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Axial Protocadherin Is a Mediator of Prenotochord Cell Sorting in *Xenopus*

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Prenotochord cell sorting is regarded as one of the first cell sorting events in early chordate development. We recently demonstrated that this sorting event occurs *in vitro,* although the mediator of this activity remains unidentified. Herein, we report the isolation of a full-length cDNA clone of *Axial protocadherin (AXPC),* the homologue of human *protocadherin-1 (PCD1). AXPC* encodes a transmembrane protein (AXPC) that is expressed exclusively in the notochord at the neurula stage and in the pronephros, somites, heart, optic vesicle, otic vesicle, and distinct parts of the brain at the tailbud stage. Cell dissociation and reaggregation assays and *in vivo* microinjection experiments demonstrated that cells overexpressing a membrane-tethered form of AXPC (MT-AXPC) acquired the same adhesive properties as prenotochord cells. Moreover, microinjection of either mRNA encoding the dominant negative form of *AXPC (DN-AXPC)* or morpholino oligonucleotides interferes with the sorting activity of prenotochord cells and normal axis formation. This study suggests that AXPC is necessary and sufficient for prenotochord cell sorting in the gastrulating embryo, and may also mediate sorting events later in development.

Key Words: **cadherin; protocadherin; AXPC; PAPC; *Xenopus*; sorting; adhesive properties.**

INTRODUCTION

The notochord is one of the most important signaling centers in early chordate development. The anterioposterior and dorsoventral axes in vertebrates are regulated in part by the organizer region of the embryo (Hamburger, 1988; Holtfreter, 1988; Spemann, 1938; Spemann and Mangold, 1924). The majority of the organizer parts, together, form the embryological notochord, which induces cell identity along the dorsoventral (Hatta *et al.,* 1991; Krauss *et al.*, 1993; Roelink *et al.*, 1994; Yamada *et al.*, 1991) and anterioposterior axes of the neural tube (Hemmati-Brivanlou *et al.,* 1990), and along the dorsoventral axis of the somites (Dietrich *et al.,* 1993; Halpern *et al.*, 1993; Pourquie *et al.*, 1993). The notochord also plays a role in the specification of cardiac left–right orientation in Zebrafish and *Xenopus* (Danos and Yost, 1996) and in the induction of pancreatic tissue following repression of endodermal signaling in chick embryos (Hebrok *et al.,* 1998; Kim *et al.,* 1997).

One of the most important roles of the notochord in chordate development may be in providing structural support for the embryo. This is achieved by elongating and straightening the embryo from gastrula to tailbud stage using a thread of notochord in the dorsal region derived from a part of the Spemann's Organizer (Adams *et al.,* 1990; Spemann, 1938). This dynamic movement from organizer to notochord depends not only on convergent extension but also on prenotochord cell sorting. When axial mesodermal cells, including the prenotochord cells, and other fated cells are brought together to form a mixed aggregate, the axial mesodermal cells have to move to the central region of the hemisphere (Townes *et al.,* 1955, Kuroda *et al.,* 1999). We propose that an adhesive molecule is required for the sorting of these axial mesodermal cells.

It is unlikely that similar cell types aggregate via heterophilic binding. We therefore propose that axial mesoderm cell sorting is achieved via homophilic binding, and ad-

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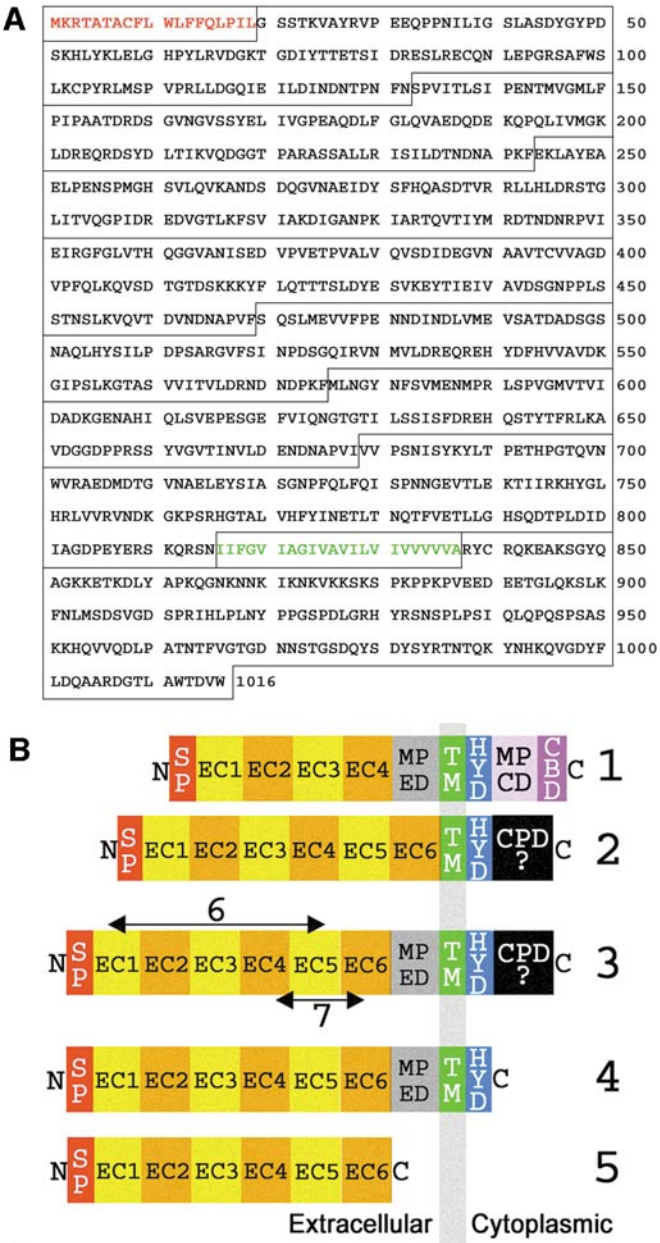


