

Two classic cadherin-related molecules with no cadherin extracellular repeats in the cephalochordate amphioxus: distinct adhesive specificities and possible involvement in the development of multicell-layered structures

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Summary

We previously reported the existence of Bb-cadherin, a molecule related to classic cadherin, in the cephalochordate amphioxus (*Branchiostoma belcheri*). The structure of Bb-cadherin is unique in that it lacks the cadherin extracellular repeats, although its cytoplasmic domain shows close similarities to those of typical classic cadherins. The extracellular region of Bb-cadherin consists of laminin globular domains and a cysteine-rich EGF-like domain that are similar to domains in nonchordate classic cadherins. In this study, we identified a second amphioxus cadherin. It was designated Bb2-cadherin (Bb2C) while the previously reported cadherin has been renamed Bb1-cadherin (Bb1C). Bb2C is very similar to Bb1C in its overall structure and amino acid sequence. Genomic BLAST searches and phylogenetic analyses suggested that these two amphioxus genes have been generated through a gene duplication that occurred after separation of the cephalochordates from the other animals. They also bear

distinct adhesive specificities. Immunohistochemical analyses showed that Bb1C and Bb2C, together with β -catenin, appear to function as adherens junction constituents in the epithelia of different germ layers of the amphioxus embryo. Differential expression of the two cadherins was also observed in the developing, multicell-layered notochord. These observations suggest that, despite their unique structures, the functions and developmental roles of Bb1C and Bb2C are comparable to those of the classic cadherins characterized to date in other animal groups, such as the vertebrate E- and N-cadherins and the *Drosophila* DE- and DN-cadherins. The possible involvement of Bb1C and Bb2C in the development of multicell-layered structures characteristic of the cephalochordate body plan is presented.

Key words: Cadherin, β -catenin, Adherens junction, Epithelium, Cephalochordate, Notochord

Introduction

Molecular systems involved in cell-cell adhesion and recognition are thought to have gained complexity during the evolutionary process that gave rise to vertebrates and to have contributed to shaping their complex bodies. One such molecular system is the classic cadherin-mediated cell-cell adhesion system. The classic cadherins constitute a molecular family of Ca^{2+} -dependent homophilic cell adhesion molecules that belongs to the cadherin superfamily, which is characterized by cadherin extracellular repeats (ECs) separated by Ca^{2+} binding pockets (Yagi and Takeichi, 2000; Nollet et al., 2000; Hirano et al., 2003). The classic cadherin family members are single-pass transmembrane proteins with a highly conserved cytoplasmic domain (CP) to which β -catenin and p120 bind (Gumbiner, 2000). The extracellular regions of the known vertebrate classic cadherins consist of five ECs. However, non-vertebrate members of the classic cadherin family do not necessarily bear this domain organization (Fig.

1) (Oda et al., 2002). In this study, the domain organization that is typical of the vertebrate classic cadherins is for convenience referred to as the vertebrate (V) form.

In vertebrates, there are a large number of subtypes of V-form cadherins that are expressed in different combinations in various tissues or cell populations (Takeichi, 1995; Hirano et al., 2003). The N-terminal ECs of the V-form cadherins confer distinct specificities of homophilic binding (Nose et al., 1990). Some of these cadherins are required in epithelialization of the cell populations and/or maintenance of the integrity of the epithelia (Larue et al., 1994; Radice et al., 1997; Masai et al., 2003). This type of function is related to the formation of adherens junctions, which are multiprotein complexes containing the cadherins, β -catenin and α -catenin (Gumbiner, 2000). The use of different cadherin subtypes in neighboring cell populations is thought to prevent the cells from intermingling (Takeichi, 1988; Inoue et al., 2001; Masai et al., 2003). The adhesive complexity characteristic of the cadherins

may be required to shape a complex body that is composed of many different cell populations.

The V-form cadherin genes are classified into type I and type II on the basis of phylogenetic differences (Takeichi, 1995). The type I cadherins are expressed rather ubiquitously, whereas the type II cadherins are expressed in more restricted cell populations. The major vertebrate type I cadherins are so-called E- and N-cadherins. In the mouse, for example, E-cadherin is expressed in the ectoderm and endoderm of the early embryo and later in most epithelial tissues, while N-cadherin is expressed in the mesoderm of the early embryo and later in neural tissues (Takeichi, 1988). These cadherins are required in major morphogenetic processes including blastocyst formation, somitogenesis and neural tube formation (Larue et al., 1994; Radice et al., 1997). The developmental roles of orthologous cadherin subtypes tend to be conserved between different groups of vertebrates. The *Drosophila* DE- and DN-cadherins also show expression profiles similar to those of the vertebrate E- and N-cadherins, although these cadherins do not have the V-form organization (Oda et al., 1994; Iwai et al., 1997).

In the urochordate *Ciona intestinalis* genome, only two classic cadherin genes are present, and these correspond to the V-form type I and type II (Sasakura et al., 2003). However, V-form cadherin genes have not been found in animals other than vertebrates and urochordates. It thus appears that the V-form cadherin genes may be specific to the chordate lineage and that these genes multiplied markedly after the vertebrate-urochordate split through a series of gene duplications and divergences.

The closest relatives of vertebrates are widely believed to be cephalochordates, while urochordates are considered as the most basal chordates (Maisey, 1986; Schaeffer, 1987; Wada and Satoh, 1994; Turbeville et al., 1994). Comparative developmental studies between these chordate groups and paleontological studies have given insights into the evolutionary history from invertebrates to vertebrates (Holland and Chen, 2001; Wada and Satoh, 2001). Assuming that these conventional phylogenetic relationships are true, it may be expected that V-form cadherin genes are present in cephalochordates and that they are used in major morphogenetic events in these organisms. Unexpectedly, however, we previously identified a classic cadherin-related molecule in the cephalochordate amphioxus, *Branchiostoma belcheri*, whose extracellular organization differed completely from that of the V-form cadherins. This cadherin was named Bb-cadherin and it contains no ECs, even though its cytoplasmic region shows close similarities to those of known classic cadherin family members (Oda et al., 2002). Instead, its extracellular region consists of two laminin globular domains (LGs) and one cysteine-rich EGF-like domain (CE) that partially matches with the sequence of the primitive classic cadherin domain (PCCD) complex that occurs in all known nonchordate members of the classic cadherin family (Fig. 1) (Oda et al., 2002). Despite its structural uniqueness, Bb-cadherin does function as an adhesion molecule (although its activity is Ca²⁺ independent) and it localizes to adherens junctions in the ectodermal tissues of the amphioxus embryo (Oda et al., 2002). These findings raised the question of whether cephalochordates have another member(s) of the classic cadherin family that, unlike Bb-cadherin, may bear the V-form organization.

In this study, we identified a second cadherin in *B. belcheri*.

This cadherin was designated as Bb2-cadherin (Bb2C) and the previously reported Bb-cadherin was renamed Bb1-cadherin (Bb1C). Bb2C was very similar to Bb1C in its domain organization. Sequence analyses suggested that the Bb1C and Bb2C genes have been generated through a gene duplication that occurred after separation of the cephalochordates from the other animals. Cell aggregation assays and immunohistochemical analyses suggested that, despite their unique structures, the functions and developmental roles of Bb1C and Bb2C are comparable to those of the classic cadherins characterized to date in other animal groups, such as the vertebrate E- and N-cadherins and the *Drosophila* DE- and DN-cadherins. The possible involvement of Bb1C and Bb2C in the development of multicell-layered structures characteristic of the cephalochordate body plan is presented.

