Specific domains drive VM32E protein distribution and integration in *Drosophila* eggshell layers

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SUMMARY

A study was made of the localization and assembly of the VM32E protein, a putative vitelline membrane component of the *Drosophila* eggshell. The results highlight some unique features of this protein compared with the other proteins of the same gene family. At the time of its synthesis (stage 10), the VM32E protein is not detectable in polar follicle cells. However, it is able to move in the extracellular space around the oocyte and, by stage 11 is uniformly distributed in the vitelline membrane. During the terminal stages of oogenesis the VM32E protein is partially released from the vitelline membrane and becomes localized in the endochorion layer also. By analyzing transgenic flies carrying variously truncated VM32E proteins, we could identify the protein domains required for the proper assembly of the VM32E protein in the eggshell. The highly conserved vitelline membrane domain is implicated in the early interactions with other components and is required for cross-linking VM32E protein in the vitelline membrane. The terminal carboxylic domain is necessary for localization to the endochorion layer. Protein with the C-end domain deleted is localized solely to the vitelline membrane and cross-linked only in laid eggs, as occurs for the other vitelline membrane proteins.

Key words: Oogenesis, Follicle cells, Eggshell, Vitelline membrane, Endochorion

INTRODUCTION

The formation of extracellular structures is a complex process that requires time-coordinate synthesis, cleavage and transport of various proteins, and, finally, cross-linking mediated by particular functional domains. Exactly how the precise features of such biological structures are constructed remains a fascinating problem. We approach this question by studying eggshell assembly in *Drosophila melanogaster*. This multilayered extracellular matrix, which forms between the oocyte and the overlying follicle cells, is made up of the following layers: a vitelline membrane, which is the innermost layer; the wax layer; the crystalline innermost chorionic layer; the tripartite endochorion (inner part, pillars, outer part); and the exochorion, which is the outer layer of the eggshell (Margaritis et al., 1980; reviewed by Waring, 2000). The eggshell proteins are synthesized and secreted by the follicle cells surrounding the oocyte during stages 8-14 of egg-chamber development. From stage 8 to stage 10, the synthesis of vitelline membrane components is predominant (King, 1970; Mahowald, 1972; Petri et al., 1976; Waring and Mahowald, 1979; Fargnoli and Waring, 1982; Spradling, 1993). During stage 9, the follicle cells begin to secrete the vitelline membrane proteins, accumulated in small vesicles called 'vitelline bodies'. At stage 10B, these vesicles fuse to form a 1.7 μm thick layer, which gradually thins down to 0.3 μm as oogenesis proceeds (Margaritis et al., 1980; Margaritis, 1985). Subsequently, during stages 11-14, the chorion proteins are synthesized and secreted (Petri et al., 1976; Waring and Mahowald, 1979). The eggshell is stabilized by a progressive cross-linking process that renders its components largely insoluble. The chorion becomes insoluble during stage 14 as a result of a peroxidase-type enzyme activity that cross-links two or three tyrosine residues of the chorion proteins (Petri et al., 1976). The vitelline membrane proteins remain soluble until stage 14 and becomes insoluble only in laid eggs (reviewed by Waring, 2000).

The analysis of mutations affecting the eggshell has suggested the structural function of specific proteins. Females homozygous for the *cor36* mutation do not synthesize the early s36 chorion protein and a defective endochorion layer is formed (Digan et al., 1979). Homozygous females for null *dec-1* mutations produce eggshells altered in both organization and stability of the endochorion layer (Bauer and Waring, 1987). The *dec-1* gene encodes multiple protein products that show distinct localization patterns in the mature eggshell. Their diverse localizations have suggested that the different *dec-1* derivatives might play different roles in the assembly and stabilization of the mature eggshell (Nogueron et al., 2000). Females homozygous for the *fs(2)QJ42* mutation fail to accumulate the vitelline membrane protein VM26A.2 and produce eggs with an altered vitelline membrane onto which the endochorion layer collapses during stage 14, suggesting that the vitelline membrane has an important role in the stabilization of the outer chorion layers (Savant and Waring, 1989; Pascucci et al., 1996). Vitelline membrane defects have been also reported for some alleles of the *nudel* gene (Hong and Hashimoto, 1995; Hong and Hashimoto, 1996; LeMosy et

al., 1998). This gene is expressed in follicle cells surrounding the oocyte and encodes a large mosaic protein with a central serine protease domain involved in the establishment of the dorso-ventral axis of the *Drosophila* embryo (Hong and Hashimoto, 1996). It has been demonstrated that *nudel* mutations that compromise Nudel protease function also result in the failure of covalent cross-linking of the vitelline membrane in the laid egg, suggesting an integral role for Nudel protease in eggshell biogenesis (LeMosy and Hashimoto, 2000). Certain mutant alleles of the *fs(1)polehole* and *fs(1)Nasrat* genes produce eggs with leaky vitelline membranes (Ambrosio, 1989; Casanova and Struhl, 1989; Degelman et al., 1990). These genes, not yet cloned, belong to the terminal group genes involved in the activation of Torso at the poles of the embryo, but nothing is known about their function in the vitelline membrane formation. A compelling question arising from these data concerns the involvement of the vitelline membrane in the localization of the maternal signals required for embryonic axis determination. The vitelline membrane might form a matrix structure necessary for the functioning of the cues relevant for embryonic development.

Four *Drosophila* vitelline membrane protein genes have been cloned so far: *VM26A.1*, *VM34C*, *VM26A.2*, and *VM32E* (Higgins et al., 1984; Mindrinos et al., 1985; Burke et al., 1987; Popodi et al., 1988; Gigliotti et al., 1989). Although the other vitelline membrane genes are expressed from stage 8 to stage 10 of oogenesis, the *VM32E* gene, expressed only at stage 10, can be considered a 'late' vitelline membrane gene (Gigliotti et al., 1989). Compared with the other members of the same family, the *VM32E* gene is under complex temporal and spatial regulation (Gargiulo et al., 1991; Cavaliere et al., 1997; Andrenacci et al., 2000). This might reflect some special functions played by the VM32E protein in eggshell formation.

In this report, we describe the distribution and fate of the VM32E protein in the initial and late stages of eggshell assembly. The results clearly show that, during the final stages of oogenesis, some VM32E protein molecules are released from the vitelline membrane and become stably integrated into the endochorion. The VM32E protein is therefore an integral component of both the vitelline and the endochorion layers. The work presented here offers new insights into the process of eggshell assembly, and allows the identification of VM32E protein functional domains required for its integration into the vitelline membrane and its recruitment in the endochorion layer.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster stocks were raised on standard cornmeal/yeast/agar medium at 25°C. The analysis of the wild-type VM32E protein was carried out in the yw^{67c23} strain (referred to as wild type in the text) because this genetic background was used for the production of the transgenic lines carrying the different chimeric constructs. The *fs(2)QJ42* mutation was also analyzed in the yw^{67c23} background.

Construction of chimeric *VM32E-MYC* genes

Standard molecular biology techniques were carried out essentially as described in Sambrook et al. (Sambrook et al., 1989). The *VM32E-MYC*, *Δ1*, *Δ2* and *Δ3* chimeric genes were produced by fusing specific PCR amplification products to DNA fragments obtained by designed endonuclease digestions of the *Hin*dIII/*Hin*dIII *VM32E* genomic clone (Gigliotti et al., 1989). The PCR reactions were performed using the same clone as template, various internal DNA primers and a designed synthetic primer (Fig. 1) carrying the coding sequences for the MYC epitope, a *Sna*BI restriction site and three stop codons.