vance the Ca^{2+}-dependent cadherins as candidates to mediate this activity. Cadherin family members play important roles in specific cell–cell adhesion events. Their expression appears to be tightly regulated during development and each tissue or cell type shows a characteristic pattern of cadherin expression (Nose *et al.*, 1988; Takeichi, 1991). Among the cadherin protein family, E-cadherin was first described as L-CAM in chicken (Gallin *et al.*, 1983) and as uvomorulin in mouse (Schuh *et al.*, 1986). Soon after the identification of E-cadherin, P-cadherin and N-cadherin were characterized (Miyatani *et al.*, 1989; Nose and Takeichi, 1986). There are approximately 50 different cadherins expressed across both vertebrates and invertebrates (Nollet *et al.*, 2000). Generally, cadherins containing one signal peptide (SP), four extracellular (EC) domains, one membrane proximal extracellular domain (MPEC), one transmembrane (TM) domain, one membrane proximal conserved domain (MPCD), and a catenin-binding domain (CBD) are known as "classical cadherins" (Fig. 1B, 1). All other cadherins that do not follow this configuration are called "protocadherins." Recently, two novel protocadherin genes were identified in the early *Xenopus* embryo (Kim *et al.*, 1998). These were *Paraxial protocadherin (PAPC)*, an adhesion molecule expressed in Spemann's organizer and paraxial mesoderm (somites) that mediates cell movement during gastrulation, and a partial sequence of *Axial protocadherin (AXPC)*, which is expressed in axial mesoderm notochord. PAPC plays a role in paraxial mesodermal cell sorting and convergent extension during *Xenopus* gastrulation. When the partial AXPC was fused in frame with an extracellular (EC) domain and transmembrane domain of *PAPC*, the hybrid protein exhibited cell adhesion activity. We therefore investigated AXPC as a candidate mediator of prenotochord cell sorting.

We report here the isolation of a full-length *AXPC* clone.

FIG. 1. Structure of the AXPC protein. (A) Amino acid sequence of AXPC. Regions highlighted in red and green demonstrate the signal peptide and the transmembrane domain, respectively. Black line indicates the position of the border between each domains. (B) Protein structures of the major cadherin family members, and the two protocadherins. 1, Classical cadherin structure. 2, Paraxial protocadherin (PAPC, Kim *et al.*, 1998). 3, Axial protocadherin (AXPC), this study. 4, Our constructed membrane-tethered form of AXPC (MT-AXPC). 5, Our constructed soluble dominant-negative form of AXPC (DN-AXPC). 6, Axial protocadherin fragment previously isolated by Kim *et al.* (1998). 7, The region of our isolated mouse *AXPC* fragment. SP, signal peptide. EC, extracellular cadherin repeats. MPED, membrane-proximal extracellular domain. TM, transmembrane domain. HYD, hydrophilic domain. MPCD, membrane-proximal conserved domain. CBD, catenin-binding domain. CPD, cytoplasmic domain. (C) Amino acid alignment of *Xenopus AXPC* (*X-AXPC*), mouse *AXPC* (*M-AXPC*), and the human homologue of *AXPC*, *protocadherin-1* (*H-PCD1*). The identities between *Xenopus*–mouse, *Xenopus*–human and mouse–human were 75, 74, and 96%, respectively.

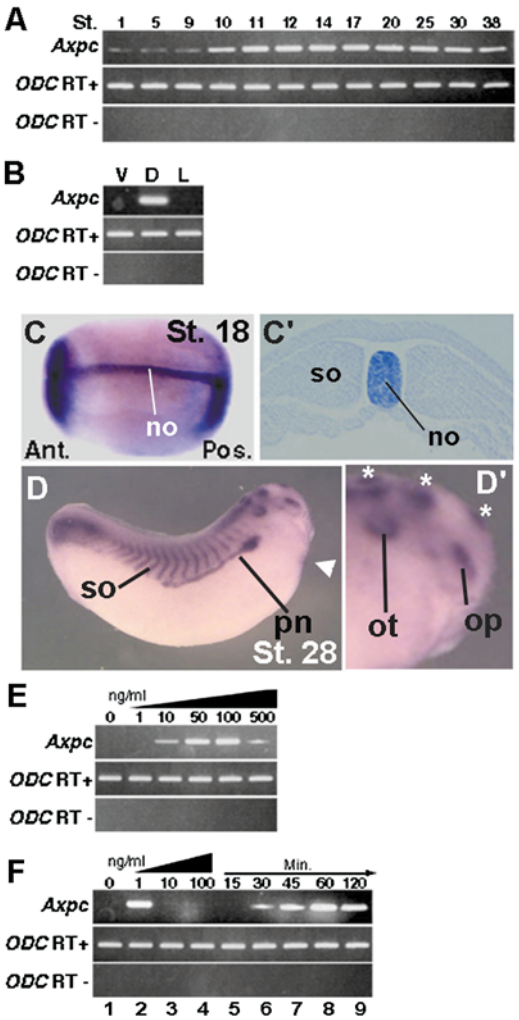


FIG. 2. Expression of *AXPC*. (A) Temporal expression analyzed by RT-PCR. Low levels of *AXPC* expression were detected before gastrulation. Expression levels increased at stage 10, with high levels of expression being maintained by stage 38. (B) *AXPC* was expressed exclusively in the dorsal marginal zone at stage 10. V, ventral marginal zone. D, dorsal marginal zone. L, lateral marginal zone. no, notochord. so, somite. (C) Whole-mount *in situ* hybridization analysis at late neurula (stage 18). *AXPC* is expressed in the notochord region at stage 18. Ant., anterior. Post., posterior. (C′) Section of (C) clearly shows the *AXPC* expression in notochord. (D) At stage 28, *AXPC* was expressed strongly in the pronephros region (pn) and weakly in the somites (so) and the heart (white arrowhead). (D′) Enlarged view of (D) shows the expression of AXPC in the optic vesicle (op), otic vesicle (ot), and some parts of central nervous system (white asterisks). (E) *AXPC* expression in activin-treated animal caps. Animal caps were treated with 0–500 ng/ml of activin for 1 h, then cultured for 5 h. High *AXPC* expression was detected in 50 or 100 ng/ml activin-treated animal caps, and low levels of *AXPC* expression were detected in 10 or 500 ng/ml activin-treated animal caps. Note: Concentrations of 50–100 ng/ml are known to most effectively induce notochord in this animal cap assay (Asashima *et al.*, 1990). (F) *AXPC* expressions in activin-treated dissociation-and-reaggregation animal caps (Fig. 3A, Method 1). Lanes 1–4 indicate *AXPC* expression in the reaggregate treated with activin for 1 h, reaggregated, and cultured for 5 h. Very high expression levels of *AXPC* were detected in the reaggregates treated with 1 ng/ml activin. No expression was detected in the other samples. Note: A concentration of 1 ng/ml induces notochord only in this dissociated and reaggregated animal cap assay (Kuroda *et al.*, 1999). Lanes 5–9 show *AXPC* expression in dissociated cells with 1 ng/ml activin-treatment. Low *AXPC* expression was detected at 30 (lane 6) and 45 min (lane 7) after the initiation of activin treatment, and high *AXPC* expression was detected at 60 (lane 8) and 120 min (lane 9) after the initiation of activin treatment.

