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The N-terminal Prodomain of sV23 is Essential for the Assembly of a Functional Vitelline Membrane Network in *Drosophila*

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The N-Terminal Prodomain of sV23 Is Essential for The Assembly of a Functional Vitelline Membrane Network in *Drosophila*

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Abstract

The vitelline membrane is a specialized extracellular matrix that surrounds and protects the oocyte. Recent studies indicate that it also serves as a storage site for embryonic pattern determinants. sV23, a major vitelline membrane protein, is essential for the morphogenesis of the vitelline membrane as sV23 protein null mutants lay flaccid, infertile eggs. By analyzing a series of sV23 mutant transgenes in the sV23 protein null genetic background, we have shown that sV23 is secreted as a proprotein in

functional excess and that C- and N-terminal prodomains are removed successively, following its deposition in the extracellular space. Although a target site for subtilisin-like convertases is essential for N-terminal processing, N-terminal processing is not necessary for the assembly of a functional vitelline membrane layer. While C-terminal truncations were tolerated, the removal of N-terminal sequences lead to the production of flaccid, infertile eggs with a soluble, rather than insoluble, vitelline membrane network. We propose that the hydrophobic N-terminal prodomain plays an early and essential role in aligning molecules within the vitelline membrane network, much like hydrophobic domains within elastin drive the assembly and alignment of molecules within elastin-based extracellular matrices.

Keywords

Extracellular assembly, Vitelline membrane formation, *Drosophila,* Mutant transgenes, sV23 eggshell gene, Prodomains

Introduction

Assembly of precise, well-defined extracellular architectures is critical for tissue integrity and function. Extracellular matrices not only provide mechanical support for tissues, but by transiently housing bioactive molecules, play a critical role in regulating a variety of cellular processes Streuli, 1999, Taipale and Keski-Oja, 1997. While significant progress has been made in identifying matrix components, how the secreted building blocks self-assemble into functional networks remains a formidable challenge. In vitro binding studies have provided a wealth of information on potential molecular interactions within several specialized extracellular matrices. However, in the absence of the spatial and temporal controls that drive assembly processes in vivo, the relevancy of these interactions to in vivo assembly remains to be established. For example, until recently, it was thought that basement membranes formed spontaneously by self-assembly. However, it is now recognized that cell membrane receptors, which are not basement membrane constituents, play an essential role in its formation in vivo (Quondamatteo, 2002). Likewise, fibronectin matrix assembly is also a cell-mediated process that requires coordination between extracellular and intracellular events (Wierzbicka-Patynowski and Schwarzbauer, 2003).

The *Drosophila* eggshell is a specialized extracellular matrix whose assembly is particularly amenable to in vivo analysis. It forms in a genetically tractable organism and defects in its assembly can be monitored at the genetic, biochemical, and morphological levels. Synthesized during the later stages of oogenesis by somatic follicle cells that overlie the developing oocyte, the eggshell consists of four morphologically distinguishable layers: an oocyte proximal vitelline membrane, a crystalline inner chorionic layer (icl), a tripartite endochorion, and an outer non-proteinaceous exochorion (Margaritis, 1985). Eggshell assembly is a complex process involving temporal as well as spatial regulation. Eggshell genes are expressed in a defined temporal sequence during the later stages of oogenesis (Waring, 2000). Vitelline membrane proteins are synthesized during the early stages of eggshell formation (stages 8–10), while endochorion proteins are synthesized later, during stages 11–14. Some eggshell proteins are secreted as proproteins that are cleaved in a stage specific manner following their deposition into the extracellular environment Mauzy-Melitz and Waring, 2003, Noguerón and Waring, 1995, Pascucci et al., 1996. Several eggshell proteins are initially housed in the vitelline membrane

layer and, either in part or in toto, become localized to the more distal inner chorionic and endochorion layers later in oogenesis Andrenacci et al., 2001, Nogueron et al., 2000, Pascucci et al., 1996. Thus, both temporal and spatial controls can be used to regulate appropriate protein–protein interactions during eggshell assembly.

The focus of the present study is the assembly of the oocyte proximal vitelline membrane layer. In addition to providing mechanical support and protecting the embryo from environmental hazards, the vitelline membrane houses informational biomolecules. Long considered a potential reservoir for follicle cell products involved in embryonic patterning, Stevens et al. (2003) have recently shown that the embryonic patterning determinant, torso-like, is a component of the vitelline membrane. Thus, the vitelline membrane provides a means by which information from ovarian follicle cells can be stored and transferred to the developing embryo (Stevens et al., 2003).

Four major vitelline membrane genes have been identified to date: sV17, sV23, VM32e, and VM34c (reviewed in Waring, 2000). All encode small proline-rich proteins that include a highly conserved 38 amino acid sequence, termed the vitelline membrane (VM) domain. sV23, this study's focus, is essential for the assembly of a functional eggshell. sV23 protein null mutants (*fs(2)QJ42*) are female sterile and the females lay flaccid eggs (Savant and Waring, 1989). The female sterile phenotype can be rescued by the introduction of wild-type sV23 transgenes that include sufficient sV23 5′ and 3′ regulatory sequences to drive its normal expression Fokta, 2000, Savant and Waring, 1989. To better understand vitelline membrane assembly and identify regions within the sV23 molecule that mediate this process, we created sV23 transgenes in which specific amino acids sequences were removed. By analyzing the effects of these mutations in *fs(2)QJ42* females, we established relationships between protein sequence, protein assembly, and genetic function. We show that C- and N-terminal propeptides are removed from the sV23 proprotein after its deposition into the vitelline membrane layer. While premature removal of the C-terminal propeptide did not have any functional consequences, premature removal of N-terminal sequences resulted in the production of an extracellular form of sV23 that could not support proper eggshell development. Extraction studies indicated that the hydrophobic N-terminal prodomain may be essential for aligning vitelline membrane molecules for assembly.

Materials and methods

Culture conditions and stocks

The Oregon R, P2 wild-type strain was maintained in mass culture; all other stocks were maintained on standard yeast, cornmeal, molasses, and agar medium. Before dissection, the standard medium was supplemented with live yeast paste for 3 to 5 days. The *fs(2)QJ42* female sterile mutants, described previously Pascucci et al., 1996, Savant and Waring, 1989, fail to produce the sV23 vitelline membrane protein. DNA sequence analysis showed that the sV23 initiating methionine codon was mutated to isoleucine in this mutant (Fokta, 2000). Transformant lines carrying mutated sV23 transgenes were created as described below.