Materials and Methods

Animals

Embryos and larvae of the Chinese amphioxus species *B. belcheri tsingtauense* were collected at Qingdao, China in 2001–2002. Embryos and larvae were staged according to Hirakow and Kajita (Hirakow and Kajita, 1991; Hirakow and Kajita, 1994).

cDNA cloning

A *B. belcheri* gastrula λZAP II cDNA library made by K. Yasui (Kumamoto Univ., Japan), H. Saiga (Tokyo Metropolitan Univ., Japan), P. J. Zhang (Institute of Oceanology, China) and Y. Wang (Institute of Oceanology, China) was used. Degenerate primers used for polymerase chain reaction (PCR) amplification were as follows:

DQ1, 5′ GAI(inosine)CA(A/G)(A/G)A(C/T)T(A/T)(C/T)GA-(C/T)TA(C/T)(C/T)T 3′ (for amino acid sequence (D/E)Q(D/N)(Y/F)DYI);

KL2, 5′ CC(A/G)TACAT(A/G)TCIGCIA(A/G)(C/T)TT 3′ (for amino acid sequence KLADMYG);

RM1, 5′ GNATGGA(A/G)GA(A/G)AT(A/C/T)GTNGA 3′ (for amino acid sequence RMEEIVE);

QD2, 5′ A(A/G)NG(G/T)(C/T)TT(C/T)TT(A/G)TA(A/G)-TC(C/T)TG 3′ (for amino acid sequence (QDYKKR(L/I)).

DQ1 and KL2 correspond to the amino acid (aa)768–774 and aa783–789 sites of Bb-cadherin, and RM1 and QD2 correspond to the aa565–571 and aa668–674 sites of mouse β-catenin. Using these primers, short fragments of classic cadherin and β-catenin cDNAs were amplified by PCR from the *B. belcheri* gastrula cDNA library. These cDNAs were cloned into the plasmid vector pCRII (Invitrogen) and sequenced. Of the classic cadherin clones, two similar but distinct sequences of 25 bp were found. One matched perfectly with the previously described Bb-cadherin cDNA but the other contained 4 bases that did not match with the Bb-cadherin cDNA. A digoxigenin-labeled DNA probe for this novel sequence was then made using a PCR DIG Probe Synthesis Kit (Roche) and this was employed to screen the *B. belcheri* gastrula cDNA library. A cDNA clone with homology to the classic cadherin CP was obtained. The complete length of the cDNA was sequenced and its open reading frame was determined. A cDNA clone for β-catenin was also isolated from the gastrula cDNA library. The nucleotide sequences of the Bb2-cadherin and Bbβ-catenin cDNAs are available from the DNA data bank of Japan (the accession numbers are AB120427 and AB120428).

Molecular phylogenetic analyses

The amino acid sequences of the classic cadherin CPs were aligned manually, and 106 amino acid sites were selected and used to construct a phylogenetic tree by the neighbor joining method

(Saitou and Nei, 1987) using PHYLIP (Felsenstein, 1993). For the phylogenetic analysis of β -catenin/plakoglobin/Armadillo, 541 amino acid sites corresponding to the aa149-435, aa442-578 and aa593-709 regions of Bb. β -catenin were used. Confidence in the phylogenies was assessed by bootstrap resampling of the data sets.

Antibody production and immunohistochemistry

A PCR-amplified fragment corresponding to the 533-amino acid extracellular region (aa32-564) of Bb2C was subcloned into the *Bam*HI site of pMAL-c2 (New England Biolabs) and into the *Bam*HI site of pGEX-4T-1 (Amersham Pharmacia). *E. coli* (BL21(DE)) was transformed with these plasmids to express the fusion proteins, which were named MAL-Bb2C and GST-Bb2C. MAL-Bb2C was separated from bacterial proteins by SDS-PAGE, electroeluted from the gel, and used to immunize two guinea pigs. On western blots, both sera specifically reacted with a fusion protein of Bb2C and GFP that was expressed in *Drosophila* S2 cells. An aliquot of one of the sera was purified through its affinity to GST-Bb2C and used at a dilution of 1:20 in the western blotting experiments shown here. For immunohistochemistry, the original antiserum was used at a dilution of 1:200 since the affinity-purified antibody did not yield sufficiently strong signals.

To detect Bb. β -catenin in embryos and larvae, a commercially available rabbit antiserum raised against a synthetic peptide (PGDSNQLAWFDL) that corresponds to the C-terminal site of human and mouse β -catenin (aa768-781) (C2206; Sigma) was used. This sequence resembles the corresponding aa847-860 C-terminal sequence of Bb. β -catenin (GGDNNQLAWFDL). The antiserum was confirmed to react with a bacterially expressed GST fusion protein that contains the Bb. β -catenin C-terminal site but not with intact GST.

For immunohistochemistry, amphioxus embryos and larvae were fixed with 3.7% formaldehyde in 0.5 M NaCl, 0.1 M HEPES (pH 7.5). Fixed samples were kept in 100% ethanol at -20°C until use. The samples were rehydrated and blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20, followed by incubation with the primary antibody. The rat antiserum to Bb1-cadherin (Oda et al., 2002) was used at a dilution of 1:200. The species-specific secondary antibodies that were used were as follows: donkey anti-rat IgG labeled with Alexa Fluor 488 (Molecular Probes), donkey anti-guinea pig IgG labeled with Cy3 (Chemicon) and donkey anti-rabbit IgG labeled with Cy5 (Chemicon). All of these antibodies were used at a dilution of 1:200. For DNA staining, DAPI (Sigma) was used. For simultaneous staining with multiple antibodies, potential cross reactions of the secondary antibodies were ruled out. The stained samples were examined with a Zeiss Axiophoto II microscope equipped with a Bio-Rad laser confocal system (MRC1024) or with an Olympus IX71 microscope equipped with a cooled CCD camera (CoolSNAP HQ, Roper Scientific) controlled by MetaMorph software (Universal Imaging Co.).

Transfection and cell aggregation assays

The Armadillo region of pCaSpeR-ubi-Arm-GFP (Oda et al., 2002) was replaced by a PCR-amplified DNA fragment corresponding to the coding region of Bb2C cDNA to produce pCaSpeR-ubi-Bb2C-GFP. This plasmid was used to express a fusion of Bb2C and GFP in *Drosophila* S2 cells by transfection. To express a fusion of Bb1C and GFP, pCaSpeR-ubi-BbC-GFP was used (Oda et al., 2002).

For the transfection, the FuGENE6 transfection reagent (Roche) was used. Cell aggregation assays were performed as described previously (Oda et al., 1994). For mixed cell aggregation assays, equal amounts of cells that had been separately transfected with pCaSpeR-ubi-Bb2C-GFP and with a mixture of pUAST-BbC and pWA-GAL4 (Oda et al., 1994) were mixed, and rotated at 150 rpm for 20 minutes, followed by fixation and immunostaining with anti-Bb1C antibody.

Results

Cloning and sequence analysis of Bb2-cadherin and Bb. β -catenin cDNAs

A cDNA clone encoding a second cadherin in *B. belcheri* was isolated (see Materials and Methods). Amino acid sequence prediction revealed that the cDNA encoded a polypeptide of 798 aa (comparable to 796 aa of Bb-cadherin) that contained a putative signal sequence, a transmembrane segment and a sequence homologous to the known classic cadherin CPs (Figs 1, 2). The amino acid sequence of this new cadherin was easily aligned to that of Bb-cadherin and no large gaps were observed (Fig. 2A). Bb-cadherin was then renamed Bb1-cadherin (Bb1C) and the new cadherin was designated as Bb2-cadherin (Bb2C). Bb2C showed 44% amino acid identity with Bb1C. The CPs of both Bb2C and Bb1C contained sequences that are highly similar to the p120-binding and β -catenin-binding sites that have been mapped in the vertebrate classic cadherins (Fig. 2B) (Thoreson et al., 2000; Stappert and Kemler, 1994). Domain searching with a PROSITE scanning tool, ScanProsite (<http://www.expasy.org/tools/scanprosite/>), identified two LG domains and a cysteine-rich segment containing EGF-like sequences in the extracellular region of Bb2C. Based on this result, the extracellular regions of Bb1C and Bb2C could be conveniently divided into three parts, namely, LG1, LG2 and CE, as shown in Fig. 1 and Fig. 2A. The sequences of the amphioxus cadherin LG2 and N-terminal half of the CE could be aligned with those of the nonchordate cadherin LG2 and CE3 as previously described (Oda et al., 2002). However, the amphioxus cadherin LG1 domain diverged highly from the nonchordate cadherin LGs in its amino acid sequence.

