Developmental Biology **217,** 352–361 (2000)
doi:10.1006/dbio.1999.9562, available online at http://www.idealibrary.com on IDEAL®

The Nudel Protease of *Drosophila* Is Required for Eggshell Biogenesis in Addition to Embryonic Patterning

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The dorsoventral axis of the *Drosophila* embryo is defined by a ventral signal that arises within the perivitelline space, an extracellular compartment between the embryo plasma membrane and the vitelline membrane layer of the eggshell. Production of the ventral signal requires four members of the serine protease family, including a large modular protein with a protease domain encoded by the *nudel* gene. Here we provide evidence that the Nudel protease has an integral role in eggshell biogenesis. Mutations in *nudel* that disrupt Nudel protease function produce eggs having vitelline membranes that are abnormally permeable to the dye neutral red. Permeability varies among mutant *nudel* alleles but correlates with levels of Nudel protease catalytic activity and function in embryonic dorsoventral patterning. These mutations also block cross-linking of vitelline membrane proteins that normally occurs upon egg activation, just prior to fertilization. In addition, Nudel protease autoactivation temporally coincides with vitelline membrane cross-linking and can be triggered in mature eggs *in vitro* by conditions that lead to egg activation. We discuss how the Nudel protease might be involved in both eggshell biogenesis and embryonic patterning. © 2000 Academic Press

Key Words: **vitelline membrane; cross-linking; serine protease; embryonic dorsoventral polarity; egg activation; Nudel.**

INTRODUCTION

The establishment of the dorsoventral axis of the *Drosophila* embryo depends upon the production of a ventral signal within the perivitelline space, an extracellular compartment that lies between the embryo plasma membrane and the innermost layer of the eggshell, the vitelline membrane (Morisato and Anderson, 1995). This signal acts as a ligand for the receptor Toll, ultimately leading to the nuclear translocation of the transcription factor Dorsal in a ventral-to-dorsal morphogenetic gradient. The absence of this signal results in the production of a dorsalized embryo, in which all cells have adopted a dorsal cell fate.

The ligand for the receptor Toll appears to be generated by proteolytic cleavage of a precursor protein, Spätzle (for recent reviews, see Anderson, 1998; LeMosy *et al.*, 1999; Roth, 1998). This event requires the activities of four members of the trypsin-like serine protease family, Nudel, Gastrulation Defective, Snake, and Easter. Studies of the Snake and Easter proteases have suggested that these proteins might act in a proteolytic cascade, with the last protease, Easter, acting directly on the ligand precursor Spätzle. Proteolytic cleavage of Spätzle also requires an unidentified localized factor that is deposited in the perivitelline space by somatic follicle cells that surround the developing oocyte and synthesize the eggshell. Recent studies have shown that expression of two genes, *pipe* and *windbeutel*, in ventral follicle cells is important for synthesis of the localized factor (Konsolaki and Schüpbach, 1998; Nilson and Schüpbach, 1998; Sen *et al.*, 1998). The role of this localized factor in the proteolytic generation of the Toll ligand is unknown, but might involve restricting the activity of one or more of the serine proteases to the ventral perivitelline space.

The somatic follicle cells also provide Nudel, one of the serine proteases required for production of the Toll ligand. Several lines of evidence suggest that Nudel acts early in the process of generating the Toll ligand. Nudel activity is required for the production of the active forms of Snake and Easter (Chasan *et al.*, 1992; Smith and DeLotto, 1994), and the active form of Nudel protease is generated by an autocatalytic mechanism that is independent of Snake, Easter, and Gastrulation Defective (LeMosy *et al.*, 1998). Also, the active form of Nudel protease is present princi-

0012-1606/00 $35.00

pally during the first hour of embryogenesis, preceding the first detection of active forms of Easter and Spätzle in the second hour (LeMosy *et al.*, 1998; Misra *et al.*, 1998; Morisato and Anderson, 1994). These findings suggest that the Nudel protease could directly initiate the proteolytic cascade, perhaps by cleaving the zymogen form of the next protease in a linear cascade.

An understanding of the precise role of Nudel in dorsoventral patterning is complicated, however, because Nudel has more than one function in development. Nudel is a large mosaic protein that is also required for the structural integrity of the egg (Hong and Hashimoto, 1995, 1996). Female flies lacking detectable *nudel* mRNA produce embryos that arrest very early in development, only rarely progressing to stages manifesting defects in dorsoventral patterning. These embryos have very fragile eggshells that collapse when touched, a phenotype similar to that seen for embryos lacking a major structural protein of the vitelline membrane layer of the eggshell (Savant and Waring, 1989). While localized to a distinct layer between the oocyte surface and the vitelline membrane, Nudel might be involved in the assembly of the vitelline membrane (LeMosy *et al.*, 1998). Certain mutant *nudel* alleles (designated Class I alleles) demonstrate the structurally defective eggs and early embryonic arrest seen for a null allele of *nudel,* while others (Class II alleles) exhibit only the dorsalized phenotype characteristic of the genes involved in the Toll signaling pathway (Hong and Hashimoto, 1996). Complementation is observed between certain Class I and Class II alleles, suggesting that Nudel's structural and patterning functions involve distinct regions of the structurally modular Nudel protein. Because the Class II (dorsalizing) alleles are characterized by defects in the function of the Nudel protease, the complementation analysis argues that Nudel protease activity is involved only in dorsoventral patterning and is dispensable for the structural integrity of the egg.