Creation of sV23 mutant transgenes

sV23 transgenes containing small internal deletions were created using a previously described PCR based strategy (Hughes and Andrews, 1996). A 1-kb *Sal*I–*Xba*I genomic fragment (5′ to 3′, respectively)

containing the sV23 coding region and 140 base pairs of 5′ and 210 base pairs of 3′ flanking DNA was cloned into a pSP73 plasmid vector. Using the pSP73 *Sal*I–*Xba*I subclone as template, sV23-specific primers, bearing tails with an engineered *Apa*I site, and pSP73-specific primers were used to generate PCR fragments that abutted the regions to be deleted. For each deletion, the 5′ *Sal*I–*Apa*I fragment was inserted into a *Sal*I–*Apa*I cut pGEM11z vector. Following digestion with *Apa*I and *Xba*I, the 3′ *Apa*I–*Xba*I fragment was inserted into the *Sal*I–*Apa*I pGEM11z subclone yielding the desired *Sal*I–*Xba*I deletion construct plus ectopic *Apa*I bases. To remove the ectopic *Apa*I bases, the deletion construct was digested with *Apa*I and the resulting 4-bp 3′ overhangs were removed with Klenow fragment (Promega, Madison, WI) or T4 DNA polymerase (Promega). The resulting blunt ends were religated and the precision of the deletion was confirmed by DNA sequencing (ABI 373a automated DNA sequencer, University of Wisconsin, Milwaukee). Previous studies had indicated that while 150 base pairs of 5′ flanking DNA was sufficient (Savant and Waring, 1989), at least 750 base pairs of 3′ flanking DNA (Fokta, 2000) was necessary to drive sufficient sV23 expression to rescue the *fs(2)QJ42* female sterile phenotype. Using these boundaries as a guide and making use of unique and appropriately positioned restriction sites, a minimum of 1.2 kb of additional sV23 5′ flanking DNA was added via a *Sal*I fragment and 2 kb of additional 3′ flanking DNA was added via a *Xba*I–*Eco*RI fragment to insure adequate expression. Utilizing a *Xho*I site in the pGEM11z linker region and the *Eco*RI site in the sV23 3′ flanking DNA, the sV23 genomic regions containing the desired deletions ($\Delta N^{24–42}$; $\Delta RVSR^{43–45}$; $\Delta C^{155–167}$; $\Delta C^{140–167}$; $\Delta C^{117–167}$) were then subcloned into *Xho*I–*Eco*RI cut pCaSpeR4, a P-element transformation vector. The recombinant pCaSpeR4 plasmid DNAs were purified using a Qiagen Plasmid Midi Kit (Qiagen, Valencia, CA), and each was coinjected with the “wings clipped” helper plasmid, pπ25.7wc, into preblastoderm embryos homozygous for the mutant eye color gene white (*w*) (Spradling and Rubin, 1982). Flies that eclosed from the injected embryos were mated with white-eyed (*w*) flies of the opposite sex and the offspring were screened for transformants. After the chromosomal linkage of the transgene was determined, a homozygous “transgene” stock was created and maintained. One or two copies of the mutant sV23 transgenes were introduced into *fs(2)QJ42* females by appropriate genetic crosses.

Western blot analysis

Ovaries were dissected and egg chambers were separated, staged, and solubilized in SDS and β-mercaptoethanol-containing buffers as previously described (Noguerón and Waring, 1995). Laid eggs from wild type and mutant flies were collected on plates made by filling 60-mm culture dishes with agar/molasses medium. Vials containing flies were capped with the plates, inverted, and allowed to sit for 50 min. Laid eggs (embryos) were manually removed from the plates and resuspended in Laemmli sample buffer containing 5% β-mercaptoethanol as above. Proteins were separated on 15% SDS polyacrylamide gels and transferred to PVDF or nitrocellulose (Bio-Rad, Hercules, CA) membranes for 50 min at 35 V using a Bio-Rad Trans-Blot Apparatus with plate electrodes. The three antibodies used in this study, sV23, sV17, and s36, have been described previously (Pascucci et al., 1996). Antigen–antibody complexes were visualized indirectly, as previously described (Mauzy-Melitz and Waring, 2003), by either autoradiography, following the addition of ^{125}I-Protein A, or enhanced chemical luminescence, following the addition of HRP-conjugated goat anti-rabbit secondary antibodies. For reprobing with different antibodies, nitrocellulose membranes were placed in Tris-buffered saline containing 0.7% β-mercaptoethanol and 2% SDS for 30 min at room temperature,

rinsed, blocked, incubated with the antiserum, and processed as above. To purify the sV23 antibody, 10 teased ovaries from *fs(2)QJ42* flies (sV23 protein null) were incubated at 4° overnight with 100 µl of serum that had been diluted ten-fold. After brief centrifugation to remove the ovaries and bound cross-reacting antibodies, the pre-adsorbed serum was diluted further and used immediately.

