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The role of Paraxial Protocadherin in selective adhesion and cell movements of the mesoderm during *Xenopus* gastrulation

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SUMMARY

Paraxial Protocadherin (PAPC) **encodes a transmembrane protein expressed initially in Spemann's organizer and then in paraxial mesoderm. Together with another member of the protocadherin family, *Axial Protocadherin (AXPC)*, it subdivides gastrulating mesoderm into paraxial and axial domains. PAPC has potent homotypic cell adhesion activity in cell dissociation and reaggregation assays. Gain- and loss-of-function microinjection studies indicate that PAPC plays an important role in the convergence and extension movements that drive *Xenopus* gastrulation. Thus, PAPC is not only an adhesion molecule but also a component of the machinery that drives gastrulation movements in *Xenopus*. PAPC may provide a link between regulatory genes in Spemann's organizer and the execution of cell behaviors during morphogenesis.**

Key words: Protocadherin, Gastrulation, Cell movement, Convergence, Extension, Cell adhesion, *Xenopus*

INTRODUCTION

One of the most influential experiments in amphibian embryology was that of Townes and Holtfreter (1955), in which cells were completely disaggregated from tissues at neurula stages and allowed to reaggregate. Initially all cells indiscriminately adhered to each other, but within as little as 2 hours they specifically sorted out according to germ layers and histotypes. This experiment led to the realization that selective cell adhesion played a central role in the formation of tissues and organs. Cells can adhere homotypically even before tissue differentiation takes place, as exemplified by studies in *Drosophila* imaginal discs (Garcia-Bellido, 1966) and *Xenopus* blastulae (Turner et al., 1989). Much effort has been invested in understanding the molecular nature of differential cell adhesion, and the cadherins have emerged as fundamental mediators of homotypic cell-cell interactions (Gumbiner, 1996; Kemler, 1993; Takeichi, 1991, 1995). Cadherins are transmembrane proteins with calcium-dependent cell adhesion properties. Classical cadherins are thought to have evolved from ancestral proteins similar to the protocadherins. Protocadherins differ from classical cadherins in several respects: they have more than five extracellular cadherin (EC) domains, contain signature amino acids in EC3 and EC5, and lack a β-catenin-binding site in the intracellular domain that is crucial for linking classical cadherins to the cytoskeleton (Suzuki, 1996). The morphogenetic role of protocadherins is largely unknown at present, but weak homotypic cell adhesion activity has been demonstrated in tissue culture (Obata et al., 1995; Sano et al., 1993), and, in the case of neural fold protocadherin, NFPC, strong adhesive activity was observed in *Xenopus* assays (Bradley et al., 1998).

Gastrulation is the process by which movements of cohesive cell sheets constituting the three germ layers – ectoderm, endoderm and mesoderm – generate the vertebrate body plan. The cell biological machinery that drives these morphogenetic (i.e., form-generating) movements is poorly understood. In another influential experiment in embryology, Spemann and Mangold (1924) showed that these complex morphogenetic movements are orchestrated by a particular region of the embryo, the dorsal blastopore lip. In an effort to isolate molecules expressed specifically in the dorsal lip, an extensive differential screen was carried out (Bouwmeester et al., 1996). Of the ten different dorsal-specific cDNAs isolated, nine encoded regulatory proteins, namely secreted or transcription factors, and have been described in previous publications (Bouwmeester et al., 1996; De Robertis et al., 1997; Leyns et al., 1997). The final gene encoded a structural transmembrane protein, Paraxial Protocadherin (PAPC), that is presented in this report.

PAPC has a dynamic pattern of expression in the paraxial mesoderm of the trunk, in regions that undergo convergence and extension movements during gastrulation. A second protocadherin gene, *AXPC*, expressed in the axial (notochordal) region of the mesoderm, in which *PAPC* is switched off, was also identified. Cell reaggregation assays showed that PAPC has potent cell adhesion activity in embryonic cells and that this activity was distinct from that of

AXPC. A dominant-negative secreted form of PAPC was able to block convergence and extension gastrulation movements and, in gain-of-function experiments, *PAPC* mRNA promoted elongation of animal cap explants treated with low doses of activin. The elongation caused by *PAPC* mRNA was accompanied by striking changes in cell morphology. The results suggest that PAPC is not merely an adhesion molecule, but also a component of the molecular machinery that drives convergence and extension movements of the paraxial mesoderm during gastrulation.

MATERIALS AND METHODS

Screening and plasmid construction

A full-length *PAPC* cDNA was subcloned into the *Eco*RI-*Xho*I sites of pCS2$^+$ (a gift of R. Rupp) to generate p*FL-PAPC*. A membrane-tethered form (p*M-PAPC*) was generated by PCR using pfu polymerase, with primers F5′-GCAGAATTCATGCTGCTTCTCTTCAGAGCC-3′ and R5′-AGCGCAGTCGACGTGTTGTTCAGGTACCTGCTT-3′ and cloned into the *Eco*RI-*Xho*I sites of pCS2$^+$. A dominant-negative secreted form (p*DN-PAPC*) was generated by amplifying the extracellular domain (amino acids 34-585, lacking the signal peptide) with primers 5′-TAATCCTCGAGCCCCCTGGCACTGTAATTGC-3′ and R5′-TACATCTAGACTACTCTTCTGCAGATGGTTGC-3′, and subcloned into the *Xho*I-*Xba*I sites of pCS2-chdSP/Flag (a kind gift from S. Piccolo). pCS2-chdSP/Flag contains the chordin signal peptide followed by a Flag epitope tag 5′ to the *Xho*I site. To clone *AXPC* cDNA, RT-PCR fragments of about 500 bp (Sano et al., 1993; Yamamoto et al., 1998) were identified as protocadherins by sequence comparison and used to screen a dorsal lip library. A partial *AXPC* cDNA of 2 kb contained the first 5 EC domains of *AXPC*, which were cloned into pCS2$^+$ to generate p*DN-AXPC*. To generate a membrane-tethered form of *AXPC* (p*M-AXPC*), a new *Bam*HI site was created at position 1360 by PCR based point mutation (pBS-*AXPC*/*Bam*), and the *Bam*HI fragment of p*M-PAPC* was replaced with that of pBS-*AXPC*/*Bam*. p*Xombi* was generated by PCR amplification of the *Xombi* open reading frame (Lustig et al., 1996) and subcloning the PCR product into the *Xho*I-*Xba*I sites of the pCS2$^+$ vector. *GFP-GAP43*, containing a palmitoylation signal (Moriyoshi et al., 1996) was kindly provided by Dr Paul Garrity and subcloned into the *Eco*RI site of pCS2$^+$.

RNA synthesis

Synthetic capped mRNA was produced using the Ambion Message Machine kit. To obtain sense mRNA, p*FL-PAPC*, p*M-PAPC*, p*DN-PAPC*, p*DN-AXPC*, p*M-AXPC*, p*Xombi*, p*GFP-GAP43* and pSP35-*cer* were digested with *Not*I and transcribed with SP6 RNA polymerase. pSP*Xnot-2* (Gont et al., 1996) was digested with *Sal*I, and transcribed with SP6 RNA polymerase. p*lacZ* and p*GFP.RN3* (Zernicka-Goetz et al., 1996) were transcribed with T3 RNA polymerase after linearization with *Pst*I and *Sfi*I, respectively.