We now report that the Class II *nudel* alleles, mutant for Nudel protease function, demonstrate a subtle defect of the vitelline membrane that renders the egg permeable to bleach and the dye neutral red. Among these alleles, the degree of vitelline membrane permeability correlates with the severity of the defects in embryonic patterning and in Nudel protease function, suggesting that the catalytic activity of the Nudel protease is required for vitelline membrane integrity. We show that this permeability is due to a failure of the covalent cross-linking that occurs at the onset of embryogenesis and that Nudel protease activation is temporally linked to the signaling event that triggers this cross-linking. To our knowledge, this observation represents the first report of a mutation specifically affecting vitelline membrane cross-linking. These findings suggest that Nudel has an integral role in the biogenesis of the vitelline membrane and raise the possibility that the Nudel protease functions in embryonic dorsoventral patterning through creation of a matrix structure necessary for activity of the dorsoventral protease cascade.

MATERIALS AND METHODS

Fly Stocks

The wild-type stock was Oregon R (OR). Embryos derived from females expressing pip^{386}/pip^{664} or $gd^{4}/Df(1)KA10$ are strongly dorsalized (Anderson and Nüsslein-Volhard, 1984; Konrad *et al.*, 1988). The ndl^{9}, ndl^{14}, ndl^{046}, ndl^{093}, ndl^{111}, ndl^{260}, $ndl^{LP\text{-}1}$, and ndl^{RM5} alleles have been described previously (LeMosy *et al.*, 2000), as have the site-directed mutant alleles ndl^{S1332A} and ndl^{R1144L} (LeMosy *et al.*, 1998). The ndl^{9} and ndl^{046} alleles have partial Nudel protease activity; the ndl^{093}, ndl^{111}, ndl^{260}, $ndl^{LP\text{-}1}$, ndl^{RM5}, ndl^{S1332A}, and ndl^{R1144L} alleles have no detectable Nudel protease activity; and the ndl^{14} allele is a Class I allele having a premature stop codon prior to the Nudel protease domain (LeMosy *et al.*, 1998, 2000). A deficiency for the *ndl* locus, *Df(3L)CH12,* was used to generate flies hemizygous for these mutant alleles (Hong and Hashimoto, 1995).

Neutral Red Assay

Egg collections (typically 0–12 h) were transferred to a wire basket and washed twice with wash solution (0.8% NaCl, 0.1% TX-100). Eggs were dechorionated by a 2-min incubation in 5% sodium hypochlorite, then washed six times in PBS. Eggs were transferred with a paintbrush to PBS in a microfuge tube and spun briefly and the PBS was removed. The eggs were resuspended gently in 100 μl of a filtered solution of 5 mg/ml neutral red (Sigma) in PBS and incubated at room temperature for 5–10 min. Eggs were washed twice with PBS to remove neutral red, then resuspended in 50% glycerol and mounted on slides for bright-field microscopy. Embryos were scored as having strong uptake (large red patches or entirely red), weak uptake (any red detectable), or no uptake; the first two categories were then combined because of variability seen for the temperature-sensitive alleles in the distribution between strong- and weak-uptake classes. This variability might result from small differences in the age distribution of the eggs or the temperature or from variability in the concentration of the commercial bleach product. A minimum of 200 eggs were scored; for some alleles, multiple independent collections were made, allowing for the determination of the variability in the assay (Fig. 2). While results are shown for only one of the alleles lacking demonstrable Nudel protease activity (ndl^{111}), similar results were obtained for the other alleles in this category.

Analysis of Vitelline Membrane Biogenesis

Antibodies to vitelline membrane proteins (Sv23, Sv17) and chorion proteins (S36, S18) were provided by G. Waring, Marquette University (Pascucci *et al.*, 1996). Ovaries were dissected in cold Ringer's solution to separate individual egg chambers. These egg chambers were staged, counted, and transferred to microfuge tubes on ice using a Pasteur pipette pretreated with Sigmacote (Sigma) to prevent sticking. To assess cross-linking, equal numbers of Stage 14 egg chambers, collected as above, or nondechorionated laid eggs (0- to 6-h collection) were transferred to microfuge tubes on ice. These were suspended in 20 mM Tris–Cl, pH 7.5/0.15 M NaCl containing either 100 mM DTT or 2% SDS and boiled 5 min to release soluble proteins, and insoluble material was removed by centrifugation. The soluble material was mixed with an equal volume of sample buffer containing 100 mM DTT and run on 15% SDS–PAGE gels. Western blots of these gels were performed as previously described (LeMosy *et al.*, 1998). Although data are

shown only for the ndl^{111} allele, solubility of vitelline membrane proteins in laid eggs was documented for several of the Class II *nudel* alleles.

Analysis of Nudel Protease Activation

To assess whether Nudel protease was activated in the absence of fertilization, 0- to 2-h collections were made of eggs laid by virgin OR females or mated OR females. These laid eggs were dechorionated, homogenized using disposable micropestles in reducing sample buffer, boiled, and run on 4–16% gradient SDS–PAGE gels, blotted to nitrocellulose, and probed with antibodies to either the protease domain or the C-terminus of Nudel (LeMosy *et al.*, 1998).

For *in vitro* egg activation, isolation, activation, and recovery buffers were prepared as described (Page and Orr-Weaver, 1997). Whole ovaries from females ages 4–6 days on yeast paste were used to enrich for mature (Stage 14) egg chambers that can respond to egg-activation conditions. The ovaries were dissected in isolation buffer at room temperature within a 15-min period and transferred to a wire basket containing isolation buffer. To activate Stage 14 egg chambers, baskets were transferred to fresh activation buffer 5 times over 5 min, then incubated in activation buffer or recovery buffer for 20–40 min. Control samples were incubated in isolation buffer for 25 min after dissection. Ovaries were then transferred to microfuge tubes on ice, homogenized in reducing sample buffer, and analyzed by Western blotting as described above.