This is the homologue of human *protocadherin-1*. We demonstrate that this molecule functions as a mediator of prenotochord cell sorting, and show that AXPC may participate in the many sorting events of later stage embryos.

MATERIALS AND METHODS

Dissociation and reaggregation assay

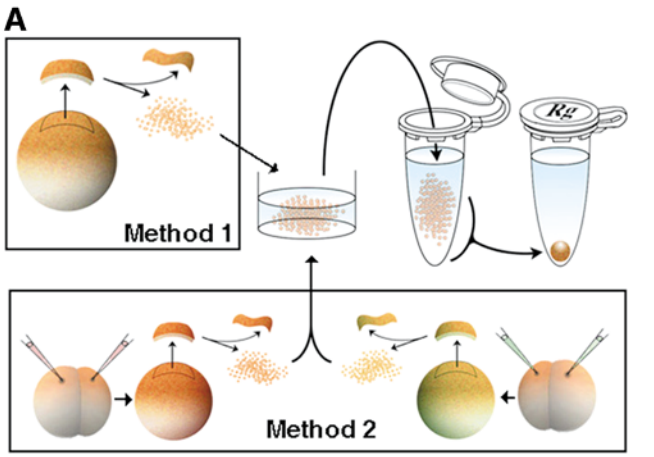
Embryos at the two- or four-cell blastomere stage were coinjected with 10 nl of 1% Texas red-dextran-amine (TRDA) or 1% fluorescein-dextran-amine (FDA) lineage tracers, and control (water) or *protocadherin* (*AXPC* or *PAPC*) mRNA (see Fig. 3A, Method 2). At stage 9, 2 h before gastrulation, animal cap explants were excised and transferred to Ca^{2+}- and Mg^{2+}-free Steinberg's solution containing 0.1% bovine serum albumin (CMFS-BSA). The cells of the inner cell layer detached in approximately 10 min. The outer layer cells were removed and transferred to CMFS-BSA supplemented with 1 ng/ml activin, incubated for 1 h, and washed three times in 100% Steinberg's solution. These treatments were essentially as described by Green *et al.* (1994, 1997). Dissociated inner layer cells from five TRDA-labeled animal caps were thoroughly mixed with those from five FDA-labeled animal caps, transferred to a 1.5-ml tube containing Steinberg's solution, and reaggregated by centrifugation at 100*g* for 5 s. The cell mixture was washed again and then incubated for 10–72 h. Embryo staging was carried out according to Nieuwkoop and Faber (1967).

Cloning of Full-Length AXPC and PAPC

A stage 12 *Xenopus* gastrula cDNA library was screened with fragments of *Xenopus AXPC* and *PAPC* cDNA clones obtained using the following primers and cycling conditions: *AXPC* (60°C, 30 cycles; forward, 5′-ATCGTGGCTGTTGACTCTGG-3′; reverse, 5′-GTAATGAAGCTGAGCGTTGG-3′); *PAPC* (60°C, 30 cycles; forward, 5′-CGTCTAGCAGATACCAAGAATTC-3′; reverse, 5′-GCTAGTTAGGTGACAGGACAATG-3′). Full-length cDNA clones for both *AXPC* and *PAPC* were cloned into pBluescript vector. The following primers and cycling conditions were used to isolate partial mouse *AXPC* cDNA clones: *M-AXPC* (60°C, 30 cycles; forward, 5′-ACACTGGACTTATCACCGTGC-3′; reverse, 5′-TAGACGTCAATGACAACGCC-3′).

RT-PCR Assays

RT-PCR was performed by using the following primers and cycling condition: *AXPC* (58°C, 28 cycles; forward, 5′-AAGGAAGCATT-



D

	red cells		green cells		n	SO	FSE
a	mt-AXPC (500 pg)	MI	non		8	7	87.5
b	mt-AXPC (500 pg)	MI	activin	TR	8	1	12.5
c	mt-AXPC (500 pg)	MI	non		8	0	0
	dn-AXPC (1 ng)	MI					
d	activin (1 ng/ml)	TR	non		8	2	25
	dn-AXPC (1 ng)	MI					
e	mt-AXPC (500 pg)	MI	non		8	0	0
	morpholino (10 ng)	MI					
f	activin (1 ng/ml)	TR	non		6	6	100
	morpholino (10 ng)	MI					
g	fl-PAPC (1 ng)	MI	non		4	4	100
h	fl-PAPC (1 ng)	MI	non		4	4	100
	dn-AXPC (1 ng)	MI					

ATGGCCTCC-3′; reverse, 5′-TTGCTTCGCTCATACTCTGG-3′); *ODC* (55°C, 25 cycles; forward, 5′-GTCAATGATGGAGTGTATG-GATC-3′; reverse, 5′-TCCATTCCCTCTCCTGAGCAC-3′).

Gene Construction

The primers T3 and 5′-CCGGCCTCGAGGGACTTCTGCAAA-CCAGT-3′ were used to construct a membrane-tethered form of *AXPC* (pCS2-*MT-AXPC*) (50°C, 25 cycles), and T3 and 5′-CC-GGCCTCGAGAACAATTACTGGGGCATT-3′ were used to con-struct a dominant-negative secreted form of *AXPC* (pCS2-*DN-AXPC*) (50°C, 25 cycles). The mutant genes were cloned into the *Eco*RI*Xho*I sites of pCS2+. Morpholino oligo, 5′-GCAGTTGCTGTCCTC-TTCATTGTTA-3′, was designed by Gene Tools, LLC Co., and the control morpholino oligo, 5′-AAACCCGGGTTTACG-3′ was pro-vided by Gene Tools, LLC Co.

