Paraxial protocadherin mediates cell sorting and tissue morphogenesis by regulating C-cadherin adhesion activity

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Introduction

Protocadherins are transmembrane glycoproteins that contain six or more conserved cadherin-repeats (EC domains) in their extracellular domains. They constitute a large subfamily of the cadherin superfamily (Nollet et al., 2000; Suzuki, 2000; Yagi and Takeichi, 2000). Classical cadherins are Ca\(^{2+}\)-dependent, homophilic cell–cell adhesion molecules with five EC domains. Their adhesion activities rely on two common features: the conserved Trp2 in the first cadherin-repeat domain (EC1), which is necessary for homophilic binding, and the conserved catenin-binding motifs in the cytoplasmic domain, which are required for signaling and linkage to the actin cytoskeleton (Gumbiner, 2005). Much less is known about the adhesion properties of protocadherins. Protocadherins do not have the Trp2 residue in the extracellular domain or the catenin-binding motifs in the cytoplasmic domain (Nollet et al., 2000). It is not even entirely clear whether they function as adhesion molecules or have evolved to perform different cellular functions. Some protocadherins exhibit weak cell aggregation activity when overexpressed in L cells, whereas others do not (Sano et al., 1993; Sago et al., 1995; Yoshida et al., 1998; Hirano et al., 1999; Yoshida, 2003). It is not clear whether the weak cell aggregation mediated by a few of these protocadherins reflects true cell adhesion function at physiological levels of expression. More direct and thorough studies, like those that have been performed on classical cadherins, are needed to establish the adhesion properties of a protocadherin.

Xenopus laevis paraxial protocadherin (PAPC) is a protocadherin that has been shown to play an essential role in the convergence and extension movements of paraxial mesoderm and in the establishment of somite boundaries during the early development of \(X.\) laevis embryos (Kim et al., 1998, 2000). It is first expressed in Spemann’s organizer at the onset of gastrulation, and is later expressed in the paraxial trunk mesoderm. By stage 14, PAPC is expressed in stripes and prefigures the forming somites. PAPC also induces the sorting out of blastomeres, which was taken as evidence that it functions as a homophilic cell-adhesion molecule. Recently, two groups reported that PAPC interacts with \(X.\) laevis Frizzled-7 (Xfz7) and can activate RhoA and JNK signaling via the noncanonical Wnt pathway to regulate tissue separation or convergent extension (Medina et al., 2004; Unterseher et al., 2004).

C-cadherin is a classical cadherin that mediates cell–cell adhesion between \(X.\) laevis blastomeres. It is expressed both
maternally and zygotically in all cell types throughout the early stages of *X. laevis* embryonic development (Choi et al., 1990; Ginsberg et al., 1991; Levi et al., 1991), and plays essential roles in the maintenance of embryo integrity (Heasman et al., 1994) and in morphogenetic cell movements (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995). Inhibition of C-cadherin adhesion activity by dominant-negative (DN) C-cadherins causes failure of blastopore closure (Lee and Gumbiner, 1995). Furthermore, the adhesion activity of C-cadherin at the blastomere surface is down-regulated during activin-induced elongation of animal cap explants, a process believed to mimic the convergence and extension cell movements during gastrulation (Brieher and Gumbiner, 1994). Disrupting the down-regulation of C-cadherin adhesion activity by an activating antibody blocks animal cap elongation (Zhong et al., 1999). These findings demonstrate that dynamic regulation of C-cadherin adhesion activity plays a pivotal role in embryonic tissue morphogenesis. However, the mechanism by which C-cadherin activity is regulated during morphogenesis is unknown.

Because PAPC, like C-cadherin, is a cadherin with a role in cell sorting and convergence and extension morphogenetic cell movements during *X. laevis* gastrulation, we chose it as an interesting model protocadherin to investigate. We first undertook a thorough examination of the adhesion properties of PAPC, including an analysis of domains required for its function. We also investigated the mechanism by which PAPC mediates cell sorting in the embryo and its relationship to C-cadherin–mediated adhesion. Finally, we asked how PAPC and C-cadherin cooperate to regulate tissue morphogenesis in the *X. laevis* embryo.