Embryo manipulations

Microinjection, in situ hybridizations and RT-PCR were carried out as described (Bouwmeester et al., 1996). For elongation assays, animal cap explants were prepared from stage 8 embryos and treated with recombinant human activin protein (a kind gift of Genentech) at a concentration of 1 ng/ml or 10 ng/ml in low Ca^{2+}/Mg^{2+} medium (Piccolo et al., 1996) containing 0.2% bovine serum albumin (BSA). After 3 hours of incubation, the explants were transferred to culture dishes with 0.4× MMR (Peng, 1991) and cultured overnight.

Dissociation and reaggregation assay

Embryos were injected with either 100 pg *GFP/RN3* mRNA or 8 ng rhodamine, and with control (*prolactin*) or protocadherin (*PAPC* or *AXPC*) mRNA into all four animal blastomeres at the 8-cell stage. As the rhodamine-dextran was not RNAse-free, it was injected separately 30 minutes before the test mRNA. At stage 9 (2-3 hours before gastrulation) animal cap explants were excised and transferred to CMFM medium, in which cells of the inner (sensorial) cell layer detached in about 10 minutes. The outer layer, which is more pigmented, did not dissociate under these conditions and was removed. Dissociated inner layer cells from three injected animal caps were thoroughly mixed with those from five uninjected caps and transferred into another dish containing 0.7× MMR, creating a heap of cells in the center of the dish. Experiments of similar design have been previously described in *Xenopus* (Detrick et al., 1990).

Open-faced animal cap explants

Animal caps were excised from stage 8 embryos and incubated for 90 minutes in recombinant activin protein (1 ng/ml or 10 ng/ml)/0.2% BSA /VLCMR (Piccolo et al., 1996) keeping the explants open and flat. The activin-treated explants were mounted on microscope slides in 0.4× MMR with restraining coverslips fixed into position by applying high-vacuum Silicon grease (Beckman) around the rim (Keller, 1991). The slides were kept immersed in 0.4× MMR to prevent drying and taken out for confocal microscopy (λ=510 nm) every 60 minutes.

RESULTS

Isolation of *Paraxial Protocadherin*

The longest *PAPC* cDNA clone was 3649 base pairs in length and was sequenced on both strands (GenBank accession number AFO42192). The 979 amino acid open reading frame contained a hydrophobic amino-terminal signal peptide and a predicted internal transmembrane domain (Fig. 1A). The extracellular domain contained six cadherin repeats of about 110 amino acids each, with conserved amino acids (indicated by asterisks in Fig. 1A), including four cysteines, that are diagnostic of protocadherin family members (Suzuki, 1996; our unpublished observations). The intracellular domain was 265 amino acids in length and had no significant homologies to other proteins in the database, including the published cadherin and protocadherin sequences. Like other protocadherins the PAPC intracellular domain lacks a β-catenin-binding site, and the only sequence similarity detected was with a zebrafish homologue of PAPC (Yamamoto et al., 1998) which has a stretch of 25 amino acids of unknown function that was 84% identical (indicated in bold in Fig. 1A).

The extracellular repeats of PAPC were most similar to *Drosophila fat*, a tumor suppressor gene containing 34 cadherin repeats (Mahoney et al., 1991); the highest identity, 40%, was shared between EC5 of PAPC and EC20 of fat. The entire PAPC protein shared 31% identity with either human protocadherin 1 or 2 (Sano et al., 1993). We conclude that *PAPC* encodes a typical member of the protocadherin family.

PAPC expression and morphogenesis

By whole-mount in situ hybridization *PAPC* mRNA is first detected at the late blastula stage about 1 hour before the start of gastrulation (stage 9.5, all stages according to Nieuwkoop and Faber, 1994). At this stage, expression is found in the dorsal marginal zone (Fig. 2A), and at early gastrula (stage 10.5) it expands to about 180º of the marginal zone (Fig. 2B). *PAPC* was isolated because of this initial dorsal-specific expression

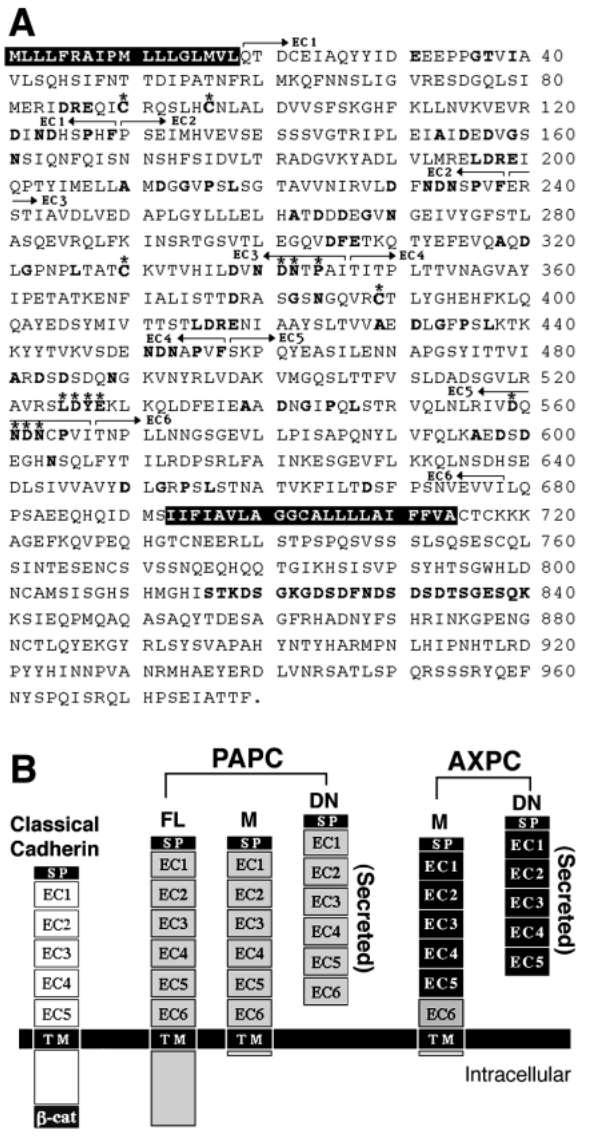

Fig. 1. *Xenopus PAPC* encodes a transmembrane protein of the protocadherin family. (A) Deduced amino acid sequence of *Xenopus* PAPC protein. The signal peptide and the transmembrane domain are boxed in black. The six extracellular cadherin repeats (EC1-6) are indicated with arrows and the conserved cadherin motif residues are in bold face. The protocadherin-specific cysteines (our observations) and the protocadherin signature amino acids (Suzuki, 1996) in EC3 and EC5 are marked by asterisks. Within the intracellular domain, a segment of 25 amino acids conserved with zebrafish *PAPC* is indicated in bold. (GenBank accession number AFO42192) (B) Schematic diagrams of PAPC and AXPC constructs used in this study.