RESULTS

Requirement for Nudel Protease in Vitelline Membrane Integrity

Eggs derived from female flies expressing the Class II *nudel* alleles were previously reported not to be fragile to handling, compared to the severely fragile Class I *nudel* mutant eggs (Hong and Hashimoto, 1996). (The term "egg," except where otherwise stated, refers to the fertilized embryo within its protective eggshell.) Using more sensitive assays, however, we subsequently found that Class II *nudel* mutant eggs exhibited subtle abnormalities of the vitelline membrane. For example, the vitelline membrane is normally impermeable to small molecules such as dyes and sodium hypochlorite (bleach), thus protecting the embryo from environmental insults (Limbourg and Zalokar, 1973). In Class II *nudel* mutant eggs, however, we found that dechorionation (removal of the outer chorion layer of the eggshell) with bleach frequently resulted in developmental arrest of the embryos, suggesting that the bleach was able to penetrate the vitelline membrane. Additionally, we found that the vitelline membranes were adherent to the embryo plasma membrane, with most (>90%) failing to be removed using a standard fixation protocol in which the eggs are shaken in a methanol–heptane mixture, although they could be removed manually (Patel, 1994).

To critically assess the integrity of the vitelline membrane, we incubated eggs laid by *nudel* mutant females with neutral red, a dye that is not normally able to penetrate the vitelline membrane. First used to demonstrate the permeabilization of wild-type eggs by octane, apparently due to solubilization of a wax layer that coats the vitelline membrane (Limbourg and Zalokar, 1973), this dye has subsequently been used to screen for vitelline membrane defects among female-sterile mutations and to demonstrate a vitelline membrane defect caused by feeding female flies α-methyl dopa (Degelmann *et al.*, 1990; Komitopoulou *et al.*, 1983; Konrad *et al.*, 1993). Such vitelline membrane defects are thought to disrupt the morphogenesis of the wax layer and thus lead indirectly to neutral red permeability. Notably, in each of these previously described cases of a vitelline membrane defect, the mutant eggs spontaneously collapse and the embryos fail to develop, phenotypes similar to those seen for the Class I *nudel* alleles but never seen for the Class II *nudel* alleles.

In contrast to wild-type eggs, eggs derived from females hemizygous for Class II *nudel* mutations showed significant uptake of neutral red. The range of staining intensities in these mutant eggs is illustrated in Fig. 1. Eggs from hypomorphic Class II alleles previously shown to have partial Nudel protease activity (ndl^{9}, ndl^{046}; LeMosy *et al.*, 2000) showed the widest range of staining intensities, with many eggs having no detectable staining and others showing weak, uniform staining or patches of intense staining (Figs. 1A–1D). The remaining Class II alleles that lack detectable Nudel protease activity typically produced eggs showing large patches of intense staining or complete staining (Figs. 1E and 1F). Morphological changes were often seen after the brief bleach treatment and dye incubation, with the more permeable eggs showing shrinkage and loss of definition of the twisted embryo shape associated with dorsalization of the embryo.

The penetrance of neutral red permeability varied among the alleles, ranging from 20 to 96%, but could be correlated with the severity of the dorsoventral patterning defect seen for the respective alleles (Fig. 2). For the strongly temperature-sensitive ndl^{9} allele, more strongly dorsalized embryos and more permeable eggs were produced at 22°C than at 18°C (Fig. 2, cf. lines 6 and 8). Furthermore, allelic interactions previously described to affect the severity of the dorsoventral patterning defect also appeared to apply to vitelline membrane permeability (Hong and Hashimoto, 1996). A complementing transheterozygous allelic combination, ndl^{9}/ndl^{46}, that rescues the dorsoventral patterning defect to give 50% wild-type development at 22°C resulted in significantly fewer neutral red-permeable eggs than did either ndl^{9} or ndl^{046} alone (Fig. 2, compare line 4 to lines 5 and 6). In contrast, the transheterozygous combinations ndl^{9}/ndl^{111} and ndl^{46}/ndl^{111}, in which the strongly dorsalizing ndl^{111} allele has negative effects on the partial function of the ndl^{9} and ndl^{46} alleles at 18°C, are as severely defective as ndl^{111} alone in both dorsoventral patterning and eggshell integrity (Fig. 2, cf. lines 8–12). Together, these observations suggest that the impaired catalytic activity of the mutant proteins is responsible for the vitelline membrane defect, rather than a less specific structural defect associated with having a mutant protease domain.