**Results**

**PAPC does not mediate homophilic cell adhesion in several different cell types**

CHO cells do not express endogenous cadherins and have been successfully used for studying the adhesion activities of classical cadherins (Brieher et al., 1996; Chappuis-Flament et al., 2001; Niessen and Gumbiner, 2002). We generated stable CHO cell lines that express full-length PAPC (FL-PAPC), a cytoplasmic tail–deleted form of PAPC (M-PAPC; Kim et al., 1998), or GFP as control, and examined their cell aggregation properties and their capacity to adhere to a substrate of purified PAPC protein. We tested M-PAPC as well as FL-PAPC because M-PAPC has been reported to have stronger cell sorting activity than FL-PAPC (Kim et al., 1998). Both FL-PAPC and M-PAPC were expressed on the surface of CHO cells, as demonstrated by accessibility to trypsinization, surface biotinylation, and immunofluorescence staining of intact cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). Surprisingly, in cell aggregation assays, neither FL-PAPC-CHO cells nor M-PAPC-CHO cells aggregated to any extent compared with mock control cells (GFP-CHO; Fig. 1 A). For comparison, a stable cell line expressing C-cadherin, C-CHO, aggregated over time in the same experiment (Fig. 1 A). We also performed cell attachment flow assays using a purified PAPC protein as an adhesion substrate (PAPC-EC.Fc, which is the extracellular domain of PAPC fused with human IgG Fc, see Fig. S2). Although C-cadherin–expressing cells (C-CHO) adhered strongly to a C-cadherin substrate (C-cad-EC.Fc), neither FL-PAPC-CHO cells nor M-PAPC-CHO cells adhered to the PAPC substrate (Fig. 1 B). In addition to PAPC-EC.Fc, we also used another adhesion substrate, purified soluble PAPC with a C-terminal 6×His-tag (PAPC-EC.His; Fig. S2), which forms higher-order oligomers rather than dimers (unpublished data) and may have a conformation different from PAPC-EC.Fc. However, PAPC-expressing cells did not adhere to PAPC-EC.His either (unpublished data). Therefore, PAPC does not mediate homophilic cell adhesion in CHO cells.

It is possible that CHO cells lack the necessary cytoplasmic factors for PAPC-mediated adhesion. Therefore, we also prepared multiple stable PAPC-expressing cell lines using different kinds of cell types, including human epithelial A431 cells (Fig. 1 C), *X. laevis* XTC cells (Fig. 1 D), MBA-MD231, SW480, and MCF7 cells, and several others (unpublished data). None of these PAPC-expressing cell lines exhibited adhesion activity to purified PAPC substrates, but they were all able to adhere strongly to either purified E- or C-cadherin substrates via their endogenous cadherins. We also examined the adhesion activity of PAPC in *X. laevis* blastomeres, in which it has been shown to mediate cell sorting (Kim et al., 1998). FL-PAPC, M-PAPC, or GFP was expressed in embryos by mRNA injection, and blastomeres dissociated from isolated animal caps were tested for adhesion to either PAPC-substrates or the C-cadherin substrate. Blastomeres expressing PAPC did not adhere to either PAPC substrate, whereas the same blastomeres were able to adhere strongly to a 10 times less concentrated C-cadherin substrate (Fig. 1 E). Adhesion assays were also performed on blastomeres from the dorsal trunk mesoderm of stage 12 embryos, in which endogenous PAPC is expressed, but these blastomeres did not adhere to PAPC substrates either (Fig. 1 F). In summary, both the blastomere adhesion assays and the cell culture adhesion assays indicate that PAPC does not function effectively as a homophilic cell–cell adhesion molecule.

**PAPC mediates cell sorting and influences gastrulation movements by down-regulating C-cadherin adhesion activity**

The lack of intrinsic cell adhesion activity for PAPC appeared inconsistent with the reported cell sorting activity of PAPC in *X. laevis* embryos (Kim et al., 1998). Therefore we tried to reproduce the cell sorting assays as described by Kim et al. (1998), using both cell dispersal assays and reaggregation assays. In cell dispersal assays, with GFP mRNA alone injected into a single blastomere at the 32-cell stage, labeled cells extensively interspersed with surrounding unlabeled cells at a later stage of development (Fig. 2 A). In contrast, cells derived from FL-PAPC or M-PAPC mRNA-injected blastomeres (with a GFP lineage tracer) formed tight patches and maintained sharp boundaries with their unlabeled neighbors (Fig. 2 B and C), confirming the cell sorting activity of both FL-PAPC and M-PAPC. Moreover, in dissociation and reaggregation assays, blastomeres dissociated from FL-PAPC or M-PAPC mRNA-injected embryos (with a GFP lineage tracer) nicely sorted out
from blastomeres obtained from uninjected embryos (Fig. 2, E, F, H, and I), whereas blastomeres from embryos in which GFP mRNA alone was injected uniformly mixed with uninjected blastomeres (Fig. 2, D and G).