All the chimeric genes contain the minimal *VM32E* promoter (up to nucleotide −465), the untranslated 5′ end, and the 3′ end including the polyadenylation site of the wild-type *VM32E* gene. The *VM32E-MYC* gene covers the full coding region of the *VM32E* gene, whereas the *Δ1*, *Δ2* and *Δ3* genes carry different deletions of the coding sequences, which are: Δ1, from amino acid residue 24 to 33; Δ2, from 50 to 59; Δ3, from 73 to 116. All the chimeric genes encode proteins with the MYC epitope at the C-terminal region. The genes were first cloned in pUC19 vector, then sequenced and subcloned in the *Xba*I site of the *pCaSpeR4* vector (Brand and Perrimon, 1993).

Transgenic lines

P-element-mediated transformation was carried out essentially as described in Spradling and Rubin (Spradling and Rubin, 1982) and Rubin and Spradling (Rubin and Spradling, 1982). Dechorionated embryos from the yw^{67c23} strain were injected with the various DNA constructs and helper plasmid *pπ25.7wc* (Karess and Rubin, 1984).

In situ hybridization

Whole-mount in situ hybridization with digoxigenin-labeled (Roche) probes was performed as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989). The 3′ end of the *VM32E* cDNA (Gigliotti et al., 1989) and the DNA encoding the MYC epitope were used as probes. The egg chambers were viewed with Nomarski optics on a Zeiss microscope.

Immunofluorescence microscopy

Fixation and antibody staining of hand-dissected ovaries were carried out as previously described (Gigliotti et al., 1998). Anti-CVM32E or anti-VMP antibodies were used at 1/50 dilution and reacted with Cy3-conjugated anti-rabbit secondary antibody (1/100 dilution). Anti-MYC monoclonal antibody were used at 1/100 dilution and reacted with Cy3-conjugated anti-mouse secondary antibody (1/100 dilution). Stained egg chambers mounted in Aquamount (Polyscience) were analyzed with conventional epifluorescence and with a Biorad laser confocal microscope attached to a Zeiss Axiophot microscope.

Immunoelectron microscopy

Ovaries were removed in cold insect Ringer's solution and fixed as described (Pascucci et al., 1996), dehydrated, and embedded in the acrylic resin Bioacryl, polymerized at 4°C under UV irradiation according to Scala et al. (Scala et al., 1992). After sectioning with an ultramicrotome, thin sections were collected on nickel grids, and incubated overnight at 4°C in the primary antibody diluted (anti-CVM32E at 1:40; anti-MYC at 1:80) in Tris-buffered saline (TBS) at pH 8.2 containing 0.1% Triton-X100 and 2% bovine serum albumin (BSA). After repeated washes in TBS, sections treated with anti-CVM32E antibody were incubated for 1 hour at room temperature with a goat anti-rabbit IgG; those treated with anti-MYC antibody were incubated in the same conditions with a goat anti-mouse IgG. In both cases, IgG were conjugated to 10 nm gold particles and used at 1:40 dilution. Controls were performed on samples from which the treatment with the first antibody was omitted. After rinsing in TBS and in distilled water, the sections were lightly stained in uranyl acetate and lead citrate for observation with a CM 100 Philips electron microscope. For double labeling, the detection of the MYC antigen with IgG conjugated to 10 nm gold particles was followed by the detection of the second antigen, VM32E, with IgG conjugated to 20 nm gold particles. As control of the quality of our immunoelectron microscopy procedure, we checked the proper distribution of the

VM26A.2 protein (Pascucci et al., 1996) using the anti-VMP antibody (data not shown).

Western blot analysis

Ovaries and staged egg chambers were quickly collected and placed in cold Ringer's solution and frozen in liquid nitrogen. Ovaries analyzed in Fig. 4B were homogenized by sonication in Laemmli sample buffer (31 mM Tris-HCl, pH 6.8, 2.5% glycerol, 0.5% SDS, 177 mM 2β-mercaptoethanol, 0.01% bromophenol blue (Laemmli, 1970)), boiled for five minutes and, following the removal of insoluble material by centrifugation (10 minutes at 15,000 ***g***), the soluble proteins were run on SDS-PAGE.

Egg chambers analyzed in Figs 4A,B and 9A were homogenized in ice-cold Ringer's solution with 0.5% Nonidet P-40, 1.3 μg ml^{-1} pepstatin A, 1.3 μg ml^{-1} antipain, 3.3 μg ml^{-1} leupeptin, 2.3 mM PMSF. Following centrifugation (10 minutes at 15,000 ***g***) the supernatant fraction was mixed with an equal volume of 2× Laemmli sample buffer and boiled for five minutes. The pellet phase was washed three times in homogenization buffer, suspended in Laemmli sample buffer and boiled for 1 hour. After boiling, the insoluble residues were removed by centrifugation and the solubilized material was run on SDS-PAGE. Two-hour-old eggs were collected in a wire basket, rinsed with distilled water and, where required, dechorionated with 50% Chlorox. The eggs were homogenized as described here and only the pellet phases were processed and loaded on the gel.

SDS solubilization of vitelline membrane proteins, shown in Figs 4C, 5B and 9B, was achieved by adding SDS to the homogenization buffer at the concentrations indicated in the text. The samples were incubated at room temperature for 10 minutes. Following centrifugation (10 minutes at 15,000 ***g***) the supernatant fraction was treated as described above. Pellets were washed three times in homogenization buffer without SDS and processed as described above.

All the samples obtained by the different procedures used were run on 15% polyacrylamide gels. Protein transfer to nitrocellulose and western blotting were performed using standard methods (Harlow and Lane, 1998). The chimeric proteins were detected using anti-MYC monoclonal mouse antibody diluted 1/500; the VM32E protein was detected by using the anti-CVM32E polyclonal rabbit antibody (diluted 1/100), whereas the VM26A.2 protein was detected by using the anti-VMP polyclonal rabbit antibody (diluted 1/100). All the primary antibodies were detected using horseradish peroxidase conjugated horse antibody (1/500 dilution) and ABC detection kit (Vector ABC Universal kit no. pk-6200).

For each set of experiments, the number of ovaries, egg chambers or eggs analyzed were exactly the same and all the material extracted was loaded into the gel. Each gel lane was loaded with four ovaries or 400 egg chambers or eggs (600 for transgenic flies). The experimental procedure used could not cause any loss of material. After the removal of the soluble phase, which was transferred to a new tube and processed for the western blot analysis, the pellet was kept in the original tube in which the egg chambers were homogenized. Furthermore, as an additional control to compare the relative amount of proteins in the various samples, filters were stained after blotting with Ponceau and gels with Coomassie blue. In all the experiments performed, the proteins detected in each lane were of comparable and highly reproducible amount.

Antibodies

The anti-VMP and the anti-CVM32E polyclonal antibodies were generated in rabbits (Primm) using synthesized peptides located at the N and C termini of the VM32E protein. The N-terminal peptide was SCPYAAPAPAYSAPAASSG (residues 18 to 36) and the C-terminal peptide was EELRGLGQGIQGQQY (residues 102 to 116). Anti-MYC mouse monoclonal antibody 9E10 was purchased from Santa Cruz Biotechnology. Cy3 conjugated anti-rabbit and anti-mouse antibodies were purchased from Sigma. 10 nm and 20 nm gold particles conjugated anti-rabbit and anti-mouse antibodies were respectively from Sigma and Chemicon.

All the images were processed in Photoshop 5.0 (Adobe Systems, Mountain View, CA, USA).