Next, we searched for genes that show significant amino acid similarities to Bb1C and Bb2C in the completed human (*Homo sapiens*), mouse (*Mus musculus*), puffer fish (*Fugu rubripes*) and ascidian (*Ciona intestinalis*) genomes. The amino acid

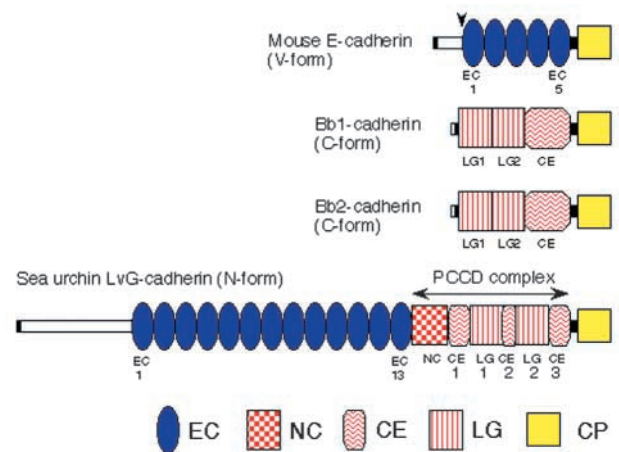


Fig. 1. Comparison of the primary structures of amphioxus Bb1C and Bb2C, mouse E-cadherin, and sea urchin LvG-cadherin. The signal sequences and transmembrane segments are indicated by filled black boxes. The arrowhead shown for mouse E-cadherin indicates a proteolytic cleavage site that is utilized in the maturation of this protein (Shirayoshi et al., 1986). The domain organization of each classic cadherin is designated in the parentheses. Domain abbreviations: EC, cadherin extracellular repeat; NC, nonchordate classic cadherin-specific domain; CE, cysteine-rich EGF-like domain; LG, laminin G-like domain; CP, cytoplasmic domain; PCCD complex, primitive classic cadherin domain complex.

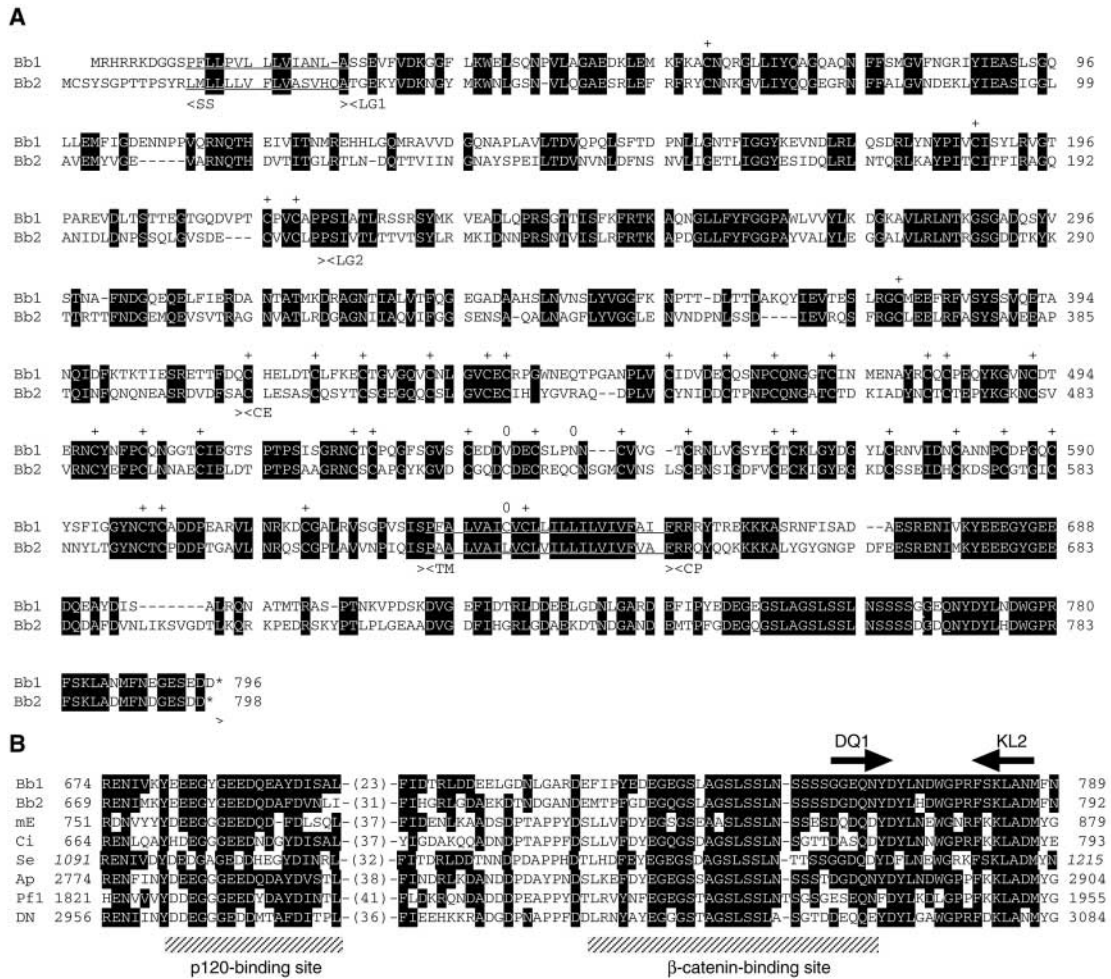


Fig. 2. Amino acid sequences of Bb1C and Bb2C. (A) Alignment of the entire amino acid sequences of Bb1C (Bb1) and Bb2C (Bb2). The putative signal sequences and transmembrane segments are underlined. The boundaries of the domains are also indicated. Cysteine residues conserved between Bb1C and Bb2C are indicated by the character '+' while those present in either Bb1C or Bb2C are indicated by the character '0'. (B) Alignment of the amino acid sequences of the CPs of Bb1C, Bb2C, mouse E-cadherin (mE), ascidian Ci-cadherin, oyster Se-cadherin, sea star Ap-cadherin, acorn worm Pf1-cadherin and *Drosophila* DN-cadherin. The abbreviations for these cadherins are shown in the legend of Fig. 3. Residues that are identical with those of Bb1C or Bb2C are highlighted. Numbers in parentheses represent the numbers of amino acid residues that were omitted at the indicated site. The p120- and β -catenin-binding sites are indicated. The position of the primers used for PCR amplification, DQ1 and KL2, is also indicated.

sequences of the LG1 and LG2 domains of Bb1C and Bb2C were subjected to Genomic BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/> or <http://aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=ciona4>). Only sequences that had weak expected values (E value >0.005) were detected. Further BLAST search analysis revealed that these detected genes were much more similar to reported non-cadherin proteins, including neurexins and the laminin α chain, than to Bb1C and Bb2C. Moreover, no candidate genes could be found in the fly (*Drosophila melanogaster*) or nematode (*Caenorhabditis elegans*) genome. Thus, the sequences of Bb1C and Bb2C appear to be unique to the cephalochordate lineage.