Notably, FL-PAPC has the same activity in inducing cell sorting as M-PAPC because the same amount of mRNA was injected in every experiment. This appears different from an earlier study that found M-PAPC mRNA to be seven times more efficient in inducing cell sorting (Kim et al., 1998). However, in that study, protein expression levels were not measured because anti-PAPC antibodies were not yet available. Indeed, the FL-PAPC construct used in the previous study produces seven times less protein in embryos than the M-PAPC construct (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). This original FL-PAPC construct differs from the M-PAPC construct because it retains both the 5' and 3' untranslated regions (UTRs). For our experiments, we removed the 3' (and 5') UTR, which results in similar protein expression levels for FL-PAPC and M-PAPC (Fig. S3 D–F) and, thus, higher cell-sorting activity for FL-PAPC (Fig. S3, G–I). Therefore, the cytoplasmic tail is not required for the cell-sorting activity of PAPC. Furthermore, expression of the membrane-bound cytoplasmic domain of PAPC had no detectable affect on cell sorting or on M-PAPC–induced cell sorting (Fig. S4).

It is important to note that the PAPC-expressing cells sorted to the outside of the aggregates in clusters. This was apparent in a surface view (Fig. 2, E and F), but was also confirmed by bisection of the aggregates (Fig. 2, H and I). According to Steinberg’s differential adhesion theory (Steinberg, 1970; Foty and Steinberg, 2004), cells with weaker adhesion strength tend to sort to the periphery of coaggregates. This suggests that PAPC-expressing cells have weaker adhesion strength than uninjected cells. Indeed, in blastomere aggregation assays, FL-PAPC– or M-PAPC–expressing cells only formed small aggregates (Fig. 2, K and L) compared with control GFP-expressing cells (Fig. 2 J), showing that PAPC-expressing cells exhibit less overall cell adhesion activity. Therefore, both FL-PAPC and M-PAPC may induce cell sorting by down-regulating the overall cell adhesion strength.

Knowing that PAPC does not mediate cell adhesion itself and yet can induce cell sorting, we hypothesized that PAPC changes the adhesion activity of other adhesion molecules. The best candidate is C-cadherin, because C-cadherin is expressed throughout the X. laevis embryo in early stages of development and has been shown to be necessary for blastomere adhesion (Heasman et al., 1994). In addition, it has been shown that C-cadherin activity can be down-regulated by growth factors such as activin (Brieher and Gumbiner, 1994). To examine
whether PAPC regulates C-cadherin adhesion, we performed blastomere adhesion assays under conditions used to detect activin-regulation of C-cadherin adhesion activity. Blastomeres obtained from FL-PAPC or M-PAPC mRNA-injected embryos exhibited significantly decreased levels of C-cadherin–mediated adhesion, which was equivalent to only \( \frac{40}{100} \) of the control level exhibited by blastomeres from GFP mRNA-injected embryos (Fig. 3 A).

A decrease in C-cadherin adhesion could be caused either by decreased C-cadherin protein level at the cell surface or by decreased intrinsic adhesion activity of C-cadherin. PAPC expression did not change the overall levels of C-cadherin protein in whole embryos (Fig. 3 B, lane 1–2) or in animal cap blastomeres used for adhesion assays (Fig. 3 B, lane 3–4). To determine whether the surface level of C-cadherin was changed by PAPC expression, we treated dissociated blastomeres with trypsin-EDTA to remove cell surface C-cadherin. PAPC expression did not alter the amount of trypsin-accessible C-cadherin (Fig. 3 B, lane 5–8). Previous work has shown that a specific C-cadherin–activating antibody, AA5, can reverse activin-regulation of C-cadherin–mediated adhesion at the cell surface, demonstrating intrinsic regulation of C-cadherin adhesion activity by activin (Zhong et al., 1999). AA5 similarly reversed PAPC-regulation of C-cadherin–mediated adhesion (Fig. 3 C). Therefore, PAPC functions by decreasing intrinsic C-cadherin adhesion activity at the cell surface. The effect of PAPC expression on C-cadherin–mediated adhesion is specific because blastomere adhesion to fibronectin or to antibodies against a nonspecific, exogenously expressed cell surface protein, human interleukin 2 receptor α (IL2Rα), is not affected by PAPC expression (Fig. 3, E and F).

To determine whether the down-regulation of C-cadherin activity is the cause of PAPC-induced cell sorting, we asked whether increasing C-cadherin expression levels could reverse PAPC-induced cell sorting. In cell dispersal assays with increasing amounts of C-cadherin mRNA coinjected with M-PAPC (and GFP) mRNA, the GFP-labeled cell population gradually changed from a tight patch to a loose patch and, eventually, to total mixing with their uninjected neighbors (Fig. 4 A). Therefore, overexpression of C-cadherin reverts PAPC-induced cell sorting. This result provides additional evidence that the down-regulation of C-cadherin adhesion by PAPC, rather than added PAPC-mediated adhesion, causes cell sorting. If PAPC increased cell adhesion, coexpression of C-cadherin should bolster cell sorting instead of blocking it.

