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Pericardin, a *Drosophila* type IV collagen-like protein is involved in the morphogenesis and maintenance of the heart epithelium during dorsal ectoderm closure

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SUMMARY

The steps that lead to the formation of a single primitive heart tube are highly conserved in vertebrate and invertebrate embryos. Concerted migration of the two lateral cardiogenic regions of the mesoderm and endoderm (or ectoderm in invertebrates) is required for their fusion at the midline of the embryo. Morphogenetic signals are involved in this process and the extracellular matrix has been proposed to serve as a link between the two layers of cells.

Pericardin (Prc), a novel *Drosophila* extracellular matrix protein is a good candidate to participate in heart tube formation. The protein has the hallmarks of a type IV collagen α-chain and is mainly expressed in the pericardial cells at the onset of dorsal closure. As dorsal closure progresses, Pericardin expression becomes concentrated at the basal surface of the cardioblasts and around the pericardial cells, in close proximity to the dorsal ectoderm. Pericardin is absent from the lumen of the dorsal vessel.

Genetic evidence suggests that Prc promotes the proper migration and alignment of heart cells. *Df(3)vin6* embryos, as well as embryos in which *prc* has been silenced via RNAi, exhibit similar and significant defects in the formation of the heart epithelium. In these embryos, the heart epithelium appears disorganized during its migration to the dorsal midline. By the end of embryonic development, cardial and pericardial cells are misaligned such that small clusters of both cell types appear in the heart; these clusters of cells are associated with holes in the walls of the heart. A *prc* transgene can partially rescue each of these phenotypes, suggesting that *prc* regulates these events. Our results support, for the first time, the function of a collagen-like protein in the coordinated migration of dorsal ectoderm and heart cells.

Key words: *Drosophila*, Extracellular matrix, Heart tube, Pericardial cells, Cardial epithelium morphogenesis

INTRODUCTION

Cell interactions between the extracellular matrix (ECM) and neighboring cells play crucial roles in the regulation of cell behavior and fate. From the very first stages of embryogenesis, these interactions are a prerequisite for the full expression of the determination-differentiation properties of cells and for tissue organization. Beyond the obvious scaffolding functions of the ECM in cell adhesion, migration and tissue morphogenesis, the matrix is also responsible for transmitting environmental cues to cells that affect essentially all aspects of the life of the cell (for a review, see Geiger et al., 2001).

In vertebrate heart development, the process by which the two lateral precardiac regions fuse along their lengths following a lateral to ventral folding is the result of an interplay between the splanchnic mesoderm and the underlying endoderm (Molkentin et al., 1997). Interactions of substrate adhesion molecules, such as those of integrins expressed by heart precursor cells with fibronectin and laminin in the extracellular matrix, are considered to be instrumental for cardiogenesis (Collo et al., 1995; Kalman et al., 1995). Likewise, both soluble morphogens such as BMP4 (TGFβ family) and extracellular matrix proteins such as fibronectin may be key actors in directing the migration of cardiomyocytes during heart development in amphibians and birds (Linask and Lash, 1988a; Linask and Lash, 1988b; Sugi and Lough, 1995)

The *Drosophila* heart, or dorsal vessel, is a hemolymph pumping organ made up of a limited number of cells and cell types (Ruggendorff et al., 1994) (reviewed by Bodmer and Frasch, 1999). It consists of a double row of cardial cells that express muscle-specific proteins, coalesce to form the heart tube enclosing a lumen and are the contractile cells of the heart. Located at the dorsal midline, the heart is flanked on either side by several types of pericardial cells. These cells are loosely associated with cardial cells and do not express muscle proteins. The precise roles pericardial cells play during heart

development as well as their physiological function have remained, until now, largely unknown (Rizki, 1978). Anteriorly, the pericardial cells terminate in the lymph glands, which are bilaterally symmetric cell clusters and, at the most anterior end, the dorsal vessel is surrounded by the ring gland, an endocrine organ of complex origin. The heart tube is covered by a network of extracellular matrix components (Ruggendorff et al., 1994) among which some are localized in specialized areas of its surface (Zaffran et al., 1995). The heart tube is affixed to the underside of the dorsal body wall by seven segmentally repeated pairs of alary muscles.

The development of the *Drosophila* heart begins with the specification of cardial precursor cells that originate from the dorsal crest of the mesoderm monolayer and involves the action of the homeobox gene *tinman* (Azpiazu and Frasch, 1993; Bodmer, 1993). This initial subdivision of the mesoderm requires additional inductive signals from the overlying dorsal ectoderm to become fated to form cardial and/or pericardial cells (for a review, see Bodmer and Frasch, 1999). In stage 11 embryos, these precursor cells are metamerically organized in clusters of mesenchymal cells. During germband retraction (late stage 11 and stage 12), the cells acquire a polarity in a typical mesenchyme-epithelium transition and reorganize their shape to form a continuous epithelial layer on each side of the dorsal opening (Ruggendorff et al., 1994; Zaffran et al., 1995; Frémion et al., 1999). Later in the process of dorsal closure, the two rows of cardial cells, together with the pericardial cells, which are attached to the basal membrane of cardial cells, migrate dorsally and fuse at the dorsal midline to form the heart tube enclosing a lumen.

Once the heart has formed in late embryogenesis, the dorsal vessel shows clear structural differences along its length (Rizki, 1978; Bate, 1993). The posterior part constitutes the heart proper. This region is broader than the rest of the heart tube and consists of three segments. It contains the pacemaker activity and three segmental pairs of ostiae or valves that allow the lateral entry of hemolymph in the heart. Anterior to the heart is a narrower section termed the 'aorta' that encompasses four segments and is devoid of ostiae (Rizki, 1978; Molina and Cripps, 2001) (M. A., unpublished).

Despite its apparent simple structure and function, the *Drosophila* dorsal vessel shares several similarities with the early-stage hearts of vertebrates (reviewed by Bodmer and Frasch, 1999). Homologues of *Drosophila* genes function during vertebrate cardiogenesis, suggesting a conservation of molecular mechanisms in the formation of these essential circulatory organs. Early in vertebrate development, uncommitted splanchnic mesoderm residing on each lateral half of the developing embryo becomes specified to a cardiogenic fate by diffusible factors released from the underlying endoderm. Once specified, the cardiogenic precursors reorganize by a mesenchyme-epithelium transition and migrate along the anterior intestinal portal (AIP) to converge at the midline of the embryo where they form the cardiac crescent that folds ventrally, resulting in the fusion of the cardiac primordia and formation of the linear heart tube. The tube, subsequently, undergoes looping morphogenesis, which does not occur in *Drosophila* (Molkentin et al., 1997).

Adhesion molecules and ECM interactions are presumably involved in the main steps – cell specification, mesenchyme-epithelium transition, acquisition of cell polarity, migration, maintenance of structure – that are necessary to build up the cardial epithelium and to form the linear heart tube. During the early stages of *Drosophila* heart development, ECM molecules (laminin, collagen IV) are not expressed at detectable levels. When the heart lumen starts to form, adhesion molecules become integrated in the apical and basal extracellular matrix that underlines both sides of the tube (Tepass and Hartenstein, 1994). Laminin participation in the maintenance of the ultrastructure of the heart and a role for the PS integrins in the movement and migration of the pericardial cells via laminin as a ligand have been proposed (Yarnitzky and Volk, 1995; Stark et al., 1997; Martin et al., 1999).

In this work, we have focused our analyses on the function that extracellular matrix-mediated adhesion could play in the coordinated dorsal migration of the heart cells and that of the overlying ectoderm during dorsal closure (see Fig. 1). A novel collagen-like component of the *Drosophila* extracellular matrix, recognized by a monoclonal antibody, EC11 (Zaffran et al., 1995), is expressed in pericardial cells and is highly concentrated in the dorsolateral part of the heart, a region in close contact with the ectoderm. We have named the protein recognized by the antibody, Pericardin (Prc). Pericardin is a good candidate to participate in the movement of the heart and ectodermal cells. Our results suggest that, during dorsal closure and migration of the cardial epithelium, Prc could serve as a link to coordinate the movements of the two cell populations, and that modification of its expression could result in a concomitant disruption of the heart tube.

MATERIALS AND METHODS

Fly stocks

Wild-type embryos were from the Oregon R strain. The *Df(3L)vin6* (Akam et al., 1978) deficiency in the region of the *prc* gene and the puc^{E69} allele (Martin-Blanco et al., 1998) were obtained from the Bloomington *Drosophila* Stock Center.