Results

N-terminal prosequences are required for sV23 function

Conceptual translation of the sV23 gene predicts a 168 amino acid protein with a modular nature. Distinctive features of the protein include a signal sequence with a predicted cleavage site at residue 21, a large central region consisting of five perfect and three degenerate copies of an octapeptide (PAYSAPAA), and a conserved 38 amino acid VM domain that is present in other vitelline membrane proteins (Fig. 1). Sequence comparison of the *Drosophila melanogaster* and *D. virilis* sV23 proproteins revealed 93% identity at the amino acid level (Fokta, 2000). The majority of the amino acid differences occurred in the central repeat region and primarily reflected insertions of alanine, proline, and tyrosine, the major constituents of the repeating motif. The N-terminal region (aa 22–53) preceding the alanine, proline-rich octapeptides, was 100% identical and included dibasic (RVSR) and GGYG motifs that are found in several other eggshell genes. To investigate the functional significance of the N-terminal region, two copies of a sV23 transgene bearing a N-terminal deletion (ΔN^{24-42}) (Fig. 1) were introduced into *fs(2)QJ42* females. Staged egg chambers were collected and analyzed by Western blot analysis using as probe a polyclonal antiserum raised against a bacterial fusion protein that included sV23 amino acids 26 through 136. The developmental Western blot in Fig. 2A shows the reactive products detected in wild type, and *fs(2)QJ42* mutant egg chambers in the absence or presence of the transgene. As expected, egg chambers from homozygous *fs(2)QJ42* females (lanes a–e) do not show reactive products in the sV23 size range. Wild-type egg chambers (lanes f–k) display stage specific differences in the mobilities of the reactive sV23 products. As described previously, sV23 migrates as a smear in the 23-kDa size range in stage 10 egg chambers (lane f) and as a smear with a leading edge in the 17 kDa size range in stages 13 and 14 egg chambers (lanes j and k; Pascucci et al., 1996). A transient smear of intermediate mobility was observed in stages 10b–12 (lanes g–i). Egg chambers from *fs(2)QJ42* females carrying two copies of the ΔN^{24-42} transgene (lanes l–q) displayed two, rather than three, smears with distinct mobilities: a slower migrating smear in stages 10a and 10b (lanes l and m) whose migration was compatible with the removal of 18 amino acids, and a faster migrating smear in stages 11–14 (lanes n–q) that appeared comparable to the “17 kDa” smear observed in wild-type stage 13 and 14 egg chambers (lanes j and k). The shift in mobility of sV23 between stages 10b and 11 in the transgenic egg chambers (lanes m and n) suggests that early processing of the sV23 proprotein does not involve the amino-terminus; moreover, the lack of a distinct shift between stages 11 and 12 (lanes n and o) suggests that late stage processing involves the removal of N-terminal sequences that are missing in the truncated ΔN^{24-42} product.

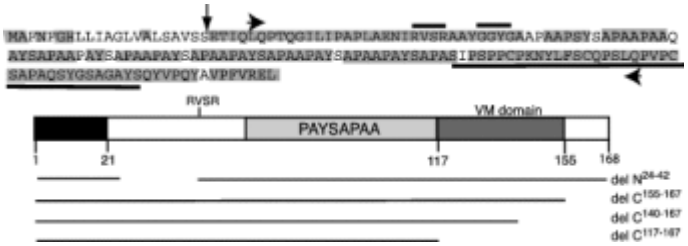

Fig. 1. sV23 vitelline membrane protein. The predicted 168 amino acid *D. melanogaster* sV23 protein is shown across the top. The vertical arrow indicates the predicted signal sequence cleavage site; the horizontal arrows demarcate the region of sV23 that was used in the production of the sV23 antiserum; the lines above the sequence indicate the dibasic and GGYG motifs that are characteristic of several eggshell proteins; the hallmark VM domain is underscored; amino acids that are identical in the *D. virilis* homolog are shaded. The schematic shown below highlights the modular structure of the sV23 gene: the signal sequence (1–21); the N-terminal region (open rectangle) with a potential subtilisin-like cleavage site (RVSR); a central region consisting of five perfect and three degenerate copies of the octapeptide indicated; the 38 amino acid VM domain (shaded rectangle); and a 13 amino acid C-terminus (open rectangle). The lines below the schematic indicate the sequences that were retained in four of the mutant sV23 transgene products analyzed in this study. The superscript numbers indicate the amino acids that were deleted in each sV23 proprotein.

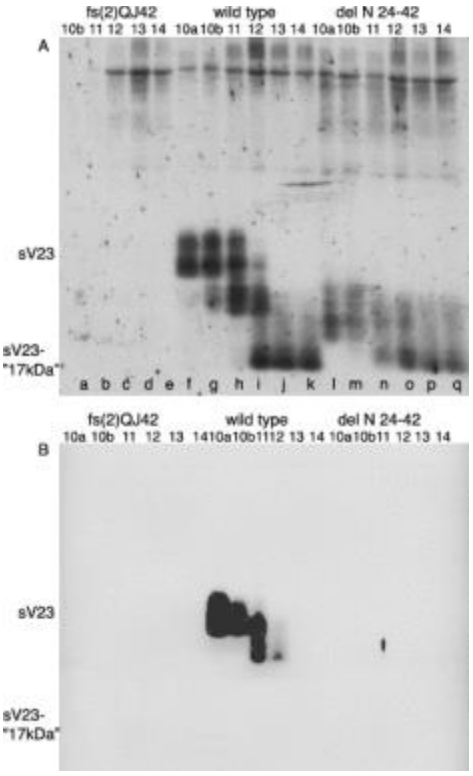

Fig. 2. N-terminal sequences are removed in processed wild-type stages 13 and 14 sV23 derivatives. (A) Western blot of egg chamber proteins from *fs(2)QJ42*, wild type, and *fs(2)QJ42* females carrying two copies of the $\Delta N^{24–42}$ transgene (del $N^{24–42}$) incubated with sV23 antiserum (1/1000 dilution). Egg chamber stages are shown across the top, lanes are lettered at the bottom; the positions of sV23 and its “17 kDa” derivative in wild-type egg chambers are shown at the left. For the *fs(2)QJ42* and wild-type samples, 10 egg chambers were used per lane; 15 egg chambers per lane were used for the $\Delta N^{24–42}$ samples. (B) Similar developmental Western blot using 10 egg chambers per lane incubated with adsorbed sV23 antiserum (1/1000 dilution). The egg chamber stages are shown across the top and the

positions of sV23 and the “17 kDa” derivative (based on superimposition of a blot incubated with the unabsorbed serum) are indicated at the left. sV23-derived signals were not detected in *fs(2)QJ42* egg chambers in either the presence of absence of the $\Delta N^{24–42}$ transgene or in wild-type stages 13 and 14 egg chambers even upon prolonged exposures (not shown).