Overexpression of M-PAPC in the animal hemisphere of the X. laevis embryo consistently caused failure of blastopore closure during gastrulation (defect rate 30/30; Fig. 4 B, middle column), which is a phenotype similar to that caused by DN C-cadherin expression (Lee and Gumbiner, 1995).
The same was observed for FL-PAPC mRNA injection (unpublished data). This phenotype is specific because injection of GFP mRNA did not cause any defect at the same stage (defect rate 0/50; Fig. 4 B, left column). Co-injection of C-cadherin mRNA, along with PAPC mRNA, rescued the blastopore closure defect significantly (defect rate 6/51; Fig. 4 B, right column). These results suggest that ectopic PAPC expression exerts its overall gastrulation phenotype by down-regulating C-cadherin activity.

To determine whether endogenous PAPC functions to inhibit C-cadherin adhesion activity, we also did loss-of-function studies. It is known that at the early stage of gastrulation, PAPC expression is limited to the dorsal marginal zone (DMZ; Kim et al., 1998). We found that the blastomeres obtained from the DMZ of stage 10.5 embryos exhibit significantly lower C-cadherin adhesion level than those from the ventral marginal zone (VMZ; Fig. 5 A, first two columns). We effectively knocked down endogenous PAPC expression using PAPC-specific morpholinos (PAPCMO; Fig. 6 B, lanes 5–6). Knocking down endogenous PAPC expression results in a significant increase in the level of C-cadherin-mediated adhesion of the DMZ blastomeres to that of the ventral blastomeres (Fig. 6 A, third column), suggesting that PAPC is responsible for the lower level of C-cadherin-mediated adhesion in the DMZ of control morpholino (COMO)–injected embryos. Moreover, the C-cadherin–activating mAb AA5 can also increase the adhesion of the DMZ blastomeres (Fig. 6 A, last column), indicating that the lower level of C-cadherin–mediated adhesion in the DMZ blastomeres is caused by specific down-regulation of the adhesion activity of the C-cadherin protein.

In the whole embryo, loss of PAPC expression by PAPCMO injection leads to a blastopore closure defect in stage 12.5 embryos (Fig. 5, B and C, PAPCMO), indicating defects in morphogenetic movements of gastrulation. This defect is specifically caused by the loss of PAPC, because it can be rescued by a morpholino-resistant form of FL-PAPC (Fig. 5, B and C, PAPCMO + FL). We then asked whether this gastrulation defect occurred because of the lack of down-regulation
of C-cadherin adhesion activity by PAPC. Indeed, a DN C-cadherin mutant, the cytoplasmic tail of C-cadherin (Lee and Gumbiner, 1995), was able to rescue the PAPCMO-defect to the same extent as the morpholino-resistant FL-PAPC (Fig. 5, B and C, PAPCMO + Ctail). Both decreased the blastopore size from ~67% of total embryo diameter in PAPCMO-embryos to ~37% of embryo diameter in rescued embryos. These results strongly suggest that PAPC functions in vivo to down-regulate C-cadherin adhesion activity and that this function of PAPC is required for proper morphogenetic cell movements during gastrulation.

**PAPC expression is induced by activin and is necessary for activin-induced regulation of adhesion and morphogenesis**

Activin, which is a TGFβ family growth factor, is a mesoderm inducer that induces elongation of animal cap explants, a process mimicking the convergence and extension movements that normally occur during gastrulation (Symes and Smith, 1987). Activin down-regulates C-cadherin activity without changing the level of C-cadherin, and this down-regulation is necessary for induction of animal cap elongation (Brieher and Gumbiner, 1994; Zhong et al., 1999). We therefore examined whether PAPC plays a role in activin regulation of adhesion and induction of morphogenesis. Activin treatment of animal caps induced PAPC expression in 1–2 h compared with untreated animal caps (Fig. 6 A), which is similar to the time required for the down-regulation of C-cadherin adhesion activity by activin (Brieher and Gumbiner, 1994). Injection of PAPC morpholinos (PAPCMO) significantly reduced both activin-induced PAPC expression and endogenous PAPC expression compared with a control morpholino (COMO; Fig. 6 B). In PAPCMO-injected embryos, activin-treatment failed to down-regulate C-cadherin adhesion activity (Fig. 6 C, columns 3 and 4), in contrast to significant decrease of C-cadherin adhesion activity in COMO-injected embryos (Fig. 6 C, columns 1 and 2). Furthermore, coinjection of a morpholino-resistant form of FL-PAPC with PAPCMO (Fig. 6 C, column 5) resulted in significant down-regulation of C-cadherin adhesion, even without activin treatment, which is similar to injection of FL-PAPC mRNA alone (Fig. 6 C, column 6). The level of down-regulation by PAPC is comparable to the down-regulation caused by activin. These results demonstrate that PAPC is necessary as well as sufficient to mediate activin-induced down-regulation of C-cadherin adhesion activity in *X. laevis* blastomeres.