(Bouwmeester et al., 1996). As gastrulation proceeds, *PAPC* expression extends to the entire mesodermal mantle including the ventral-most mesoderm and is switched off in the dorsal midline as the notochordal anlage starts to form (Fig. 2C). As the mesodermal mantle completes its involution at stage 13, a sharp anterior border of *PAPC* mRNA separates head and trunk mesoderm (Fig. 2D). Histological sections at this stage (Fig. 2G) confirm that *PAPC* is expressed exclusively in the mesodermal layer, except for the notochord (Fig. 2G).

By stage 14, *PAPC* transcripts become more intense in two stripes prefiguring the appearance of somites (Fig. 2E). Once somitogenesis begins, *PAPC* expression is turned off in mature somites and new stripes appear more posteriorly (Fig. 2F). This cycle continues until completion of somite formation, with *PAPC* expression disappearing as the last somites form at the tip of the tail (stage 40, data not shown). During somitogenesis *PAPC* is expressed throughout the unsegmented paraxial mesoderm, a region also called the segmental plate (Fig. 2F). Histological sections show that the two most intense bands of *PAPC* expression are located within the segmental plate (sp); weaker bands of expression are found in the anterior boundaries of the forming somite (fs) budding off from the segmental plate and in the last (newly completed) somite, as indicated in Fig. 2H. The two strong stripes of *PAPC* expression are located in the region corresponding to the somitomeres that precede overt somitogenesis (Jen et al., 1997; Meier, 1979). One noteworthy aspect of this expression is that the stripes can extend laterally well beyond the external boundary of the presomitic mesoderm (arrowheads in Fig. 2F). Similar wide stripes that extend into the lateral plate mesoderm, which is supposedly unsegmented, have been previously noted in the case of *X-Delta-2* (Jen et al., 1997). We conclude that *PAPC* is dynamically expressed in paraxial mesoderm undergoing morphogenesis during gastrulation.

Axial Protocadherin in notochord

Since expression of *PAPC* is downregulated in the future notochord at midgastrula (Fig. 2C), we asked whether another member of the protocadherin family might be expressed in the axial midline. To investigate this, the notochord precursor region of about 100 gastrulae (stage 12) was dissected and RNA prepared. Protocadherin fragments were isolated using the PCR strategy of Sano et al. (1993). A partial *axial protocadherin (AXPC)* cDNA clone spanning EC1 through 5 was isolated (GenBank accession number AF053469). The predicted amino acid sequence of this AXPC clone was 33% identical to PAPC. By in situ hybridization, localized expression of *AXPC* was not detectable during early gastrula stages, but from stage 13 onwards was found specifically in the notochord (Fig. 2I-K). These results show that the early mesodermal mantle can be subdivided into two complementary regions that express specific protocadherins, *AXPC* and *PAPC*. Despite several attempts, we were unable to isolate a longer *AXPC* cDNA. We therefore generated a hybrid protein consisting of EC1 through 5 of AXPC fused in frame with EC6 and the transmembrane domain of PAPC (Fig. 1B). This membrane-tethered construct, *M-AXPC*, had cell adhesion activity and proved invaluable as a specificity control in functional assays (see below).

PAPC mediates cell sorting

To investigate whether PAPC has cell adhesion activity, a dissociation and reaggregation assay with animal cap cells was used (Fig. 3A, see Materials and Methods for experimental details). After placing in Ca^{2+}, Mg^{2+}-containing solution, animal cap cells reaggregated forming large spherical aggregates; cells co-injected with control *prolactin* mRNA and a GFP lineage tracer were uniformly interspersed, whereas cells injected with full-length *PAPC* (*FL-PAPC*) mRNA sorted out into multiple clumps after incubations overnight (compare

Fig. 3B and C). Formation of these patches of sorted cells was first detectable 1-3 hours after aggregation (data not shown). When the aggregates were cut in halves, it became evident that the *FL-PAPC*-injected cells tended to concentrate in the center of the aggregate (Fig. 3D).

As additional controls, we constructed carboxy-terminal deletions, generating membrane-tethered forms of *PAPC* (*M-PAPC*) and *M-AXPC* truncated 17 amino acids after the transmembrane domain (Fig. 1B). Similar deletions of the intracellular domain of classical cadherins lack adhesion activity in cell culture (Takeichi, 1991) or are even inhibitors of cadherin adhesion (Lee and Gumbiner, 1995). Surprisingly, we found that *M-AXPC* and *M-PAPC* mRNAs were active in reaggregation assays (Fig. 3E,F). Importantly, strong cell adhesion by M-PAPC could be obtained with 7-fold less *M-PAPC* mRNA in molar terms than the minimal amount of mRNA required for *FL-PAPC* (Fig. 3F). This suggests that the intracellular domain of PAPC is not only not required for, but may even have an inhibitory effect on cell-cell adhesion. To test whether PAPC is a homotypic cell adhesion molecule, aggregates were prepared by mixing cells injected with *M-PAPC* and rhodamine, with cells injected with *M-AXPC* and *GFP*, and with uninjected cells (n=12 aggregates). The three cell types sorted out from each other (Fig. 3F-H). When such aggregates were bisected, M-PAPC-expressing cells were found in deep layers, whereas M-AXPC cells were found in the periphery (Fig. 3I-K). We conclude that PAPC and AXPC, two cell adhesion molecules expressed in complementary domains of the mesoderm, can mediate mutually exclusive cell adhesion via their extracellular domains.

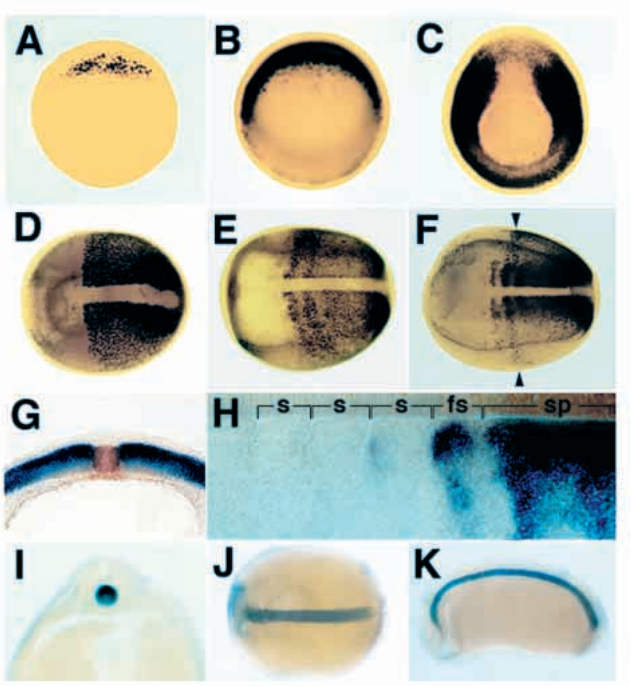

Fig. 2. *PAPC* is expressed during mesodermal mantle morphogenesis. (A) *PAPC* expression in the dorsal marginal zone at late blastula (stage 9½). (B) Expression expands around the marginal zone at early gastrula (stage 10½). (C) Repression of *PAPC* expression in the axial mesoderm prefiguring the segregation between axial and paraxial mesoderm (stage 11½). (D) A sharp anterior border separates head and trunk mesoderm (stage 13). (E) At early neurula (stage 14), stripes of *PAPC* expression prefigure the future somites. (F) As the first mature somite is segmented (stage 17), the anterior stripe disappears and new posterior stripes appear. *PAPC* expression is also found in lateral plate mesoderm and in the forming otic vesicle (not shown). (G) Transverse section of stage 13 embryo stained for *PAPC* in blue and *chordin* in brown by double in situ hybridization; note *PAPC* expression exclusively in paraxial mesoderm. (H) *PAPC* stripes during somite segmentation; the most anterior *PAPC* stripe is in the mature somite (s), the second stripe marks the forming somite (fs) and the more intense two posterior stripes are located in the segmental plate (sp). (I) Transverse section of stage 18 embryo showing *AXPC* expression exclusively in the notochord. (J) Dorsal view of *AXPC* expression in the notochord (stage 14), anterior to the left. (K) Lateral view of *AXPC* expression in the notochord (stage 22). At later tailbud stages *AXPC* was transiently expressed in developing heart and pronephros (data not shown).