RESULTS

VM32E protein synthesis and distribution

As previously reported, the *VM32E* gene is transcribed only at stage 10 of egg chamber development (Gargiulo et al., 1991; Cavaliere et al., 1997; Andrenacci et al., 2000). The expression is first detected at stage 10A in a group of ventral columnar follicle cells (Fig. 2A) and progressively extends, involving all the main body follicle cells by stage 10B. The most anterior and posterior cells remain silent (Fig. 2B). To clarify whether or not the polar regions of the vitelline membrane layer remain devoid of the VM32E protein, we have studied the distribution of VM32E protein during egg chamber development. For this purpose, a polyclonal antibody was raised against the C-terminal VM32E peptide EELRGLGQGIQGQQY (anti-CVM32E) that was specific to this protein.

Analysis by confocal microscopy showed that, at stage 10B, the VM32E protein is synthesized in the columnar cells and secreted into the extracellular space between the follicle cells and the oocyte, where the vitelline membrane is forming (Fig. 2C,D). No synthesis of the protein was detectable in the anterior and posterior follicle cells. In these regions, only a faint signal of reacted antibody was visible (Fig. 2E,G). By stage 11, although the pole follicle cells remain silent, the VM32E protein was also present at the poles of the oocyte, which appeared to be uniformly surrounded by it (Fig. 2F,H). This indicates that, once secreted, the VM32E protein moves to the poles. In comparison to this, we analyzed the distribution of the VM26A.2 protein, which has been reported to be expressed in all follicle cells surrounding the oocyte (Popodi et al., 1988). To detect this protein we used a polyclonal antibody (anti-VMP) originally raised against the N-terminal SCPYAAPAPAYSAPAASSG peptide of the VM32E protein that can also recognize the VM34C and the VM26A.2 proteins. It reacts most strongly with VM26A.2 and barely with the other two proteins (data not shown), probably because a PAYSAPAA peptide is repeated four times in VM26A.2. As shown in Fig. 2I,L, the signal was detected in all follicle cells surrounding the oocyte.

The distribution of VM32E was also studied at the ultrastructural level by immunogold electron microscopy on egg-chamber thin sections. At stage 10, the immunogold particles were detected in the secretory vesicles of the follicle cells and appeared to be widely distributed in the vitelline bodies (Fig. 3A), with the exception of those present at the poles, in which a very low density of gold particles was scored (Fig. 3B). At the late stage 10B, the immunogold particles

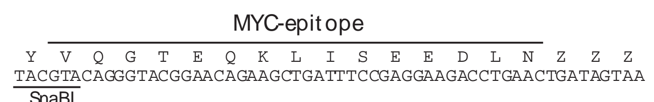

Fig. 1. Structure of the primer used to construct chimeric *VM32E-MYC* genes, showing the MYC epitope and the *Sna*BI restriction site.

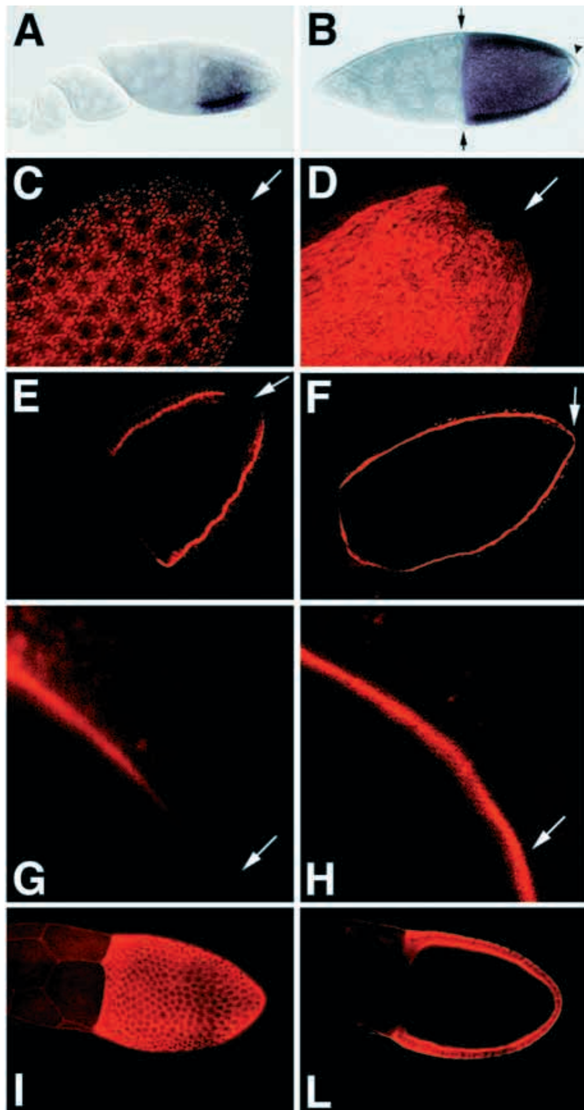

Fig. 2. *VM32E* gene expression and VM32E protein distribution. (A,B) Whole mount in situ hybridization showing the spatial distribution of the *VM32E* transcript. (A) At stage 10A, the expression is evident in a group of ventral columnar follicle cells. (B) At stage 10B, all main body columnar follicle cells express the gene; most anterior (arrows) and posterior (arrowhead) follicle cells are silent. (C-H) Whole-mount egg chambers stained with anti-CVM32E antibody and examined laterally by confocal microscopy. The white arrows indicate the posterior polar domain. (C) Surface view of a stage 10B egg chamber in which the VM32E protein appears within the main body follicle cells, except the most posterior ones. (D) Section of the same egg chamber showing the VM32E protein in the extracellular space between the follicle cells and the oocyte, and the absence of this protein from the posterior domain. (E) Sagittal section of a stage 10B egg chamber at a lower magnification. (F) Sagittal section of a stage 11 egg chamber showing, at a lower magnification, the presence of the VM32E protein in the polar domains. (G) The posterior polar domain of a stage 10B egg chamber at a higher magnification. (H) The posterior polar domain of a stage 11 egg chamber at a higher magnification. (I,L) Whole-mount egg chambers stained with anti-VMP antibody and examined laterally by confocal microscopy. (I) Surface view of stage 10B egg chamber in which the VM26A.2 protein appears within all the main body follicle cells. (L) Sagittal section of the same egg chamber showing the VM26A.2 protein in the extracellular space between the follicle cells and the oocyte.

strongly labeled the vitelline membrane (Fig. 3C). At stage 12 (Fig. 3D), immunogold particles were detected in the vitelline membrane and in the forming endochorion pillars. Finally, at stage 14, gold particles labeled both the vitelline membrane and the endochorion (Fig. 3E). The presented data clearly indicate that, at the end of egg chamber development, the VM32E protein is a component not only of the vitelline membrane but also of the endochorion layer.

Western blot analysis of VM32E protein

We analyzed the VM32E protein during egg chamber development using a protein extraction procedure that allowed us to follow up its integration into the eggshell. Staged egg chambers were homogenized and, after centrifugation, the pellet and supernatant phases were separated and analyzed by western blot. Proteins recovered in the pellet phase should be those assembled within the eggshell, whereas proteins in the supernatant phase should be free, or aggregated in small macromolecular complexes not yet integrated in the eggshell.