DNA techniques

Standard molecular biology methods were used (Sambrook et al., 1989). Three EST clones CK 02611 (1.2 kb), CK 01593 (0.95 kb) and CK 02594 (0,9 kb) were obtained from the Berkeley *Drosophila* Genome Project (BDGP) (Kopcynski et al., 1998) and were used to screen a Canton-S 12-24 hour embryonic cDNA library (Brown and Kafatos, 1988). No clone could be identified when using the EST 02594 as probe and the EST expression profile described by BDGP could not be reproduced. That EST, therefore, was not considered as relevant and was not used further. The two other ESTs identified a cDNA clone – *prcV2* – that was sequenced on both strands (Genome Express, Grenoble, France). High density filters of P1 clones from the P1 *Drosophila* library were purchased from Genome Systems (St Louis, MO). Hybridization of the filters with the EST clones was carried out as described in 'P *Drosophila* filter overview' (provided by Genome Systems). The P1 clone DS 00169 (BDGP) was obtained from Dr Ashburner's laboratory

A 4.5 kb genomic fragment (*prc4.5*) located upstream of the 5′-end of *prcV2* (see Fig. 4A) was PCR amplified from DS 00169 by using as primers T7 and a specific primer: 5′-CCGATTTGCTT-CCGATCGCG-3′, complementary to the 5′-end of *prcV2* and containing the first *Pvu*I restriction site of the cDNA, subcloned in pGEM-T Easy (Promega, France) and finally cloned in the *Not*I site of the pCaSpeR-AUG-β-gal polylinker (Thummel et al., 1988).

The cDNA *prcV2* was excised from pNB40 by *Bgl*II digestion and inserted into *Bgl*II-cut pUAST (Brand and Perrimon, 1993). This

construct was injected in flies and independent UAS-prc lines were established and crossed to engrailed-GAL4 flies (a generous gift from Dr Gallet).

GenBank Accession Number for *prc* is AF203342.

Construction of a *prc* minigene and rescue experiments

prc4.5 was amplified with a modified T7 primer that included an additional *Not*I site in its 5′ end, purified, digested with *Pvu*I, and ligated in a 1/1 molar ratio to *Pvu*I-*Not*I cut and purified *prcV2*. The presence of a full-length minigene after subcloning in pBlueScript (Stratagene), was assessed according to three criteria: (1) restriction enzyme mapping, (2) PCR amplification with primers scattered randomly on the total length of the minigene and (3) Southern blotting with probes from different regions of the minigene. It was finally inserted into *Not*I cut CaSpeR 4 (Pirotta, 1988).

The construct MN-prc was injected with the Δ (2-3) helper plasmid (Robertson et al., 1988) in *yw* embryos to generate transgenic flies by standard methods (Rubin and Spradling, 1982). For rescue experiments, flies of genotypes *MN-prc; Df(3L)vin6/+* were constructed and crossed.

RNA-mediated interference (RNAi)

As described by Kennerdell and Carthew (Kennerdell and Carthew, 2000), two *prc* PCR amplified products [1025 bp from *prcV2* 3′-region (3446-4471); see Fig. 4A] were made with differing ends. One product had an *Eco*RI site at the 3446 end and a *Sfi*I site at the 4471 end (first pair of primers: a and b, 5′-CGGAATTCGGACAA-CCTGGAATAGGCGG-3′ and 5′-GGCCAAGATGGCCGGATT-GTGCAGCACCATGGT-3′; top strand sequence, GGCCATCTT-GGCC). The second product had a *Xba*I site at the 3446 end and a different *Sfi*I site at the 4471 end (second pair of related primers: a′ similar to a with a different 5′ end, 5′-CGTCTAGA….; b′ similar to b with a different 5′ end, 5′-GGCCTTCTC…..; top strand sequence CCGGAAGAGCCGG). Underlined sequences indicate the central non palindromic core of each site. After digestion with *Sfi*I, the two amplified products were ligated, the dimers purified and subcloned in *Eco*RI-*Xba*I cut pBlueScript (Stratagene). The resulting *Sfi*I site created in the dimer has a central non palindromic ATCTC sequence. Finally, the dimers were subcloned in *Eco*RI-*Xba*I cut pUAST (UAS-IR prc) (Brand and Perrimon, 1993). All the ligated products were transformed into the DH5α strain of *E. coli* to maximize the stability of the inverted repeats (Kennerdell and Carthew, 2000). The spacer between the two repeats was only 13 bp long, which could explain the somewhat inefficient rate of cloning of the inverted repeat in *E. coli*.

A GAL4-driver gene specific for pericardial cells was constructed by using the *prc4.5* genomic fragment. The GAL4 coding sequence excised from pGATB (Brand and Perrimon, 1993) and *prc4.5* recovered from pCaSpeR-AUG-β-gal were inserted sequentially into CaSpeR4 (prc-GAL4).

In situ hybridization and antibodies staining of whole-mount embryos

According to the protocol described by Frémion et al. (Frémion et al., 1999), DIG-labeled DNA probes were used for whole-mount in situ hybridization and fixed staged embryos were stained with primary and secondary antibodies as follows: mouse or preadsorbed rabbit anti-β-galactosidase (Promega and Cappel, respectively) 1:1000; rabbit anti-Tinman (Azpiazu and Frasch, 1993) 1:800, preadsorbed; mouse anti-Pericardin (EC11) (Zaffran et al., 1995) 1:2; rabbit anti-Mef2 (Nguyen et al., 1994) 1:1000, preadsorbed; rabbit anti-Oddskipped (Ward and Skeath, 2000) 1:1000, preadsorbed; anti-α-Spectrin (Lee et al., 1993) 1:500, preadsorbed; mouse anti-Nrt (Piovant and Léna, 1988) 1:500. Affinity-purified secondary antibodies were either coupled to alkaline phosphatase or to biotin (Jackson Immuno Research Laboratories) and used at a 1:1000 dilution or were either Alexa-488 or Alexa-594 conjugated (Molecular Probes) and used at a 1:500 dilution. In some cases, the signal was amplified with the aid of a 'Tyramide Signal Amplification' kit (NEN life sciences). The stained embryos were mounted in Geltol medium (Immunotech, France) or, when fluorescent, in Vectashield (Vector Laboratories) for further observation under an Axiophot Zeiss microscope or a LSM 410 Zeiss confocal microscope.

Immunogold electron microscopy

Stage 17 embryos and first instar larvae were fixed as described (Berryman and Rodewald, 1990) and embedded in LR Gold resin (TAAB Laboratories equipment). Ultrathin sections were incubated with undiluted anti-Prc antibody overnight at 4°C and then, with 10 nm gold-labeled goat anti-mouse IgG (1:25) (Aurion) for 1 hour at room temperature. The sections were post-fixed in 2% glutaraldehyde and observed under a Leo 912 electron microscope.

Prc immunoprecipitation and tryptic peptides analysis

Ten to 16 hours old embryos were homogenized in PBS buffer containing 1 mM EDTA, 2 M urea, 1.5% Triton X-100 and protease inhibitors. The proteins were incubated for 2 hours at 4°C with the EC11 monoclonal antibody coupled with dimethylpimelimidate (Pierce chemicals) to protein A-Sepharose 4B (Pharmacia, Uppsala). The bound antigen(s) was eluted with diethylamine (pH 11.5) and further purified by preparative electrophoresis on 5% polyacrylamide gels and western blotting. The nitrocellulose-bound antigen was submitted to trypsin digestion, and the peptides were purified by HPLC and sequenced. These experiments were carried out in Dr K. Williams' laboratory (Yale University, New Haven). Owing to the high molecular weight of the protein, the peptides were difficult to purify. Three of them were sequenced but only one has yielded, with reasonable confidence, a eight amino acid stretch whose sequence (NFQSTYYTK) can be found in Prc. The two other sequences had too many ambiguities to be considered.

RESULTS

In embryos mutant for *puckered* (*puc*), the migration of heart cells can be uncoupled from dorsal closure

During dorsal closure, the migration of the two rows of epithelial cardioblasts that will join to form the dorsal vessel underneath the dorsalmost ectodermal leading edge (LE) cells is coupled to that of the dorsal ectoderm (Fig. 1). Cell-ECM interactions through local receptor mediated signaling between mesoderm and ectoderm have been suggested as a mechanism to ensure such a coordinated movement. The cardioblasts and the attached pericardial cells are close enough to the overlying ectoderm (Ruggendorff et al., 1994) to engage efficiently in these interactions via the extracellular matrix network that surrounds the heart tube (Zaffran et al., 1995).

Fig. 2 illustrates the relative positions of the dorsalmost ectodermal cells that express the *puckered* (*puc*) gene (Ring and Martinez-Arias, 1993; Martin-Blanco et al., 1998) and the heart tube. During dorsal closure (Fig. 2A), the two rows of cells expressing Prc on their surface followed the same direction as the *lacZ*-expressing cells (*puc* cells) but with a slight asynchrony in their movement (Fig. 2B) that did not persist after closure because the ectodermal cells are, by then, perfectly aligned with the cardioblasts (Fig. 2C). These results suggest that the interaction of the heart tube with the dorsal ectoderm involved cells that are in a more lateral position than the LE cells, in agreement with previous observations by Ruggendorff et al. (Ruggendorff et al., 1994) (see also Fig. 3E,D).