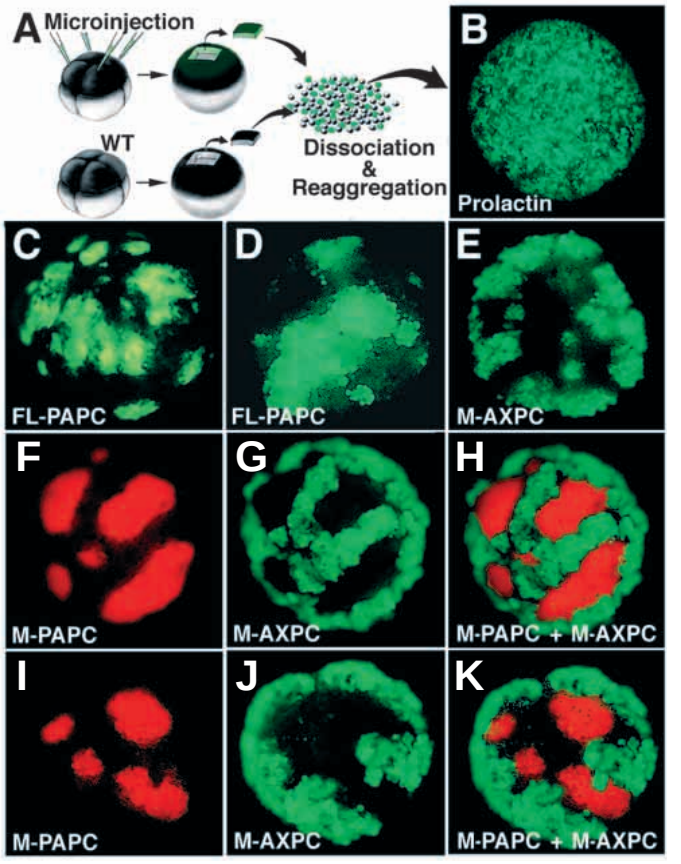

Fig. 3. PAPC causes homotypic cell sorting in reaggregation experiments. (A) Experimental design of cell sorting assays, which are described in more detail in Materials and Methods. (B) Complete mixing between cells injected with *prolactin* control mRNA (800 pg per blastomere) and *GFP* mRNA (100 pg) and uninjected cells (12/12 aggregates). (C) *FL-PAPC* mRNA (800 pg)-injected cells (labeled with GFP) sort out from uninjected cells (20/21). (D) Bisection of the *FL-PAPC* aggregates showing that *FL-PAPC*-injected cells tend to migrate to the center of the aggregates (10/12). (E) Bisection of *M-AXPC* (100 pg) aggregates showing cohesive patches in the periphery of the aggregates (11/11). (F-K) Aggregates containing *M-PAPC* (100 pg, in red), *M-AXPC* (green) and uninjected cells demonstrating homotypic cell sorting (12/12). (F-H) External views; (I-K) bisected aggregates.

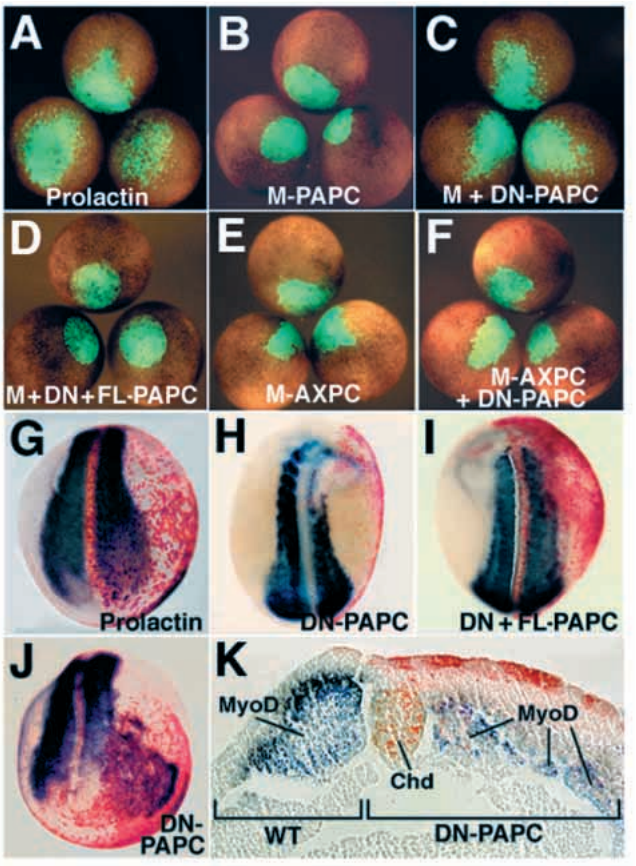

Fig. 4. Dominant-negative PAPC affects paraxial mesoderm morphogenesis. (A) Control *prolactin* mRNA (200 pg) injection into an animal blastomere at the 32-cell stage showing cell mingling at stage 12. (B) *M-PAPC* (100 pg) injection generated a cohesive patch with a sharp clonal boundary (54/54). (C) *DN-PAPC* (1.6 ng) interfered with the formation of the cohesive patch induced by 100 pg *M-PAPC* (38/43). (D) Additional *FL-PAPC* mRNA (400 pg) rescued formation of cohesive patches (48/51), indicating that the dominant-negative effects are reversible. (E) *M-AXPC* (200 pg) generated cohesive patches with sharp clonal boundaries (36/38). (F) Coinjection of *DN-PAPC* mRNA (1.6 ng) did not interfere with *AXPC* patches (3/32); coinjection of 1.6 ng of *DN-AXPC* dispersed *M-AXPC* patches (31/38, not shown). (G) Unilateral injection of control *prolactin* mRNA (1.6 ng) and *lacZ* tracer (100 pg) at the 2-cell stage does not affect paraxial mesoderm marked by *MyoD* (*n*=44). (H) *DN-PAPC* (1.6 ng) caused defects in anterior somites on the injected side at stage 20 (105/151). (I) Rescue of paraxial mesoderm morphology by co-injection of *FL-PAPC* (600 pg) together with *DN-PAPC* (42/47). (J) When injected posteriorly, *DN-PAPC* (1.6 ng) caused defects in midline convergence of paraxial mesoderm on the injected side. (K) Transverse section showing effect of *DN-PAPC* on *MyoD* convergence, but not on notochord tissue marked by *chordin*. In G-K, *MyoD* is marked in blue, *lacZ* in red and *chordin* in brown.