According to the mRNA open reading frame, the VM32E protein should be 116 amino acids long with an expected molecular mass of 12 kDa, but because the first 17 amino acids follow all the rules for a predicted cleavage site of a signal peptide (Von Heijne, 1984), the secreted protein must be shorter and with a molecular mass of 10.4 kDa. A prominent band of ~10 kDa, corresponding to the hypothetic mature VM32E protein, was observed in all the stages analyzed (Fig. 4A). Two additional bands of slightly different size were detected by the antisera and might represent unknown modifications of the protein. At stage 10, most of the VM32E protein was detected in the pellet phase, suggesting its incorporation into the vitelline membrane. VM32E protein was recovered in the supernatant fraction until stage 12. This might suggest that the VM32E protein is gradually integrated into the eggshell, because no VM32E transcript is detectable after stage 11 (Gigliotti et al., 1989). By stage 13, no protein was detected in the supernatant phase and the amount of VM32E protein extracted from the pellet was lower than at the earlier stages, and even more so at stage 14. In comparison to this, using the same extraction procedure described above, we analyzed the vitelline membrane protein VM26A.2 (Fig. 4B). As reported by Pascucci et al. (Pascucci et al., 1996), the VM26A.2 pattern is quite complex because the protein undergoes proteolytic cleavage in the late stages of oogenesis. At stage 9, the ongoing synthesis of the VM26A.2 protein was clearly visible and, by stage 10, most of this protein was recovered from the pellet phase. At stage 14, the VM26A.2 protein was fully releasable from the pellet phase. Therefore, the observed reduced level of VM32E protein detected at stage 14 (Fig. 4A) might be due to the cross-linking with other endochorion components.

It is thought that vitelline membrane protein cross-linking occurs in two steps. First, during oogenesis, the vitelline membrane proteins are bound by disulfide cross-linking, and treatments with reducing agents result in solubility of the membrane. Second, successive non-disulfide cross-linking, which renders the vitelline membrane completely insoluble, occurs around the time of oviposition (Petri et al., 1979). To determine whether or not the VM32E protein undergoes

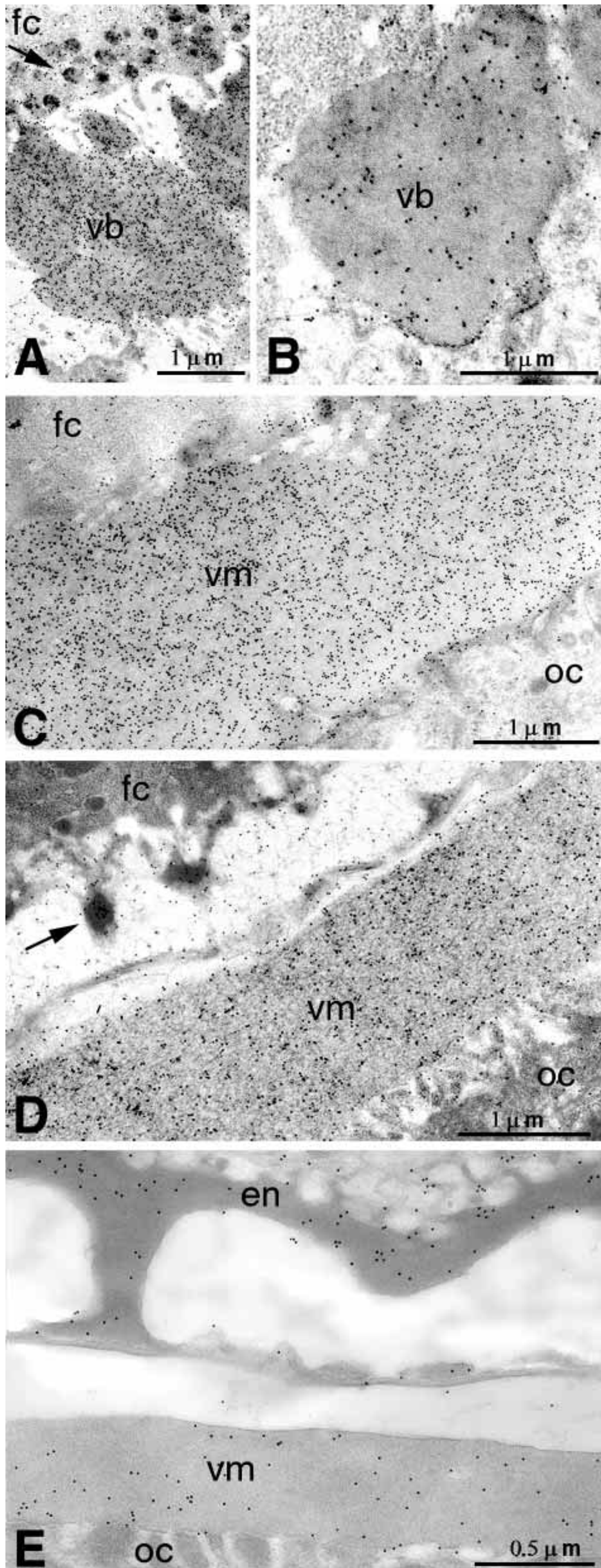

Fig. 3. Multilayered distribution of VM32E protein detected by immunoelectron microscopy using anti-CVM32E antibody. (A) At stage 10A of egg chamber development, immunogold particles are seen in the vitelline bodies and in secretory vesicles of the follicle cells (arrow). (B) At the same stage, in the posterior domain, a very low density of gold particles is visible inside the vitelline body. (C) At stage 10B, immunogold particles are clearly visible in the vitelline membrane. (D) At stage 12, the gold particles are localized in the vitelline membrane and in the forming endochorion pillars (arrow). (E) Stage 14 egg chamber showing immunogold particles on the endochorion layer and on the vitelline membrane. Abbreviations: en, endochorion; fc, follicle cell; oc, oocyte; vb, vitelline body; vm, vitelline membrane.

disulfide cross-linking, the proteins from ovaries and stage 14 egg chambers were extracted in presence of 2% SDS, which permits the release of the non-covalently bound proteins. After centrifugation, the pellet and supernatant phases were separated, submitted to SDS-PAGE and analyzed by western blot (see Materials and Methods). To compare the extraction patterns of VM32E and VM26A.2, we cut the membrane into two parts after blotting that were separately reacted with the proper antibodies. As shown in Fig. 4C, the mature form of the VM26A.2 protein, present only at the terminal stages of oogenesis, was held in the pellet phase, indicating its cross-linking by disulfide bridges. The VM32E protein extracted from the whole ovary was mostly solubilized by the SDS but, at stage 14, this protein was detected only in the pellet phase. These data clearly indicate that, by the end of oogenesis, the VM32E protein too is cross-linked by disulfide cross-linking into the vitelline membrane.

Analysis of VM32E in *fs(2)QJ42* mutant

To investigate the interaction occurring among different structural components at the early stage of vitelline membrane assembly, we have analyzed the distribution of VM32E in egg chambers obtained from *fs(2)QJ42* females that fail to accumulate the VM26A.2 protein and have vitelline membrane defects (Savant and Waring, 1989). The VM32E protein was present in both the anterior and posterior regions of vitelline layer already at stage 10B (Fig. 5A). Then, in the absence of the VM26A.2 gene product, this protein can move to the poles as soon as it is secreted from the follicle cells, perhaps because of the looser structure of the mutant vitelline membrane. However, as assayed by western blot analysis, the extraction pattern of the VM32E protein was the same as that of wild-type or *fs(2)QJ42* ovaries (Fig. 5B, the first two lanes), which indicates that this protein keeps its ability to bind to the other vitelline membrane components. To ascertain whether or not the increased mobility of the VM32E protein in *fs(2)QJ42* vitelline envelope could be due to a change of its non-covalent interactions, extraction from ovaries was performed in the presence of increasing amounts of SDS. As shown in Fig. 5B, in the mutant, the VM32E protein was totally released from the vitelline membrane with 0.5% SDS, whereas 2% of SDS was required in the wild type completely to solubilize this protein. Although these data do not prove their direct interaction, the VM32E and VM26A.2 proteins might fit together in the assembling vitelline membrane.