RESULTS

Cloning of AXPC cDNA in Xenopus and Mouse

An *AXPC* fragment was amplified by PCR using primers designed to hybridize to the partial *AXPC* clone previously described by Kim *et al.* (1998). This fragment was used to screen a *Xenopus* gastrula-stage cDNA library. A full-length *AXPC* clone was isolated (5643 bp, Accession No. AB066361) with a single open reading frame encoding 1016 amino acids (Fig. 1A), including a signal peptide (SP), six extracellular cadherin repeats (EC 1-6), a membrane-

FIG. 3. AXPC mediates homophilic cell sorting in cell dissociation and reaggregation assays. (A) Steps involved in the cell dissociation and reaggregation procedure. Reaggregates were made using blastula ectodermal cells (animal cap cells). Method 1: to induce particular tissue types. For example, if dissociated cells were treated with 1 ng/ml of activin for 1 h, reaggregates differentiate into only notochord. Method 2: to examine the sorting activity of cells. (B) Prenotochord cells show potent sorting activity. (a) The 3-day cultured reaggregate derived from the cells of Method 1 in (A) differentiated into only notochord. Note: The vacuolated structure is the typical feature of notochord tissue. (b) The reaggregate mixed with untreated cells using Method 2 in (A) did not show sorting, but (c) the red-labeled cells treated with 1 ng/ml of activin mimicked the prenotochord cells and demonstrated potent sorting activity. In this case, a vacuolated structure could not be detected because the culture time was reduced (10 h). The frequency of this sorting event was 100% (n = 8). (C) Dissociation and reaggregation assay using *AXPC* constructs (Fig. 1B, 4, 5) and morpholino oligo. (a) When reaggregates were made from cells injected with 500 pg of *MT-AXPC* mRNA (red) and untreated cells (green), the frequency of a sorting event (FSE) was 87.5% (n = 8). The details of (b–h) are summarized in (D). (D) Table of the results in Fig. 3C, a–h. MI, microinjection of mRNA or morpholino oligo in a four-cell stage embryo. TR, activin treatment to dissociated cells. non, no injection and no treatment. n, total number of experiments. So, the total number of reaggregates to show sorting. FSE, frequency of the sorting event (%).

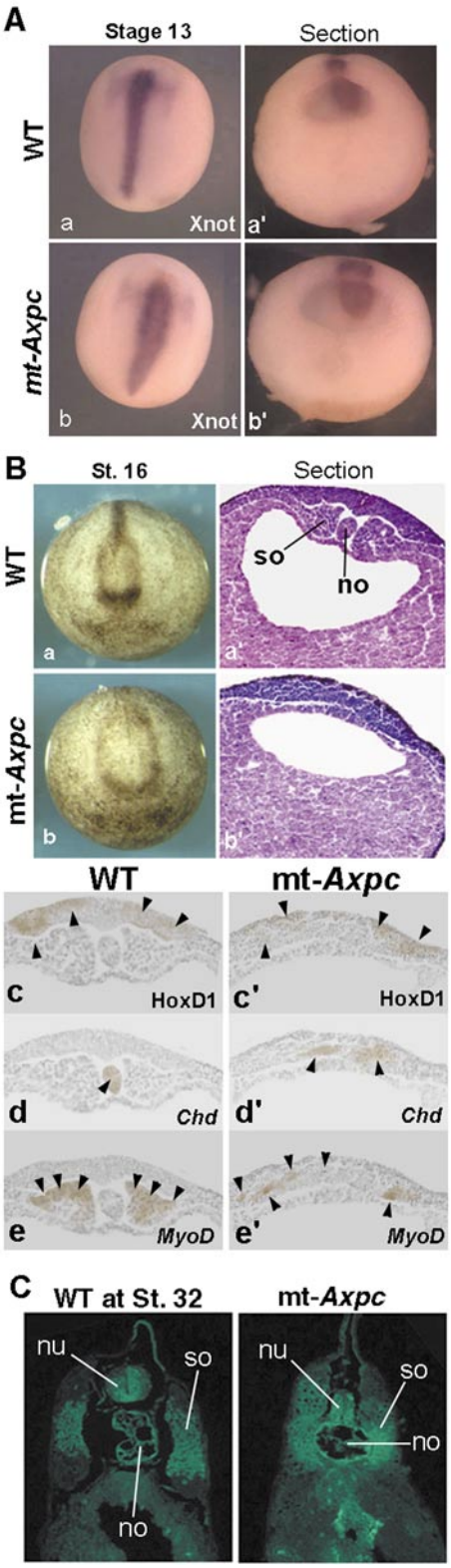


FIG. 4. Gain-of-function experiments using *MT-AXPC* mRNA led to the acquisition of adhesive properties by cells around the prenotochord. (A) (a) *Xnot* expression in nontreated embryo at stage 13 and (a′) transversal view. (b) *Xnot* expression in 500 pg of *MT-AXPC* mRNA-injected embryo at stage 13 and (b′) transversal view. *Xnot* expressed region was expanded in the case of (b). (B) (a) Control embryos at stage 16 and (a′) transversal view stained by hematoxilin and eosin. (b) Microinjection of 500 pg of *MT-AXPC* mRNA into two dorsal blastmeres at the four-cell stage, which inhibited normal neural formation and interfered with notochord formation at stage 16 (87.5%, $n = 24$), and (b′) transversal view of (b). (c–e), (c′–e′) Section *in situ* hybridization analysis using the posterior neural marker, *HoxD1* (c), notochord marker, *Chordin* (d), and somite marker, *MyoD* (e). The control embryos and *MT-AXPC*-injected embryos were fixed, frozen, sectioned at stage 16, and hybridized with probes. Black arrowheads indicate marker-detected regions. no, notochord. so, somite. (C) Later effects of *MT-AXPC*. The left panel shows a section of a stage 32 control embryo, which had been injected with fluorescein-dextran amine (FDA) lineage tracer into two dorsal blastomeres at the four-cell stage. The green regions are cells derived from the dorsal sites of four-cell stage embryos. The right panel indicates a section of 500 pg of *MT-AXPC* mRNA and FDA coinjected embryo. Since many embryos died by stage 30, we collected the surviving embryos at stage 30 and continued culturing of sibling embryos at stage 32. Inhibition of the notochord separation from the neural tube, somites, and endoderm was detected (57.9%, $n = 19$). nu, neural tube; no, notochord; so, somite.