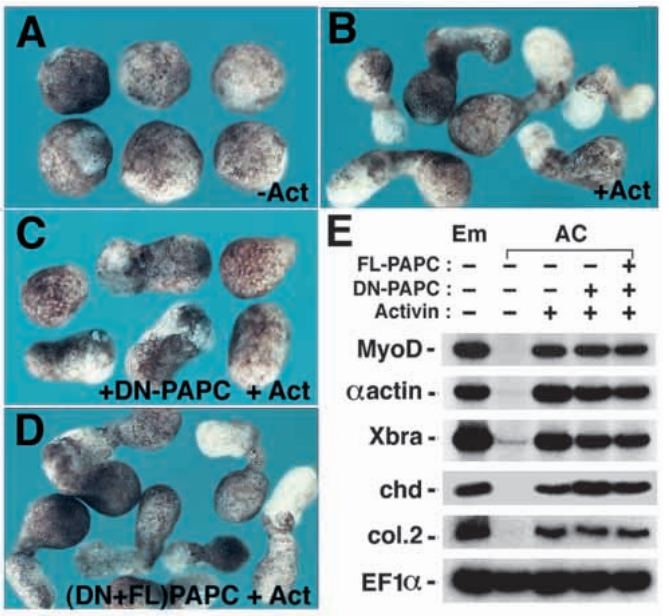

Fig. 5. *DN-PAPC* mRNA inhibits animal cap extension by activin. (A) Animal caps without any treatment (*n*=32, three independent experiments). (B) High Activin (10 ng/ml) induced elongation of the explants (33/35). (C) *DN-PAPC* mRNA (1.5 ng per blastomere at the 4-cell stage) injection interfered with explant elongation (31/35). (D) Coinjection of *FL-PAPC* mRNA (400 pg per blastomere) together with *DN-PAPC* mRNA (1.5 ng) rescued the elongation of animal caps (36/38). (E) RT-PCR of RNA extracted from these caps showed no significant effect of *PAPC* mRNA injection on the expression of mesodermal markers such as *Xbra*, *α–actin*, *MyoD*, *chordin* and *collagen 2*; *EF1(*serves as loading control.

Dominant-negative PAPC interferes with gastrulation movements

Based on the data indicating that the extracellular domains are responsible for homotypic adhesion, overexpression of a secreted form of the PAPC extracellular region would be predicted to dominantly interfere with the activity of endogenous PAPC molecules by binding to them in the extracellular space. In the case of classical cadherins, stable secreted forms have been generated and found to have homotypic adhesive activity (Brieher et al., 1996). Therefore, a construct encoding a secreted form of PAPC was generated by truncating the cDNA seven amino acids before the transmembrane domain and designated *DN-PAPC* (Fig. 1B). The activity of DN-PAPC was tested in an in vivo cell adhesion assay. Microinjection of control (*prolactin*) mRNA together with GFP lineage tracer into a single animal blastomere at the 32-cell stage resulted in extensive cell mingling by midgastrula (Fig. 4A). When 100 pg of *M-PAPC* mRNA was injected, cohesive patches formed, which could be dispersed by coinjection of 1.6 ng of *DN-PAPC* mRNA (Fig. 4B,C). This effect of *DN-PAPC* could be reversed by the addition of 400 pg of *FL-PAPC*, which restored formation of cell adhesion patches (Fig. 4D). The dominant-negative effect was specific, because *DN-PAPC* was unable to disperse patches of adhesion generated by the related molecule *M-AXPC* (Fig. 4E,F). In similar conditions, 1.6 ng of *DN-AXPC* mRNA prevented cell adhesion by *M-AXPC* (data not shown).

To investigate the effects of *DN-PAPC* microinjection in gastrulating mesoderm, embryos were coinjected into one blastomere at the 2-cell stage with 1.6 ng of *DN-PAPC* mRNA and *lacZ* lineage tracer. On the injected side (marked by red-gal staining), the paraxial mesoderm extended less anteriorly, resulting in loss or reduction of anterior somites in 70% of the injected embryos (*n*=151) as visualized with the *MyoD* marker

(Fig. 4H). A loss of anterior somites caused by defective convergence movements of paraxial mesoderm has been previously noted in a zebrafish mutant called *spadetail* (Kimmel et al., 1989). Paraxial mesoderm morphology was not affected by microinjection of 1.6 ng of *prolactin* (Fig. 4G, *n*=44) or *DN-AXPC* mRNA (not shown, *n*=42), indicating that the effects of DN-PAPC were specific. The somite defects could be rescued by coinjection of 600 pg of *FL-PAPC* (Fig. 4I, 95%, *n*=47), as expected from a dose-dependent dominant-negative effect. When the microinjected *DN-PAPC* mRNA was delivered into posterior mesoderm (by injecting into the ventral-marginal region of 4-cell embryos), *MyoD*-positive cells failed to converge to the dorsal midline, extending into more lateral mesodermal regions (Fig. 4J,K). These effects of *DN-PAPC* are specific for paraxial mesoderm, since the axial mesoderm marked by double staining with a *chordin* probe had normal morphology (Fig. 4G,J,K). We conclude that the effects of a secreted dominant negative form of PAPC are consistent with a reduction of convergence and extension movements in paraxial mesoderm during gastrulation.

DN-PAPC interferes with explant elongation

In *Xenopus*, mesodermal convergence and extension movements are a primary driving force of gastrulation (Keller et al., 1992). To test further the effect of *DN-PAPC* on gastrulation movements, we made use of a standard *Xenopus* assay in which convergence and extension movements are induced by treating animal caps with activin (Symes and Smith, 1987). Addition of high doses of activin protein (10 ng/ml) caused elongation of the explants (Fig. 5A,B), and injection of *DN-PAPC* mRNA reduced significantly this elongation (Fig. 5C, three independent experiments, *n*=35). Co-injection of *FL-PAPC* (*n*=38) rescued the inhibitory effect of *DN-PAPC* (Fig. 5D), as expected for a dominant-negative effect. Molecular analyses of the same samples showed that mesodermal differentiation (determined by *MyoD*, *α-actin* and *Xbra* expression) remained unchanged (Fig. 5E). The mesodermal markers *collagen 2* and *chordin* (Su et al., 1991; Piccolo et al., 1996), which are expressed in notochord, were not inhibited by *DN-PAPC* (Fig. 5E), indicating that the lack of elongation is not due to an inhibition of notochord differentiation. The presence of a notochord is not required for elongation of paraxial mesoderm in a number of experimental situations in *Xenopus* (Piccolo et al., 1996; Steinbeisser et al., 1993), nor in the zebrafish *floating-head* mutation that lacks a notochord (Talbot et al., 1995). Thus, the block of elongation by *DN-PAPC* is most likely due to inhibition of convergence and extension of paraxial mesoderm in the explants. As an additional control, *DN-AXPC* mRNA failed to block explant elongation (data not shown). We conclude from these results that endogenous PAPC is required for convergence-extension movements in explants but not for histotypic specification of mesoderm.