Dissection of VM32E protein domains

All the putative *Drosophila* vitelline membrane proteins so far identified are rich in proline and alanine, and contain a highly conserved hydrophobic domain (VM domain) of 38 amino acids (Scherer et al., 1988; Gigliotti et al., 1989) (Fig. 6B). A similar domain, showing conserved amino acid residues, is also present in the vitelline membrane proteins 15a-1, 15a-2 and 15a-3 of the mosquito *Aedes aegypti* (Lin et al., 1993; Edwards,

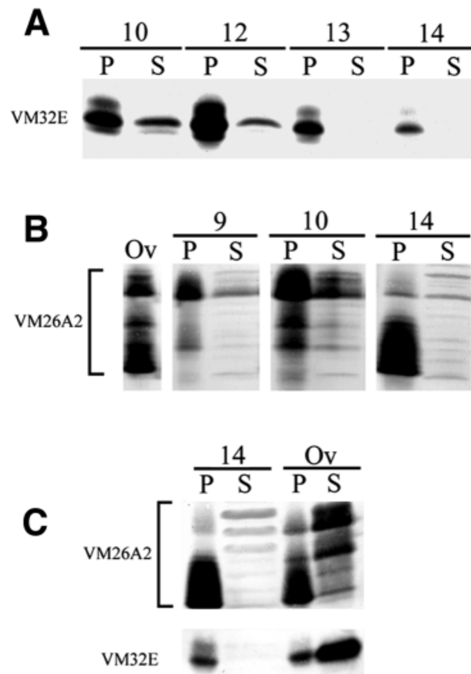

Fig. 4. Western blot analysis of VM32E and VM26A.2 vitelline membrane proteins. Staged egg chambers (stages 9-14) and ovaries (Ov) were processed as described in Materials and Methods. The pellet phase (P) contains the proteins integrated into the eggshell whereas the supernatant phase (S) contains those not yet incorporated into the eggshell. The VM32E protein was detected using the anti-CVM32E antibody, whereas the VM26A.2 protein was detected by anti-VMP antibody. (A) By the end of oogenesis (stages 13-14), a significant reduction of the VM32E signal is detected compared with the earlier stages (10-12). (B) At stage 10, the VM26A.2 protein is detected mainly in the pellet phase. At stage 14, this protein is still fully releasable from the pellet phase. (C) Stage 14 egg chambers and ovaries were extracted in presence of 2% SDS.

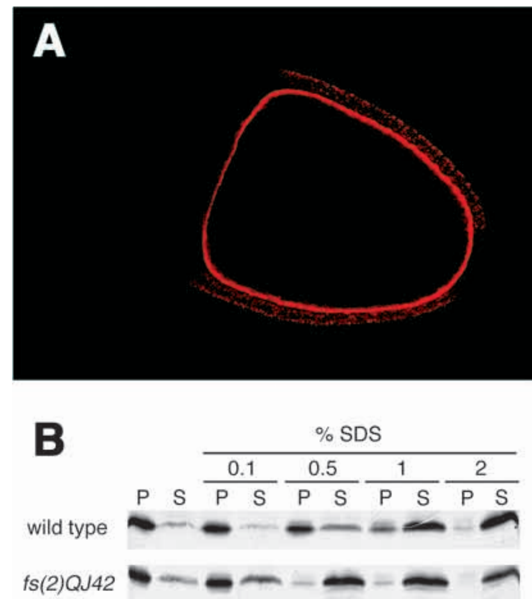

Fig. 5. Analysis of the VM32E protein in *fs(2)QJ42* egg chambers. (A) Stage 10B egg chamber stained with anti-CVM32E antibody and examined laterally by confocal microscopy. The sagittal section shows the antibody staining in the anterior (to the left) and posterior domains. (B) Western blot analysis of VM32E protein in wild-type and *fs(2)QJ42* ovaries extracted in the absence of SDS (the first two lanes from the left) and with increasing amounts of this detergent. The pellet (P) and supernatant (S) phases were separated and analyzed by western blot using the anti-CVM32E antibody. In the *fs(2)QJ42* mutant, the VM32E protein appears to be quickly solubilized by SDS at low concentration.

1996; Edwards et al., 1998). An additional conserved region of ten amino acids is also present in the VM32E, VM34C and VM26A.2 proteins, but absent from VM26A.1. These homologies might reflect some common structural features of these proteins relevant to building up the vitelline membrane. In order to investigate the hypothesis that these protein domains are required for the integration of VM32E in the eggshell layers, we produced transgenic flies encoding two differently deleted VM32E proteins (Fig. 6A) fused at the C terminus with a MYC epitope of 14 amino acids. As control, we analyzed the localization and integration of a chimeric protein containing the complete VM32E coding region fused to the MYC epitope (VM32E-MYC). The expression of these chimeric genes in the same cell types involved by the native *VM32E* gene was driven by the minimal promoter, 5′ and 3′ flanking regions of *VM32E*, and analyzed through in situ hybridization (data not shown). Because the activity of the minimal promoter used is lower than that of the wild type (Gargiulo et al., 1991), although they are very similar, the expressed amounts of the different chimeric proteins were lower than the wild-type VM32E protein (data not shown). As judged by egg permeability and egg eclosion, no effect on the vitelline membrane protein structure was produced by the expression of the different chimeric genes (data not shown). As shown in Fig. 7A,B, the localization of the VM32E-MYC protein at stage 10B was identical to that of native VM32E and, by stage 11, this chimeric protein was also localized at the poles (data not shown). Immunoelectron microscopy of double labeled sections of stage 10B egg chambers showed a wide distribution of both the MYC-tagged and wild-type VM32E proteins in the vitelline membrane (Fig. 7C). At the terminal stages of oogenesis, by using only the anti-MYC antibody, we detected the VM32E-MYC protein in the vitelline membrane and in the endochorion (Fig. 7D), as observed for wild-type VM32E protein. In addition, the VM32E-MYC protein also appeared in vesicles within the oocyte (Fig. 7E). In these cellular structures, we had also detected the wild-type VM32E protein (data not shown); the high efficiency and specificity of the anti-MYC antibody allow us to be more confident in pointing out this feature.

In the Δ1 construct, the deletion removes ten amino acids that are almost identical in the VM32E, VM34C and VM26A.2 proteins. The removal of this region did not cause any alteration of the localization of the Δ1 protein, as assayed by confocal and electron microscopy analysis (data not shown). In the Δ2 construct, the deletion removes ten of the 38 amino acids of the highly conserved hydrophobic VM domain (Figs 6A,8F). Confocal analysis of egg chambers from flies carrying Δ2 construct revealed an altered distribution of the chimeric protein; in fact, the protein was already present in both anterior and posterior regions of vitelline layer at stage 10B (Fig. 8A,B). It is worth noticing that the early mobility of the Δ2

