 1-2 mitotic divisions (Fig. 5A). The staining intensity rapidly declined over the early mitotic divisions, however, by the syncytial blastoderm divisions leaving only weak staining of the pseudocleavage furrows between protruding nuclei (divisions 10-13; Fig. 5B). Similar staining was seen with N-terminal antibody (not shown). In contrast, protease domain antibody stained punctate structures that lined the embryo periphery during the early mitotic divisions (Fig. 5D), but this staining disappeared gradually during mitotic divisions 8 and 9 (not shown). Double-labeling with protease domain antibody and either C-terminal antibody (Fig. 5E) or rhodamine-phalloidin (Fig. 5F) showed that the punctate structures stained with protease domain antibody are entirely distinct from C-terminal staining at the embryo surface and lie within the cortical cytoplasm of the embryo. This result indicates that these structures do not
contain the 250 kDa polypeptide recognized by both Nudel antibodies (Fig. 2).

We found that Nudel distribution was altered in embryos containing either of the site-directed mutant forms of Nudel. The protease domain antibody failed to stain punctate cytoplasmic structures in these embryos, instead staining the embryo surface (cf. Fig. 5G and H), suggesting that Nudel protease function is required for entry into the punctate cytoplasmic structures. In addition, surface staining with C-terminal and N-terminal antibodies persisted much longer than in wild-type embryos, with cellularizing embryos still showing strong surface staining (division 14; Fig. 5C), suggesting that the uniform decline in surface staining seen in wild-type embryos is dependent upon Nudel protease function. This analysis provides evidence that the wild-type staining patterns reflect global activation of Nudel protease and subsequent processing of N- and C-terminal regions of Nudel at the embryo surface.

In addition to surface staining, we noticed that N- and C-terminal antibodies showed staining of internal vesicles (Fig. 5A,B). By several criteria (described in Methods), this staining represents the movement of follicle cell-derived Nudel from the plasma membrane to the interior of the embryo, which may reflect a more general turnover of plasma membrane occurring near the onset of embryogenesis (Bretscher, 1996). In contrast to surface staining with these antibodies, the internal staining was not altered in the absence of functional Nudel protease (cf. Fig. 5C with Fig. 5A,B), suggesting that this process is not regulated by Nudel’s protease activity.

**DISCUSSION**

We have utilized site-directed mutagenesis to define an in vivo requirement for catalytic activity and zymogen cleavage of Nudel’s central serine protease domain in the establishment of embryonic dorsoventral polarity. By comparing the processing and localization of wild-type and site-directed mutant forms of Nudel, we found that Nudel protease is activated by an apparent autocatalytic mechanism and that this activation appears to occur uniformly within the perivitelline space of the...
early embryo. These results suggest that Nudel protease activation could trigger the serine protease cascade that generates the signal for ventral development, but that spatial restriction of the signal likely occurs at a downstream step.

**Nudel protease appears to be activated by an autocatalytic mechanism**

Secreted as a protease zymogen into the future perivitelline space in mid-oogenesis, Nudel protease appears to be activated during early embryogenesis (Fig. 3). In contrast to the Snake and Easter zymogens whose activation requires the products of several upstream genes (Chasan et al., 1992; Smith and DeLotto, 1994; Misra et al., 1998), the Nudel zymogen is activated independently of the other dorsal-group genes. The dependence of this activation on Nudel’s own proteolytic activity suggests that Nudel is activated by an autocatalytic mechanism. A direct autocatalytic cleavage is plausible because Nudel protease is predicted to cleave after basic residues and the Nudel zymogen is predicted to be activated by cleavage after an arginine residue (Hong and Hashimoto, 1995).

While typical protease zymogens of the trypsin family are activated by a single cleavage N-terminal to the catalytic domain (Hartley, 1970), an additional C-terminal cleavage is involved in Nudel protease activation. The C-terminal cleavage appears to occur first and is independent of the Nudel, Gastrulation Defective, Snake and Easter proteases known to be involved in generation of the Toll ligand. Thus, this cleavage appears to be performed by an uncharacterized proteolytic activity present within the perivitelline space and rapidly followed by cleavage of the N-terminal zymogen cleavage site. One role of the unique C-terminal extension of Nudel may be to inhibit an intrinsic autoactivating potential of the Nudel zymogen, preventing premature activation of Nudel protease during oogenesis.

What triggers this sequence of proteolytic cleavages in the embryo? One clue is provided by the finding that a change in Nudel protease localization that depends upon Nudel protease activation was detected in the earliest laid embryos (Fig. 5D). The activated patterns of Nudel polypeptides and immunolocalization are also detected in laid unfertilized eggs (E. L., unpublished data), suggesting that ovulation may somehow lead to Nudel protease activation. Ovulation normally immediately precedes fertilization and results in many physiologic changes in the egg, such as resumption of meiosis (Page and Orr-Weaver, 1997 and references therein), so linking Nudel protease activation to ovulation could ensure that Nudel protease is active in early embryogenesis.

**What is the substrate of Nudel protease?**

The Nudel and Gastrulation Defective serine proteases are required for the sequential zymogen activation of Snake and Easter (Chasan et al., 1992; Smith and DeLotto, 1994). Because Gastrulation Defective is not required for activation of Nudel protease, Nudel protease appears to act upstream of, or parallel to, Gastrulation Defective. Nudel protease is unlikely to directly activate Snake, which is predicted to be activated by cleavage after a leucine residue (Smith et al., 1994). Thus, it is attractive to suggest that autoactivation of Nudel protease functions as the initiating event in a linear serine protease cascade similar to the blood clotting cascade (Furie and Furie, 1988), wherein Nudel protease activates Gastrulation Defective and Gastrulation Defective activates Snake. However, it is not clear if Gastrulation Defective activity is regulated by zymogen cleavage, because Gastrulation Defective lacks a typical zymogen cleavage consensus site (Konrad et al., 1998).