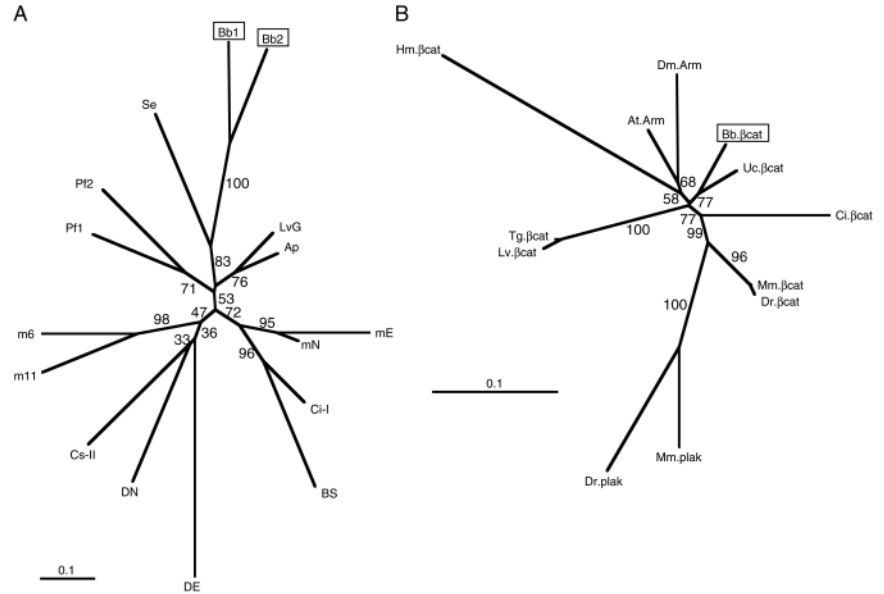
In addition to the Bb2C cDNA clone, a cDNA clone coding for *B. belcheri* β -catenin (Bb. β -catenin) was isolated (see Materials and Methods). The predicted amino acid sequence reveals an 860 aa polypeptide that bears 71% identity to mouse β -catenin, 75% identity to ascidian β -catenin, and 68% identity

to sea urchin β -catenin and *Drosophila* Armadillo. No marked difference in the overall structure was observed between Bb. β -catenin and the other β -catenins.

Molecular phylogenetic analyses of classic cadherins and β -catenin/plakoglobin/Armadillo

To understand the phylogenetic context of Bb1C and Bb2C, a molecular phylogenetic tree was constructed using the amino acid sequences of the known classic cadherin CPs (Fig. 3A). Bb1C and Bb2C were separated from the other classic cadherins by a bootstrap value of 100%, which is consistent with the differences these proteins show in the organization of their extracellular domains. Combined with the results of the genomic searches, this observation strongly suggests that the Bb1C and Bb2C genes have been generated through a gene duplication that occurred after separation of the cephalochordates from the other animals. The tree also

Fig. 3. Molecular phylogenetic trees of classic cadherin and β -catenin/plakoglobin/Armadillo generated by the neighbor joining method. Numbers indicate bootstrap values. (A) A tree constructed using the CPs of selected classic cadherin family members. Bb1, Bb1C (AB075366); Bb2, Bb2C (AB120427); DE, *Drosophila* DE-cadherin (BAA05942); DN, *Drosophila* DN-cadherin (T00021); LvG, sea urchin LvG-cadherin (U34823); Ap, sea star Ap-cadherin (AB075365); Pf1, acorn worm Pf1-cadherin (AB075368); Pf2, acorn worm Pf2-cadherin (AB075369); Se, oyster Se-cadherin (AB075367); BS, ascidian BS-cadherin (U61755); Ci-I, ascidian *Ciona intestinalis* type I cadherin (AB031540); Cs-II, ascidian *Ciona savignyi* type II cadherin (AB057736); mE, mouse E-cadherin (X06115); mN, mouse N-cadherin (M31131); m6, mouse cadherin 6 (NM_007666); m11, mouse cadherin 11 (D31963). (B) A tree constructed using 541 amino acid sites of β -catenin/plakoglobin/Armadillo. Bb. β cat, Bb. β -catenin (AB120428); Dm.Arm, *Drosophila melanogaster* Armadillo (P18824); At.Arm, spider *Achaearanea tepidariorum* Armadillo (AB120624); Hm. β cat, *Hydra magnipapillata* β -catenin (U36781); Uc. β cat, spoon worm *Urechis caupo* β -catenin (S33793); Tg. β cat, sea urchin *Tripneustes gratilla* β -catenin (P35223); Lv. β cat, sea urchin *Lytechinus variegatus* β -catenin (AAC06340); Ci. β cat, ascidian *Ciona intestinalis* β -catenin (BAA92185); Dr. β cat, fish *Danio rerio* β -catenin (NP_571134); Dr.plak, fish *Danio rerio* plakoglobin (NP_571252); Mm. β -cat, mouse *Mus musculus* β -catenin (NM_007614); Mm.plak, mouse *Mus musculus* plakoglobin (XP_126747).



supported the notion that Bb1C and Bb2C are more closely related to the nonchordate classic cadherins than to the vertebrate and urochordate V-form cadherins.

Another phylogenetic tree was constructed for β -catenin/plakoglobin/Armadillo. This tree supported the idea that Bb. β -catenin is more closely related to nonchordate β -catenin than to vertebrate β -catenin and plakoglobin and urochordate β -catenin (Fig. 3B). The two phylogenetic trees presented here are consistent with each other in that compared to the cephalochordate proteins, the urochordate proteins are more closely related to the vertebrate proteins.

Production of a Bb2C-specific polyclonal antibody

We previously described a rat polyclonal antibody raised against an extracellular portion of Bb1C (Oda et al., 2002). This antibody did not cross-react with a fusion protein of Bb2C and GFP (Bb2C-GFP), which was expressed in S2 cells by transient transfection (Fig. 4A). To enable double labeling of Bb1C and Bb2C, we produced a guinea pig polyclonal antibody against a similar extracellular portion of Bb2C (see Materials and Methods). This antibody reacted with Bb2C-GFP without cross-reacting with Bb1C-GFP on western blots (Fig. 4A). When amphioxus embryos were stained with the anti-Bb2C antibody, areas of cell-cell contact in endodermal tissues, in which Bb1C is not expressed (Oda et al., 2002), were observed (Fig. 4B). Pre-immune guinea pig sera did not yield such staining patterns (data not shown). The stainings were highly restricted to the apical portions of the lateral surfaces of the cells. The same areas

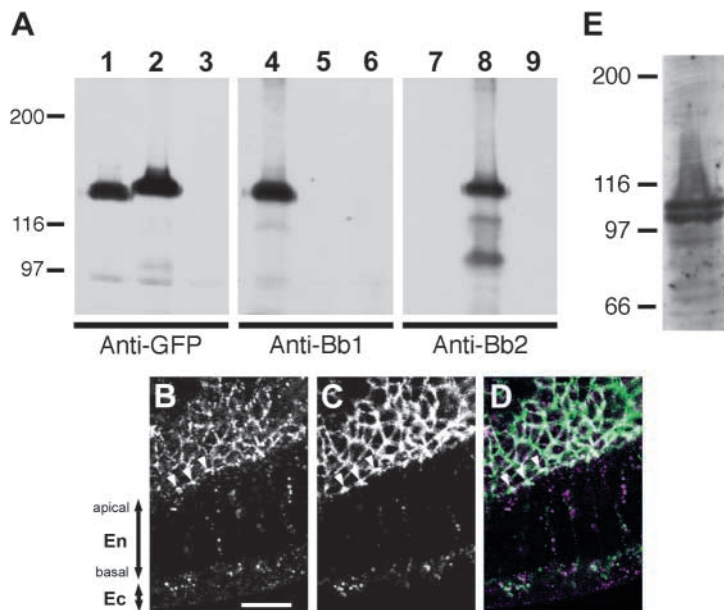


Fig. 4. The specificities of polyclonal antibodies to Bb1C, Bb2C and β -catenin. (A) Western blot analysis of S2 cells transiently transfected with plasmids for Bb1C-GFP (lanes 1, 4 and 7), Bb2C-GFP (lanes 2, 5 and 8) or no insert (lanes 3, 6 and 9). The same blot was repeatedly used for detection with polyclonal antibodies to GFP (lanes 1-3), Bb1C (lanes 4-6) and Bb2C (lanes 7-9). (B-D) Endodermal epithelium of an amphioxus stage N3 embryo double-stained with the anti-Bb2C (B) and anti- β -catenin (C) antibodies. (D) The two images were colored and merged (B in purple and C in green). The signals yielded by the two antibodies were coincidentally detected at the apical portions of the lateral cell surfaces as seen in white (arrowheads). En, endoderm; Ec, ectoderm. (E) Western blot analysis of amphioxus stage L1 larvae (18 hour) to indicate the specificity of the anti- β -catenin antibody. Two bands of about 100 and 106 kDa were detected.