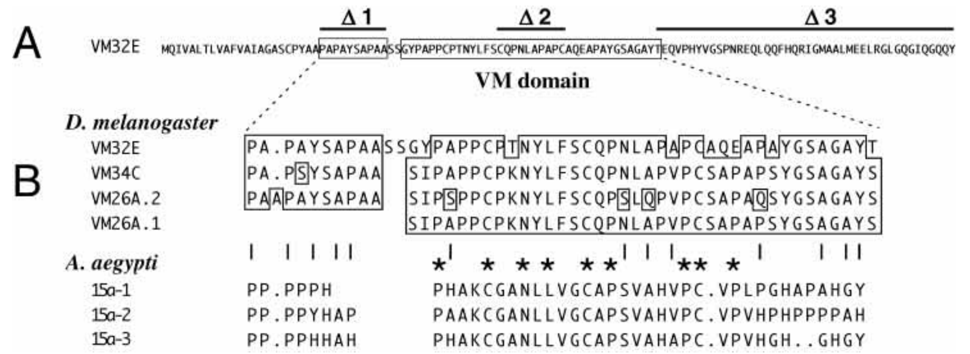

Fig. 6. (A) Sequence of the VM32E protein deletions analyzed. (B) Alignment of the protein sequences of the conserved regions in *D. melanogaster* and *A. aegypti* vitelline membrane proteins. Residues that are conserved between the two species are indicated by bars. Highly conserved residues are indicated with asterisks. Small gaps that improve the alignment are shown as dots. References: VM32E (Gigliotti et al., 1989; Adams et al., 2000), VM34C (Mindrinos et al., 1985), VM26A.2 (Popodi et al., 1988; Adams et al., 2000), VM26A.1 (Burke et al., 1987), 15a-1 and 15a-2 (Lin et al., 1993), 15a-3 (Edwards et al., 1988).

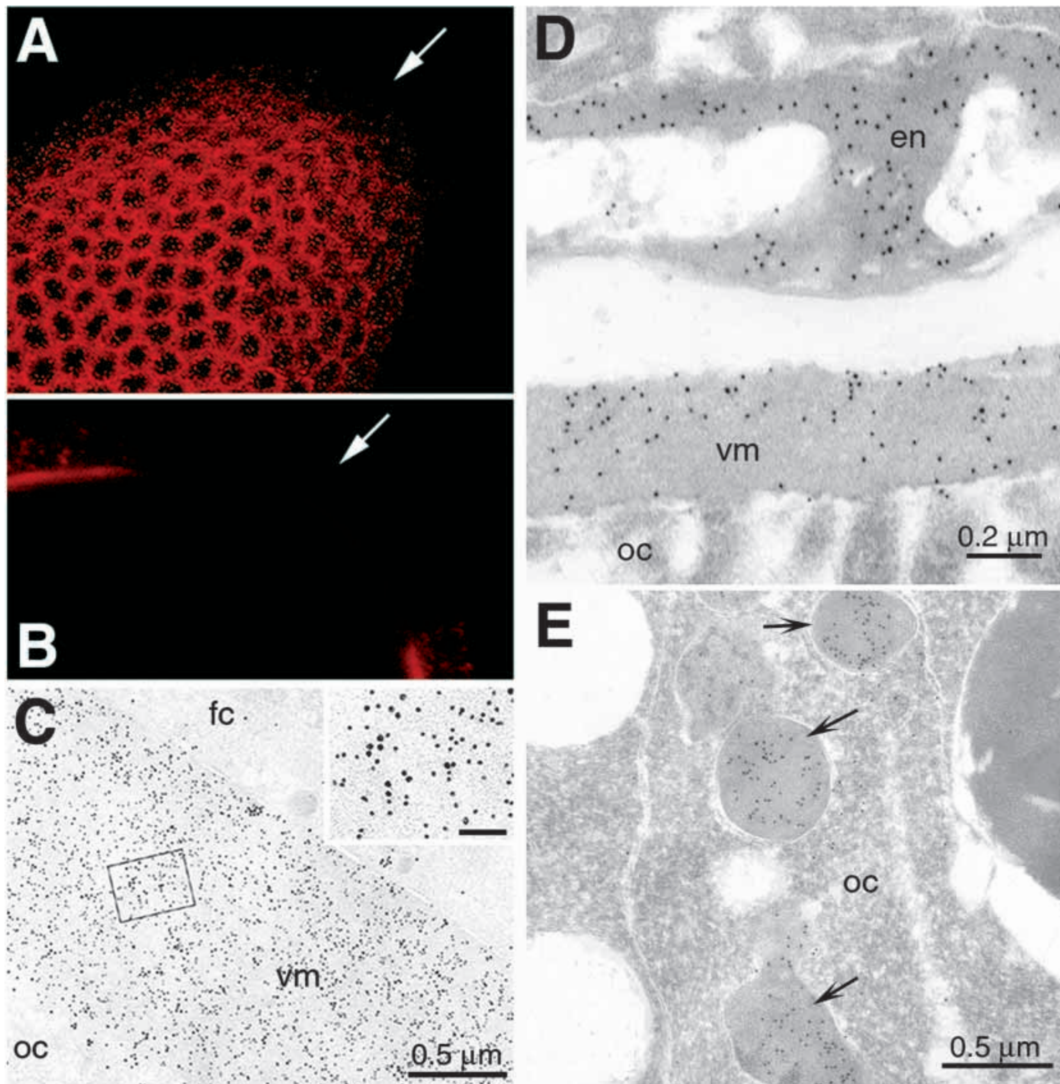

Fig. 7. Immunolocalization of the VM32E-MYC protein. (A,B) Stage 10B egg chamber stained with anti-MYC monoclonal antibody and examined by confocal microscopy. The white arrows indicate the posterior polar domain. (A) Surface view in which the VM32E-MYC protein appears within the main body follicle cells except the most posterior ones. (C) Immunoelectron microscopy of VM32E and VM32E-MYC proteins by double immunogold staining in stage 10B VM32E-MYC egg chamber. The 10-nm gold particles label the VM32E-MYC protein, whereas the 20 nm particles label both the wild-type and the VM32E-MYC proteins. The two proteins appear uniformly distributed in the vitelline membrane. The scale bar in the insert is 0.1 µm. Abbreviations: fc, follicle cell; oc, oocyte; vm, vitelline membrane. (D) In a stage 14 egg chamber, the VM32E-MYC protein is localized in both the vitelline membrane and endochorion. (E) At the same stage, the gold particles are also detected in vesicles localized in the subcortical region of the oocyte (arrows).