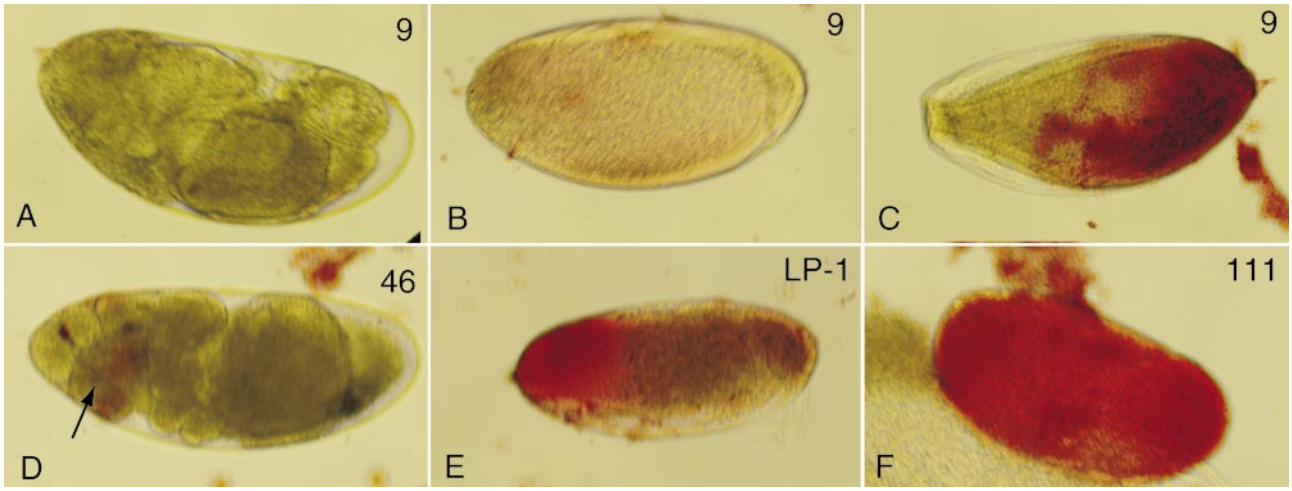

FIG. 1. Eggs lacking function of Nudel protease (Class II *nudel* alleles) are permeable to neutral red dye. Dechorionated eggs derived from female flies hemizygous for a *nudel* mutant allele were incubated in a neutral red solution as described under Materials and Methods. The ndl^{9} allele has partial Nudel protease activity and produces eggs with a broad range of neutral red staining, from no staining (A) through weak staining (B) to intense, usually patchy, staining (C). In (D), an egg from another partially active allele, ndl^{46}, shows only a small patch of neutral red uptake (arrow). Severely affected alleles lacking detectable Nudel protease activity produce eggs that show intense, patchy staining (E, $ndl^{LP\text{-}1}$) and often complete staining (F, ndl^{111}). While eggs showing little or no uptake of neutral red typically contain embryos showing the characteristic twisted shape of the dorsalized phenotype (A, D), more severely affected eggs often show shrinkage and sometimes loss of definition of the embryo shape after dye incubation (C, F).

The Class I *nudel* alleles also showed permeability to neutral red (not shown); however, over 75% of these eggs typically burst upon brief exposure to bleach. Eggs derived from females expressing strongly dorsalizing mutations of the *pipe* or *gastrulation defective* genes were only rarely permeable (Fig. 2, lines 2 and 3), suggesting that neutral red permeability is not a general characteristic of embryos lacking function of the Toll signaling pathway.

Specific Requirement for Nudel Protease in Covalent Cross-Linking of the Vitelline Membrane

We next wanted to explore the nature of the vitelline membrane defects associated with Class I or Class II *nudel* mutations. The vitelline membrane is a homogeneous, proteinaceous structure about 300 nm in thickness (Margaritis *et al.*, 1980). While little is known about how the several protein components of the vitelline membrane are assembled within this structure, certain steps in vitelline membrane biogenesis can be assayed biochemically. Using antibodies to two major vitelline membrane proteins, Sv17 and Sv23, we examined posttranslational processing and cross-linking of these proteins in two mutant *nudel* alleles, one lacking catalytic activity of the Nudel protease domain (ndl^{111}, Class II) and one expressing a greatly reduced amount of a truncated Nudel protein that physically lacks the Nudel protease domain (ndl^{14}, Class I).

Following secretion in midoogenesis, the vitelline membrane proteins undergo proteolytic processing at defined stages of egg chamber development to generate successively smaller polypeptides (Pascucci *et al.*, 1996). We detected no alteration in the expression or stage-specific processing of either Sv17 (Fig. 3A) or Sv23 (not shown) in egg chambers from either *nudel* mutant compared to wild-type egg chambers (illustrated for ndl^{14}). These findings indicate that the Nudel protease is not required for proteolytic processing of the vitelline membrane.

Covalent cross-linking renders the vitelline membrane of laid eggs insoluble in denaturing or reducing agents (Fargnoli and Waring, 1982; Petri *et al.*, 1976). Petri and colleagues (1979) have reported in abstract form that this cross-linking occurs in two steps, including disulfide cross-linking in the latter portion of oogenesis that results in solubility of the vitelline membrane in reducing agents but not detergents, followed by further non-disulfide cross-linking around the time of oviposition that renders the vitelline membrane completely insoluble. Using the more sensitive and specific antibody reagents, we confirmed that the Sv17 and Sv23 proteins present in wild-type Stage 14 egg chambers could be solubilized in 100 mM DTT (Fig. 3B, lanes 1) but were much less soluble in 2% SDS (Fig. 3B, lanes 4). Like wild type, Stage 14 egg chambers mutant for either Class I or Class II *nudel* function could be solubilized in DTT (Fig. 3B, lanes 2 and 3), but were much less soluble in SDS (Fig. 3B, lanes 5 and 6).