We also asked whether PAPC expression is required for activin-induced elongation of animal cap explants. Animal caps excised from COMO-injected embryos fully elongated (20/20) in response to activin treatment (Fig. 6 D, 1), whereas explants from PAPCMO-injected embryos fell into two groups: no elongation (18/30; Fig. 6 D, 2b) and partial elongation without significant narrowing (12/30; Fig. 6 D, 2a). As shown in Fig. 6 D
(bottom), the explants from COMO-injected embryos had high levels of PAPC, whereas the “partial-elongation” group of explants from PAPCMO-injected embryos had lower, but detectable, levels of PAPC expression, and the “no-elongation” group of explants from PAPCMO-injected embryos had no detectable PAPC expression. Hence, strong inhibition of PAPC expression blocked elongation, whereas partial reduction in its expression partially blocked elongation. On the other hand, animal caps from PAPC mRNA-injected embryos did not elongate in the absence of activin treatment (unpublished data). Therefore, PAPC expression is necessary, but not sufficient, for activin-induced animal cap elongation.

**PAPC-regulation of C-cadherin is independent of Frizzled-7 signaling**

Two recent studies reported that PAPC functionally interacts with *X. laevis* Frizzled-7 (Xfz7) -mediated Wnt/planar cell polarity pathway to control tissue separation behavior (Medina et al., 2004) and convergent extension movements (Unterseher et al., 2004). Because M-PAPC, unlike FL-PAPC, does not induce tissue separation when coexpressed with Xfz7 (Medina et al., 2004), but is still capable of decreasing C-cadherin-mediated adhesion suggests that the mechanism of PAPC-dependent tissue separation is different from that of PAPC-inhibition of C-cadherin adhesion activity. Nonetheless, we decided to directly test whether Xfz7 mediates the C-cadherin down-regulation activity of PAPC. Two methods were used to disrupt Xfz7 function: Xfz7 morpholinos (Xfz7MO) and cytoplasmic domain–deleted DN form of Xfz7 (DN-Xfz7). Both Xfz7MO and DN-Xfz7 had been successfully used previously to interfere with Xfz7 function in *X. laevis* embryos (Sumanas and Ekker, 2001; Sumanas et al., 2000). Xfz7MO- or DN-Xfz7–injected embryos developed severe gastrulation defects, and failed to form a normal axis at the neurula stage (Fig. 7 A), indicating that both the morpholinos and the DN construct effectively interfered with Xfz7 function. However, coinjection of Xfz7MO or DN-Xfz7 mRNA with PAPC mRNA into embryos had no affect on the ability of PAPC to decrease C-cadherin adhesion activity compared with COMO coinjection (Fig. 7 B). Moreover, Xfz7MO or DN-Xfz7 coinjection did not block PAPC-mediated cell sorting (Fig. 7 C). Thus, interference with Xfz7 function does not affect
PAPC-mediated down-regulation of C-cadherin adhesion. Furthermore, we did not observe any change of C-cadherin adhesion activity because of Xfz7 expression or the sorting out of Xfz7-expressing cells (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1). Therefore, PAPC regulates C-cadherin adhesion activity and cell sorting independent of Xfz7 signaling.

Discussion

PAPC does not function by directly mediating cell–cell adhesion in X. laevis embryos

Previous studies on protocadherins, including X. laevis PAPC, have either assumed or suggested that they function by mediating cell–cell adhesion (Nollet et al., 2000; Suzuki, 2000). This notion has been based primarily on the presence of cadherin EC domains, but in some cases also based on limited evidence for adhesive function. In the case of PAPC, the evidence for adhesion was that it caused cell sorting out, which is a common consequence of adhesion molecule function (Kim et al., 1998). To our surprise, we found no evidence that PAPC functions as a bona fide homophilic cell–cell adhesion molecule. First, cells that express PAPC at their surfaces, either in tissue culture or from X. laevis embryos, exhibit no detectable adhesion to purified PAPC proteins. Second, there is no detectable aggregation of PAPC-expressing cells, indicating no adhesive interactions between PAPC molecules, even when both are presented on the surface of living cells. Third, PAPC-expressing blastomeres exhibit less aggregation activity than non–PAPC-expressing blastomeres and sort to the outside of coaggregates with non–PAPC-expressing blastomeres, suggesting a decrease, rather than an increase, in the cell-adhesive strength of PAPC-expressing cells. Furthermore, overexpression of C-cadherin counteracts, rather than reinforces, PAPC-mediated cell sorting, consistent with the notion that PAPC does not mediate cell adhesion. Although we cannot exclude the possibility that PAPC has weak homophilic binding activity undetectable in our adhesion assays or that PAPC mediates adhesion in some cell systems other than the ones we tested, it is clear that its cell sorting activity in the X. laevis embryo is not mediated by PAPC-mediated increase in cell–cell adhesion.
PAPC down-regulates C-cadherin adhesion activity