In previous studies we showed that the sV23 antiserum detects immunoreactive species in the vitelline membrane of *fs(2)QJ42* egg chambers (Pascucci et al., 1996). This indicates that sV23 and other vitelline membrane proteins share common epitopes. Antibodies directed against the common epitopes were removed from the sV23 serum by pre-adsorbing the serum with acetone extracts from *fs(2)QJ42* ovaries. With the pre-adsorbed serum, immunoreactive products were detected in situ and by Western blotting in wild-type stages 10–12 egg chambers. Immunoreactive products were not detected in wild-type stage 14 egg chambers. These data suggested that sV23-specific epitopes were removed during late oogenesis (Pascucci et al., 1996). Fig. 2B shows a Western blot of staged egg chambers from wild type, *fs(2)QJ42* females, and *fs(2)QJ42* females carrying two copies of the $\Delta N^{24–42}$ transgene incubated with sV23 antiserum that had been pre-adsorbed with teased *fs(2)QJ42* ovaries. Immunoreactive products were not detected in wild-type stages 13 and 14 egg chambers, *fsQJ42* egg chambers, or egg chambers from *fsQJ42* females carrying the $\Delta N^{24–42}$ transgene. These data suggest that the sV23-specific epitopes recognized by the pre-adsorbed serum are located within the region defined by the $\Delta N^{24–42}$ deletion, and supports the conclusion from Fig. 2A above that in wild-type egg chambers N-terminal sequences are removed from sV23 during late stage processing.

Although N-terminal sequences appear to be removed during late stage processing, amino-terminal sequences are required for sV23 function. The $\Delta N^{24–42}$ transgene from two independent lines failed to rescue the female sterility of *fs(2)QJ42* females. The intensity of the sV23 signal in $\Delta N^{24–42}$ stage 14 egg chambers was reduced relative to wild type. Nevertheless, sV23 accumulated at levels well in excess of what is needed to support the formation of a functional eggshell (discussed later).

The failure to detect N-terminal epitopes in sV23 derivatives from stages 13 and 14 suggests that the N-terminal sequences function in the context of the stage 10 proprotein or intermediate stage 11/12 derivative. Prodomains are typically used to maintain extracellular proteases in a latent form. Failure to remove the prodomain results in inactive proenzymes that fail to cleave their substrates. While the function of the sV23 N-terminal prosequence remains unknown, the evolutionary conservation of sV23 stage-specific processing (Pascucci et al., 1996) suggests that the removal of the sV23 prodomains is functionally significant. Many secreted proteins are excised from inactive proproteins by cleavage at pairs of basic residues. The cleavages are catalyzed by serine proteases that belong to the kex2/subtilisin-like family of proprotein convertases (Van de Ven et al., 1993). Inspection of the sV23 sequence revealed a potential dibasic cleavage site (RVSR) at a position (amino acids 43–46) whose use would be compatible with the reactivity of the “17 kDa” stage 13/14 derivative. To determine if the dibasic cleavage site is utilized, the 12 nucleotides encoding the dibasic RVSR site (Fig. 1) were deleted from a wild-type sV23 transgene yielding ΔRVSR. The Western blot in Fig. 3 shows that *fs(2)QJ42* females carrying two copies of the ΔRVSR transgene accumulate wild-type quantities of a slightly faster migrating form of sV23 in stage 10 egg chambers. The comparable shifts in the mobilities of the wild type and ΔRVSR sV23 proteins in stage 11 egg chambers indicate that the removal of the dibasic site does not impair biogenesis of the intermediate sV23 derivative. The

absence of the faster migrating “17 kDa” species and the presence of the intermediate sV23 derivative in stage 14 egg chambers from *fs(2)QJ42* females carrying the ΔRVSR transgene show that the dibasic sequences are essential for the removal of the N-terminal prodomain and that the removal of the N-terminus is likely catalyzed by a subtilisin-like proprotein convertase. While prodomain removal is essential for the function of many extracellular proteins, the removal of the sV23 N-terminus is not essential for its function as the non-cleavable substrate generated by the ΔRVSR transgene from three independent lines was capable of rescuing the *fs(2)QJ42* female sterile phenotype.

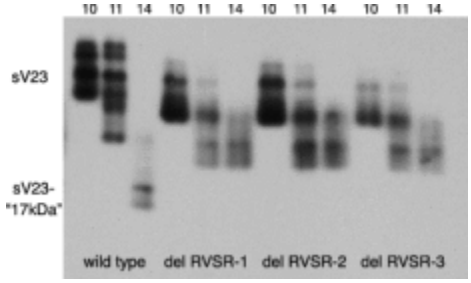

Fig. 3. Late stage processing of sV23 does not occur when the dibasic RVSR sequence is deleted. Western blot of egg chamber proteins from wild type and *fs(2)QJ42* females containing two copies of the ΔRVSR transgene. The egg chamber stages are shown at the top; del RVSR-1, del RVSR-2, and del RVSR-3 indicate *fs(2)QJ42* females carrying the ΔRVSR sV23 transgene from three independent transformant lines. The blot was incubated with sV23 antiserum diluted 1/1500. The positions of the sV23 and “17 kDa” derivatives in wild-type egg chambers are shown at the left.

The evolutionarily conserved C-terminal domain of sV23 is not required for female fertility

The reactivity of the intermediate sV23 derivative in wild-type egg chambers with the pre-adsorbed sV23 antiserum (Fig. 2B, stages 11 and 12) and the shift in the mobility of the N-terminally truncated proprotein ($\Delta N^{24–42}$) at stage 11 (Fig. 2A) both suggested that the sV23 proprotein undergoes C-terminal processing before the removal of the N-terminal amino acids. To substantiate this hypothesis and evaluate the importance of the evolutionarily conserved C-terminus, a sV23 transgene in which the nucleotides encoding amino acids 155 through 167 were deleted ($\Delta C^{155–167}$, Fig. 1), was introduced into homozygous *fs(2)QJ42* mutant females. Fig. 4 shows that a smaller product, commensurate with the removal of 13 amino acids, accumulated in $\Delta C^{155–167}$ stage 10 egg chambers (lanes g–h). The mobility of the truncated product was similar to that of the intermediate sV23 derivative seen in wild-type stage 11 and 12 egg chambers (lanes c and d), suggesting that biogenesis of the intermediate derivative involves the removal of about 13 amino acids. The absence of a perceptible size change in the $\Delta C^{155–167}$ proprotein between stages 10b and 11 (lanes h and i) suggests that normal processing involves the early removal of C-terminal residues. The shift in the mobility of the $\Delta C^{155–167}$ proprotein during stages 12–14 to a derivative whose migration is essentially indistinguishable from that of the wild-type “17 kDa” stage 14 derivative indicates that the truncated $\Delta C^{155–167}$ protein undergoes normal N-terminal processing. *fs(2)QJ42* females carrying either one or two copies of the $\Delta C^{155–167}$ transgene from four independent lines laid turgid eggs that gave rise to viable progeny. Taken together, the data indicate that C-terminal amino acids are removed from the wild-type sV23 proprotein between late stages 10 and 11 and that the site of cleavage is at or very close to the boundary between the VM and C-terminal

domains (Fig. 1). Premature removal of the C-terminal amino acids does not appear to accelerate late N-terminal processing or impair the function of sV23.