At stage 11, Decapentaplegic (Dpp), a member of the TGFβ

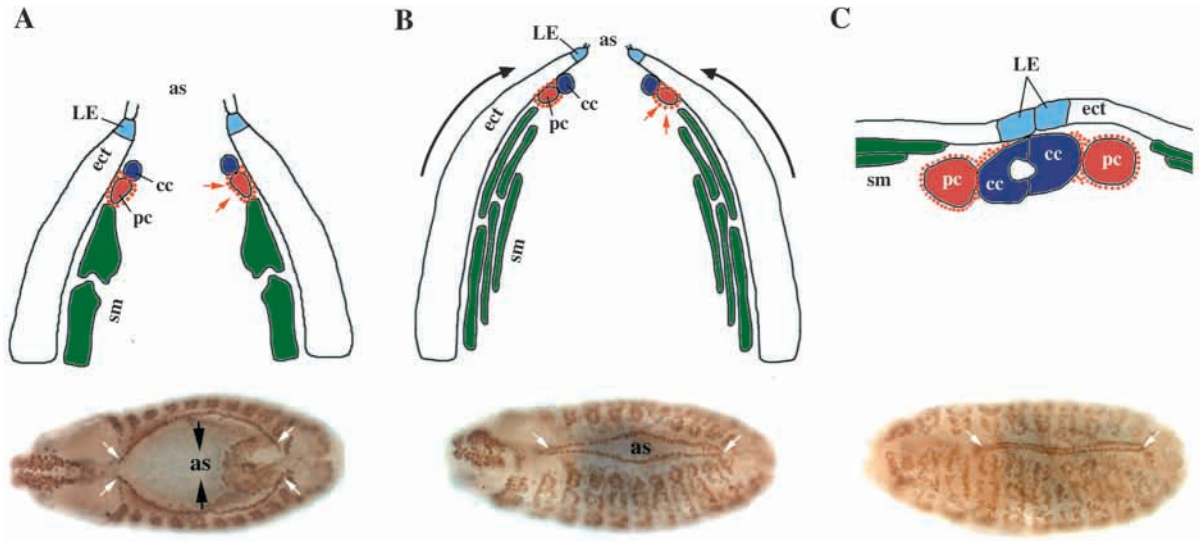

Fig. 1. Coordinated movement of the dorsal ectoderm and of the pericardial and heart cells during dorsal closure. In the schemes, the dorsal ectoderm (ect) is in white and the dorsalmost leading edge (LE) cells in blue. The ectoderm overlays the somatic mesoderm (sm, in green), the pericardial cells (pc in red) and the cardial cells (cc, in purple). as, amnioserosa. The embryos labeled with anti-Mef2 show the cardial cells (white arrows) and the amnioserosa (as, black arrows). (A) In a stage 13 embryo, the LE cells are one or two cells ahead with respect to the pericardial and cardial cells in close proximity to the ectoderm. The hypothesis of a link by the extracellular matrix (red dots and red arrows around the pc) between the two cells layers implies the existence of specific receptors localized in the dorsal ectoderm as well as on the heart cells. (B) The migration of the ectoderm in the dorsal direction (black arrow) carries along the somatic mesoderm and the heart cells. The extracellular matrix is represented as in A. (C) Dorsal ectoderm has closed. The heart tube is formed and encloses the lumen. The LE cells are now aligned with the heart cells. The extracellular matrix covers the basal face of the cardioblasts.

superfamily, is expressed in the *Puc*-LE cells. Puc negatively regulates *dpp* expression through the Jun kinase (JNK) pathway that is involved in dorsal closure. Whereas *dpp* provides an effector of dorsal closure, *puc* encodes a regulatory element that controls the amount of signaling through the pathway (Martin-Blanco et al., 1998). In puc^{E69} embryos, the JNK pathway remains constitutively activated resulting in Dpp overexpression in the ectodermal cells and in an abnormal expansion of Puc expression to several rows of cells (Fig. 2D) (Martin-Blanco et al., 1998). In homozygous puc^{E69} mutants, dorsal closure takes place but the LE cells do not differentiate properly. Under these conditions, the two rows of cardioblasts are no longer able to migrate and to join at the dorsal midline to form the heart tube (Fig. 2D-F) resulting in a cardia bifida phenotype as described for MesP1 GATA4 mutant mice (Molkentin et al., 1997; Saga et al., 1999) and for various zebrafish mutants (Stainier, 2001). As shown in Fig. 2D,E, the cardial epithelium is confined to the boundary between *puc* mutant cells and more lateral ectodermal cells, as if the two rows of cardioblasts were repelled from the *Puc*-expressing cells. This observation indicates that a change in the fate specification of LE cells had resulted in a concomitant alteration of the coordinated movements of the dorsolateral ectodermal cells and cardioblasts cells during dorsal closure.

Pericardin, an extracellular matrix component, is a candidate for participation in the dorsal ectoderm/heart epithelium interaction during dorsal closure

As mentioned above, the interaction taking place between dorsal ectoderm and the heart epithelium to coordinate cell movement during dorsal closure probably involves components of the extracellular matrix (Fig. 1). The spatiotemporal expression profile of Pericardin suggests that it could coordinate these movements (Zaffran et al., 1995). Prc is clearly detectable in early stage 13 embryos, its concentration increases in late-stage embryos and remains abundant in adults (Zaffran et al., 1995).

During dorsal closure and after the heart tube has finally formed, Prc is located around the periphery of the pericardial cells and outlines the basal surface of the cardioblast epithelium (Fig. 3A,B). As depicted in Fig. 3C, in embryos double labeled for Prc and α-Spectrin, a specific marker for the basal lateral membrane of epithelial cells, Prc appears particularly abundant at the boundary between the cardioblasts and the dorsal ectoderm. A better illustration of Prc localization was gained from sections focused either on the heart tube (Fig. 3E) or on the ectoderm (Fig. 3D). In that latter view, after the dorsal ectoderm has fused at the dorsal midline, Prc expression is concentrated in a position that corresponds to a layer of cells situated in a more lateral position (second or third row of ectodermal cells) than dorsal ectodermal cells.

Electron microscopy observation of a stage 17 embryo (Fig. 3F,G) in which the heart lumen is still very small and the ECM in a nascent state, shows that the expression of Prc is detectable only in the extracellular space around the pericardial cells and on the basal surface of cardiomyocytes. At the first instar larval stage (Fig. 3H,I), the lumen has considerably enlarged but Prc is totally absent from luminal ECM, while its concentration increases concomitantly with the maturation of the basal ECM, thus confirming the disymmetric distribution of Prc.

In conclusion, the specific location of Prc in close proximity to the dorsal ectoderm and around the heart tube could be consistent with its participation in the proper migration of the