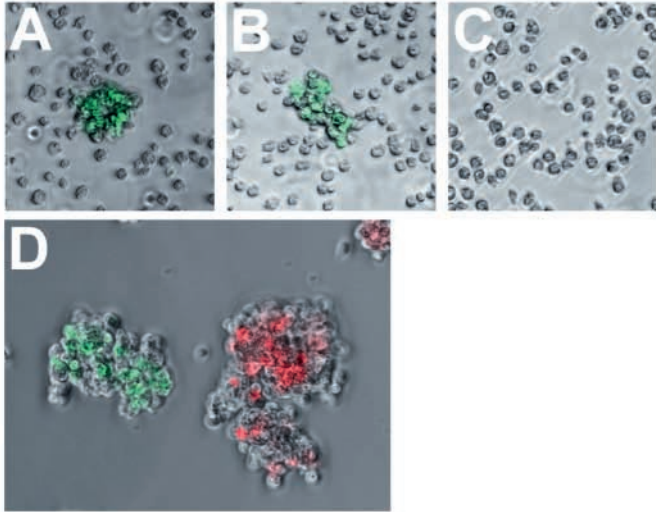


Fig. 5. Cell aggregation assays to test the adhesive specificities of Bb1C and Bb2C. (A-C) S2 cells transiently transfected with plasmids for Bb1C-GFP (A,B) and no insert (C) were tested for aggregation in the presence of 1 mM Ca^{2+} (A,C) or 1 mM EDTA (B). The cells expressing Bb2C-GFP were aggregated in a Ca^{2+} -independent manner. (D) S2 cells separately transfected with plasmids for Bb1C and Bb2C-GFP were mixed and tested for aggregation. The resulting aggregates were fixed and stained for Bb1C (red). The cells expressing Bb1C (red) and those expressing Bb2C-GFP (green) aggregated separately.

were also labeled with a commercially available rabbit polyclonal antibody to vertebrate β -catenin (Fig. 4B-D). This anti- β -catenin antibody reacts with the C-terminal site of bacterially expressed Bb. β -catenin (data not shown). Western blot analysis of amphioxus larva lysates revealed that the anti- β -catenin antibody recognizes two polypeptides of about 100 and 106 kDa (Fig. 4E), which are comparable to the deduced

molecular mass of Bb. β -catenin (94.3 kDa). In simultaneous western blot analysis, however, we failed to detect endogenous Bb1C and Bb2C with the polyclonal antibodies. This failure might be due to weak reactivities of the anti-BbC antibodies we used in relation to the anti- β -catenin antibody, or it may be because only small amounts of the BbC proteins are present in the tissues compared to Bb. β -catenin. Alternatively, it might be due to less efficient solubilization of the BbC proteins. The biochemical nature of endogenous Bb1C and Bb2C remains to be studied. Nevertheless, since the staining patterns yielded by the anti-Bb1C, anti-Bb2C and anti- β -catenin antibodies are very consistent with the established knowledge regarding classic cadherins and β -catenin, we believe that these antibodies faithfully visualized the endogenous proteins in at least the immunohistochemical assays.

Distinct adhesive specificities of Bb1C and Bb2C

Different subtypes of the vertebrate V-form cadherins show distinct adhesive specificities in cell aggregation assays, which show that the cells expressing the same cadherins will aggregate selectively with each other (Nose et al., 1988). Similar assays were conducted for Bb1C and Bb2C using transfected *Drosophila* S2 cells. Bb1C was previously shown to have a Ca^{2+} -independent cell-cell adhesion activity (Oda et al., 2002). To test the adhesive activity of Bb2C, Bb2C-GFP was expressed in S2 cells by transient transfection. When these cells were incubated with rotation for 20 minutes, aggregates formed in a Ca^{2+} -independent manner (Fig. 5A-C). When the cells expressing Bb2C-GFP and the cells expressing Bb1C (not fused to GFP) were mixed and allowed to aggregate, aggregates consisting of GFP-positive and GFP-negative cells were formed separately (Fig. 5D). The GFP-negative, but not GFP-positive, aggregates were stained with the anti-Bb1C antibody. These results suggest that Bb1C and Bb2C, like the vertebrate E- and N-cadherins, bear distinct specificities of homophilic binding, at least in vitro.

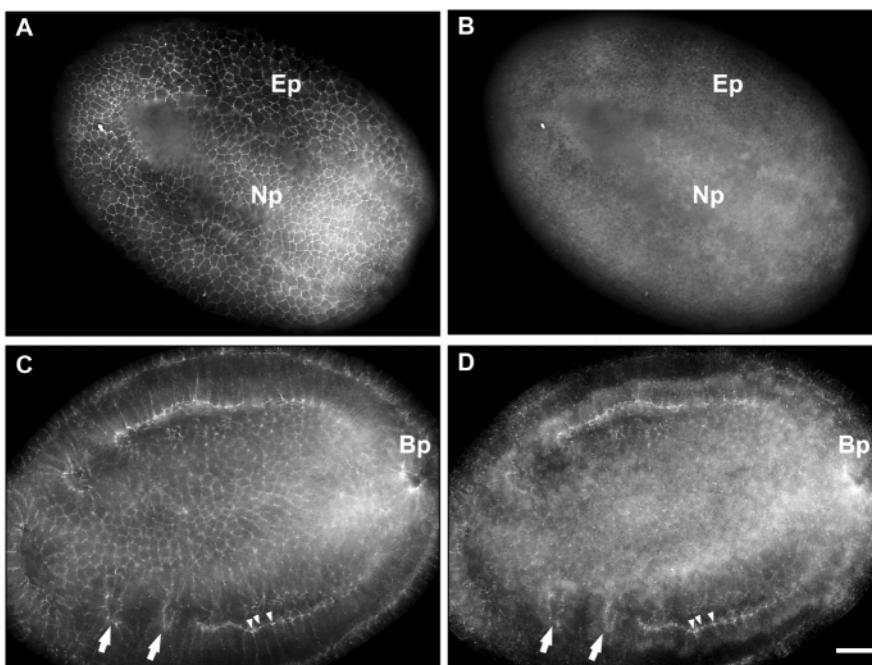


Fig. 6. Expression of Bb. β -catenin and Bb2C in the early neurula. Embryos were double-stained for Bb. β -catenin (A,C) and Bb2C (B,D). (A,B) Dorsal surface view of a stage N1 embryo at the same focal plane. Anterior is to the upper left. The epidermal ectoderm (Ep) has started to spread over the neural plate (Np). Staining for Bb2C yielded no specific signal in the epidermal ectoderm or the neural plate. (C,D) Internal view of a stage N1 embryo at the same focal plane. Anterior is to the lower left. The mesendodermal cell layer is undergoing somitogenesis (arrows). Since the observed embryo is compressed, the apical surfaces of some of the ventrally located, prospective endodermal cells are also in focus. Bb2C and Bb. β -catenins were detected at the apical zones of the lateral surfaces of the mesendodermal cells (arrowheads). The strongest signals were detected at the blastopore region (Bp). Scale bar: 20 μm .