In contrast to the similarity of vitelline membrane biogenesis in wild-type and *nudel* mutant ovaries, we found a dramatic difference upon examination of laid eggs. Extracts

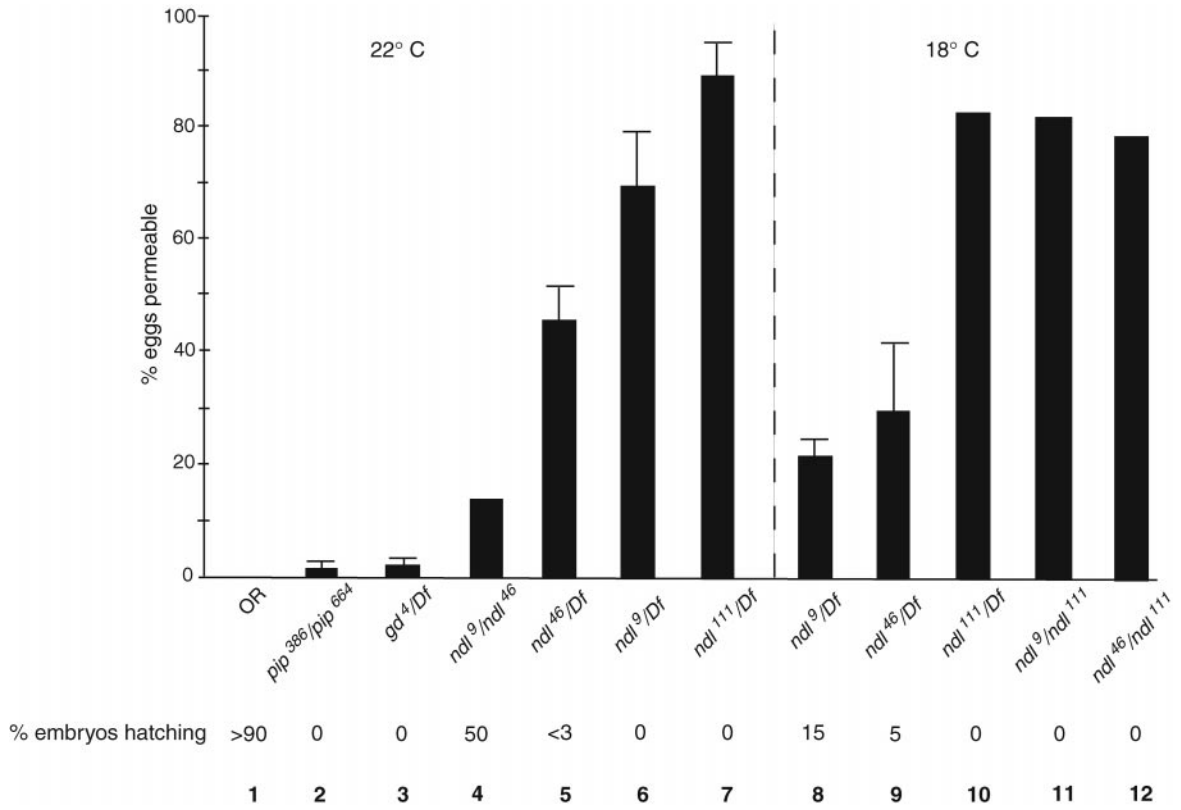

FIG. 2. Penetrance of neutral red permeability varies according to the catalytic activity of mutant Nudel protease. Eggs showing any uptake of neutral red were scored as permeable. The percentage embryos hatching is provided below the bar graph as a measure of gene function in dorsoventral patterning. Collections made at 22°C, a nonpermissive temperature for the ndl^{9} and ndl^{46} alleles, allowed comparison of the egg permeability caused by the ndl^{46} (line 5) and ndl^{9} (line 6) mutations to that found for the complementing allelic combination ndl^{9}/ndl^{46} (line 4). Collections at 18°C, a permissive temperature for ndl^{9} and ndl^{46}, allowed comparison of the permeability of ndl^{9} (line 8) and ndl^{46} (line 9) eggs with the permeability of eggs produced by the allelic combinations ndl^{9}/ndl^{111} (line 11) and ndl^{46}/ndl^{111} (line 12) that are as severely defective in both dorsoventral patterning and egg integrity as eggs resulting from expression of ndl^{111} alone (line 10). The wild-type eggs (OR) never showed neutral red permeability (line 1), while eggs derived from females expressing strongly dorsalizing alleles of *pipe* (line 2) and *gastrulation defective* (line 3) were rarely permeable; an additional strong *gastrulation defective* allele, *gd(VM90)*, failed to show permeability (not shown). Error bars are shown for the genetic backgrounds in which several independent collections were scored to establish the reproducibility of the assay; for transheterozygous *nudel* backgrounds, collections of >200 eggs were scored in parallel with the relevant hemizygous controls.

of laid, fertilized eggs derived from *nudel* mutant females showed levels of DTT-soluble Sv17 and Sv23 proteins indistinguishable from levels present in Stage 14 egg chambers (Fig. 3B, lanes 8 and 9), while extracts of wild-type eggs did not contain DTT-soluble Sv17 or Sv23 (Fig. 3B, lanes 7). This result suggests that the catalytic activity of the Nudel protease is essential for non-disulfide covalent cross-linking of the vitelline membrane. It is possible that a small amount of covalent cross-linking occurs in the *nudel* mutants based upon the presence of slightly slower migrating forms of Sv17 in extracts of laid eggs compared to Stage 14 egg chambers (Fig. 3B, compare lanes 8 and 9 to lanes 2 and 3). Similar covalent cross-linking of the chorion, the outer eggshell layer, occurs during Stage 14 of oogenesis and is unaffected by mutation of the *nudel* gene (not shown; Petri *et al.*, 1976).