PAPC promotes convergence-extension movements and cell shape changes

During the course of experiments in which *DN-PAPC* effects were rescued with *FL-PAPC* (as in Fig. 5D) we noted that some explants elongated more than the controls treated with activin alone (data not shown). To test more systematically whether the PAPC transmembrane protein was sufficient to promote convergence and extension movements in a gain-of-function situation, we resorted to *Xenopus* explants treated with low doses of activin protein (1 ng/ml). In these conditions, animal

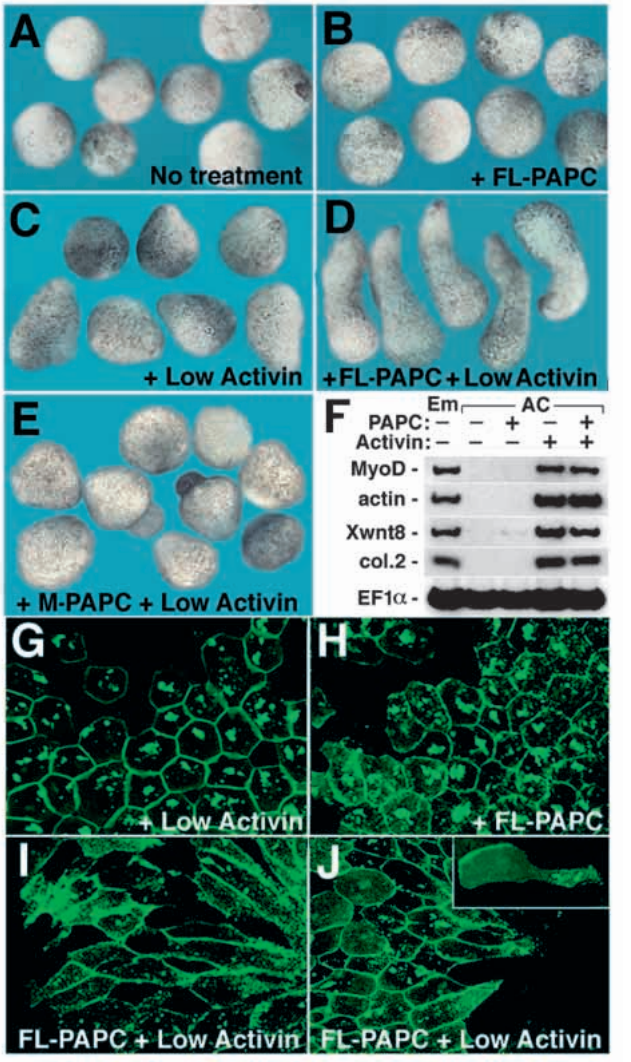

Fig. 6. *FL-PAPC* can promote animal cap elongation and changes in cell morphology. Embryos were injected with or without *PAPC* in each animal blastomere at the 8-cell stage and animal cap explants were treated with low activin at stage 8 (1 ng/ml). (A) Control animal cap explants with no activin treatment (26/26). (B) Microinjection of *FL-PAPC* mRNA (400 pg) without activin treatment (18/18). (C) Low activin (1 ng/ml) treatment was not sufficient to induce explant elongation (21/24). (D) *FL-PAPC* mRNA (400 pg) promoted elongation of animal caps treated with low activin (22/27, three independent experiments). (E) Microinjection of *M-PAPC* (400 pg) did not promote explant elongation (18/18), suggesting a requirement for the intracellular domain. (F) RT-PCR showed no significant effect of *FL-PAPC* mRNA on the expression of the mesodermal markers, *MyoD*, *α-actin*, *Xwnt8* and *collagen 2*. (G-J) Confocal microscopy of open-faced animal caps (see Materials and Methods). (G) Low activin treatment as in C did not induce changes in cell polarity (41/43). (H) *FL-PAPC* mRNA (400 pg) injection as in B had no effect on ectodermal cell morphology (10/10). (I) *FL-PAPC* mRNA (400 pg) with low activin treatment, as in D, caused cells to elongate into fusiform or (J) monopolar cells (34/51) at stage 12 (the inset shows a particularly asymmetric cell with membrane vesicles within the thin end).

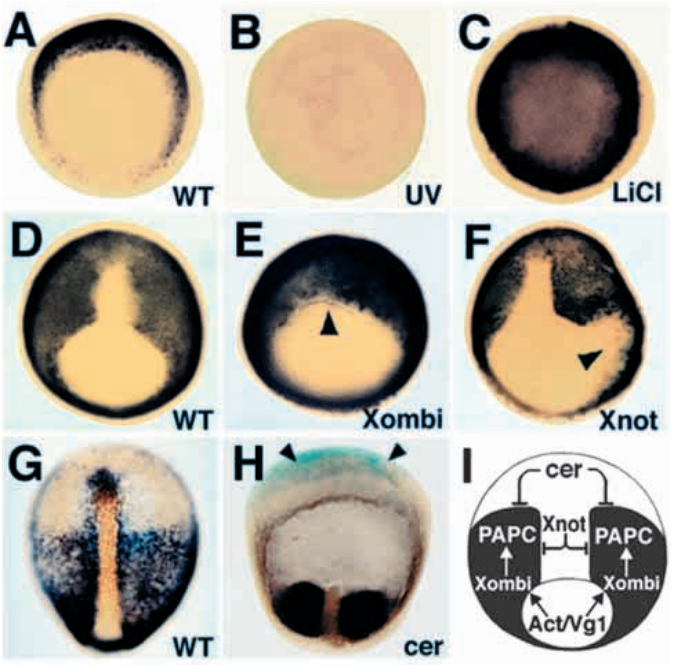

Fig. 7. Multiple regulatory steps control *PAPC* expression. (A) Wild-type *PAPC* expression at stage 10.5 in dorsal mesoderm. (B) Ventralizing UV treatment eliminates early dorsal expression. (C) Dorsalizing LiCl treatment expands *PAPC* expression around the marginal zone. Thus, initial *PAPC* expression is activated by factors from Spemann's organizer. (D) Wild-type *PAPC* expression during midgastrula (stage 11.5). (E) Dorsal injection of *Xombi* mRNA (50 pg) at 32-cell stage induces ectopic *PAPC* expression in the dorsal midline (arrowhead) at stage 11.5. (F) Lateral injection of *Xnot-2* mRNA (100 pg) induces ectopic repression of *PAPC* (arrowhead) suggesting that *Xnot-2* may be a upstream repressor of *PAPC* in the dorsal midline. (G) Wild-type *PAPC* expression (blue) and *chordin* (brown) at stage 13. (H) Microinjection of *Xcer* mRNA (50 pg) coinjected with *lacZ* as lineage tracer (arrowheads) into a vegetal site at the 4-cell stage displaces posteriorly the anterior border of *PAPC* expression. (I) Model summarizing spatial regulation patterns suggested by the mRNA injection studies.

cap explants were induced to form mesoderm but did not elongate (Fig. 6A-C). However, when injected with *FL-PAPC* mRNA and treated with low activin, the explants elongated (Fig. 6D). The amount of *FL-PAPC* mRNA required for this effect was 400 pg per animal blastomere at the 8-cell stage; this amount was lower than that required for the formation of strong adhesive patches by *FL-PAPC* (800 pg) in whole embryos. The effects of *FL-PAPC* are dose-sensitive and all mRNA preparations were titrated in injection assays before use. *FL-PAPC* mRNA promoted explant elongation without changing the expression of mesodermal markers such as *MyoD*, *α-actin*, *Xwnt-8* and *collagen 2* (Fig. 6F). This activity of FL-PAPC requires the intracellular domain, as indicated by the fact that *M-PAPC* mRNA was unable to elicit explant elongation in the same conditions (Fig. 6E).