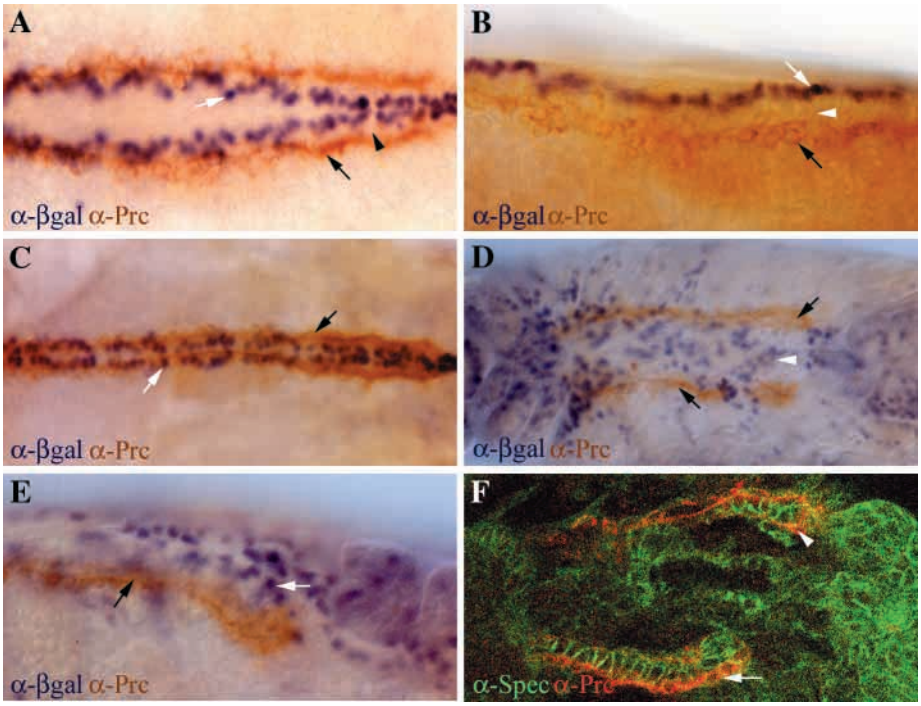

Fig. 2. Alteration of the fate of the *puc*-expressing LE cells hampers the migration of the two rows of cardioblasts to form the heart tube. The embryos have been double labeled to detect the nuclear β-gal expression (blue) of a P(*lacZ*) insertion in the *puc* gene and, with the EC11 monoclonal antibody, to detect the expression of Prc (brown), a component of the extracellular matrix outlining the periphery of the heart tube. (A) A dorsal view of an embryo (end of stage 13) during dorsal closure. *puc* is expressed in the dorsal ectoderm (Ring and Martinez Arias, 1993; Martin-Blanco et al., 1998) and Prc is localized at the basal surface of the cardial cells (Zaffran et al., 1995) (see Fig. 3). Migration of the two rows of cardial cells (black arrow) towards the dorsal midline is coordinated with that of LE cells (white arrow) during dorsal closure. Notice (arrowhead) the space separating the two types of cells. (B) In a lateral view of the same embryo, the pericardial cells (black arrow) coincide with ectodermal cells located in a position that appears one or two rows more lateral than LE cells (white arrow) (Ruggendorff et al., 1994), indicating a shift in the migration of the pericardial cells with respect to the LE cells. The white arrowhead indicates the distance between the two layers. (C) At the end of dorsal closure (dorsal view), LE (white arrow) and Prc-expressing cells (black arrow) are aligned (Martin-Blanco et al., 1998) and the two rows of cardial cells that are not visible have joined at the dorsal midline below the ectoderm. (D) Dorsal view of a puc^{E69} mutant embryo bearing a P(*lacZ*) insertional mutation in the *puc* gene. The dorsal ectoderm is properly closed but the heart tube is not closed and the two rows remain separated (black arrows). More cells express β-gal (white arrowhead) and occupy, in the dorsal ectoderm, a domain from which the cardial cells seem to be excluded. (E) In a lateral view of the same puc^{E69} mutant embryo, only one side of the embryo is depicted and, consequently, only one row of cardioblasts is visible (black arrow). The labeling by anti-Prc is excluded from the *puc*-expressing cells territory (white arrow). (F) Dorsal view. Optical confocal sections of a fluorescently double stained puc^{E69} mutant embryo for α-Spectrin (green) and Pericardin (red). The embryo is closed dorsally (not seen in the plane of focus) but the two rows of cardioblasts have not joined to form the heart tube. The polarity of the cardioblasts has not, however, been altered, as judged from the concentration of Prc on their basal surface (white arrow) where are attached the pericardial cells. In one exception (white arrowhead), Prc has been detected on the apical face of the cardioblasts. In all the views, anterior is towards the left and posterior is towards the right.

two layers of cells and/or in consolidating morphogenesis of the heart once formed.

The *prc* gene encodes a type IV collagen-like protein

In a search of *Drosophila* databases for ESTs that displayed expression profiles resembling those described above for Prc (in BDGP, 3000 individual cDNAs screened by in situ hybridization, 19 expressed in the dorsal vessel), three such ESTs were noticed that labeled pericardial cells and oenocytes, another site of Prc expression. For reasons mentioned in the Materials and Methods, one of them was not considered as relevant. The two other ESTs identified two overlapping DS phages on high density filters of P1 clones that allowed the cytological mapping of the gene to 68 E2-E3 (Fig. 4A).

Screening of a cDNA library (Brown and Kafatos, 1988) with the two ESTs led to the recovery of an almost full length cDNA – *prcV2* (5.6 kb) – that hybridized to a single 5.8 kb transcript on northern blots (data not shown), appeared in 8- to 12-hour-old embryos and increased in abundance in older embryos. No transcript was detected in preblastoderm stage embryos, thus indicating strict zygotic expression of the gene.

Sequence analysis of the genomic region upstream of the first exon (Fig. 4A) identified several putative promoter sites and a TATA box that positioned the transcriptional start in that region. This was further supported, as will be discussed below (see also Fig. 5E,F), by the observation that the *prc4.5* fragment, when inserted upstream of a *lacZ* reporter gene, is able to direct β-gal expression in a pattern superimposable on that described for the staining with the anti-Prc antibody.

The *prcV2* cDNA contains a long open reading frame of 1729 amino acids (Fig. 4B) with a calculated molecular mass of 165 kDa, consistent with size estimates from western blots revealed by the anti-Prc antibody (Zaffran et al., 1995).

The presumptive initiation codon has been ascribed to the first in-frame ATG (Fig. 4A) that was preceded by an in-frame TAA stop codon. The initiating methionine is followed by a sequence (20 amino acids) containing structural features characteristic for a signal sequence (Fig. 4B) (von Heijne, 1986). No other long hydrophobic regions indicative of transmembrane spanning segments were found, suggesting that this protein is a secreted protein.

The sequence of a particular stretch of eight amino acids in the C-terminal region was identical to that in a peptide obtained by trypsin-mediated cleavage of the EC11-immunoprecipitated and purified Prc antigen (see Materials and Methods) (Fig. 4B). Prc is the unique protein in *Drosophila* that contains this motif