Nudel Protease Activation Appears to Temporally Coincide with Vitelline Membrane Cross-Linking

The non-disulfide cross-linking of the vitelline membrane appears to be triggered by passage of the mature egg through the oviduct and does not require fertilization (Page and Orr-Weaver, 1997; Spradling, 1993). During transfer through the oviduct, the egg is exposed to a chemical environment that causes the egg to swell by hydration. This chemical environment also triggers several signaling events known collectively as egg activation. In addition to cova-

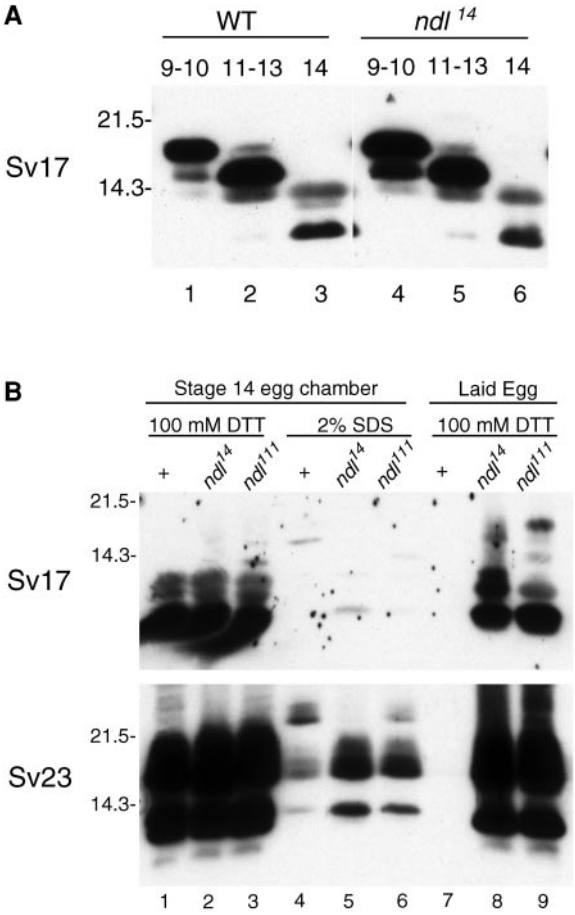

FIG. 3. The Nudel protease is required for cross-linking of vitelline membrane proteins. (A) Western blot analysis of extracted proteins from wild-type (WT) or Class I *nudel* mutant (ndl^{14}) egg chambers with the Sv17 antibody shows that stage-specific proteolytic processing of vitelline membrane proteins does not require *nudel* function. Represented developmental stages include midoogenesis (stages 9–10), late oogenesis (stages 11–13), and mature eggs (stage 14). (B) Stage 14 egg chambers derived from wild-type, Class I *nudel* mutant (ndl^{14}), or Class II *nudel* mutant (ndl^{111}) females contain vitelline membrane proteins that can be solubilized by boiling in 100 mM DTT (lanes 1–3), but not 2% SDS (lanes 4–6). Vitelline membrane proteins are not solubilized by DTT extraction of laid, fertilized eggs derived from wild-type females (lane 7), but remain soluble in laid, fertilized eggs derived from either Class I or Class II *nudel* mutant females (lanes 8 and 9), indicating a failure of non-disulfide covalent cross-linking in the absence of Nudel protease function. The equivalent of 10 eggs/lane was run on 15% SDS–PAGE and blotted with Sv17 (top) or Sv23 (bottom) antibodies.

lent cross-linking of the vitelline membrane, these events include the translation of *bicoid* mRNA, release of a meiosis I block with completion of meiosis, and changes in the cortical microtubule cytoskeleton (for references, see Page and Orr-Weaver, 1997). We carefully examined the timing of Nudel protease activation to determine if the generation of the active form of Nudel protease occurs in response to egg activation and not fertilization, similar to vitelline membrane cross-linking.

Activation of the Nudel protease can be detected by the appearance of characteristic fragments of the Nudel protein on Western blots and by changes in the subcellular localization of the Nudel protease (LeMosy *et al.*, 1998). We previously observed that the active form of the Nudel protease is generated very early in embryogenesis, as judged by activation-associated changes in Nudel localization (LeMosy *et al.*, 1998). To determine if Nudel protease activation is triggered by fertilization, we compared the patterns of Nudel polypeptides found in 0- to 2-h fertilized eggs and in 0- to 2-h unfertilized eggs laid by virgin females. The active Nudel protease and two C-terminal Nudel polypeptides that are dependent on the presence of active Nudel protease could be detected in unfertilized eggs as well as in fertilized eggs, indicating that fertilization is not required for Nudel protease activation (Fig. 4A).

Mature Stage 14 egg chambers can be induced to undergo egg activation *in vitro* by exposure to an "activating buffer" (Mahowald *et al.*, 1983; Page and Orr-Weaver, 1997). Using such a buffer (Page and Orr-Weaver, 1997), we asked whether Nudel protease activation can be triggered by *in vitro* egg activation. The active form of Nudel protease and two C-terminal Nudel polypeptides that depend on Nudel protease function were generated only in egg chambers incubated in activating buffer, with or without subsequent incubation in a physiological buffer, and were not seen in egg chambers incubated only in isolation buffer (Fig. 4B). These findings suggest that Nudel protease activation is triggered, directly or indirectly, by the same chemical environment that triggers the previously characterized events of egg activation.

DISCUSSION

We have demonstrated that *nudel* mutations that compromise Nudel protease function also result in the failure of non-disulfide covalent cross-linking of the vitelline membrane that normally occurs at the onset of embryogenesis. We have shown that the Nudel protease is activated in response to egg activation and thus appears to temporally coincide with vitelline membrane cross-linking. These findings argue for an integral role for Nudel protease in eggshell biogenesis.

Two Structural Roles for Nudel?