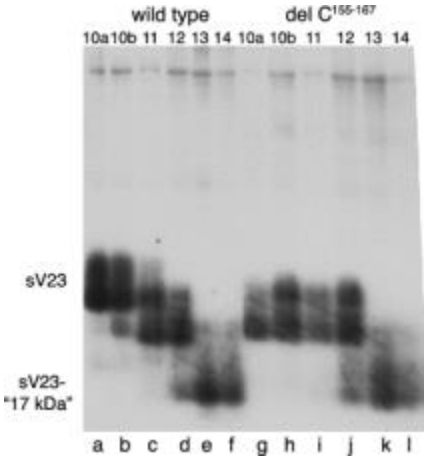

Fig. 4. Removal of the sV23 C-terminus precedes cleavage of its N-terminus in late stage egg chambers. Western blot of egg chamber proteins from wild-type females and *fs(2)QJ42* females containing two copies of the $\Delta C^{155–167}$ transgene (del $C^{155–167}$) incubated with sV23 antiserum (1/2000 dilution). Egg chamber stages are indicated at the top, lanes are lettered at the bottom. The positions of the sV23 and "17 kDa" derivatives in wild-type egg chambers are shown at the left.

Sequences within the highly conserved VM domain are required for proper accumulation of sV23

The 38 amino acid VM domain (Fig. 1) is highly conserved among different members of the vitelline membrane gene family. A 10 amino acid deletion centered within the VM domain of the VM32e protein affected its initial spatial distribution within the vitelline membrane (Andrenacci et al., 2001). In early wild-type stage 10 egg chambers, the VM32e protein is excluded from the pole regions. VM32e proteins bearing the deletion were distributed homogeneously within the vitelline membrane layer. Since wild-type VM32e proteins displayed a similar aberrant behavior in *fs(2)QJ42* stage 10 egg chambers, it was postulated that the VM domain is required for protein–protein interactions among vitelline membrane proteins. To test the functional significance of sequences within the sV23 VM domain, the C-terminal truncation was extended 15 amino acids into the VM domain ($\Delta C^{140–167}$Fig. 1). Fig. 5A shows a developmental Western blot of staged egg chamber proteins from *fs(2)QJ42* females carrying two copies of the $\Delta C^{140–167}$ transgene incubated with sV23 antiserum. The migration of the smear observed in stage 10 egg chambers is consistent with the removal of 28 amino acids, and the absence of a shift in mobility between stages 10 and 11 is compatible with a C-terminal truncation. The increase in the mobility of the leading and trailing edges of the smear in stages 12–14 is compatible with normal N-terminal processing. To verify N-terminal processing, a developmental blot was incubated with the pre-adsorbed N-terminal-specific sV23 antiserum (Fig. 5B). As expected, in both the wild-type and transgenic egg chambers, only the slower migrating species found in stages 10 and 12 were recognized. The blot shown in Fig. 5B was stripped and re-probed with the sV23 antiserum to confirm that sV23-reactive material was present in the stage 14 egg chambers (data not shown). Taken together, these data indicate that N-terminal processing is not affected by the

removal of these twenty-eight C-terminal amino acids, although minor differences in the position of the cleavage cannot be excluded.

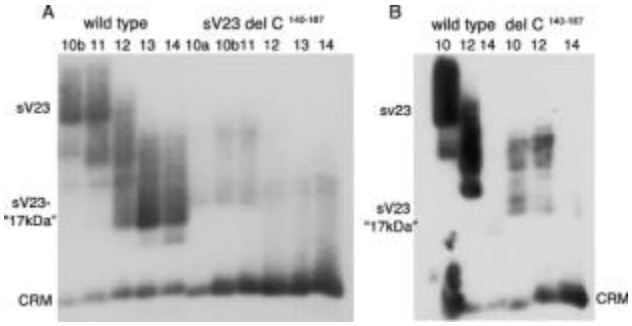

Fig. 5. Extracellular accumulation and N-terminal processing of sV23 derivatives produced by the $\Delta C^{140–167}$ transgene. (A) Western blot of egg chamber proteins from wild-type females (2 egg chambers/lane) and *fs(2)QJ42* females carrying two copies of the $\Delta C^{140–167}$ transgene (del $C^{140–167}$; 10 egg chambers/lane) incubated with sV23 antiserum (1/2000 dilution). Egg chamber stages are indicated at the top; the positions in wild-type egg chambers of sV23, its "17 kDa" derivative, and cross-reacting material (CRM—defined by its presence in *fs(2)QJ42* egg chambers) are indicated at the left. (B) Developmental Western blot of wild-type egg chamber proteins (4 egg chambers/lane) and egg chamber proteins from *fs(2)QJ42* females carrying two copies of the $\Delta C^{140–167}$ transgene (20 egg chambers/lane) incubated with pre-adsorbed sV23 antiserum (1/2000 dilution). The positions of sV23, the CRM, and the "17 kDa" sV23 derivative in wild-type egg chambers are shown. The position of the latter was determined by re-incubating the blot with unabsorbed sV23 serum. Although CRM is evident with the pre-adsorbed serum, its relative intensity is considerably reduced when compared with equivalent exposures using the unadsorbed serum (not shown).

Relative to wild type, there was a considerable reduction in the intensity of the sV23-reactive products in egg chambers from *fs(2)QJ42* females carrying two copies of the $\Delta C^{140–167}$ transgene (Fig. 5A). To estimate the extent of the reduction, the signal intensities of the sV23 derivatives from varying numbers of wild-type and mutant stage 10b egg chambers were compared (Fig. 6). Stage 10b egg chambers were used for the analysis since sV23 synthesis is essentially complete and processing of the sV23 proprotein has not yet begun. Stage 10b egg chambers from *fs(2)QJ42* females carrying two copies of the $\Delta C^{155–167}$ transgene were included for comparison. Fig. 6 shows that one wild type, and 10 *fs(2)QJ42*;$\Delta C^{140–167}$ stage 10b egg chambers gave comparable sV23 signals with the sV23 antiserum. Since similar results were observed with independent transformant lines (lines 1 and 2), wide spread differences in gene expression due to the different and ectopic sites at which the $\Delta C^{140–167}$ transgenes integrated did not appear to be a significant factor. The sequences deleted in the C-terminal truncations were not included in the open reading frame used to generate the polyclonal sV23 antibodies (Fig. 1) therefore differences due to missing epitopes were not a consideration. The similar intensities of the wild type and $\Delta C^{155–167}$ signals with equivalent numbers of egg chambers (Fig. 6) indicate that the truncated $\Delta C^{155–167}$ derivatives accumulate at near wild-type levels.