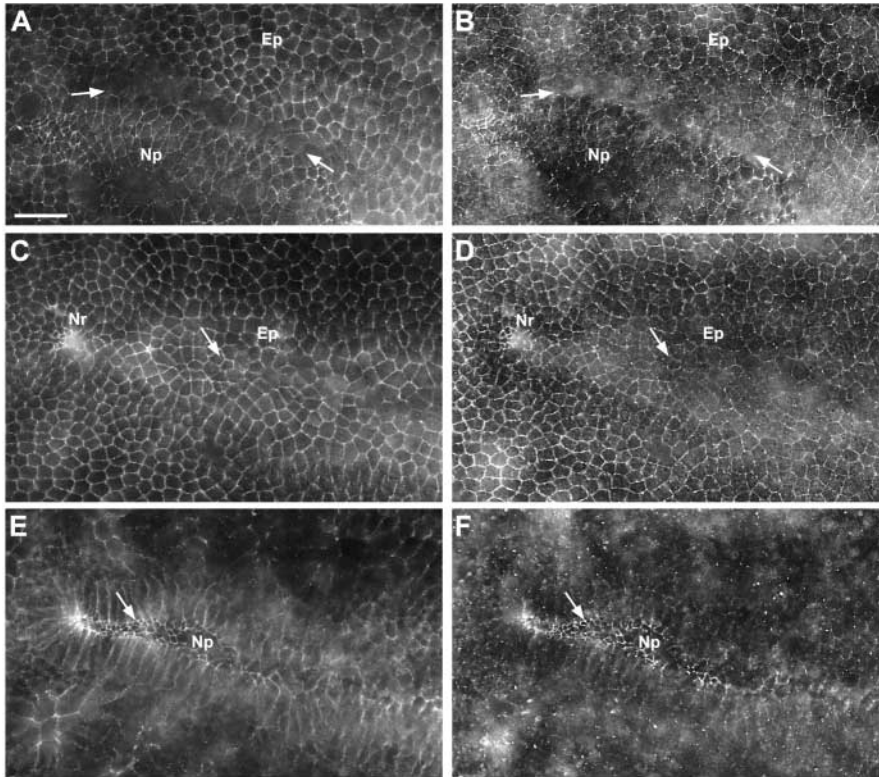


Fig. 7. Expression of Bb.β-catenin and Bb1C during neurulation. The embryos were double-stained for Bb.β-catenin (A,C,E) and Bb1C (B,D,F). Anterior is to the left. (A,B) Dorsal surface view of a stage N1 embryo at the same focal plane. Signals for Bb.β-catenin and Bb1C were detected at cell-cell contact sites in both the epidermal ectoderm (Ep) and the neural plate (Np). In cells at the edges of the epidermal ectoderm spreading over the neural plate (arrows), the characteristic concentrations of Bb1C and Bb.β-catenin were poorly observed. Note that Bb2C was not detected during neurulation as shown in Fig. 6B. (C-F) Dorsal surface (C,D) and internal (E,F) views of a stage N2 embryo. The focal plane of E and F is separated from that of C and D by 9 μm. At the dorsalmost epidermal ectoderm, weaker levels of Bb1C were observed compared to more lateral areas, although the levels of Bb.β-catenin showed no apparent differences (arrows in C and D). In the folded neural plate, Bb1C, together with Bb.β-catenin, was highly concentrated at the apical sites of cell-cell contact (arrows in E and F). Nr, neuropore. Scale bar: 20 μm.

Expression of Bb1C, Bb2C and Bb.β-catenin in the developing germ layers

Amphioxus embryos and larvae at different stages were immunostained for Bb1C, Bb2C and Bb.β-catenin. Bb.β-catenin was detected at cell-cell contact sites in all cells of neurula-stage embryos and larvae (Fig. 6A,C, Fig. 7A,C,E, Fig. 8A, Fig. 9A). Although Bb1C is present in the ectodermal cell layer of stage N1 embryos (Oda et al., 2002), specific signals for Bb2C were not detected in this germ layer (Fig. 6A,B). Instead, Bb2C was detected in the mesendodermal cell layer (Fig. 6D). Bb1C is not present in this layer (Oda et al., 2002). The Bb2C proteins were highly concentrated at the apical ends of the lateral surfaces of the cells, as was Bb.β-catenin (Fig. 6C,D, arrowheads). The strongest concentrations of Bb2C and Bb.β-catenin were observed at the blastopore region (Fig. 6C,D, Bp). In the mesodermal epithelia undergoing somitogenesis, high apical concentrations of Bb2C and Bb.β-catenin were persistently observed (Fig. 6C,D, arrows).

During neural plate closure, both Bb1C and Bb.β-catenin were detected in both the epidermal and neural plate cells (Fig. 7B). In cells at the edges of the epidermal cell layers, the characteristic concentrations of Bb1C and Bb.β-catenin were not observed (Fig. 7A,B, arrows). This is probably related to the observation that partially mesenchymalized cells with lamellipodia are present at these sites (Holland et al., 1996). Around the dorsalmost area at which the epidermal cell layers were fused to enclose the neural plate, weaker levels of Bb1C were observed than in more lateral areas of the epidermal ectoderm, although there were no apparent differences in the levels of Bb.β-catenin in this area (Fig. 7C,D, arrows). As the neural plate cells constricted their apices, the strongest concentrations of Bb1C and Bb.β-catenin were observed at the

apical parts of the lateral cell surfaces (Fig. 7E,F, arrows). The expression of Bb1C persisted in the epithelium of the established nerve cord into the larval stages (Fig. 8B,D, Fig. 9B). The signals for Bb1C in the epidermal ectoderm became ambiguous after stage N2 (data not shown).

At stage N3 and later, the expression of Bb2C was barely detectable in the differentiating myotomes, although Bb.β-catenin was weakly seen as thin lines between the elongating muscle cells (data not shown). Apparently, Bb2C continued to be expressed in the endodermal cells into the larval stages (Fig. 4B, Fig. 8C, Fig. 9C). It was persistently observed on the apical parts of the larval intestinal cells (Fig. 9C, thin arrows).

Expression of Bb1C and Bb2C during notochord development

In the development of the amphioxus embryo, the anlage of the notochord segregates from the dorsal roof of the archenteron around stage N2 (Hirakow and Kajita, 1994; Stach, 1999). Three distinct cell types have been described in the early notochord tissue (Conklin, 1932; Stach, 1999; Urano et al., 2003). Here we call these cell types notochord dorsal (NoD), notochord mid (NoM), and notochord ventral (NoV) cells. NoD cells are rather cuboidal in shape and are aligned in a single row just below the ventral midline of the nerve cord (Fig. 8E,G) (Urano et al., 2003). NoV cells are also aligned in a single row on the ventral side of the notochord tissue. Between the NoD and NoV cell layers, NoM cells differentiate to become thin and interdigitated (Fig. 8E). The cells eventually intercalate and show a 'stack of coins' configuration (Fig. 9A). In the posterior part of the notochord, this differentiation is delayed (Fig. 8A,G). In the established larval notochord, the