The expression of Nudel containing a protease active site mutation predicted to eliminate all protease activity results in neutral red-permeable eggs containing dorsalized embryos, rather than the severely fragile eggs containing developmentally arrested embryos typical of Class I *nudel* alleles (this work and LeMosy *et al.*, 1998). This finding

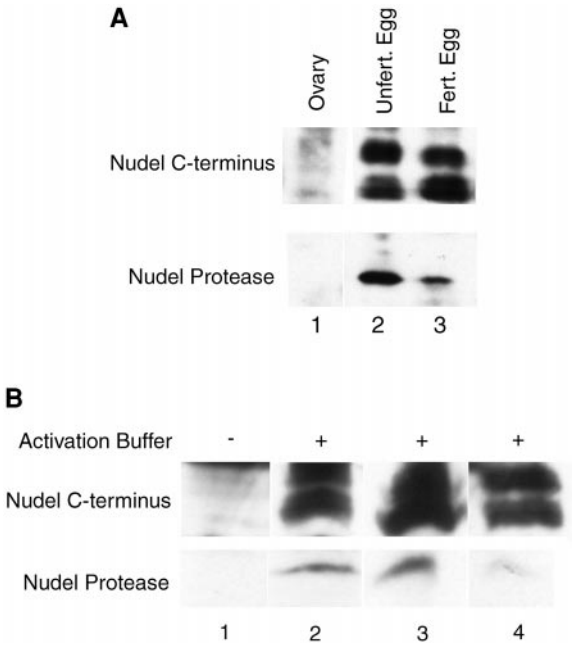

FIG. 4. Active Nudel protease appears to be generated at egg activation. Antibodies to the extreme C-terminus of Nudel and to the protease domain were used in Western blot analysis of egg extracts run on 4–16% SDS–PAGE gels. Shown are 50- to 60-kDa C-terminal forms that are found only when Nudel protease is active and a 33-kDa Nudel protease form believed to be the fully active Nudel protease (LeMosy *et al.*, 1998). (A) Western blotting of wild-type ovaries (lane 1), a 0- to 2-h collection of unfertilized eggs (lane 2), and a 0- to 2-h collection of fertilized eggs (lane 3) demonstrates that extracts of unfertilized eggs and fertilized eggs contain active Nudel protease, while ovary extract does not. (B) Generation of the Nudel protease in Stage 14 egg chambers activated *in vitro*. Following dissection of ovaries in isolation buffer, egg chambers were incubated in (1) isolation buffer, 25 min; (2) activation buffer, 5 min, followed by recovery buffer, 20 min; (3) activation buffer, 25 min; and (4) activation buffer, 5 min, followed by recovery buffer, 40 min. Generation of the active Nudel protease occurs only in samples exposed to activation buffer. Comparison of lanes 2 and 4 shows that the abundance of the active Nudel protease declines over time.

suggests that there is a significant, additional structural requirement for the nonprotease regions of Nudel. However, our biochemical analysis of vitelline membrane biogenesis did not detect any additional defect in the ndl^{14} mutant generally deficient for Nudel protein when compared to the ndl^{111} mutant lacking only Nudel protease function. One possibility is that Nudel is structurally required only for vitelline membrane cross-linking, with the Class I phenotype representing a more complete and catastrophic loss of cross-linking. Alternatively, there may be an earlier structural requirement for a non-protease region of Nudel during oogenesis that was not detected by our biochemical assays. Consistent with the latter possibility, we have observed modest structural abnormalities in Class I *nudel* mutant egg chambers, such as the presence of F-actin-containing inclusion bodies within the oocyte and separation of the oocyte plasma membrane from the vitelline membrane, that could reflect abnormalities of the extracellular matrix or of oocyte adhesion (E.K.L., unpublished).

Nudel Protease Function in Vitelline Membrane Cross-Linking

Cross-linking of the vitelline membrane at the onset of embryogenesis is thought to be performed by a peroxidase-type enzyme, based upon the presence of cross-linked dityrosine and trityrosine residues in hydrolysates of vitelline membranes prepared from laid eggs, but not ovaries (Petri *et al.*, 1976, 1979). Supporting this mechanism of cross-linking in *Drosophila,* there is strong evidence that the outer chorion layer of the eggshell is cross-linked by an endogenous peroxidase in late oogenesis (Mindrinos *et al.*, 1980).

Cross-linking reactions are tightly regulated by controlling the availability or activity of the cross-linking enzyme or by controlling the availability of the substrate. For example, the chorion peroxidase is incorporated into the forming eggshell but does not act until the follicle cells secrete H_2O_2, a hydrogen acceptor required for the cross-linking reaction (Margaritis, 1985; Mindrinos *et al.*, 1980). In contrast, the sea urchin fertilization envelope is rapidly generated and cross-linked by an ovoperoxidase that is secreted together with other structural components of the envelope and H_2O_2 (Foerder *et al.*, 1978; Foerder and Shapiro, 1977). While the vitelline membrane is preformed prior to cross-linking, like the chorion, no peroxidase appears to be associated with this structure during oogenesis (Mindrinos *et al.*, 1980). The vitelline membrane might be cross-linked by a mechanism involving incorporation of an active peroxidase into a preexisting scaffold at the onset of embryogenesis.