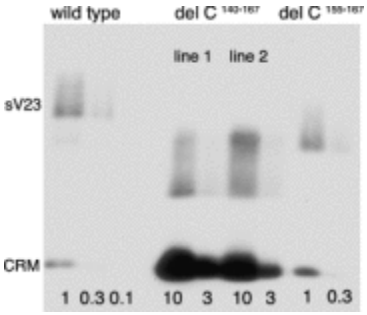

Fig. 6. sV23 produced by the ΔC$^{140–167}$ transgene accumulates at approximately 10% of wild-type levels. Western blot of proteins from varying numbers of stage 10b egg chambers (indicated at the bottom of each lane) from wild type or *fs(2)QJ42* females carrying two copies of either the ΔC$^{140–167}$ or ΔC$^{155–167}$ sV23 transgene. The blot was incubated with sV23 antiserum (1/2000 dilution). Females carrying the ΔC$^{140–167}$ transgene from two independent transformant lines (lines 1 and 2) were examined. The positions of sV23 and the CRM in wild-type egg chambers are shown at the left. The increased mobility of the sV23 smear in the ΔC$^{140–167}$ and ΔC $^{155–167}$ lanes reflects the deletion of 28 and 13 amino acids, respectively. The relative intensities of the CRM signals indicate that the accumulation of the CRM is not affected by the presence of the truncated sV23 derivatives.

Despite the significant reduction in the ΔC$^{140–167}$ derivatives that accumulated in the *fs(2)QJ42* mutant egg chambers, fertility was restored in some *fs(2)QJ42* females carrying two copies of the ΔC$^{140–167}$ transgene (33% and 60% for lines 1 and 2 females, respectively). When fertile, approximately 50% of the females produced progeny at wild-type levels. All *fs(2)QJ42* females carrying one copy of the transgene were infertile. The simplest explanation for the dosage dependence and variable fertility of females carrying two copies of the transgene is that threshold quantities of functional sV23 derivatives are required to assemble an eggshell that is compatible with fertility. Provided that threshold levels accumulate (approximately 10% of wild type), truncated ΔC$^{140–167}$ sV23 derivatives are able to support the assembly of a functional eggshell.

The *D. melanogaster* VM domain includes eight amino acids whose identity and spacing have been strictly conserved in vitelline envelope genes from the mosquito, *Aedes aegypti*(Edwards et al., 1998). These evolutionarily conserved residues lie between amino acids 121 and 140 of the sV23 VM domain. A transgene that encoded sV23 with a C-terminal truncation which encompassed the entire VM domain along with the C-terminus (ΔC$^{117–167}$) failed to rescue the *fs(2)QJ42* female sterile phenotype. Furthermore, unlike ΔC$^{140–167}$, ΔC$^{117–167}$ sV23 derivatives were not detected by Western blot analysis, even after prolonged exposures (data not shown). Similar results were observed with three independent transformant lines indicating that the VM domain, either alone or in concert with the C-terminus, is required for the accumulation of stable sV23 products.

During late oogenesis, vitelline membrane proteins, including sV23, are extractable provided reducing agents are present. After the mature oocyte passes through the oviduct, vitelline membrane proteins form non-disulfide cross-links and become completely insoluble, even in the presence of reducing agents Heifetz et al., 2001, Waring, 2000. Based upon the presence of cross-linked di-tyrosine and tri-tyrosine residues in vitelline membrane hydrolysates prepared from laid eggs, but not ovaries, it is thought that the non-disulfide cross-links are catalyzed, at least in part, by a peroxidase type enzyme (Petri et al., 1976). To determine whether late non-disulfide based cross-linking is impaired

in *fs(2)QJ42* eggs bearing mutant sV23 derivatives, eggs laid over a 50-min time interval were collected from either wild-type or transgenic *fs(2)QJ42* females, boiled in SDS- and β-ME-containing solutions, and analyzed by Western blot analysis. In Fig. 7, products detected with the sV23 antiserum in stage 14 egg chambers are compared with those detected in laid eggs. Whereas sV23 derivatives as well as cross-reacting products (defined by their presence in *fs(2)QJ42* egg chambers) are apparent in wild-type stage 14 egg chambers, immunoreactive products were not visible, as expected, in laid eggs. On the other hand, a slightly faster migrating form of the cross-reacting product remained soluble in eggs laid by *fs(2)QJ42* females. While the identity of the cross-reacting material (CRM) is not known, based on several criteria, it appears to be a vitelline membrane component: (1) its accumulation profile (Fig. 5A) is consistent with its synthesis during the period of vitelline membrane formation, (2) its insolubility in early embryos is consistent with an eggshell protein (Fig. 7A), and (3) sectioned *fs(2)QJ42* egg chambers (stages 10–14) showed specific, and intense immunogold labeling over the vitelline membrane with the sV23 antiserum (Pascucci et al., 1996). As shown in Fig. 7D, the sV17 vitelline membrane protein also remains soluble in eggs laid by *fs(2)QJ42* females. Taken together, these data indicate that the absence of sV23 affects the behavior of other eggshell proteins. The solubility of the sV23-reactive eggshell proteins in transgenic *fs(2)QJ42* females that laid turgid, fertile eggs (ΔRVSR and $\Delta C^{155–167}$) was indistinguishable from wild type. In contrast, sV23, the cross-reactive product, and sV17 remained soluble in the flaccid eggs produced by *fs(2)QJ42* females carrying the $\Delta N^{24–42}$ transgene (Figs. 7A and D). A mixture of turgid and flaccid eggs were laid by *fs(2)QJ42* females carrying two copies of the $\Delta C^{140–167}$ transgene. Even after prolonged exposures (Fig. 7B), sV23-reactive products were not detected in either the flaccid (E3) or turgid eggs (E1 and E2). This indicates that defective cross-linking is not linked to the flaccid egg phenotype.