Convergence and extension movements in *Xenopus* are characterized by changes in cell morphology (Keller et al., 1992). To investigate the effect of PAPC on cell shape, four animal blastomeres were injected at the 8-cell stage with *FL-PAPC* mRNA. At midblastula animal caps were excised, treated with 1 ng/ml activin for 90 minutes and then mounted under a coverslip as open-faced explants (Keller, 1991). To demarcate cell outlines, one blastomere was injected at the 32-cell stage with mRNA encoding GFP fused to the 20 amino acid palmitoylation signal of the neuronal protein neuromodulin/GAP43 which targets the fusion protein to the *trans*-Golgi and plasma membrane compartments (Liu et al., 1994). This GFP-GAP43 fusion has proven to be a useful membrane marker in axon guidance studies (Moriyoshi et al., 1996) and provides a previously unavailable marker for the polarity of gastrulating mesodermal cells in *Xenopus* (see below).

Fluorescence microscopy of the explants did not reveal any morphological alterations until midgastrula. At this time, within a period of about 1 hour, dramatic changes in cell morphology were observed. Explants that received either *PAPC* mRNA or activin alone remained as polyhedral cells containing a prominent organelle, presumably the *trans*-Golgi (Fig. 6G,H). Explants injected with *PAPC* mRNA and treated with low activin contained groups of parallel elongated cells in which the organelle became dispersed into multiple vesicles. The cells adopted an elongated shape that was sometimes fusiform or bipolar (Fig. 6I) but more commonly had a thick end and a thin end (Fig. 6J). In this population of monopolar cells, membrane vesicles were usually seen within the thin end (Fig. 6J, see inset).

Changes in cell shape were found in 68% (n=51, three independent experiments) of the explants that received both *FL-PAPC* and low activin. The open-faced explants themselves did not elongate, presumably due to the restraining force of the coverslip, but elongation of sibling animal caps explants in culture took place concomitantly with the cell shape changes observed. Since notochord patches occurred only rarely in elongated explants treated as in Fig. 6D (determined by MZ15 antibody staining, data not shown), it seems likely that the cell shape changes seen in Fig. 6I and J mimic those of gastrulating paraxial mesoderm rather than those of notochordal precursors. In conclusion, these gain-of-function studies show that injection of *FL-PAPC* mRNA can promote elongation of explants and cell shape changes reminiscent of gastrulating mesoderm. The results suggest that PAPC, in addition to its cell adhesion properties, can promote cell movements.

Regulation of *PAPC* expression

In *Xenopus* there is a wealth of information concerning regulatory factors in Spemann's organizer. At some point, however, these regulatory mechanisms must start building an embryo composed of three germ layers. Since PAPC appears to be involved in the execution of mesodermal morphogenesis, we carried out tests of the relationship between its expression pattern and upstream regulatory mechanisms. *PAPC* is expressed in dorsal mesoderm during early gastrulation. At this stage, two well-known treatments, UV and LiCl, can decrease or increase the amount of Spemann organizer tissue (Kao and Elinson, 1988). As seen in Fig. 7A-C, *PAPC* early expression behaves as that of a typical Spemann organizer gene.

By midgastrula, *PAPC* expression is found in the mesodermal mantle but is repressed in the future notochordal region (Fig. 7D). The PAPC pattern of expression closely parallels that of a T-box transcription factor independently isolated by four research groups and variously designated as

Xombi, *VegT*, *Antipodean* or *Brat* (Lustig et al., 1996; Zhang and King, 1996; Stennard et al., 1996; Horb and Thomsen, 1997). Microinjection of *Xombi/VegT* mRNA caused ectopic *PAPC* expression in the dorsal midline (Fig. 7E). The repression in the notochord parallels the expression of *Xnot*, a gene that promotes notochord formation (Gont et al., 1996), and injection of *Xnot* mRNA repressed *PAPC* expression (Fig. 7F). *Cerberus* encodes a secreted factor expressed in anterior endomesoderm that promotes head formation (Bouwmeester et al., 1996); injection of *cerberus* mRNA (together with *lacZ* lineage tracer) led to the reduction of *PAPC* expression in the trunk region (compare Fig. 7G,H). Taken together, these gain-of-function experiments suggest that multiple regulatory pathways control the spatial pattern of expression of the *PAPC* structural gene in the mesodermal mantle of the trunk (Fig. 7I).

DISCUSSION

PAPC and *AXPC* subdivide gastrulating mesoderm

Despite large efforts expended, the molecular mechanisms that drive morphogenetic movements during gastrulation remain largely unknown. *PAPC* encodes a transmembrane protein of the protocadherin family that is expressed at the right time and place to participate in gastrulation movements of the mesodermal layer of the trunk. *PAPC* is initially expressed in the entire mesodermal mantle and subsequently switched off in the prospective notochordal region. A second protocadherin gene, *AXPC*, is expressed in a complementary pattern in axial mesoderm. Thus, gastrulating mesoderm starts as a uniform cell layer and is then subdivided into two regions, axial and paraxial, by the expression of two protocadherins. In cell dissociation and reaggregation assays, PAPC was shown to have potent cell adhesion activity, as in the Townes and Holtfreter (1955) experiment. This adhesion is homotypic, for cells expressing extracellular domains of AXPC on their surface selectively sort out from PAPC-expressing cells (Fig. 3F-K). The homotypic cell adhesion properties and the complementary expression patterns of *PAPC* and *AXPC* suggest they could play an important role in maintaining the mesoderm as a cohesive layer during morphogenetic movements and in subdividing trunk mesoderm into axial and paraxial domains.

PAPC and gastrulation movements

The existence of a transmembrane molecule providing homotypic cell-cell adhesion between cells in gastrulating mesoderm, although previously unreported, is not in itself surprising (Takeichi, 1995; Townes and Holtfreter, 1955). What makes PAPC unique is its unexpected ability to promote cell movements in addition to providing cell adhesion.