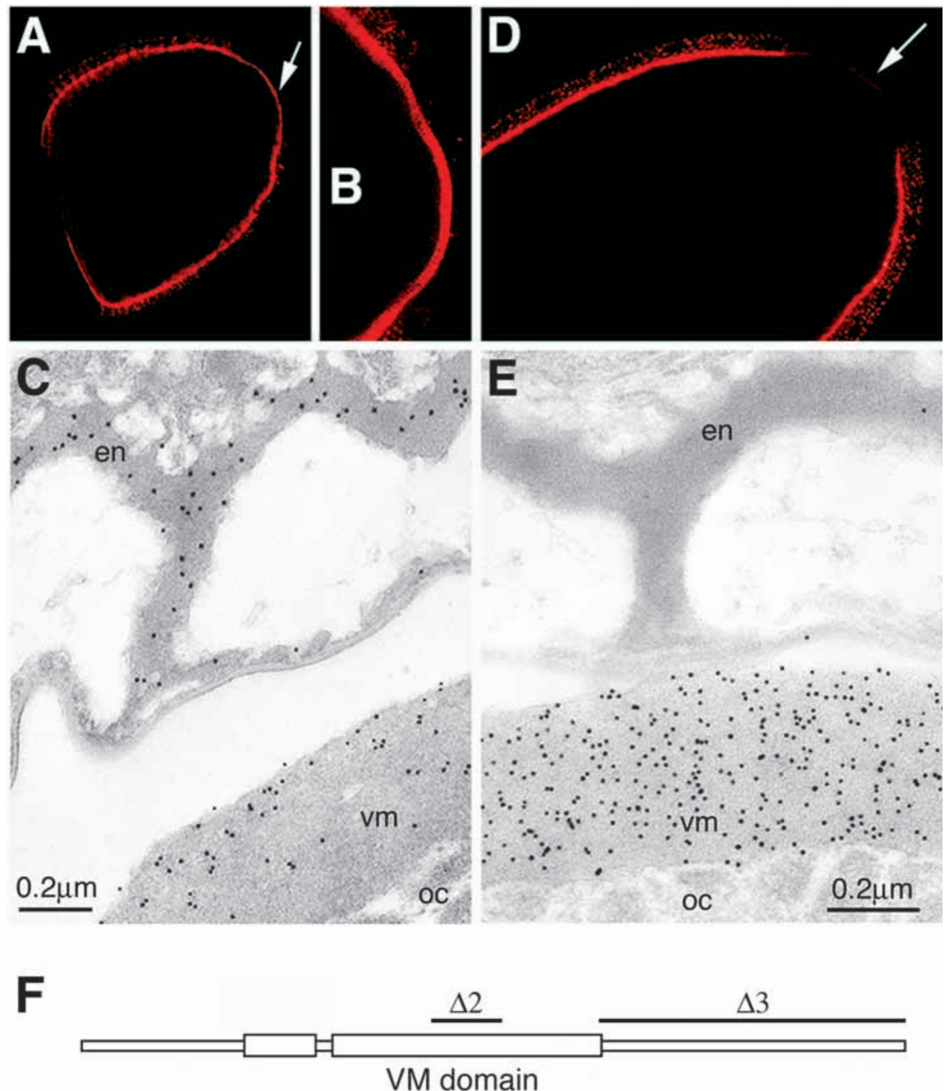

Fig. 8. Distribution of Δ2 and Δ3 proteins. (A) Stage 10A egg chamber from a female expressing the Δ2 protein stained with anti-MYC monoclonal antibody and examined laterally by confocal microscopy. The sagittal section of the egg chamber clearly shows the presence of the Δ2 protein in the anterior and posterior domains. (B) Higher magnification of the posterior pole of the same egg chamber. (C) Electron microscopy of stage 14 egg chamber showing the presence of the Δ2 protein in both the vitelline membrane and endochorion layer. (D) Stage 10B egg chamber from a female expressing the Δ3 protein stained with anti-MYC monoclonal antibody and examined laterally by confocal microscopy. The sagittal section of the egg chamber clearly shows the proper localization of the Δ3 protein. (E) Electron microscopy analysis of stage 14 egg chamber showing the absence of Δ3 protein from the endochorion layer. Strong positive labeling is visible in the vitelline membrane. The white arrows indicate the posterior polar domain. Abbreviations: en, endochorion; oc, oocyte; vm, vitelline membrane. (F) Structures of theVM32E deletions analyzed here.

protein at stage 10 appeared similar to that of the wild-type VM32E protein when the VM26A.2 protein was missing (Fig. 5A). This result suggests that the VM domain is required for protein-protein interaction among the various vitelline membrane proteins. Migration of the Δ2 protein into the endochorion was not affected (Fig. 8C), indicating that the integrity of VM domain is not necessary for VM32E movement from the vitelline membrane to the endochorion.

The VM32E protein presents a carboxyl end of 44 amino acids that does not share significant homology with the other vitelline membrane proteins. This region might provide the protein with its special feature. Therefore, we analyzed in transformed flies the localization and integration into the eggshell of a chimeric VM32E protein, deleted of its carboxyl end (Δ3, Figs 6A,8F). Confocal analysis of stage 10B egg chambers showed for the truncated protein the same distribution pattern as the wild-type VM32E (Fig. 8D). In stage 11 egg chambers, Δ3 protein was also localized at the poles (data not shown). This suggests that the C-terminal deletion does not alter the proper localization of the protein at these stages of egg chamber development. Electron-microscopy analysis of Δ3 protein distribution in the eggshell revealed that, at the terminal stages of oogenesis, it was localized only in the vitelline membrane, which showed a very strong immunogold signal (Fig. 8E). Therefore, the C-terminal domain is indispensable for localizing the VM32E protein in the endochorion. As a control, we analyzed the wild-type VM32E protein in sections from the same egg chambers and, as expected, it appeared in both the vitelline and the endochorion layers (data not shown). As shown for the VM32E-MYC protein, these chimeric proteins were found also in the ooplasm of stage 14 egg chambers (data not shown).

The various VM32E chimeric proteins (Figs 6A,9D) were analyzed by western blot at stages 10B and 14 of egg-chamber development (Fig. 9A). The VM32E-MYC control and the Δ1 product generated the same pattern as the wild type VM32E protein (Fig. 9A). The strong signal of the Δ2 protein detected in the supernatant phase of stage 10B egg chambers indicates that a considerable amount of this protein is not integrated into the membrane. These data agree very well with confocal analysis showing a high mobility of this protein during stage 10. At stage 10B, the Δ3 protein showed an extraction pattern similar to the wild type VM32E protein. The complex distribution pattern of the Δ3 protein is probably due to some

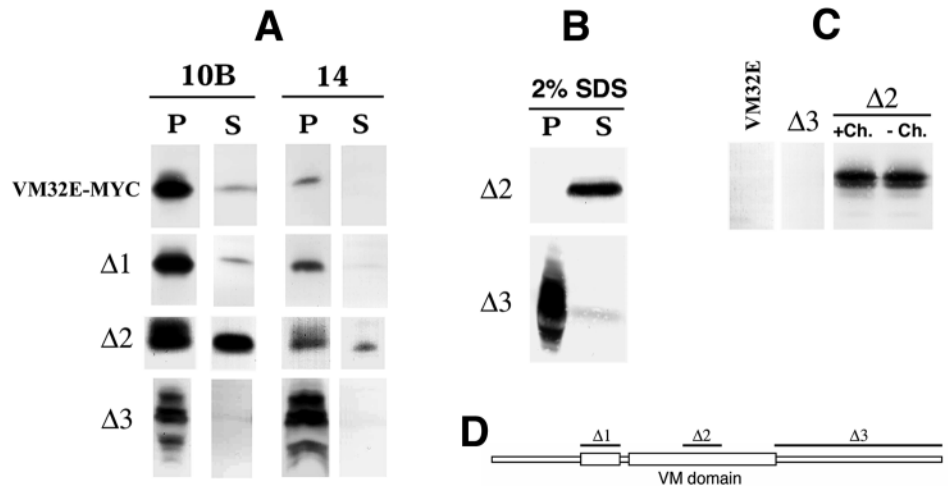

Fig. 9. Western blot analysis of various truncated VM32E-MYC chimeric proteins. (A) Staged egg chambers treated as described in Materials and Methods. The VM32E-MYC and Δ1 constructs show the same protein extraction pattern as the wild-type VM32E protein. The Δ2 protein shows a strong signal in the S phase of stage 10B, indicating that a significant proportion of its molecules are not yet integrated into the membrane. A remarkable feature of Δ3 protein is the high amount extracted from the pellet of stage 14. (B) Stage 14 egg chambers processed in presence of 2% SDS. The Δ2 protein is fully solubilized by SDS, whereas the Δ3 protein appears cross-linked by disulfide bridges. (C) Western blot analysis of Δ2 and Δ3 proteins in laid eggs. As with the wild-type VM32E protein, the Δ3 protein appears tightly integrated in the vitelline membrane. The released amount of Δ2 protein is the same from whole (+Ch.) or dechorionated (–Ch.) eggs, suggesting tight cross-linking only in the chorion layer. (D) Structure of the three VM32E deletions analyzed here.

unknown modifications, because all the major bands were larger than the predicted one. Also the VM32E-MYC, Δ1, and Δ2 proteins might undergo modifications, because their migration on the gel was slower than expected. Much Δ3 protein was extracted from stage 14 egg chambers, probably owing to its inability to localize in the endochorion layer. It is worth noticing that, at stage 14, the extraction pattern of Δ3 was identical to that of VM26A.2 (Fig. 4B), which was totally extracted from the pellet fraction. To assess whether or not the Δ2 and Δ3 proteins can form disulfide cross-links, stage 14 egg chambers were homogenized in presence of 2% SDS and the pellet and supernatant fractions were analyzed by western blot. As shown in Fig. 9B, the Δ2 protein was fully solubilized by the detergent, indicating that it is not cross-linked by disulfide bridges. Instead the Δ3 protein, which contains an intact VM domain, was recovered in the pellet phase, indicating that it forms disulfide cross-links.