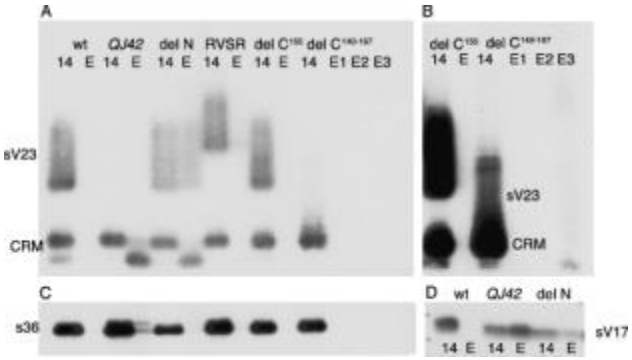

Fig. 7. Solubility of vitelline membrane proteins in eggs laid by females producing mutant sV23 proteins. (A) Western blot of proteins from 10 stage 14 egg chambers or 10 eggs laid by wild-type females, *fs(2)QJ42* females, or *fs(2)QJ42* females carrying two copies of the indicated sV23 mutant transgenes incubated with sV23 antiserum (1/1000 dilution). The positions of sV23 and the CRM in wild-type egg chambers are indicated at the left. The slower mobility of sV23 in stage 14 egg chambers from *fs(2)QJ42* females carrying the ΔRVSR transgene (RVSR) reflects the absence of N-terminal processing. A mixture of turgid and flaccid eggs were laid by *fs(2)QJ42* females carrying the $\Delta C^{140–167}$ transgene. E1 and E2 represent the turgid egg population, E3 the flaccid eggs. (B) A longer exposure of the last six lanes shown in panel A. (C) Blot shown in A, stripped and incubated with s36 antiserum (1/1000). The region of the blot encompassing the s36 signal is shown. (D) A similar blot showing stage 14 egg chambers and eggs from wild type (wt); *fs(2)QJ42* females, and *fs(2)QJ42* females carrying two copies of the $\Delta N^{24–42}$ transgene (del N) incubated with sV17 antiserum (1/1000). The region of the blot in the sV17 size range is shown.

While vitelline membrane proteins become cross-linked via non-disulfide covalent bonds after ovulation, proteins in the endochorion layer become cross-linked and insoluble in late stage 14 egg chambers. To determine if the absence of sV23 affected the behavior of s36, an endochorion protein that is transiently housed within the vitelline membrane during the early stages of choriogenesis (Pascucci et al., 1996), the Western blot in Fig. 7A was stripped and incubated with a s36-specific serum. As shown in Fig. 7C, s36 remained insoluble in laid eggs despite the absence of sV23 (*fs(2)QJ42*) or the presence of mutant sV23 derivatives (ΔN, RVSR, ΔC^{140}, ΔC^{155}). These data indicate that perturbations in the vitelline membrane layer that result from either the absence or presence of abnormal sV23 derivative do not prevent the insolubilization of endochorion proteins such as s36 that are initially housed in this layer.

Discussion

During stages 8–10, ovarian follicle cells synthesize and secrete the sV23 vitelline membrane proprotein in excess of what is needed for function. The fertility of *fs(2)QJ42* females carrying the truncated C-terminal derivative $\Delta C^{140–167}$ indicates that accumulation at approximately 10% of wild-type levels is sufficient to support the assembly of a functional eggshell. During eggshell formation, sequential removal of C- and N-terminal sequences produces the mature sV23 protein that becomes stably integrated into the vitelline membrane layer via disulfide and non-disulfide covalent bonds. Although stage-specific processing of sV23 is evolutionarily conserved, neither the premature removal of the C-terminal sequences ($\Delta C^{155–167}$) nor the retention of the N-terminal sequences (ΔRVSR) is detrimental to sV23 function. The C-terminal prodomain was removed in a timely manner from the truncated N-terminal proprotein, $\Delta N^{24–42}$, indicating that the presence of the N-terminal prodomain is not required for proper C-terminal cleavage. Conversely, N-terminal sequences were removed at the proper time from the truncated C-terminal derivatives, $\Delta C^{155–167}$ and $\Delta C^{140–167}$, indicating that the removal of the C-terminal prodomain does not provide the cue for the timing of the N-terminal cleavage.

Sequences within the C-terminal half of the vitelline membrane domain appear to be required for efficient exit of sV23 from the cell (Fig. 5, next section), while sequences in the N-terminal prodomain appear critical for its extracellular function. Females that secrete sV23 with a deleted N-terminal domain produce flaccid eggs in which the mature sV23 protein fails to become properly integrated into the highly cross-linked, insoluble vitelline membrane layer characteristic of wild type (Fig. 7). This assembly defective form of sV23 is associated with a fully penetrant female sterile phenotype.

Role of C-terminal region of the VM domain

The hallmarks of the Drosophila VM domain are its hydrophobic nature and the presence of three precisely spaced cysteine residues (Fig. 1). Similar VM-like domains have been found in the 15a-1, 15a-2, and 15a-3 vitelline membrane proteins of the mosquito, *Aedes aegypti*(Edwards et al., 1998). Based on changes in the spatial distribution of the VM32e protein in MYC-tagged derivatives bearing a 10 amino acid deletion in the central part of the VM domain, it has been postulated that this domain is involved in protein–protein interactions within the vitelline membrane layer (Andrenacci et al., 2001). The functional significance of the aberrant behavior of the tagged mutant derivative could not be

assessed, as the endogenous wild-type VM32e gene was expressed along with the mutant transgene. In this study, sV23 derivatives bearing a deletion that extended into the C-terminal third of the VM domain ($\Delta C^{140–167}$) were associated with a dramatic reduction in sV23 accumulation. Since truncated sV23 products accumulated at near wild-type levels in sV23 protein null mutants carrying the $\Delta C^{155–167}$ transgene, sequences within the VM domain (140–154) appear to be critical for the stabilization of sV23. N- and C-terminal processing of sV23 occurs after its secretion from the cell, in the vitelline membrane layer. N-terminal processing of the ($\Delta C^{140–167}$) derivative (Fig. 5) indicates that truncated sV23 proproteins are secreted from the cell. Since the signal intensity of the accumulated sV23 products was maintained after the completion of sV23 synthesis during late stage 10 (Fig. 5), extracellular turnover does not appear to be problematic. Taken together, these results suggest that sequences within the VM domain are needed for the efficient exit of sV23 from the cell.