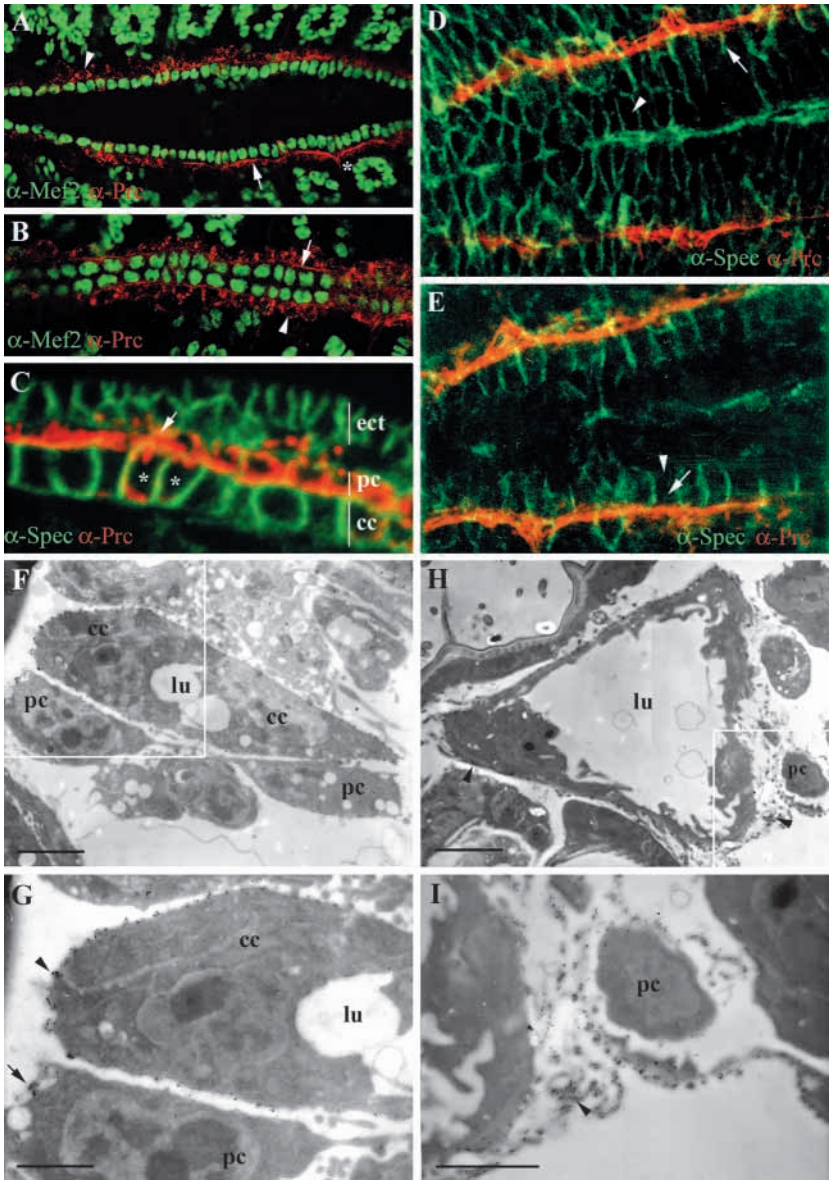

Fig. 3. Pericardin is located around the pericardial cells and at the basal surface of the cardioblasts in close proximity to the ectoderm. Embryos were double labeled by using either anti-Prc (red) and anti-Mef2 (green) that stained the nuclei of all myogenic cells (A,B) or anti-Prc (red) and anti-α-Spectrin (green) that is specific for the basolateral membrane of epithelial cells (C-E). (A) Dorsal view of a stage 14 embryo during dorsal closure and (B) of a stage 15 embryo in which the heart tube is formed. At both stages, Prc is located around the periphery of the pericardial cells (white arrowhead) and at the basal surface of the cardioblasts (white arrow). Attachment sites of alary muscles are also decorated by Prc (asterisk in A). (C-E) Confocal microscope sections. (C) Transverse section of the dorsal side of an embryo that shows successively from the top to the bottom, the polarized epithelial cells of the dorsal ectoderm (ect), the pericardial cells (pc) and the cardioblasts (cc), whose basolateral surface is stained with anti-α-Spectrin. Prc is concentrated at the boundary between the basal surface of the ectodermal epithelial cells and that of the cardial epithelium. The yellow color (white arrow) results from the superimposition of the two markers in the thickness of the section. Prc is also expressed in two out of every six cardial cells (stars) that correspond to the *seven-up* (*svp*)-expressing cells (Gajewski et al., 2000; Lo and Frasch, 2001). (D,E) Sections in the dorsal part of an embryo in which the ectoderm but not the heart tube is closed. (D) Superficial section through the dorsal ectodermal cells (arrowhead). Prc (red) is concentrated to the basal face of the cardioblasts whose basolateral membranes labeled with anti-α-Spectrin (green) are weakly detectable (arrow). In several positions (yellow color), Prc is in contact with the ectodermal cells. (E) Deeper section at the level of the cardioblasts (white arrow). Prc is still located at the basal face of the polarized cardioblasts arranged as a typical epithelium whose apical surface is not stained by anti-α-Spectrin (arrowhead). (F-I) Immunogold staining of heart ultrathin sections with anti-Prc as probe. (F) Cross section of a stage 17 embryo treated as described in the Materials and Methods and (G) enlargement of the insert. It shows two opposing cardiomyocytes (cc) enclosing a small lumen (lu) in which no gold particles are visible in the nascent basement membrane underlining the luminal surface. Particles are present (arrows) along the basal surface of the cardial cells and around the pericardial cells (pc), indicating the presence of Prc in the extracellular space. (H) Cross section of a larval heart focused on the enlarged lumen, which is still devoid of gold particles in the luminal basement membrane. In the mature basal extracellular matrix (I, enlargement of the boxed area in H), a high concentration of gold particles (arrowheads) is detected. Scale bars: 2 μm in F; 4 μm in G; 2.5 μm in H; 1 μm in I.

(Blast analysis, pattern search), thus confirming that the cloned cDNA probably codes a protein corresponding to the antigen recognized by anti-Prc (EC11).

An ARG-GLY-ASP (RGD) sequence, a well known potential cell attachment site mostly found in extracellular matrix components (for a review, see Hynes, 1992), is present within the extreme C-terminal moiety. In addition, an ARG-GLU-LYS-ARG tetrapeptide corresponding to the Furin consensus sequence for efficient cleavage of its substrates (Molloy et al., 1999) resides in the N-terminal extremity of Prc. Furin is a serine proteinase that can regulate the composition and, thereby, the function of extracellular matrix by processing matrix components and/or by activating metalloproteinases (Molloy et al., 1999).

Several characteristics raised the possibility that Prc could be a type IV collagen-like protein. Similar to type IV collagens, Prc has a high content of Gly (29%), Gln (13%) and Pro (10%) residues, its transcript is large in size (5.8 kb) and it is organized in three characteristic domains that include a leader peptide and short N-terminal non-collagenous segment, a long collagen-like domain and a C-terminal non-collagenous domain (NCI) (Vuorio and de Crombrugghe, 1990). The amino acid sequence of the collagen-like domain displayed high scores of homology (about 35% identity) with type IV collagen α-chains from various species, including *Drosophila*, *Ascaris*, *C. elegans* and human. The typical $(Gly\text{-}X\text{-}Y)_n$ triplet repeat in which X and Y can be any amino acid but X is often proline, and which is responsible for the triple helical structure of

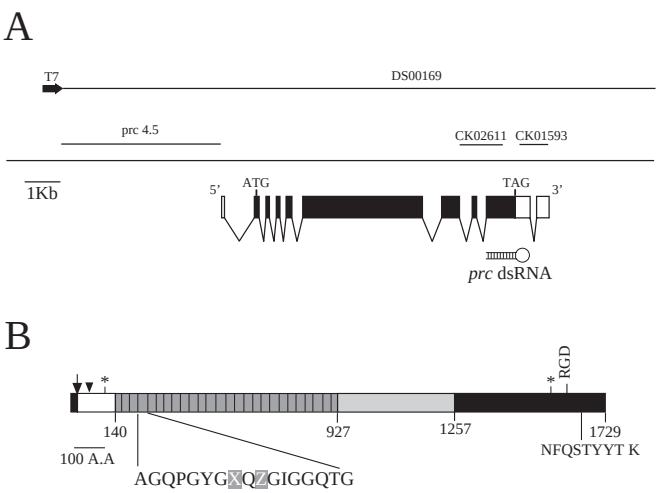

Fig. 4. Molecular characterization of the *prc* locus and schematic representation of the Prc protein. (A) The 68 E locus drawn to scale. The locus is encompassed by the DS 00169 P1 phage. *prc4.5* has been prepared as described in the Materials and Methods, and this fragment contains the regulatory sequences necessary for the expression of *prc*. Black boxes represent the coding regions of the mRNA, while empty boxes represent untranslated parts. The translation start and stop codons are indicated by ATG (145 in the cDNA) and TAG. The intron-exon structure of *prc* as deduced from the *Drosophila* genome sequence (BDGP) reveals nine exons and eight introns. The locations of CK 02611 and CK 01593 whose sequences match to the 3′-end of the cDNA are shown above the gene. CK 01593 extends further downstream in 3′ of the *prcV2* cDNA. prc dsRNA indicates the position of the 1 kb sequence used to construct UAS-IR prc. (B) The Prc protein. Small black box indicates signal peptide; arrow above the black box indicates putative signal peptide cleavage site (in 21); arrowhead indicates putative furin cleavage site (in 38); stars (72 and 1570) indicate the two N(I/G)S sequences that may serve as sites for N-linked glycosylation; large black box indicates C-terminal non-collagenous region. The potential attachment site for integrin is RGD (1652-1654). NFQSTYYTK is the tryptic peptide sequence (1707-1714). The collagen-like domain (140-1267) contains 26 atypical repeats illustrated by vertical bars (140-927) and the typical $(Gly\text{-}X\text{-}Y)_n$ repeat (920-1267 moiety, gray box). The consensus sequence of one of the atypical repeats is shown below.

collagens, is present in that domain. The length of the repeat is especially short as *n* never exceeds five and interruptions are very frequent. One function of these discontinuities observed in other collagens, such as type IV (Vuorio and de Crombrugghe, 1990) or type XVIII (Oh et al., 1994) collagens, is to provide flexibility between triple helical regions.

In this same domain, the $(Gly\text{-}X\text{-}Y)_n$ triplet repeat is preceded by another repeat of the type $(AGQPGYGXQZGIGGQTG)_n$, where n=26. Interruptions that varied in composition and length could be noticed flanking either side of 14 consecutive strictly identical repeats. The sequence of the repeat is unique in *Drosophila* and within the whole phylum.

Search for predicted secondary structures with the aid of a PSIPRED Program (Jones, 1999) revealed no striking differences between the Prc collagen-like domain and those of *Drosophila* or human type IV collagens, suggesting that, in spite of its atypical repeats, Prc may have conserved the potential for a triple helical structure.

Only two putative N-linked glycosylation sites were detected, indicating that Prc is a poorly glycosylated protein as is the case for other collagens and as was previously inferred from in vitro deglycosylation experiments that did not modify the electrophoretic migration of the protein (Zaffran et al., 1995).

The short N-terminal and long C-terminal domains displayed no homologies with any known proteins, including the NCI domain of type IV collagens.

Prc may, therefore, constitute a new extracellular matrix component possessing characteristic hallmarks of basement membrane proteins.

prc is expressed in pericardial cells, in oenocytes and in a subset of cardioblasts

The expression of the *prc* transcript is shown in Fig. 5A,B. It specifically appears at stage 13, at the onset of dorsal closure, in the two rows of pericardial cells that are attached to the surface of the cardial cells (Fig. 5A). In the mature embryonic heart, the *prc* transcript is very abundant in the pericardial cells and in the ring gland (Fig. 5B). *prc* is also expressed in oenocytes and in small cells in the anterior part of the embryo that seem to arise from the ring gland and whose identity has not been determined (Fig. 5B). This pattern of expression is superimposable on that of the protein. As depicted in Fig. 5C,D,L, the anti-Prc antibody detects an epitope expressed in the pericardial cells, the ring gland and in the oenocytes as well, thus confirming that the EST probably encodes the protein identified by EC11.

In a transformed line of flies (*prclacZ*) carrying the *prc4.5* genomic fragment inserted upstream of the *lacZ* reporter gene (see Fig. 4A), *lacZ* activity (Fig. 5E,F) was detected in the same cells as that of the *prc* mRNA, indicating that *prc4.5* contains all the regulatory elements necessary for *prc* expression.