A key finding of this study is that PAPC down-regulates C-cadherin adhesion activity to cause cell sorting and contribute to morphogenetic movements. PAPC expression causes a significant decrease in blastomere adhesion to purified C-cadherin protein (Fig. 3). In addition, PAPC-induced cell sorting in embryos is reversed by coexpression of C-cadherin, consistent with the view that decreased C-cadherin adhesion in PAPC-expressing cells is the cause of cell sorting (Fig. 4 A). Furthermore, PAPC-induced gastrulation defects in embryos phenocopies the adhesion defect caused by DN C-cadherin (Lee and Gumbiner, 1995), and overexpression of C-cadherin rescues PAPC-induced gastrulation defects (Fig. 4 B). More importantly, knocking-down of endogenous PAPC results in increased C-cadherin adhesion activity in corresponding tissue, and loss-of-PAPC-function defects can be rescued by decreasing C-cadherin–mediated adhesion using a DN C-cadherin construct (Fig. 5).

The effect of PAPC on blastomere adhesion is specific to C-cadherin and not caused by an overall interference with the capacity for cell adhesion. Neither the integrin-mediated adhesion to fibronectin nor attachment via IL2Rα to anti-IL2Rα antibodies is affected by PAPC expression. Most important is the finding that a specific anti–C-cadherin–activating mAb can reverse the affects of PAPC on C-cadherin adhesion, demonstrating that the adhesive change is intrinsic to the C-cadherin protein. Therefore, PAPC specifically down-regulates C-cadherin adhesion activity, resulting in changes in cell sorting behavior.

We find that both M-PAPC, the cytoplasmic domain deletion mutant of PAPC, and FL-PAPC have the same activity in cell sorting and regulation of adhesion. This differs from a previous study in which M-PAPC was found to have higher cell-sorting activity than wild type FL-PAPC (Kim et al., 1998). Our results using anti-PAPC antibodies indicate that this was caused by differences in the levels of PAPC protein expression. Removal of the 3′ UTR from the FL-PAPC mRNA resulted in higher protein expression and similar sorting and adhesion regulation activity as M-PAPC. Therefore, the cytoplasmic domain of PAPC is not required for its function in regulation of adhesion and induction of cell sorting.
The cytoplasmic domain of PAPC is probably involved in other signaling functions of PAPC. Recent studies found that FL-PAPC interacts and cooperates with X. laevis Frizzled-7 (Xfz7) and activates RhoA and JNK in regulation of tissue separation, as well as convergent extension movements (Medina et al., 2004; Unterseher et al., 2004). The cytoplasmic domain appears to be required because M-PAPC cannot induce tissue separation together with Xfz7 (Medina et al., 2004). Furthermore, an earlier study has found that FL-PAPC, but not M-PAPC, promotes elongation of animal cap explants that are treated with low activin, suggesting a requirement for the cytoplasmic domain in induction of morphogenetic movements (Kim et al., 1998). The cytoplasmic domain of PAPC contains a region of 25 amino acid residues (aa 816–840) that is highly conserved across species and present in other protocadherins. This region might be important for mediating interactions with unknown cytoplasmic factors involved in Xfz7-mediated signal transduction events.

Although Xfz7 can mediate signaling events induced by PAPC (Medina et al., 2004; Unterseher et al., 2004), it does not appear to be involved in PAPC regulation of C-cadherin adhesion activity. The regulation of C-cadherin by PAPC does not require the cytoplasmic domain, in contrast to Xfz7-mediated PAPC-control of tissue separation and convergent extension movements (Fig. 8 A). Moreover, interference with Xfz7 expression or function has no affect on the ability of PAPC to down-regulate C-cadherin adhesion activity or to induce cell sorting (Fig. 7). Furthermore, overexpression of Xfz7 does not affect C-cadherin-mediated adhesion nor induce cell-sorting behavior in embryos (Fig. S5). Therefore, PAPC regulates C-cadherin adhesion activity and cell sorting independent of Xfz7.