proximal extracellular domain (MPED), a transmembrane domain (TM), a hydrophilic domain (HYD), and a cytoplasmic domain (CPD) (Fig. 1B) as described by Nollet *et al.* (2000). However, AXPC lacks a membrane-proximal conserved domain (MPCD) and a catenin-binding domain (CBD), both of which play a crucial role in lateral clustering, cytoskeletal interactions, and adhesion of cadherins (Stappert *et al.*, 1994; Yap *et al.*, 1998). A gene with this domain structure (6 ECs and 1 MPED) has never been reported in *Xenopus*. Full-length human protocadherin-1 (H-PCD1, Sano *et al.*, 1993) has a 72% amino acid identity with full-length *Xenopus* AXPC, and was therefore regarded as the human homologue of AXPC (Fig. 1C). We also isolated a partial mouse homologue of *AXPC* (*M-AXPC*) from embryonic day 7 mouse cDNA libraries by PCR using primers designed to the mouse EST database sequence. *M-AXPC* encodes a partial EC4, full EC5, and partial EC6 domain (663 bp, 221 aa; Fig. 1B, 7). Amino acid identities of these regions of *Xenopus*–mouse, *Xenopus*–human, and mouse–human were 75, 74, and 96%, respectively (Fig. 1C). The identities of 72% in the full-length gene (*Xenopus* and human) and more than 74% in the partial sequence (*Xenopus*–human–mouse), indicating that *AXPC* must be a conserved gene throughout vertebrate evolution.

AXPC Expression and Morphogenesis

Temporal expression analyzed by RT-PCR (Fig. 2A) revealed a low abundance of transcripts from stage 1 to approximately stage 9. After the start of gastrulation (stage

10), the level of *AXPC* expression increased by one order of magnitude (Fig. 2A). *AXPC* was also expressed abundantly in gastrula (stages 10–12 in Fig. 2A), neurula (stages 14 and 17 in Fig. 2A), and tailbud stages (stages 20, 25, 30, and 38 in Fig. 2A). In addition, further RT-PCR analyses at gastrula (stage 10) showed that *AXPC* was expressed in the dorsal marginal zone but not in the ventral or lateral marginal zones (Fig. 2B). Thus, although we could not detect expression by *in situ* hybridization at early gastrula (data not shown), RT-PCR showed that AXPC gene expression must be dorsal-specific at the beginning of zygotic expression. RT-PCR provided us with a semiquantitative measure of the AXPC expression, and whole-mount *in situ* hybridization detected a weak but clear specific expression of *AXPC* in the notochord at stage 14 (data not shown) and strong expression at stage 18 (Figs. 2C and 2C′). At the late tailbud stage (stage 28), *AXPC* was expressed strongly in the pronephros and also detectable in the somites, heart (Fig. 2D), optic vesicles, otic vesicles, and some parts of the anterior central nervous system (Fig. 2D′).

RT-PCR analyses using the potent mesoderm inducer activin and intact animal caps or dissociated animal cap cells revealed a more specific *AXPC* expression pattern in the early stages (Figs. 2E and 2F). Activin used at 50–100 ng/ml is known to strongly induce notochord tissue (Asashima *et al.*, 1990, 1991, 1999; Green *et al.*, 1990) as well as many mesodermal and neural tissues. When we cut animal caps at stage 9, treated them with 50–100 ng/ml of activin for 1 h, and cultured them for 5 h, *AXPC* expressions reached their maximum (Fig. 2E). Since this *in vitro* condition of 6 h culturing after stage 9 is representive of the late gastrula (stage 12–12.5) *in vivo*, *AXPC* must be expressed in prenotochord cells in stage 12–12.5 embryos. When dissociated animal cap cells are treated with activin for 1 h, washed, reaggregated, and then cultured for 3 days (Fig. 3A, Method 1), the reaggregates differentiate into notochord only with vacuole structure of the feature as notochord (Fig. 3B, a) (Kuroda *et al.*, 1999). Thus, 0- to 5-h cultured reaggregates after 1 h of treatment with 1 ng/ml are thought to mimic the presumptive notochord. It is not known why the activin response of dissociated animal cap cells is much higher than that of intact animal cap cells. One reason may be that dissociated cells have more free receptors to bind activin than intact animal caps. The other may be that inhibitors, such as activin (Tanegashima *et al.*, 2000) and BMP4 (Sasai *et al.*, 1995; Piccolo *et al.*, 1996) are lost when animal cap cells are dissociated. We detected high expression of the transcripts in reaggregates derived from cells treated for 1 h and cultured for 5 h in this way (Fig. 2F, lane 2). We could not detect *AXPC* expression in the conditions designed to induce atypical epidermis (Fig. 2F, lane 1) and endodermal yolk-rich tissue (Fig. 2F, lanes 3 and 4). These results also strongly indicate that *AXPC* is expressed in the prenotochord cells. To determine when the expression of *AXPC* is initiated by activin, cells were cultured in 1 ng/ml activin for up to 2 h. *AXPC* expression was detected from 30 min of treatment (Fig. 2F, lane 6), suggesting that *AXPC* was not only downstream of activin induction but also must be expressed very early in the prenotochord region (Fig. 2E). Taken together with the *in situ* hybridization and RT-PCR data, these results indicate that *AXPC* is expressed