In the absence of direct evidence for Gastrulation Defective cleavage by Nudel protease, we offer for consideration an alternative role for Nudel protease suggested by our data, that the biologically relevant substrate for Nudel protease is Nudel itself. Processing of both N-terminal and C-terminal portions of Nudel is blocked in the absence of Nudel protease but occurs normally in the absence of the downstream dorsal-group proteases, suggesting that Nudel protease may itself proteolyze other regions of the Nudel protein. Cleavage of extracellular matrix proteins by matrix metalloproteases has been shown to reveal functions of these proteins that are not present in the intact molecules (Fukai et al., 1995; Giannelli et al., 1997). Similarly, cleavage of Nudel by Nudel protease could activate a distinct portion of Nudel, such as the LDL-receptor ligand-binding motifs, that subsequently interacts with other proteases in the Toll signaling pathway (Fig. 1A; Hong and Hashimoto, 1995).

**Potential significance of Nudel localization at the cell surface**

We previously proposed that Nudel could be a component of the vitelline membrane, where it might bind and localize serine proteases involved in generating the Toll ligand (Hong and Hashimoto, 1995). However, our current work suggests that
Nudel is associated with the plasma membrane of the oocyte and early embryo. By immunofluorescence (Fig. 4A,C) or immunoperoxidase staining (not shown) of ovarian egg chambers showing maximal synthesis of Nudel, we never detected secreted Nudel in the region of the future perivitelline space containing vitelline membrane proteins. Strong preferential association of newly secreted Nudel with the oocyte surface, just as vitelline membrane association, may serve to limit diffusion of Nudel within the future perivitelline space during oogenesis. This observation highlights the possibility that the positional cue for the Toll signaling pathway, generated by the pipe and windbeutel genes (Nilson and Schüpbach, 1998), could similarly be assembled on the ventral surface of the oocyte.

Nudel protease activation and subsequent Nudel processing appear to occur at the embryo surface, based upon our finding that Nudel protease is required for the rapid decline in surface staining seen with all Nudel antibodies during early embryogenesis (Fig. 5). While we rarely detected surface staining with protease domain antibody in wild-type embryos, this antibody recognized punctate structures lying just beneath the embryo plasma membrane that could represent endocytosed Nudel protease targeted for degradation or a sequestered form of the active Nudel protease. A portion of the active Nudel protease may also be released into the perivitelline space, as we find both soluble and membrane-associated pools of the 33 kDa Nudel protease by biochemical fractionation experiments (E. L., unpublished data).

Activation of Nudel protease at the embryo surface, rather than the vitelline membrane, raises the possibility that Nudel protease function in the Toll signaling pathway (and perhaps that of the downstream proteases) is required at the plasma membrane. Involvement of the plasma membrane in the assembly of macromolecular complexes containing serine proteases is known to greatly increase both catalytic efficiency and resistance to inhibitors of bound proteolytic enzymes when compared to soluble forms (Mann et al., 1988; Werb, 1997). In the case of the Toll signaling pathway, such an association could also result in generation of the Toll ligand in close proximity to its receptor, limiting diffusion of the ventral signal. The localization of the active forms of the other dorsal-group serine proteases is not known, so it would be interesting to see if there is colocalization of these proteins and Nudel at the embryo surface or within the cytoplasmic punctate structures.

**How can uniform activation of Nudel protease lead to dorsoventral asymmetry?**

The activation of Nudel protease early in embryogenesis, independent of other dorsal-group genes, suggests that Nudel protease activation could be a triggering event that activates the protease cascade responsible for generating the Toll ligand. However, Nudel protease activation appears to occur uniformly around the embryo circumference, based upon the finding that Nudel protease function is required for symmetric changes in Nudel localization that occur during early embryogenesis (Fig. 5). These observations appear to be inconsistent with a model in which the serine protease cascade that generates the Toll ligand is initiated only within the ventral perivitelline space (Morisato and Anderson, 1995). This model was based upon several observations suggesting that the zymogen forms of Snake and Easter are freely diffusible in the perivitelline space and are activated ventrally in a process requiring the pipe, windbeutel, nudel and gastrulation defective genes (Chasan et al., 1992; Stein and Nüsslein-Volhard, 1992; Smith and DeLotto, 1994; Misra et al., 1998).

Uniform activation of Nudel protease can be reconciled with these findings if an event lying downstream of Nudel protease activation in the Toll signaling pathway, such as the generation of the active form of Gastrulation Defective, is restricted to the ventral side of the embryo. In contrast to the Snake and Easterzymogens, which are freely diffusible within the perivitelline space, Gastrulation Defective activity cannot be transferred in perivitelline fluid (Stein and Nüsslein-Volhard, 1992). It is possible that Gastrulation Defective is fixed within the perivitelline space, perhaps by binding to a ventral site created through the actions of the pipe and windbeutel genes (Nilson and Schüpbach, 1998). Nudel protease could directly cleave Gastrulation Defective or activate a distinct part of the Nudel protease that is directly involved in activating Gastrulation Defective. In either case, Nudel protease, while generated globally, would only participate ventrally in the formation of a productive complex in which Gastrulation Defective is activated (illustrated in Fig. 6).

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