The molecular mechanism by which PAPC down-regulates C-cadherin activity is not yet understood. One possibility is that PAPC could interact with C-cadherin directly and influence its adhesive conformation and activity, but in preliminary experiments, we have not yet observed significant amounts of C-cadherin coimmunoprecipitated with PAPC from detergent lysates of X. laevis embryos or PAPC/C-cadherin–expressing CHO cells (unpublished data). Moreover, stable expression of PAPC in C-cadherin–expressing CHO cells does not appear to significantly change C-cadherin adhesion activity, suggesting that a more complicated mechanism is involved in regulation in X. laevis embryo blastomeres. For example, it is possible that PAPC interacts with another membrane protein that either links PAPC to C-cadherin or transduces a signal from PAPC to regulate C-cadherin. If we are able to identify such a membrane protein in future studies, it will be interesting to determine whether it can reconstitute PAPC regulation of C-cadherin in CHO cells, as it does in X. laevis blastomeres.

**PAPC mediates activin-induced down-regulation of C-cadherin adhesion activity and convergent extension movements**

Activin is a TGFβ family member that induces mesodermal gene expression in X. laevis embryos. Activin treatment triggers X. laevis animal cap explants to elongate, a process mimicking convergence and extension movements during gastrulation. Little is known about the mechanism of activin-induced animal cap elongation. Activin has been reported to decrease C-cadherin adhesion activity in animal cap explants (Brieher and Gumbiner, 1994), and reversing this down-regulation with a C-cadherin–activating antibody blocks activin-induced animal cap elongation (Zhong et al., 1999). In the present study, we demonstrate that activin induces PAPC expression and that PAPC expression is necessary for activin-regulation of C-cadherin adhesion activity, as well as activin induction of animal cap elongation. PAPC has also been reported to be required for animal cap elongation induced by another TGFβ family growth factor, BVg1 (Medina et al., 2004).
However, PAPC expression alone is not sufficient to induce animal cap elongation (unpublished data). These results suggest that additional signals resulting from activin induction are required to elicit morphogenetic cell movements.

We propose a model for how activin or other TGFβ family members present in the embryo regulate cell adhesion and induce morphogenesis (Fig. 8 B). Activin induces PAPC expression and PAPC down-regulates C-cadherin adhesion activity. Dynamic regulation of C-cadherin-mediated cell-cell adhesion is required for convergence and extension cell movements (Zhong et al., 1999). PAPC probably also contributes to morphogenesis via Frizzled-7–mediated planar cell polarity pathway, perhaps via cell polarization. Because PAPC expression alone is not sufficient to induce animal cap elongation, activin probably induces expression of additional factors that participate in the generation of convergent extension movements.

Do most protocadherins function as adhesion molecules?
Protocadherins represent a huge subfamily of molecules in the cadherin superfamily in vertebrates, and have been implicated in several biological processes, especially in the nervous system (Nollet et al., 2000; Suzuki, 2000; Yagi and Takeichi, 2000). Protocadherins have neither the known conserved cadherin interfaces for homophilic adhesion, including the Trp2 residue, that mediate adhesion of classical cadherins nor do they have the catenin-binding motifs required for cytoskeletal interactions in their cytoplasmic domains (Nollet et al., 2000).

To date, the protocadherins that have been studied either exhibit no adhesion activity or have been suggested to mediate weak adhesion based on limited evidence (Sano et al., 1993; Sago et al., 1995; Yoshida et al., 1998; Hirano et al., 1999; Yoshida, 2003). Whether the weak interaction between some of these protocadherins represents bona fide cell–cell adhesion or is actually involved in other functions such as signal transduction remains unclear. The protocadherin α proteins (Pcdha) bind the secreted protein reelin and mediate reelin signaling via the nonreceptor tyrosine kinase Fyn that binds to their cytoplasmic domains (Kohmura et al., 1998). Therefore, protocadherins can function as receptors for extracellular ligands that mediate signal transduction into the cell.