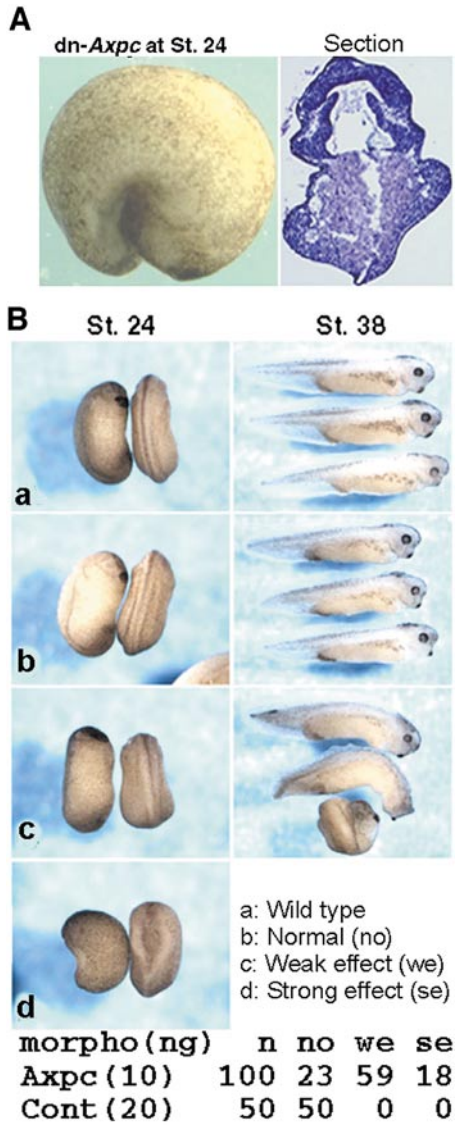


FIG. 5. Loss-of-function experiment using dominant negative *DN-AXPC* mRNA or morpholino antisense oligo. (A) Microinjection of 1 ng of *DN-AXPC* mRNA resulted in a rounded outward form (left, 25.0%, $n = 24$) and loss of normal notochord formation (right, 16.7%, $n = 24$). (B) (a) Control wild-type embryos. (b–d) 10 ng of morpholino oligo-injected embryos. Twenty-three percent of injected embryos developed normally (b). Fifty-nine percent of injected embryos produced abnormal axis formation (c). Eighteen percent of embryos produced the dominant negative-like effects, unclosed neural tissue, and died by stage 30 (d). All embryos injected with 20 ng of control morpholino developed normally (see table).

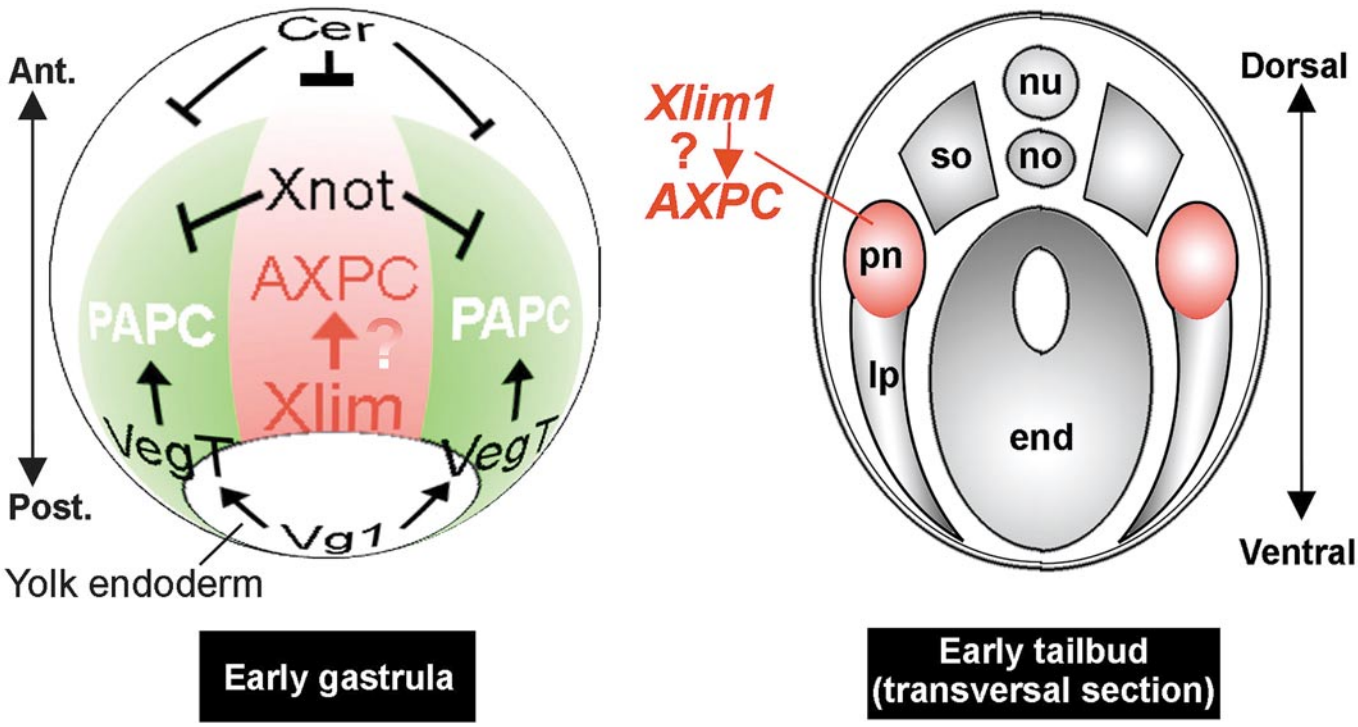


FIG. 6. Model summarizing spatial regulation patterns in early gastrula (left figure) and early tailbud stage (right figure). The model of early gastrula is shown in the dorsal view and above-below indicates anterior-posterior in future morphogenesis. Small circle indicates the yolk endoderm. Green region indicates the PAPC expressed region, and the red region indicates the *Xlim1* and *Xnot* expressed region. The model of late stage is shown in the transversal view and above–below indicates dorsal–ventral. nu, neural tube; no, notochord; so, somite; pn, pronephros tube; lp, lateral plate; end, endoderm. Red region indicates the *Xlim1* expressed region (Chan *et al.*, 2000).

in prenotochord at gastrula, notochord at neurula, and in several tissue types at the late tailbud stage.