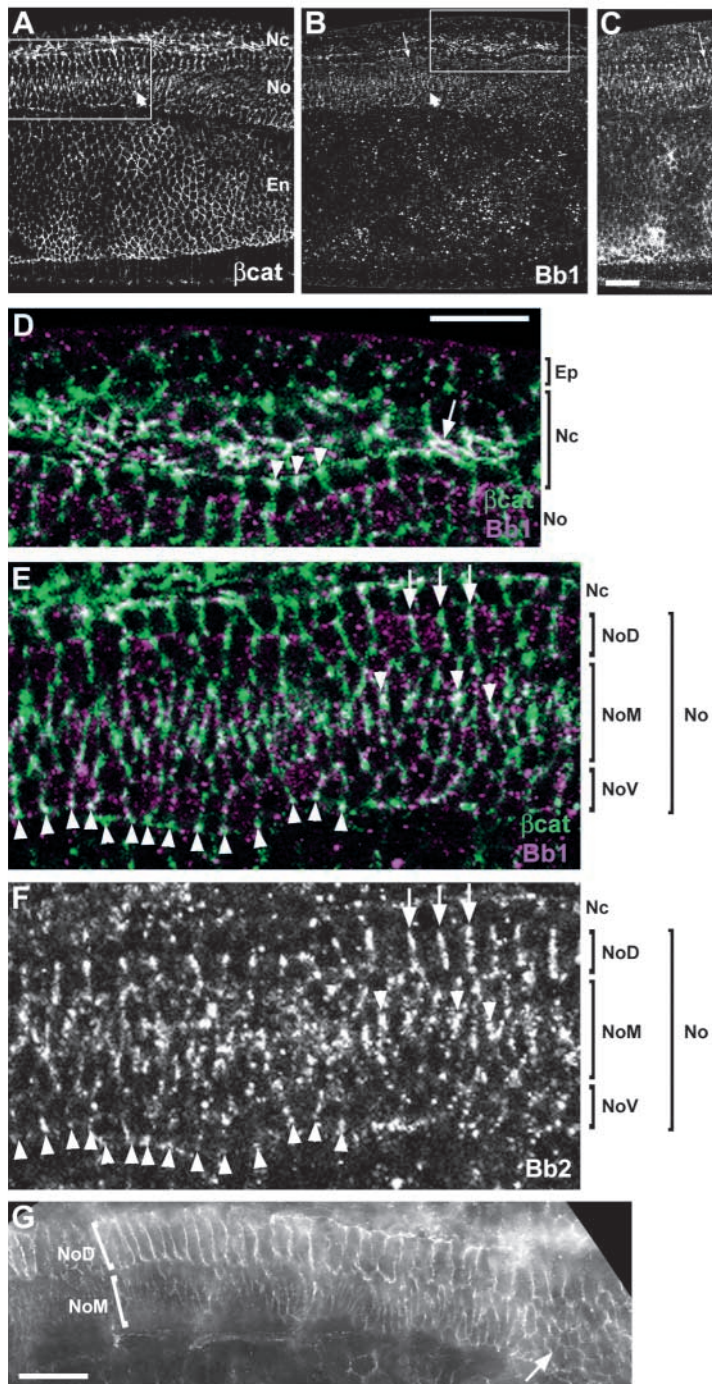


Fig. 8. Expression of Bb1C and Bb2C in the late neurula. (A–C) A stage N3 embryo was triple-stained for Bb.β-catenin (A), Bb1C (B) and Bb2C (C). Anterior is to the left. Dorsal is to the top. Bb1C was detected in the nerve cord (Nc) and differentiating notochord (No), but not or only faintly in the endoderm (En). Bb2C was detected in the notochord and endoderm, but not or little in the nerve cord. Note that in the notochord, the pattern of Bb1C expression differs from that of Bb.β-catenin and of Bb2C (fat and thin arrows). (D) High magnification of the area boxed in B. The images for Bb.β-catenin (green) and Bb1C (purple) were colored and merged. Bb1C colocalized with Bb.β-catenin appears white (arrow). Arrowheads indicate the apical concentrations of Bb1C in the nerve cord (Nc) epithelial cells. (E,F) High magnification of the area boxed in A. In E, the images for Bb.β-catenin (green) and Bb1C (purple) were colored and merged. In F, the image for Bb2C is shown. Arrows indicate interfaces between notochord dorsal (NoD) cells, at which Bb2C was detected, and arrowheads indicate interfaces between notochord ventral (NoV) cells and between notochord mid (NoM) cells. Some of the Bb1C and Bb2C signals were closely located. Note that NoM cells are interdigitated between the NoD and NoV cell layers. (G) Dorsolateral view of a stage N3 embryo stained for Bb.β-catenin. Anterior is to the left. A single line of NoD cells is seen. The arrow points to the posterior region of the notochord, where NoM cells have not interdigitated. Scale bars: 20 μm.

in these cells became reduced. In the larval notochord, the differential expression of Bb1C and Bb2C became prominent (Fig. 9B–D). Bb1C was detected at the interfaces between NoM cells, between NoV cells, and between NoM and NoV cells (Fig. 9F). It tended to accumulate more profoundly at the tricellular contact sites. Bb2C was instead detected at the interfaces between NoD cells. It was also detected, but was weaker, at the interfaces between NoD and NoM cells, which indicates that the NoM cells were still expressing Bb2C. At the posterior end of the developing larval notochord, the highest levels of Bb2C and Bb.β-catenin were detected, whereas no Bb1C was detected at this site (Fig. 9A–D, large arrows).

numbers of NoD, NoM and NoV cells within a given region were at an approximate ratio of 1:5:2 (Fig. 9A,E). Bb2C was initially expressed in all or most prospective mesodermal cells, including the notochord anlage, and was found in all the cell types of the notochord in stage N3 embryos (Fig. 8C,F). In contrast, Bb1C gradually appeared in NoM and NoV cells, but not in NoD cells, during stage N3 (Fig. 8B,E). In the undifferentiated (posterior) region of the notochord, Bb1C was not detected at all. Some of the signals of Bb1C and Bb2C in NoM and NoV cells were closely located (Fig. 8E,F, arrowheads). The expression of Bb1C in NoM and NoV cells became progressively stronger, while the expression of Bb2C

Discussion

Structure and adhesive function of Bb1C and Bb2C

In this study, we identified Bb2C in the cephalochordate amphioxus, *Brachiostoma belcheri*. This protein structurally resembles the classic-cadherin-related molecule, Bb1C, which we reported previously in the same animal species (Oda et al., 2002). Both Bb1C and Bb2C differ markedly from typical members of the classic cadherin family in that they lack ECs (Fig. 1). However, they have a CP domain bearing p120-binding and β-catenin-binding sites, which is characteristic of the classic cadherin family. In addition, the extracellular domains in these two cephalochordate proteins consist of LGs and CEs typical of all known nonchordate members of the classic cadherin family. Therefore, it is justifiable to include Bb1C and Bb2C in the classic cadherin family. The domain organizations of the classic cadherins that have been discovered to date can be classified into three forms, namely, the vertebrate (V) form, the cephalochordate (C) form and the

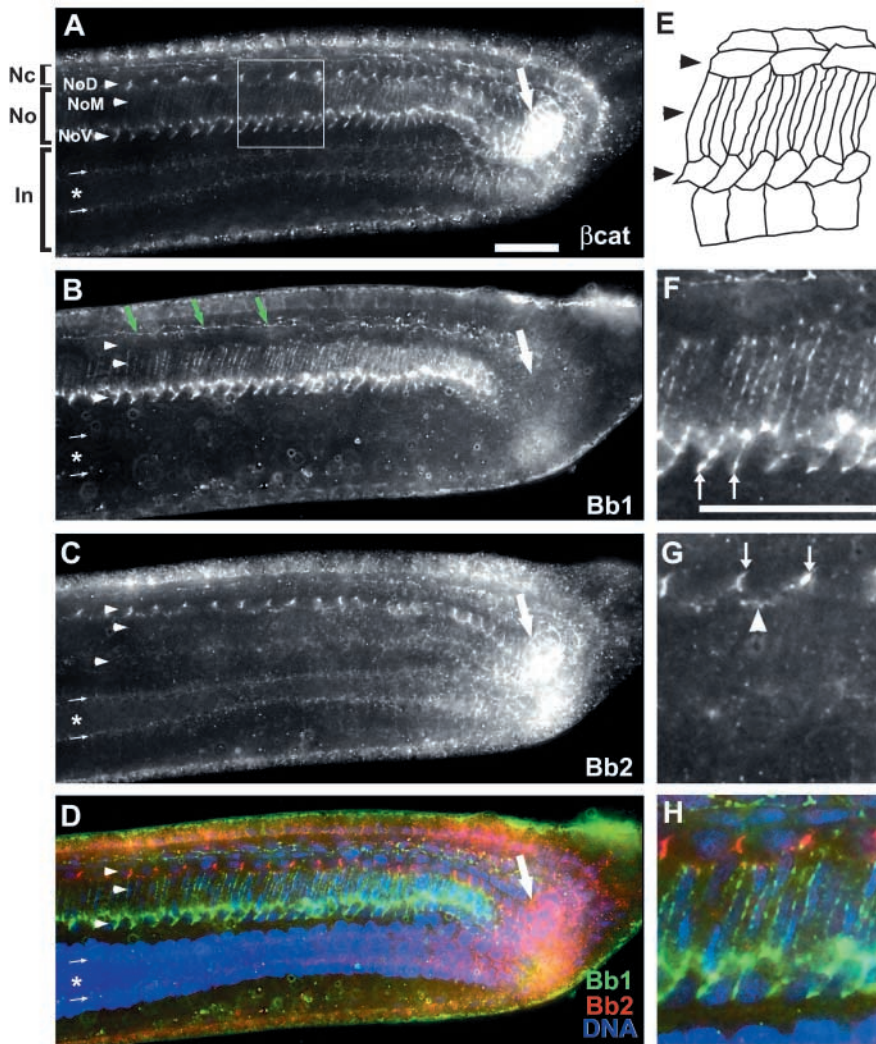


Fig. 9. Expression of Bb1C and Bb2C in the knife-shaped larva. (A–D) A stage L1 larva (24 hour) was simultaneously stained for Bb.β-catenin (A), Bb1C (B,D in green), Bb2C (C,D in red) and DNA (D in blue). Arrowheads indicate the NoD, NoM and NoV cell layers of the notochord (No). Asterisks and thin white arrows indicate the lumen of the intestine (In) and the apical surfaces of the intestinal epithelial cells, respectively. Large white arrows point to the posterior end of the notochord. Green arrows in B point to lines of Bb1C concentration in the nerve cord (Nc). (E) Schematic representation of the area boxed in A. (F–H) High magnifications of B–D corresponding to the area boxed in A. In F, the arrows point to high concentrations of Bb1C at the interfaces between NoV cells. In G, the arrows point to high concentrations of Bb2C at the interfaces between NoD cells, while the arrowhead indicates the weaker concentrations of Bb2C between a NoD cell and NoM cells. Scale bars: 20 μm.

nonchordate (N) form (Fig. 1). The N-form cadherins also show variations in the number of ECs and the organization of the PCCD complex (Oda et al., 2002).