Co-expression of Odd and Prc is observed in all the Odd-pericardial cells (Ward and Skeath, 2000). These Odd-expressing pericardial cells are large cells particularly easy to recognize in third instar larvae and are considered to be ‘classical’ pericardial cells (Rizki, 1978; Ward and Skeath, 2000) (Fig. 5G). These cells have been shown to express the Mab3 epitope (Yarnitsky and Volk, 1995; Ward and Skeath, 2000), whose expression pattern is identical to that of EC11, suggesting that the two epitopes may be shared by the Prc protein, although no attempt has been made to formally demonstrate this point. Prc is also expressed in Tin-positive pericardial cells (Jagla et al., 1997) in the heart region of the dorsal vessel (Fig. 5H) that do not express Odd (compare Fig. 5G with 5H). It has been verified that the absence of anterior signals did not result from sectioning through different planes along the anteroposterior axis. *prc* could also be detected, although more weakly, around the cells in seven segmentally repeated clusters that were cardioblasts, as judged from their staining with anti-Mef2 (data not shown). They correspond to the *seven-up* (*svp*)-expressing cardioblasts (Gajewski et al., 2000; Ward and Skeath, 2000; Lo and Frasch, 2001) (Fig. 5I,J; see also Fig. 2), which are the precursors of the larval ostiae (Molina and Cripps, 2001). At the anterior end of the aorta, Prc is expressed in the ring gland while Odd is expressed in the lymph glands (Fig. 5K).

Finally, as shown in Fig. 5M, when the *prc* cDNA was driven

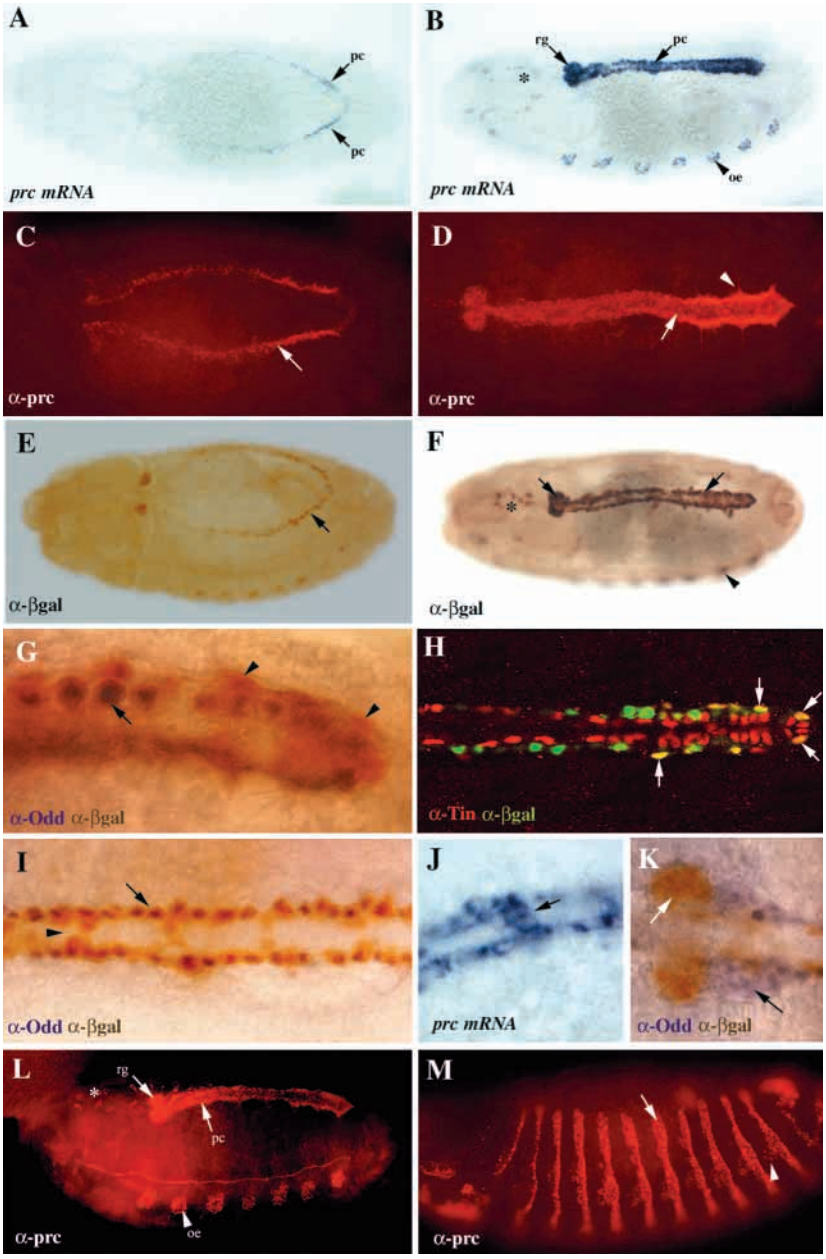

Fig. 5. Expression of the *prc* transcript and of the protein. (A,B) In situ hybridization to whole-mount stage 13 (A) and stage 17 (B) embryos with *prcV2* as probe. The mRNA is already detected before dorsal closure (A) in the pericardial cells. When the heart is formed (B), the mRNA is expressed in the pericardial cells and ring gland (arrows), the oenocytes (arrowhead) and in some unidentified cells in the anterior part of the embryo (star). This pattern of expression can be superimposed on that observed with the anti-Prc (EC11) monoclonal antibody (C,D,L). White arrow, pericardial cells; white arrowhead, alary muscles. (E,F) In a transformed line carrying the *prc4.5* genomic fragment inserted upstream of the *lacZ* reporter gene, anti-β-gal staining reflecting Prc expression is detected in the same cells as the *prc* mRNA (compare with A,B, same symbols). (G) Double staining of a stage 17 embryo with anti-Prc (brown) and anti-Odd (blue) showing only the heart. All the Odd-expressing cells express also Prc (arrow). The Tin- and Prc-expressing cells (arrowhead) do not express Odd (compare with H). (H) Confocal section of a stage 17 embryo fluorescently double labeled for Tin (red) and Prc (green), as monitored by anti-β-gal staining in the *prclacZ* transformed line. The arrows indicate yellow pericardial cells that co-express Tin and Prc in the heart. (I,J) In I (double staining as in G), clusters of segmentally repeated cardioblasts (arrowhead) do not express Odd but they express *lacZ* and the *prc* mRNA (J, arrow) (in situ hybridization as in B); they are *svp*-expressing cardioblasts. (K) Double staining as in G. Prc stains the ring gland (white arrow) and Odd labels the lymph glands (black arrow). (L) In a stage 17 embryo, labeling with anti-Prc detects an epitope present in the pericardial cells (arrow), the ring gland (arrow), the oenocytes (arrowhead) and unidentified cells (asterisk). (M) Embryos in which the *prc* cDNA is driven under UAS control with engrailed-GAL4. EC11 immunoreactivity is detected in a pattern in stripes (arrow) in addition to the endogenous labeling of oenocytes (arrowhead). In all views, anterior is leftwards and dorsal is upwards.

under UAS control in a distinct pattern, such as stripes with engrailed-GAL4, EC11 immunoreactivity detected an epitope ectopically expressed in stripes in addition to its endogenous pattern, exemplified by the labeling of oenocytes. These observations strongly support that fact that the protein encoded by the *prcV2* cDNA is identical to the antigen recognized by the EC11 antibody.

Interference by double stranded (ds) RNA of Prc expression results in a disorganization of the cardial epithelium during its coordinated migration with the dorsal ectoderm

Further characterization of Prc function required a detailed description of a mutant phenotype. *Df(3L)vin6* (Akam et al., 1978) uncovers the *prc* gene and abolishes the expressions of the *prc* transcript and of the protein (not shown). This deficiency is, however, large and uncovers several other genes. However, available lethal alleles in the region maintained *prc* expression and no P-element insertion close enough to the *prc* locus has been identified. To circumvent the absence of mutants in the *prc* gene, we have chosen to use RNA interference (RNAi) to trigger degradation with double-stranded RNA (dsRNA) of the mRNA bearing the same sequence. This process was first developed for use in invertebrates and later, in vertebrates (for a review, see Baulcomb, 1999). Methods have been devised in *Drosophila* and in *C. elegans* that use a heat-inducible promoter (Lam and Thummel, 2000; Tavernarakis et al., 2000) or the GAL4/UAS system to express extended hairpin-loop RNA in a controlled temporal and spatial pattern and flexibly induce inhibition at any time of the life cycle or in a specific tissue (Kennerdell and Carthew, 2000; Martinek and Young, 2000; Piccin et al., 2001).