In laid eggs, the vitelline membrane hardening does not permit the solubilization of its structural proteins. Therefore, we determined by western blot whether the deleted Δ2 and Δ3 proteins were properly integrated into the eggshell (Fig. 9C). The wild-type VM32E protein appeared to be tightly incorporated into the eggshell, as expected. The Δ3 protein was also fully insoluble, suggesting that it must be integrated by covalent cross-links. The analysis of the Δ2 protein revealed an altered cross-linking of this protein in the vitelline membrane; the same amount of Δ2 protein was in fact extracted from whole or dechorionated eggs. These results indicate that stable integration by cross-linking of the VM32E protein, and probably of any vitelline membrane protein, is based on the VM domain. By contrast, the absence of a functional VM domain did not affect the localization (Fig. 8C) or integration of the Δ2 protein in the endochorion layer (Fig. 9C).

DISCUSSION

Our structural and molecular analyses of the VM32E protein demonstrate several features of this protein that add more detail on the complex process of eggshell morphogenesis. At stage 10, this protein is synthesized in the columnar follicle cells, except the most anterior and posterior ones, and secreted in the extracellular space between the follicle cells and the oocyte surface. Thereafter, it moves to the poles and, by stage 11, it appears uniformly distributed in the vitelline membrane. This movement might involve unbound secreted VM32E molecules or protein aggregates. In any case, this result clearly suggests a highly dynamic state of the vitelline membrane at the time of its formation. The absence of *VM32E* expression from the polar follicle cells is an uncommon feature among the vitelline membrane proteins, and the *VM26A.1* and *VM26A.2* genes are expressed in all the columnar follicle cells (Burke et al., 1987; Popodi et al., 1988; Jin and Petri, 1993). Absence of expression from terminal follicle cell domains has, however, been observed for the *VM34C* gene (Bryant et al., 1999). It is not known whether the VM34C protein can also move to the poles, but it would be no surprise if it were to behave similarly to VM32E. The regulatory mechanism that represses the expression of the *VM32E* and *VM34C* genes at the poles might be part of a complex molecular strategy ruling the assembly of the vitelline membrane. Follicle cells are engaged in depositing some signals for proper embryonic development (Savant-Bhonsale and Montell, 1993; Ray and Schupbach, 1996). Programmed local delays in the assembly of vitelline membrane might allow the proper embedding into the vitelline membrane, or the deposition of positional cues elaborated by the follicle cells into the perivitellinic space.

The proper assembly of the VM32E protein in the membrane requires the activity of the VM26A.2 protein. In the *fs(2)QJ42* mutant egg chambers, which lack the VM26A.2 protein, movement to the poles of the VM32E protein occurs as soon as it is secreted. Therefore, the VM32E-VM26A.2 interaction also appears to regulate the timing of VM32E protein movement to the poles. The VM domain is necessary for the assembly of the VM32E protein in the vitelline membrane. We have shown that a deletion of ten amino acids within this domain impairs the early integration of the VM32E protein into the vitelline membrane and its cross-linking at the final stages of oogenesis. Interestingly, this domain is present in all the *D. melanogaster* vitelline membrane proteins cloned to date and

shares homology with the vitelline membrane proteins of the mosquito *A. aegypti* (Scherer et al., 1988; Gigliotti et al., 1989; Lin et al., 1993; Edwards, 1996; Edwards et al., 1998). In both species, this domain contains three precisely spaced cysteine residues, two of which are ablated by the small deletion analyzed (Δ2). This strongly suggests that the VM domain should fulfill a general function in holding together the various vitelline membrane proteins by disulfide cross-links.

In laid eggs, vitelline membrane hardening is thought to be performed by a peroxidase-type enzyme that cross-links the tyrosine residues of the different structural proteins (Petri et al., 1976; Petri et al., 1979). Because the VM32E protein contains seven tyrosine residues, this protein might also undergo such cross-linking. Our data indicate that the Δ2 protein is not tightly integrated into the vitelline membrane of laid eggs. Because this deletion does not remove any tyrosine residues, we suggest that the proper vitelline membrane assembly by disulfide bridges during oogenesis is necessary for any further covalent protein cross-linking in the egg.

Our data show for the first time that a vitelline membrane component, the VM32E protein, also participates in the assembly of the endochorion layer. This implies that VM32E protein molecules are released from the vitelline membrane and move outwards to the chorion layer. Transient storage of eggshell components within the vitelline membrane has been reported for some *dec-1* derivatives (Nogueron et al., 2000) and for the chorion protein s36, which is produced during early choriogenesis (Pascucci et al., 1996; Trougakos and Margaritis, 1998). Moreover, it is not known how these proteins can migrate to the chorion layers nor particularly whether there are any signal peptide to guide their movement. The presented results allowed the definition for the first time of an interacting motif of an eggshell protein involved in its outward movement. Our deletion analysis indicates that the VM32E C-terminal region is required for the recruitment of this protein by the endochorion layer. If this region is deleted, the truncated VM32E protein behaves like the other vitelline membrane proteins, appearing widely distributed only in the vitelline layer and becoming cross-linked only in the laid egg. The C-terminal domain might be the target of an unknown component that will carry the VM32E to the endochorion layer. Alternatively, this protein domain might allow the interaction of the VM32E protein with other chorionic components to form protein complexes that will move to the chorion layer. Based on the presence of nine glutamine residues in the C-terminal region, cross-linking of the VM32E protein could be also performed by a transglutaminase-type enzyme, as occurs with the proteins shaping the cornified cell envelope of mammals, in which glutamyl-lysine cross-links are formed by transglutaminases (Hohl et al., 1993). Even though transglutaminase activity in egg chamber extracts is still awaiting documentation, the VM32E protein might be bridged to the s36 and s38 chorion proteins by cross-links of the glutamine residues in the VM32E carboxyl domain to the lysine residues of these proteins. It has also been supposed that some *dec-1* derivatives that include the central glutamine-rich repeats might be cross-linked to other eggshell proteins by this type of cross-linking (Waring et al., 1990).

At stage 14, the VM32E also appears in vesicles within the oocyte, and the same is true for the VM32E chimeric proteins. Besides the endocytotic uptake of yolk proteins during vitellogenic stages (8-10) (Engelmann, 1979; Schonbaum et al., 1995), a late endocytotic activity at stage 14 has been reported for some *dec-1* derivatives (Nogueron et al., 2000). Although the functional significance of the uptake of eggshell components is not known, our finding that some vesicles loaded with VM32E are contained in the ooplasm might suggest that, as for the yolk proteins, this protein is stored to supply nutrients to the developing embryo. A programmed oocyte uptake of eggshell constituents could be part of the process of eggshell assembly in order to prevent inappropriate overload of specific proteins in the different eggshell layers.

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