AXPC Mediates Cell Sorting in Vitro

Functional analyses of AXPC were performed by using two mutant constructs; a membrane-tethered form, which lacks the CPD (Fig. 1B, 4, MT-AXPC), and a dominant-negative secreted form (Fig. 1B, 5, DN-AXPC). A modified cell dissociation and reaggregation assay was used to determine whether AXPC mediates cell adhesion (Fig. 3A, Method 2). From preliminary experiments, we knew that full-length AXPC did not work well as an adhesive mediator (data not shown). This was not unexpected because PAPC is also an adhesive molecule, and if it has the cytoplasmic region intact, sorting activity goes down (Kim *et al.*, 1998). The sorting activity of AXPC or PAPC, like protocadherin molecules, may be negatively regulated by the cytoplasmic region under normal conditions. Therefore, we constructed MT-AXPC to study the sorting activity of the AXPC molecule alone. Within reaggregates, cells injected with either lineage tracer alone were evenly interspersed (Fig. 3B, b), but if red cells were treated with 1 ng/ml of activin to induce notochord, red cells associated together in the central region of the reaggregates during the 10-h culture (Fig. 3B, c; Kuroda *et al.*, 1999). The yellow region indicates mixed red- and green-labeled cells.

The mutant *AXPC* constructs were also tested for cell adhesion activity by using the method described in Fig. 3A, Method 2. Cells coinjected with *MT-AXPC* mRNA and TRDA sorted into reaggregates after 10 h of incubation (Fig. 3C, a), as did cells treated with 1 ng/ml activin (Fig. 3B, c). These aggregating cells could be regarded as having potent adhesive properties. However, neither cells injected with *MT-AXPC* mRNA alone nor those treated with activin showed sorting (Fig. 3C, b). These results suggest that MT-AXPC can induce the same adhesive properties in animal cap cells as in presumptive notochord cells. The EC domain of *PAPC* acts as an efficient dominant-negative inhibitor of endogenous *PAPC* (Kim *et al.*, 1998). This property was exploited to block AXPC function in light of its likely homophilic adhesive properties (Kim *et al.*, 1998; Brieher *et al.*, 1996). Overexpression of *DN-AXPC* inhibited sorting induced by MT-AXPC or activin in a specific manner (Fig. 3C, c, d). This dominant-negative form was effective only against AXPC because DN-AXPC did not interfere with the sorting induced by FL-PAPC (Fig. 3C, g, h). From these results, we concluded that AXPC could

mediate notochord-specific cell adhesion via its extracellular domains.

Gain-of-Function Experiments in Vivo

To investigate whether and how AXPC mediates cell sorting *in vivo*, two different types of *AXPC* mRNA, *MT-AXPC* and *DN-AXPC*, were injected into the two dorsal blastomeres at the four-cell stage. *Xnot* is a marker gene expressed in the notochord region at neurula stage (Fig. 4A, a, a′; Gont *et al.*, 1996). We detected much wider expression of *Xnot* in the *MT-AXPC*-injected embryos (Fig. 4A, b, b′). In *Xenopus*, the notochord cells have already separated from the surrounding somitic mesoderm tissue by stage 16 (Fig. 4B, a, a′). However, embryos injected with 500 pg of *MT-AXPC* mRNA showed no evidence of a notochord distinct from the surrounding somitic mesoderm tissue at stage 16 (Fig. 4B, b, b′). To address the fate of the notochord cells, somitic mesoderm cells, and neural cells, we conducted *in situ* hybridization on sections of the injected embryos to detect expression of the posterior neural marker, *HoxD1* (Kolm and Sive, 1992), notochord marker, *Chordin* (Sasai *et al.*, 1994), and paraxial mesoderm marker, *MyoD* (Harvey, 1992). *HoxD1* expression was weaker than in the controls and located along the Chordin-expressing region (Fig. 4B, c, c′, d, d′). *MyoD* was expressed at control levels but was located more in paraxial regions (Fig. 4B, e, e′). It may be interpreted from these results that changes in the location of notochord cells induced by the *MT-AXPC* caused the abnormal formation of surrounding tissues. More than half of the embryos injected with *MT-AXPC* did not develop past stage 20, although some did continue development after that stage. Figure 4C (left) shows a control embryo injected with FDA lineage tracer into two dorsal blastomeres at the four-cell stage; clear spaces can be seen between the notochord and other tissues. Embryos injected with *MT-AXPC* and the FDA tracer showed no obvious separation of notochord from surrounding tissues (Fig. 4C, right). These data directly indicate that *Xenopus* embryogenesis is strongly affected by the extracellular domain of AXPC.

Loss-of-Function Experiments in Vivo

The failure of the notochord to correctly separate from mesodermal cells may be due to a marked change in cell adhesion properties resulting from the experiments using *MT-AXPC*. As mentioned, *DN-AXPC* was used in similar experiments to examine the loss-of-function effects *in vivo*. Some embryos injected with 1 ng of *DN-AXPC* mRNA appeared to be malformed and shortened after stage 14, and sections showed these embryos to lack normal notochord formation (Fig. 5A). The frequency of this inhibited phenotype was 25% (6/24). We could not show rescue experiments because coinjections of *MT-AXPC* (500 pg) and *DN-AXPC* (1 ng) induced an exogastrulation phenotype. Exogastrulation is the typical phenotype resulting from an excess of injected mRNA injection. An antisense approach to inject morpholino oligo and inhibit the translation of the *DN-AXPC* (Heasman *et al.*, 2000) supported the data of the DN-AXPC-injected phenotypes (Fig. 5B). The phenotypic abnormalities observed in the morpholino oligo-injected embryos were milder than those of *DN-AXPC*-injected embryos, but the efficiency of inhibition was increased. More than half of the embryos did not show straight axis formation (77%, Fig. 5B, c, d), and 18% showed an unclosed neural tube and died by stage 30 (Fig. 5B, d). The injection volume of 10 ng is not toxic for normal development as 20 ng was injected into control morpholino-injected embryos and these developed normally (Fig. 5B, table). These results suggest that loss-of-function of the isolated AXPC can cause abnormal axis formation and neural induction, but that the level of this abnormality was greater in some of the *DN-AXPC*-injected embryos. It is possible that genes homologous to *AXPC are* expressed at the protein level, but are not inhibited by the morpholino oligo at the translational level. This is supported by the cell dissociation and reaggregation assays, where morpholino oligos inhibited sorting in response to MT-AXPC but not activin treatment (Fig. 3C, e, f). AXPC may be one of the adhesive molecules working downstream of activin or in the prenotochord region. Since prenotochord cell sorting is an important phenomenon, it is not surprising that many genes with similar functions exist in the prenotochord region.