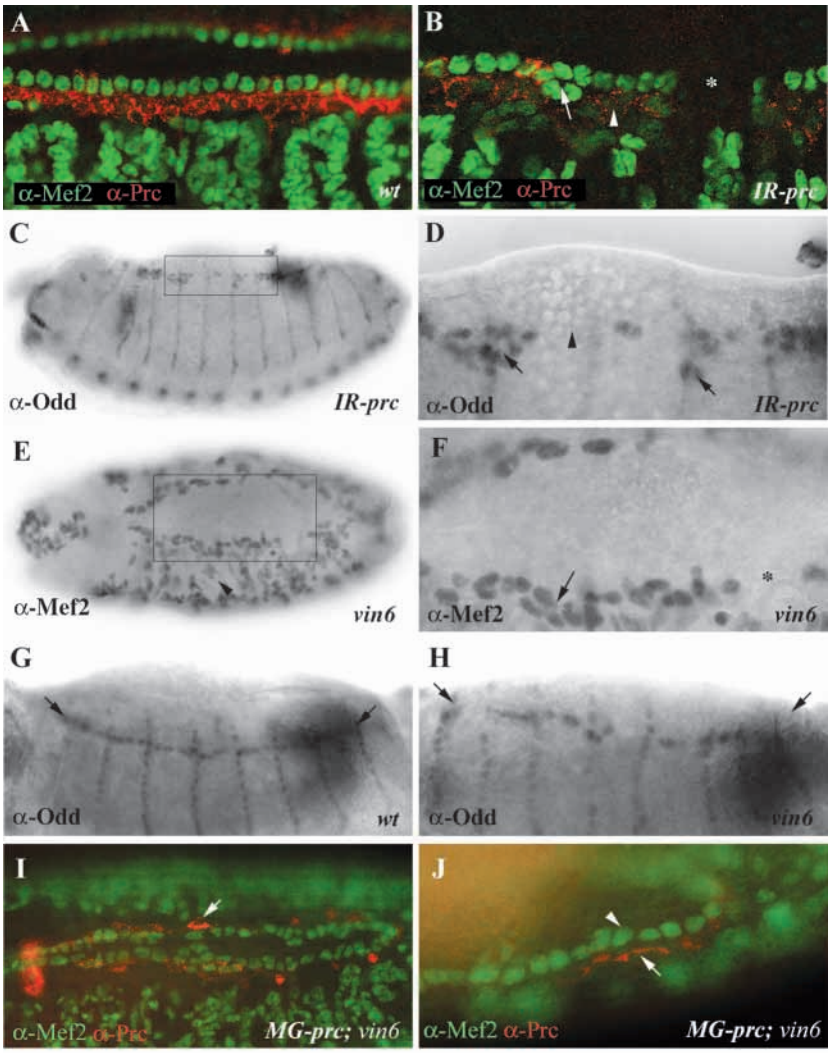

Fig. 6. Perturbation in *prc* expression results in a disorganization of the cardial epithelium. (A-D) Hairpin-loop RNA interference experiments. (A,B) Confocal microscope observations of embryos double-labeled for Mef2 (green) and Prc (red) expression. A dorsal view of a stage 16 embryo (B) shows an important decrease in Prc expression (arrowhead, compare with a wild-type embryo in A), a cluster of cardioblasts (arrow) and a hole in the cardial epithelium (asterisk). (C,D) In embryos stained for Odd (D, which is an enlargement of the insert in C), holes (arrowhead) and clusters (arrows) of cells are visible. (E,F,G-J) *vin6*-deficient embryos. (E and box area enlarged in F) Disorganization of the cardial epithelium, visualized using anti-Mef2, with holes in the cardial epithelium (asterisk in F) and clusters of cardioblasts (arrow in F). The cardiac phenotype is very similar to that observed in B. The number of pericardial cells stained with anti-Odd is reduced in *vin6*-deficient embryos (H, compare with a wild-type embryo in G). Black arrows indicate the row of pericardial cells. (I,J) Rescue of the cardiac*vin6* phenotype with the minigene MG-prc. In embryos double-labeled for Mrf2 (green) and Prc (red), the staining with anti-Prc (I) shows a restoration of Prc expression (arrows in I and J). As the number of pericardial cells is still reduced, the staining with anti-Prc is not completely normal. The structure of the cardial epithelium is less disorganized (arrowhead; compare with B,F). In all views, anterior is leftwards and dorsal is upwards.

We have attempted hairpin-loop RNA interference of *prc* mRNA with a prc- GAL4 driver (see Materials and Methods) aimed at a specific reduction of the function of *prc* in pericardial cells. The inverted repeat sequence was chosen in the C-terminal domain of Prc in a region that was completely free of the N-terminal repeated motifs (see Fig. 4A) and that shared no homologies with any other *Drosophila* protein. The length of a single repeat was close to 1 kb as recommended by Fire et al. (Fire et al., 1998).

As shown in Fig. 6B, dsRNA interference of *prc* mRNA resulted in a significant decrease in protein expression (compare Fig. 6B with 6A). The partially silenced embryos display a clear disorganization of the cardial epithelium during its migration in concert with the dorsal ectoderm (Fig. 6B). In many places, the alignment of the cardial cells is interrupted by holes from which they are absent (compare Fig. 6A with 6B) and cells accumulate in small clusters around the holes along the rows of cardioblasts (Fig. 6B). As shown in Fig. 6C,D, disorganization of the pericardial cells also results in interruptions in the dorsal vessel and, as in the case of the cardioblasts, in repeated clustering of the cells, even though their total number did not seem to be modified. The interference was not total and ~50-60% of embryos were silenced. Each affected embryo displaying a reduced expression of Prc exhibited all the phenotypes described above with somewhat variable numbers of interruptions and, in some instances, interruptions in only one side of the tube.

The phenotypes obtained by RNA interference of *prc* were similar to those provoked by a total lack of *prc* expression and of EC11 immunoreactivity (data not shown) in *vin6*-deficient embryos, at least with regard to the heart tube morphogenesis (Fig. 6E,F). The cardial epithelium formed properly but fell apart with holes and clustering of cardial cells during its coordinated migration with the dorsal ectoderm. The phenotypes in deficient embryos were not much stronger than in silenced embryos. The incomplete penetrance of the phenotype and the absence of EC11 immunoreactivity could suggest that other molecules may play a redundant role with Prc in the formation of the heart tube. However, the comparable phenotypes strongly support the idea that the inverted repeat inactivation of the *prc* gene is specific. In *vin6*-deficient embryos, which are grossly perturbed in their development, only a small percentage of individuals survived to the stage of complete closure of their dorsal ectoderm and presented severe defects in their muscles. Generally, the size and the number of cells were different from those in a wild-type animal. In

particular, the number of pericardial cells was reduced and the cells were larger (Fig. 6G,H).

As a consequence, the heart phenotypes of *vin6*-deficient embryos could only be partially rescued by a MN-prc minigene. Prc expression was restored although to a lesser extent than in a wild-type animal (Fig. 6I,J), probably because of the reduced number of pericardial cells. The cardial epithelium is less frequently disrupted, especially in the places where Prc is expressed (Fig. 6I,J). It is thus reasonable to postulate that the cardial phenotypes obtained both by RNA interference and in the *vin6* deficiency are primarily due to a reduced expression of Pericardin. In addition, the recovery (even partial) of EC11 immunoreactivity in rescued embryos reinforces the hypothesis that *prc* encodes the protein identified by EC11.

A more detailed analysis of the cardial phenotypes in silenced and *prc* loss-of-function (*vin6*) embryos is depicted in Fig. 7 to gain a better understanding of the function of Prc in morphogenesis of the heart epithelium. At the onset of dorsal closure (Fig. 7A), the alignment of cardial cells appears to be as regular, as in a wild-type embryo. As dorsal closure progresses, the epithelium becomes disorganized, as if the interaction with dorsal ectoderm has loosened and it eventually collapsed (Fig. 7B). A concomitant disorganization of dorsal muscles but not of the entire somatic mesoderm should be noted. This disorganization might result from an indirect effect of the disruptions in the attachment of the bilateral primordia of the dorsal vessel to dorsal ectodermal cells and does not necessarily imply a function of Prc in muscle morphogenesis. In rare occasions in which the mutant embryos completed the closure of their heart tubes, the correct organization of the cardial cells in two rows was often not respected and, in several positions, the shape of the cells was distorted and their polarity seemed incorrect (Fig. 7D). It should be noted that, in these cases, the cells have detached from the dorsal ectoderm, which could explain the loss of structure.