All the classic cadherins detected in a wide range of nonchordate bilaterian animals are N-form cadherins (Oda et al., 2002). Recently, even in several vertebrate species, but not in the urochordate *Ciona intestinalis*, genes for N-form cadherins were found (Tanabe et al., 2004; Sasakura et al., 2003). Therefore, it is likely that in the earliest chordates, an N-form cadherin gene(s) existed that acted as a precursor for the V-form and C-form cadherin genes. Two independent structural simplifications may account for the generation of the V-form and C-form cadherins in chordate evolution (Oda et al., 2002). We could not find any genes that show significant similarities to Bb1C and Bb2C in the completely sequenced genomes of the vertebrates and urochordate or in nonchordate animals. Thus, the C-form cadherins are likely to be an innovation of cephalochordates, although the possibility is not excluded that such cadherin genes were lost in some non-cephalochordate lineages. Whether a V-form cadherin(s) is present in the cephalochordates is the key to reconstructing the phylogenetic relationships between Vertebrata, Urochordata and Cephalochordata (Oda et al., 2002), but remains unanswered.

The ECs play essential roles in the homophilic interaction of the V-form cadherins and the N-form cadherins. However, despite their lack of ECs, Bb1C and Bb2C can function in cell-cell adhesion. Aggregation assays using a mixture of cells expressing Bb1C and Bb2C-GFP suggested that the amphioxus cadherins bear distinct adhesive specificities (Fig. 5D), which is similar to what has been observed for the vertebrate E- and N-cadherins and the *Drosophila* DE- and DN-cadherin (Nose et al., 1988; Oda and Tsukita, 1999). The N-terminal ECs of the V-form cadherins are involved in generating this specificity in homophilic binding (Nose et al., 1990). However, LGs display in general a conserved structural fold that is suitable for generating ligand-binding diversity (Rudenko et al., 2001). Thus, in the C-form cadherins, the LGs may play the same role as that of the N-terminal ECs of the V-form cadherins. It will be necessary to investigate the mechanistic similarities and differences between the V-form, C-form and N-form cadherins to understand why the cadherins evolved into such drastically diverse forms.

Immunohistochemical analyses showed that Bb1C and Bb2C were localized at the apical areas of cell-cell contact in the polarized epithelia of amphioxus embryos. This subcellular localization indicates that these proteins play a role at the adherens junctions. We know that Bb1C at least can complex with the *Drosophila* catenins (Oda et al., 2002), which is consistent with the colocalization of Bb1C and Bb2C with Bb.β-catenin that we observed in immunohistochemical analysis of amphioxus embryos and larvae. Thus, despite their unique structures, Bb1C and Bb2C appear to function as major adherens junction constituents in a manner similar to the epithelial classic cadherins characterized in other animal species (Oda et al., 1994; Miller and McClay, 1997).

Developmental roles of Bb1C and Bb2C

Bb1C and Bb2C were complementarily expressed in amphioxus embryos. The expression of Bb1C was specific to the ectodermal epithelial cell layer, whereas the expression of Bb2C was specific to the mesendodermal epithelial cell layer. This differential expression of Bb1C and Bb2C is reminiscent of that of the vertebrate E- and N-cadherins (Takeichi, 1988) and the *Drosophila* DE- and DN-cadherins (Oda et al., 1994; Iwai et al., 1997). Notably, however, there are also clear differences. Bb1C is similar to the E-type cadherins in that it is initially expressed in the ectoderm, but it also differs from the E-type cadherins in that it is persistently expressed in the neural cell population. Moreover, Bb2C is similar to the N-type cadherins in that it is initially expressed in the mesoderm, but it also differs from the N-type cadherins in that it is expressed in the endoderm and not in the initial neural cell population. These differences in the germ layer-dependent and complementary expression of the cadherin gene pairs may be related to the possibility that the cadherin gene pairs in the vertebrate, cephalochordate and insect lineages arose by independent gene duplications (Fig. 3A).

In addition to the ectodermal versus mesendodermal expression of Bb1C and Bb2C, differential expression was observed in the notochord tissue, which develops from the Bb2C-expressing archenteron. Three cell types of notochord cells that were denoted as NoD, NoM and NoV cells were observed, consistent with recent work that isolated and examined notochord-specific genes (Suzuki and Satoh, 2000; Urano et al., 2003). The NoD and NoV cells are probably precursors of the Müller's cells, which are found at the dorsal and ventral ends of the amphioxus adult notochord (Conklin, 1932; Ruppert, 1997b; Stach, 1999). During the early phase of notochord development, Bb2C in the NoM and NoV cells, but not in the NoD cells, was replaced by Bb1C. A potentially mechanistically similar switching, from Bb2C to Bb1C expression, was observed in the posterior end of the elongating larval notochord. This dynamic regulation of cadherin expression may be associated with the rearrangement of the notochord cells and the formation of the multicell-layered structure. In addition, the patterns of Bb1C and Bb2C localization in the formed notochord are consistent with a previous electron microscopic observation that the Müller's cell precursors are interconnected by adherens junctions (Stach, 1999). Bb1C and Bb2C appear to be required to maintain the multicell-layered organization of the cephalochordate notochord, which somewhat differs from the vertebrate and urochordate notochords in structure and function (Ruppert, 1997a; Ruppert, 1997b; Burighel and Cloney, 1997; Suzuki and Satoh, 2000; Nishino and Satoh, 2001; Urano et al., 2003).

It is widely believed that the cephalochordate somites are homologous to the vertebrate somites. The cephalochordate somites form as ordered foldings of the polarized epithelia. Bb2C function at the adherens junctions may be prerequisite to this epithelial morphogenesis. In contrast, the epithelialization of the vertebrate somites involves N- and 11-cadherins (Radice et al., 1997; Horikawa et al., 1999). Considering that the C-form Bb2C and the V-form N- and 11-cadherins have different phylogenetic backgrounds, when and how the mesoderm was epithelialized in phylogenetic evolution is the issue of interest.

Our immunohistochemical data suggest possible involvement of Bb1C and Bb2C in the major morphogenetic events characteristic of the cephalochordate body plan. The developmental roles of Bb1C and Bb2C appear to be comparable with those of the classic cadherins characterized to date in other animal groups, such as the vertebrate E- and N-cadherins and the *Drosophila* DE- and DN-cadherins. Combined with the structural relationships of these classic cadherins, it is also suggested that these comparable conditions in the different animal groups are the result of parallel evolution. Because of an increase in the complexity of classic cadherin-based cell adhesion after separation from the hypothetical vertebrate-plus-urochordate lineage (Jefferies, 1986; Oda et al., 2002), the precursor of extant cephalochordates may have complexed its body structure in its own way. Compared to the cephalochordates, the urochordates appear to more faithfully reflect the primitive state of the vertebrates at least with respect to intercellular junctional systems (Lane et al., 1994; Sasakura et al., 2003). Further comparative studies from the viewpoint of cell biology may contribute to a better understanding of the vertebrate and chordate origins.

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