A likely role for the Nudel protease in cross-linking is the proteolytic activation of a cross-linking enzyme. Such proteolytic activation has been documented for several types of cross-linking enzymes but remains more speculative for the peroxidases (Johansson and Soderhall, 1996; Smith-Mungo and Kagan, 1997; Takahashi *et al.*, 1986). Proteolytic cleavage upon secretion has been demonstrated for the sea urchin ovoperoxidase (Deits *et al.*, 1984; LaFleur *et al.*, 1998). While the significance of this cleavage is unknown, conservation of the cleavage site among three sea urchin ovoperoxidases and a *Drosophila* peroxidase, peroxidasin, suggests that cleavage might be important for peroxidase function (LaFleur *et al.*, 1998; Nelson *et al.*, 1994; Nomura *et al.*, 1999). Alternatively, the Nudel protease could be involved in another aspect of a cross-linking reaction, such as the release of H_2O_2 from the oocyte or cleavage of a vitelline membrane protein to generate a form capable of being cross-linked (Margaritis, 1985; Smith-Mungo and

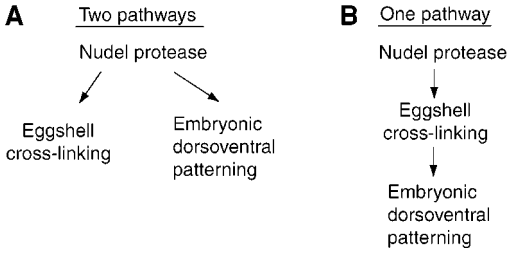

FIG. 5. Alternative models for Nudel protease function in dorsoventral patterning. In (A), the Nudel protease acts in two distinct pathways that lead to eggshell cross-linking and to the establishment of embryonic dorsoventral polarity. This could occur if the Nudel protease acts on two substrates, e.g., a peroxidase involved in cross-linking and a protease zymogen in the dorsoventral protease cascade. In (B), the Nudel protease functions in one pathway in which cross-linking is somehow required for embryonic dorsoventral patterning. Cross-linking of the eggshell (or at the plasma membrane) could generate a matrix structure required for activity of the dorsoventral protease cascade. In this one-pathway model, Nudel protease does not directly cleave a protease zymogen in the dorsoventral protease cascade.

Kagan, 1997). In any case, the identification of *nudel* as a gene required for cross-linking of the vitelline membrane and the description of a mutant phenotype associated with a defect in this specific step of vitelline membrane biogenesis should facilitate future biochemical and genetic studies of this process.

Relationship of Nudel Protease Function in Eggshell Cross-Linking to Embryonic Dorsoventral Patterning

A compelling question arising from this work is, what is the relationship between Nudel's functions in eggshell cross-linking and embryonic patterning? Our previous work established that catalytic activity of the Nudel protease domain is essential for embryonic dorsoventral patterning (LeMosy *et al.*, 1998), while we have now shown that Nudel protease activity is also required for vitelline membrane cross-linking. One possibility is that the Nudel protease cleaves distinct substrates that act independently in vitelline membrane cross-linking and in dorsoventral patterning, e.g., a peroxidase and a protease zymogen in the dorsoventral protease cascade. In this two-pathway model (Fig. 5A), the involvement of the Nudel protease in eggshell biogenesis is irrelevant to its role in dorsoventral patterning.

A potentially more interesting possibility is that Nudel protease acts in only one pathway with dorsoventral patterning dependent upon Nudel's activity in a cross-linking reaction (Fig. 5B); in this model, the Nudel protease would not directly cleave a protease zymogen in the dorsoventral protease cascade. Several lines of evidence suggest that if cross-linking is required for dorsoventral patterning, this requirement is likely to be specific rather than due to general leakiness of the vitelline membrane. Studies of the downstream components of the dorsoventral protease cascade have shown that preactivated forms of the Snake and Easter proteases can function in the perivitelline space of *nudel* mutant embryos and suggest that the endogenous components are not lost by leakage through the defective vitelline membrane of these embryos (Chasan *et al.*, 1992; Misra *et al.*, 1998; Morisato and Anderson, 1994; Smith and DeLotto, 1994). This argument is further supported by the finding that certain mutant alleles of the terminal-group gene, *fs(1)Nasrat*, produce eggs with leaky vitelline membranes within which embryos develop with normal dorsoventral polarity (Degelmann *et al.*, 1990).

Cross-linking of the vitelline membrane could directly lead to the creation of a specific matrix structure that is necessary for the function of one or more of the dorsoventral proteases. An analogy for this is found in the fibrinolytic protease cascade in mammals, in which cross-linked fibrinogen and fibrin act as catalysts to dramatically increase the conversion of a serine protease zymogen, plasminogen, to its active form, plasmin (Mosesson *et al.*, 1998; Nieuwenhuizen, 1994). Cross-linked fibrinogen and fibrin have this property, while monomeric fibrinogen does not, because polymerization of fibrinogen exposes binding sites for the serine proteases tissue plasminogen activator (tPA) and plasminogen that appear to orient the active site of tPA with the zymogen activation site of plasminogen. Similarly, vitelline membrane cross-linking involving Nudel protease could expose binding sites on the inner surface of the vitelline membrane that are involved in the formation of a zymogen activation complex for the dorsoventral protease cascade. A variant of the one-pathway model is that the Nudel protease activates cross-linking of not only the vitelline membrane but also a matrix structure at the plasma membrane where Nudel resides and that dorsoventral patterning is dependent on the latter cross-linking event (LeMosy *et al.*, 1998). Distinguishing among these possibilities will be important to the long-term goal of understanding how the Toll signaling pathway is initiated and ventrally restricted within the embryonic perivitelline space.

ACKNOWLEDGMENTS

We thank Gail Waring for generously providing antibodies to chorion and vitelline membrane proteins and Lynn Cooley, Mark Solomon, and Sandra Wolin for critically reading the manuscript. This work was supported by postdoctoral fellowships from the National Institutes of Health (HD-08041) and the American Heart Association, Heritage Affiliate, to E.K.L. and by National Institutes of Health Grant GM-49370 to C.H.

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Received for publication September 14, 1999
Revised November 4, 1999
Accepted November 4, 1999