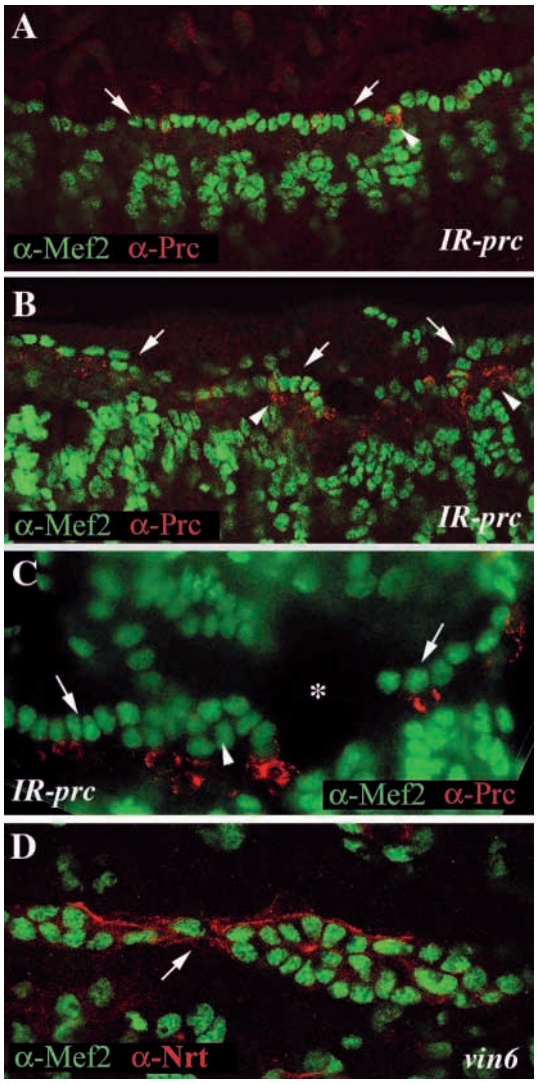

Fig. 7. Cardial phenotypes in *prc* silenced embryos and in *prc* loss-of-function embryos. (A,B) Staining with anti-Mef2 and anti-Prc of *prc* ds RNA silenced embryos. (A) At the onset of dorsal closure, the alignment of the cardioblasts appears normal (arrows). (B) When the dorsal closure progresses, the epithelium is disorganized (arrows) and clusters of cells are shown along the row of cardioblasts (arrowheads). (C) Stage 16 ds RNA silenced embryo double stained for Mef2 (green) and Prc (red). Owing to the low level of expression of Prc, the cardial epithelium (arrows) has collapsed and displays holes (asterisk) from which the cells are absent. Clusters of cardioblasts appear along the row of cardial cells (arrowhead). The red color for Prc has been artificially increased to become visible. (D) Stage 16 *vin6*-deficient embryo double stained for Mef2 (green) and Nrt (red). The polarity of the cardial cells in the epithelium seems to be affected (arrow). These cells appeared to be detached from the overlying ectoderm. In all views, anterior is leftwards and dorsal is upwards.

DISCUSSION

A novel protein, Pericardin, which is expressed in pericardial cells and is a component of the extracellular matrix, could mediate the crosstalk between the dorsal ectoderm and cardioblasts required to insure their coordinated movement during dorsal closure.

The homology of Prc with type IV collagen α-chains, including those of the two *Drosophila* collagens encoded by *Dcg1* (Cecchini et al., 1987) and *viking* (Yasothornsrikul et al., 1997) was primarily due to its high content of Gly, Gln and Pro residues. The typical $(Gly\text{-}X\text{-}Y)_n$ collagen repeat is, however, highly divergent and another atypical repeat is present in several copies. This observation challenged the classification of Prc as a type IV collagen, even though its primary structure could be divided into the three conventional collagen domains, its predicted secondary structure was compatible with a characteristic triple helical folding and it contained only two N-linked glycosylation sites (Vuorio and de Crombrugghe, 1990). We propose, therefore, to call Prc a type IV collagen-like extracellular matrix component.

As no specific mutants for Prc could be generated in spite of several unsuccessful attempts at creating P-element induced alleles, dsRNA interference of Prc expression (Kennerdell and Carthew, 2000) was attempted by using a prc-GAL4 driver that led to hypomorphic phenotypes. Prc expression was efficiently and sufficiently reduced to provoke alterations in the heart epithelium. However, it was not completely abolished and a small amount of protein might have already been synthesized,

because of the late activation of UAS-IR prc. In *vin6*-deficient embryos, which have totally lost Prc function, the alterations in the cardial epithelium were similar to those obtained by RNA interference. In addition, an architecture of the heart tube resembling that in a wild-type embryo could be almost fully rebuilt by expressing a *prc* minigene in *vin6*-deficient embryos. All these observations suggest that the lack of Prc synthesis was the primary cause of the defects.

Prc does not participate in the specification of the cardial cells precursors or in the mesenchyme-epithelium transition because it is synthesized after these two events take place. Furthermore, in *vin6*-deficient and in partially silenced *prc* RNAi embryos, the cardial epithelium appeared normal at the onset of dorsal closure, thus excluding a role of the protein in the acquisition of cell polarity. In places where Prc concentration had been locally diminished, the interaction between cardial epithelium and dorsal ectoderm might have been interrupted while it remained unchanged elsewhere, causing distorsions during dorsal ectoderm migration that led to breaks in the heart tube. The cardial and pericardial cells no longer affixed to the ectoderm fell apart and formed clusters on either side of the breaks. The function of Prc in maintaining the structural integrity of the heart tube and in coordinating the migration of the dorsal ectoderm and of the cardial epithelium might, however, not be the unique actor in these processes. Because, in the absence of that function, some cardial cells conserve their epithelial polarity and migrate in concert with the dorsal ectoderm, even in *vin6*-deficient embryos, additional partners whose functions could be partially redundant with Prc are likely to be present.

Drosophila mutants with a dorsal hole that are affected in dorsal closure, are also affected in the migration of the two rows of epithelial cardioblasts and pericardial cells. This has hampered the study, independently, of the two movements, and alteration in heart morphogenesis may be due indirectly to defects in the dorsal ectoderm. Mutants in the *puc* gene proceed to a complete dorsal closure even though the *puc*-expressing cells have acquired a more lateral cell fate (for a review, see Noselli, 1998). In *puc* mutants, we have shown that it was possible to uncouple dorsal closure from cardial epithelium migration: the two rows of cardial epithelial cells and the attached pericardial cells did not join at the dorsal midline and a cardia bifida phenotype was obtained. This defect in heart tube formation, resulting from a change in LE cells identity, may reflect defects in the morphogenetic signals that are normally required for the specific interaction between dorsal ectoderm and cardial epithelium.

The precise nature of these signals has not been elucidated, but extracellular matrix proteins, such as laminin or fibronectin, and their receptors, such as integrins, may be efficient partners in cell-substrate adhesion during dorsal closure. Expression of integrin molecules in flies is suggestive of their role in movements of tissues. For example, in *myospheroid* (*mys*) mutant embryos (the *mys* gene encodes one of the two integrin β subunits), the pericardial cells appear to dissociate, migrate randomly and be sparse (Stark et al., 1997). Likewise, in *scab* mutant embryos (*scab* encodes an integrin α subunit, which is expressed at the edge of the epidermis and in cells of the dorsal vessel) mislocalization of the pericardial cells and reduced numbers of these cells have been observed (Stark et al., 1997). Both mutants were identified as the result of a failure in dorsal closure, suggesting that the two integrin subunits are involved in that latter process as well. Laminin could be a ligand for these integrins although its late expression in the cardiac tube precludes a role in the migration of the cardial epithelium (Yarnitzky and Volk, 1995).

Pericardin localized expression on the basal surface of the cardioblasts and around the pericardial cells, in the extracellular matrix and in close proximity to the dorsal ectoderm, as well as the disorganization of the cardial epithelium when Pericardin activity was reduced or abolished, strongly favor the hypothesis that Prc participates in a link between the two layers of cells. Such an interaction seems necessary to maintain the structure of the bilateral heart epithelium and to coordinate its migration in concert with the dorsal ectoderm during dorsal closure. No tools are yet available to examine the role for Prc in the maintenance, once formed, of the structural integrity of the cardiac tube because the phenotypes associated to a loss of Prc function appear during the migration step before the tube is completed.

The early events in heart formation have been extremely well conserved in invertebrates and vertebrates. The heart origin can be traced back to two primordia located on either side of the vertical axis that marks the embryo center. As development progresses, the lateral wings of the precardial splanchnic mesoderm with the underlying endoderm fold inward ventrally to converge at the midline of the embryo and develop the centrally located single primitive heart tube. The yolk sac, which is contiguous with the underlying endoderm, is pulled from its lateral position to a ventral position. Several mutations in vertebrates, caused by a fundamental loss in ventral folding, seem to affect the migration of heart cells to the midline and cause two heart tubes to form resulting in a cardia bifida phenotype. In zebrafish, among the eight cardia bifida mutations that have been reported and grouped in three categories, based on the process they control, the *miles apart* gene, for example, seems to control the migration of the myocardial cells to the midline but not their differentiation (for a review, see Driever, 2000; Stainier, 2001). Likewise, mouse *Mesp1* mutant cells seem to be slower than wild-type cells at leaving the primitive streak and reaching the anterior-lateral regions of the embryo (Saga et al., 1999). Homozygous *Gata4*-null mice most notably lacked a primitive heart tube. The embryos developed splanchnic mesoderm that differentiated into primitive cardial myocytes. The two promyocardial primordia failed to migrate ventrally but, instead, remained lateral and generated two independent heart tubes. The overlying endoderm and somatic mesoderm never moved ventrally, so that the amnion remained dorsal and did not surround the embryo (Kuo et al., 1997; Molkentin et al., 1997). Wild-type endoderm abrogates the ventral developmental defects associated with GATA4 deficiency, including heart tube formation (Narita et al., 1997). These observations point towards a crucial role for GATA4 in regulating the rostral to caudal and lateral to ventral folding of the embryo that is needed for normal cardiac morphogenesis (Kuo et al., 1997).

All these examples underline the importance of a relationship between endoderm (or ectoderm in invertebrates) and mesoderm in the morphogenesis of the primitive linear heart tube. We propose that extracellular matrix components such as Pericardin serve, at least partially, as partners in the relationship.

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