Secreted proteins begin synthesis in the ER. Through the interactions with a host of resident ER folding proteins and chaperones proteins, newly synthesized proteins are folded properly so that they can be transported to their appropriate destinations. Beyond interacting with general folding enzymes and chaperones, some proteins interact with protein-specific chaperones. HSP47 is a molecular chaperone that plays an unknown but essential role during the assembly and transport of procollagen (Hendershot and Bulleid, 2000). The low steady state level of sV23 observed with the ($\Delta C^{140–167}$) transgene is compatible with intracellular retention and turnover. Considering the roles of known protein-specific chaperones (Hendershot and Bulleid, 2000), sequences within the VM domain may interact with proteins that facilitate the transport of sV23, and perhaps other vitelline membrane proteins, through the secretory pathway. Proper transport of sV23 may be dependent upon its assembly into an oligomeric complex. Vitelline membrane proteins are secreted constitutively in electron dense secretory vesicles. It is not known if, or to what extent, vitelline membrane proteins oligomerize within the ER, Golgi, or secretory vesicles. If oligomerization is required for efficient export, sV23 assembly and transport may be compromised by the removal of sequences within the VM domain. Alternatively, sequences within the VM domain may be necessary to prevent the formation of aggregates or higher-order complexes within the ER that would be subject to intracellular degradation. In any case, sequences within the C-terminal region of the VM domain appear to have an important role in proper sV23 accumulation. Interestingly, the removal of 10 amino acids from the center of a VM domain of another eggshell protein, MYC-tagged VM32e, did not lead to a notable difference in its accumulation (Andrenacci et al., 2001).

Role of sV23 N-terminus

The N-terminal prodomain is an essential assembly domain. Females expressing the N-terminally truncated form of sV23 lay collapsed eggs characterized by defects in the formation of non-disulfide covalent cross-links. Normally insoluble, sV23, a cross-reactive VM component, and to a lesser extent sV17, are recovered in monomeric form when eggs laid by sV23 null mutant females carrying the $\Delta N^{24–42}$ transgene are solubilized in Laemmli sample buffer in the presence of a reducing agent (Fig. 7). Cross-linking of the latter two proteins is also defective in sV23 null mutants. Taken together, these results indicate that the alignment of cross-linking sites on sV23 is dependent upon the N-terminal prodomain, and in turn, the alignment of cross-linking sites on other vitelline membrane proteins is dependent on sV23. The N-terminal prodomain is a hydrophobic, highly conserved segment. After removing the predicted signal peptide, hydrophobicity plots indicate that the sV23 proprotein is

headed by a 21 amino acid hydrophobic segment that extends to the predicted RVSR cleavage site. As shown at the top of Fig. 1, this segment is identical in sV23 homologs from species that have been separated in evolution for over 60 million years (*D. melanogaster* and *D. virilis*). If the hydrophobic sV23 prodomain plays a critical role in aligning molecules within the vitelline membrane, it must do so before its removal during late oogenesis (stages 12–14). Since non-disulfide cross-links of sV23 form as the egg moves through the oviduct and uterus (Heifetz et al., 2001), alignments that are established during the early stages of eggshell formation (10–12) must either be stable or maintained during the later stages by a mechanism that does not depend on the presence of the N-terminal prodomain.

The alignment of monomers for polymer formation is critical for major extracellular matrix proteins such as elastin and collagen. Elastin is synthesized as a monomer, tropoelastin, and is assembled into polymeric elastin in the extracellular matrix. Elastin consists of alternating hydrophobic and cross-linking domains. Recent studies using small recombinant elastin polypeptides, consisting of different combinations of the hydrophobic and cross-linking domains, showed that the ability of these recombinant polypeptides to self-assemble, align, and form cross-links was dependent upon aggregation driven by the hydrophobic domains (Keeley et al., 2002). The hydrophobic N-terminal prodomain of sV23 may play a similar role in aligning sV23 sequences. Mature sV23 essentially consists of the VM domain and the central octapeptide repeat region. Precise alignment of the octapeptide repeats within oligomeric complexes may be essential for proper cross-link formation. We propose that the hydrophobic N-terminal prodomain aggregates and aligns sV23 molecules so that the tyrosine residues within the repeat domain are positioned properly for subsequent cross-link formation. In its absence, misaligned molecules fail to form non-disulfide covalent linkages in the oviduct and uterus.

Amino terminal processing of sV23

The amino terminus of sV23 is removed during stages 13 and 14 of oogenesis. N-terminal processing of sV23 is evolutionarily conserved, as sV23 in *D. virilis* displays similar stage specific mobility shifts during the terminal stages of oogenesis (Pascucci et al., 1996). The production of partially cleaved substrates following the deletion of the RVSR motif suggests that a subtilisin-like protease catalyzes the cleavage of the N-terminal prodomain. Subtilisin-like proteases appear to be involved in the assembly of the *Caenorhabditis elegans* cuticle, a complex multilayered extracellular structure composed primarily of collagens. SQT-1 collagen is synthesized as a procollagen whose amino-terminal domain is cleaved before its incorporation into the cuticle. Mutations in the putative subtilisin-like cleavage site block cleavage. The uncleaved pN-SQT-1 molecules dominantly disrupt normal cuticle structure (Yang and Kramer, 1999). In contrast, sV23 molecules that retain the N-terminal prodomain neither disrupt nor impair eggshell development, as both wild-type and sV23 protein null mutants that carry two copies of the ΔRVSR transgene lay fertile eggs with insoluble vitelline membranes. Given the evolutionary conservation of sV23 amino-terminal processing and the putative subtilisin-like cleavage site, the lack of a functional consequence was not anticipated. Another vitelline membrane protein, sV17, undergoes stage-specific processing and contains a dibasic cleavage site whose position and use would be compatible with the size and antigenicity of the processed sV17 product. Perhaps, utilization of the ovarian subtilisin-like processing machinery is critical for the function of this or an as yet unidentified extracellular substrate. Subtilisin-like proprotein convertases are maternally expressed

in *Drosophila*(Roebroek et al., 1995) and extracellular forms of subtilisin-like convertases have been reported.

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