DISCUSSION

Xenopus, Humans, and Mice Have the AXPC Gene

In this study, we isolated the full-length *Xenopus AXPC* gene and a partial fragment of the mouse *AXPC* gene, and identified human *protocadherin-1* from sequenced databases as a homologue of *AXPC*. The sequence identities between *Xenopus*–mouse, *Xenopus*–human, and mouse–human *AXPC* were 75, 74, and 96%, respectively (Fig. 1C). These scores are reasonable in terms of the evolutionary distance between amphibians and mammals for these genes to be homologues. The existence of high sequence identities for *AXPC* between *Xenopus* and other vertebrates suggests that this molecule may be essential for notochord formation in early vertebrate development. As to whether other animals also have this gene, the *paraxial protocadherin* gene is expressed in the somite region next to the notochord in *Xenopus* (Kim *et al.*, 1998, 2000), zebrafish (Yamamoto *et al.*, 1998), and mice (Yamamoto *et al.*, 2000); therefore zebrafish must have the *axial protocadherin* gene. We propose that all vertebrates may use this gene for notochord formation, and isolating the homologues of this gene is an important focus of future research.

Sorting Activity by AXPC

We demonstrated sorting to occur in reaggregates cultured for 10 h with *MT-AXPC* mRNA-expressing cells. This time course is representative because the sorting event

caused by activin also happens at approximately 10 h after treatment (Kuroda *et al.*, 1999). Interestingly, although the sorting activity mediated by activin is continuously maintained, the activity induced by *MT-AXPC* later loses effectiveness, and cells cultured for more than 20 h did not exhibit potent sorting activity (data not shown). These findings indicate two possibilities. One is that there is no *AXPC* mRNA left in reaggregates cultured for 20 h with the *MT-AXPC*-expressing cells, and *AXPC* is unlikely to induce additional adhesive molecules. Initially, we expected that *AXPC* would have some additional activities, as paraxial protocadherin can induce the paraxial mesoderm marker, *myoD*, and promote convergent-extension movements and cell shape changes (Kim *et al.*, 1998). However, *AXPC* did not show such additional activity. Therefore, all mRNA must be translated and degraded between 10 and 20 h of culture. The other possibility is that cell–cell binding in the prenotochord requires another similar type of adhesive molecule. We showed that *DN-AXPC* can readily interfere with the sorting activity mediated by both *MT-AXPC* and activin (Fig. 3C, c, d), as well as *in vivo* (Fig. 5A). However, morpholino oligos of AXPC did not inhibit the activity mediated by activin (Fig. 3C, e, f) and the effects due to the morpholino oligo were weaker than those seen with the dominant negative *AXPC* (Fig. 5). Consistent with our results, mouse dominant negative paraxial protocadherin is effective, but the null mutant of paraxial protocadherin has no effect on mouse development (Yamamoto *et al.*, 2000). These molecules may have similar genes playing the same role in the expressed region. If this is true, the mouse *AXPC* knock out mutant might also be ineffective as a *PAPC* null mutant.

Hypothesis of Early Morphogenesis Using the Protocadherin Model

Zygotic *AXPC* expression starts at stage 10 (Fig. 2A) and we detected localized expression in the dorsal marginal zone at stage 10 (Fig. 2B), prenotochord region at gastrula (Figs. 2E and 2F), notochord region at stage 18 (Figs. 2C, C′), and pronephros region at stage 26 (Fig. 2D). This expression pattern is quite similar to the LIM class homeobox gene *Xlim-1*. *Xlim-1* is expressed in *Xenopus* embryos in the dorsal lip at stage 10, notochord at neurula, and the pronephros and certain cells of the central nervous system at tailbud stage (Taira *et al.*, 1992, 1994a,b; Chan *et al.*, 2000). In preliminary experiments using the animal cap assay, we have shown that some organizer genes, including *Xlim1*, induce *AXPC* overexpression. Thus, *Xlim1* is one of the strongest candidates to induce *AXPC in vivo* and thereby regulate cell sorting activity. In fact, *AXPC* and *Xlim1* expression in the notochord and pronephros must require tight regulation of sorting activity to form the tissue structure because prenotochord cells must separate from surrounding mesodermal cells, and pronephros tube must separate from the lateral plate cells. Thus, the relationship between *Xlim1* and *AXPC* is clearly an important subject for future studies.

At early gastrula, *PAPC* is strongly expressed while *AXPC* is weakly expressed in the dorsal marginal zone (Kim *et al.*, 1998, Fig. 2B). By midgastrula, *PAPC* expression is found in the mesodermal mantle but is repressed in the future notochord region, where *Xlim1* is strongly expressed. *PAPC* expression is induced by *Vg1* (Zhang and King, 1996) and *VegT* (Zhang *et al.*, 1998), but inhibited by *Xnot* (Kim *et al.*, 1998), while *Xlim1* is probably upstream of *AXPC*. *Cerberus* encodes a secreted factor expressed in anterior endomesoderm that functions to inhibit the Wnt, Nodal, and BMP signals that promote head formation (Bouwmeester *et al.*, 1996, Picollo *et al.*, 1999). Taken together, these results suggest that multiple signaling pathways regulate the spatial pattern of expression of the *AXPC* and *PAPC* structural genes in the early gastrulating mesoderm. In early-stage embryos, *PAPC* is expressed in the dorsal lip region, but moves laterally due to inhibition of *Xnot*. *Xlim1*, which is coexpressed with *Xnot*, induces *AXPC* expression, perhaps initiating AXPC to induce the axial mesodermal region via its potent sorting activity (Fig. 6). Alternatively, *Xenopus* may utilize the *AXPC* sorting activity to induce pronephros formation along with other structures (Fig. 6). The *AXPC* isolated here will allow us to test whether regulatory cell interactions, such as those highlighted in this study, are conserved across the other tissues in *Xenopus*, and during the developments of other animals.

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