The expression of *PAPC* sharply demarcates the region of trunk mesoderm that undergoes convergence and extension movements (Fig. 2D) from the region of head mesoderm that undergoes crawling-migration movements in the anterior direction (Keller et al., 1992). This spatial distribution led us to explore whether *PAPC* might participate in convergence and extension movements. A dominant negative construct of *PAPC* consisting of a secreted form of the extracellular domain was generated and shown to interfere specifically with PAPC-, but not AXPC-, mediated cell adhesion. Microinjected *DN-PAPC* mRNA interfered with mesoderm gastrulation, leading to loss of anterior somites and defective convergence towards the midline in posterior paraxial mesoderm in *Xenopus* embryos (Fig. 4). In animal cap explants, the convergence and extension movements of gastrulation can be mimicked by treatment with high doses of activin that cause elongation (Symes and Smith, 1987). In a loss-of-function situation, injection of *DN-PAPC* mRNA was able to block this elongation without affecting histotype differentiation, in particular notochordal markers (Fig. 5). Presumably multiple components are required for a complex cellular behavior such as explant elongation, for cells must interact with the extracellular matrix as well as with the surfaces of neighboring cells. The best indication that PAPC is an actual part of the cell biological machinery that drives gastrulation movements comes from gain-of-function experiments. At low activin concentrations, animal caps do not elongate but, remarkably, injected full-length *PAPC* mRNA was able to promote convergence and extension of these explants starting at the midgastrula stage. This activity of *PAPC* mRNA did not affect histotypic differentiation and required the presence of the intracellular domain (Fig. 6A-F).

In addition, striking changes in cell shape were observed concomitantly with the start of explant elongation at midgastrula. Cells treated with low activin and *FL-PAPC* mRNA changed from polyhedral to elongated cells (Fig. 6G-J). In some cases, the cells adopt a fusiform morphology reminiscent of the parallel arrays of bipolar cells undergoing mediolateral intercalation behavior (Shih and Keller, 1992). In most cases, however, cells were asymmetric and monopolar. Polarity could be assessed by the use of a GFP-GAP43 lineage tracer carrying a palmitoylation signal that marks the *trans*-Golgi compartment (Liu et al., 1994). Membrane vesicles were usually observed in the thin end of monopolar cells. Although these elongated cells appear to display only part of the phenotypes observed in the bipolar intercalating cells seen in embryos (Shih and Keller, 1992), the observation of polar vesicles may provide a useful tool to study how directional cell movement takes place during normal *Xenopus* gastrulation. Indeed, although bipolar cells appear symmetric during convergence and extension, their movements are directional as cells converge in the direction of the dorsal midline, leading to cell intercalation (Keller et al., 1992; Shih and Keller, 1992). The GFP-GAP43 lineage tracer may now allow the analysis of cell polarity in living gastrulating *Xenopus* cells.

Protocadherins and classical cadherins

The mechanism by which PAPC, a cell adhesion molecule, might promote cell movement is intriguing. One possible explanation may lie in the mechanistic differences noted between the function of the intracellular domains of PAPC and cadherins. In classical cadherins, the intracellular domain provides an anchorage to the cytoskeleton via β-catenin, which is required for cell adhesion (Kemler, 1993; Takeichi, 1995), whereas, in PAPC, deletion of most of the intracellular domain results in even stronger cell adhesion, which can be obtained with 7-fold less mRNA than the amount required for *FL-PAPC*. In classical cadherins, a current model proposes that the role of the cytoskeletal anchorage is to permit clustering of cadherins at the cell surface, thus facilitating the formation of zipper-like junctions between adjoining cells (Takeichi, 1995;

Yap et al., 1997). In the case of PAPC, lateral clustering and cell adhesion would occur without anchorage to the cytoskeleton.

In the course of mesodermal convergence and extension movements, the ability to break cell attachments could be a particularly important part of the morphogenetic process. Indeed, the embryo elongates because cells intercalate, moving past each other in directional movements toward the midline (Keller et al., 1992). To achieve this, they associate with neighbors via cellular protrusions, but these relationships are transient and must be resolved before new associations are formed during intercalation. Perhaps PAPC participates in processes requiring transient associations between the surfaces of migrating cells. It is attractive to suggest that the 25 amino acid region conserved between the *Xenopus* and zebrafish PAPC homologues (Fig. 1A; Yamamoto et al., 1998) may provide a binding site for a protein that would inhibit lateral clustering and cell adhesion; the cloning of such a protein would help unravel the molecular mechanisms that drive convergence and extension.

An important aspect of convergence and extension is the asymmetric movement of cells towards the midline. Organizer grafts by which a second trunk is induced suggest that the polarity signal should emanate from the dorsal midline (Spemann and Mangold, 1924). The present studies on *PAPC* do not explain what leads to this asymmetric cell behavior. However, it seems possible that additional signals, independent of protocadherins, may participate in cell polarity. It is interesting to note that a recent report has shown that *dachsous*, a *Drosophila* gene related to *fat*, which contains 27 extracellular cadherin repeats, has tissue polarity phenotypes in the fly wing (Adler et al., 1998). This phenotype requires a functional tissue polarity *frizzled* pathway. Thus, *dachsous* might affect tissue polarity indirectly, perhaps by modulating cell adhesion and affecting the activity of the frizzled cell polarity pathway (Adler et al., 1998).

Regulatory versus structural factors in gastrulation

In *Xenopus*, treatments with LiCl and UV showed that *PAPC* behaves as a typical zygotic gene activated by Spemann's organizer during early gastrulation (Fig. 7). Microinjection of *Xombi/VegT* mRNA induced ectopic expression of *PAPC* in axial mesoderm. Important functions in the determination of the endodermal and mesodermal germ layers have been recently reported for maternally stored *VegT/Xombi* mRNA (Zhang et al., 1998). The expression pattern of PAPC closely follows the zygotic *Xombi/VegT* transcripts, first in Spemann's organizer and subsequently in paraxial mesoderm (Zhang and King, 1996). The effects of microinjected *Xombi/VegT* mRNA would mimic the zygotic expression of this gene. In zebrafish, cloning of the *PAPC* homologue has permitted the analysis of the genetic control of *PAPC* expression in a loss-of-function situation (Yamamoto et al., 1998). Transcription of zebrafish *PAPC* in the organizer and in paraxial mesoderm of the anterior trunk requires the *spadetail* T-box gene, which is a homolog of *Xenopus Xombi/VegT* (Griffin et al., 1998). Interestingly, zebrafish *spadetail* function is required for convergence of paraxial mesodermal cells towards the dorsal midline (Kimmel et al., 1989; Yamamoto et al., 1998). Microinjection of *Xnot* mRNA, which encodes a homeodomain protein that promotes notochord development (Gont et al., 1996), represses expression of *PAPC* in *Xenopus*. In zebrafish, loss-of-function mutations in the *Xnot* homologue *floating-head* (Talbot et al., 1995) cause ectopic expression of *spadetail* and of *PAPC* in the notochordal midline (Yamamoto et al., 1998). Thus, both gain- and loss-of-function experiments suggest that *PAPC* is downstream, directly or indirectly, of important transcription factor genes involved in the regulation of gastrulation.

In conclusion, PAPC encodes a cell adhesion molecule expressed at the right time and place to participate in the convergence and extension movements of paraxial mesoderm. The PAPC cell adhesion protein has two novel properties. First, it can promote cell movements in overexpression experiments. Second, the intracellular domain of PAPC is not required for cell adhesion and indeed seems to decrease cell adhesion mediated by PAPC, suggesting a mechanism for the release of cell-cell contacts during gastrulation convergence movements. Despite the plethora of transcription and secreted regulatory factors uncovered in recent years in vertebrate gastrulation, the link with the actual structural machinery that converts this regulatory information into physical cell-cell interactions and movements in the embryo has remained elusive; genes such as *PAPC* may provide such a link.

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