An important finding of our study is that a protocadherin can modify cell adhesion by regulating the adhesion activity of a classical cadherin. This could be a potential general mechanism for how protocadherins affect cell adhesion. In fact, Angst et al. speculate that Pcdha may regulate N-cadherin function in neurons (for review see Angst et al., 2001), but it would be interesting to test whether Pcdha regulates N-cadherin–mediated adhesion. Two other X. laevis protocadherins, neural fold protocadherin and axial protocadherin, also induce cell sorting like PACP (Bradley et al., 1998; Kim et al., 1998; Kuroda et al., 2002), but their intrinsic adhesion activities have not been directly tested. One possibility is that they, like PACP, regulate adhesion activities of other adhesion molecules. To determine whether any of these protocadherins function as bona fide adhesion molecules, direct careful examination of their adhesive functions will be required.
previously described (Zhong et al., 1999). Blastomere adhesion assays were performed as previously described (Niesen and Gumbiner, 2002), with the following modifications: (a) 15 µl of 0.1 mg/ml purified PAPC-EC.Fc, 0.1 mg/ml PAPC-EC.His, 3–10 µg/ml C-cad-EC.Fc, 50 µg/ml fibronectin, or 0.5 mg/ml anti-IL2R mAb BB10 was used for substrate coating, and 1% BSA was used for substrate blocking; and (b) X. laevis animal caps (at least five) were excised at stage 9 to obtain blastomeres. As needed, the dissociated blastomeres were treated with 5 ng/ml activin for 1 h and/or with 1 µg/ml AAS Fab for 30 min. The adhesion strength of blastomeres was measured by the ratio of the number of blastomeres remaining attached after shaking (Nt) versus the number before shaking (No). At least four independent experiments were performed for each sample. The SEM was plotted as error bars.

In vitro transcription and morpholinos
Capped mRNAs were synthesized using the Riboprobe in vitro transcription systems (Promega). Two PAPC morpholinos (Medina et al., 2004) and two Xfz7 morpholinos (Sumanas and Ekker, 2001) have been described, which were ordered from Gene Tools, LLC. In each case, a 1:1 mix of the two morpholinos was used for injection. The same amount of standard control morpholino (Gene Tools, LLC) was injected to control embryos.

X. laevis embryo manipulations
All experimental protocols that involved the use of X. laevis were approved by the Animal Care and Use Committee at the University of Virginia. X. laevis eggs and embryos were obtained and handled by standard techniques (Newport and Kirschner, 1982). Standard Nieuwkoop staging of embryos was used (Nieuwkoop and Faber, 1967). Microinjection of mRNAs or morpholinos was performed at the 2–4–cell stage, as previously described (Lee and Gumbiner, 1995). Typically, 1–2 ng mRNA or 40–80 ng mRNAs or morpholinos was performed at the 2–4–cell stage, as previously described. Typically, 1–2 ng mRNA or 40–80 ng mRNAs or morpholinos was injected into the animal pole of the embryos. For animal cap elongation assays, animal caps were excised at stage 8, treated with 5 ng/ml activin for 30 min. The adhesion strength of blastomeres was measured by the ratio of the number of blastomeres remaining attached after shaking (Nt) versus the number before shaking (No). At least four independent experiments were performed for each sample. The SEM was plotted as error bars.

Blastomere reaggregation assay and dispersal assay
Both assays were performed as previously described (Kim et al., 1998). NLS-GFP mRNA (200–400 pg/embryo) was coinjected with PAPC mRNA as a lineage tracer. In brief, for reaggregation assays, RNAs (with tracer) were injected into 4–cell stage embryos. At stage 9, a single cap was excised and the cap–blastomeres dissociated from injected and uninjected embryos were mixed at a 1:2 ratio and rocked overnight in Ca2+–containing media. The aggregates, with or without bisected, were examined under fluorescence microscope. For dispersal assays, sample mRNA, together with tracer, was injected into one blastomere at the animal hemisphere of 32-cell stage embryos. After stage 13, the injected embryos were observed under fluorescence microscope for distribution of GFP-labeled blastomeres.

Image acquisition
All images were acquired at room temperature. Light images of embryos were acquired with a digital camera (model G6; Canon; at ~4× optical zoom) mounted on a dissecting microscope (Nikon) with an objective lens and Cys3 filter (all from Carl Zeiss MicroImaging, Inc.). Images were acquired with a digital camera (model G6; Canon; at 4× magnification). Fluorescence images of embryos were acquired with a color LCD camera (SPOT RT; Diaphot; Nikon) with an objective lens (Plan 4; Nikon) and a Z-V filter. Immunofluorescence microscopy was performed on an Axiosplan2 microscope with a Neofluar 20× objective lens and Cys3 filter (all from Carl Zeiss MicroImaging, Inc.). Images were acquired with a digital camera (model C4742-95; Hamamatsu) and with OpenLab 4.0 (Improvision) software.

Online supplemental material
Fig. S1 shows surface expression of PAPC in CHO cells. Fig. S2 shows the purity of PAPC adhesion substrates. Fig. S3 shows the 3’UTR of FL-PAPC inhibits its protein expression. Fig. S4 shows the membrane-bound cytoplasmic domain of PAPC does not induce cell sorting or affect M-PAPC–induced cell sorting. Fig. S5 shows that Xfz7 expression does not decrease C-cadherin–mediated adhesion or induce cell sorting. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200602062/DC1.

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