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Evidence for the Heparin-Binding Ability of the Ascidian Xlink Domain and Insight into the Evolution of the Xlink Domain in Chordates

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Abstract The vertebrate Xlink domain is found in two types of genes: lecticans and their associated hyaluronan-and-proteoglycan-binding-link-proteins (HAPLNs), which are components of the extracellular matrix, and those represented by CD44 and stabilins, which are expressed on the surface of lymphocytes. In both types of genes, Xlink functions as a hyaluronan binding domain. We have already reported that protochordate ascidians possess only the latter type of gene. The present analysis of the expression of ascidian Xlink domain genes revealed that these genes function in blood cell migration and apoptosis. While the Xlink domain is found in various metazoans, including ascidians and nematodes, hyaluronan is believed to be specific for vertebrates. In comprehensive genome surveys for hyaluronan synthase (HAS), we found no HAS gene in ascidians. We also established that hyaluronan is absent from the ascidian body biochemically. Therefore, ascidians possess the Xlink domain, but they lack HA. We recovered one ascidian Xlink domain gene that encoded a heparin-binding protein, although it shows no affinity for hyaluronan. Based on these findings, we conclude that in invertebrates, the Xlink domain serves as heparin-binding protein domain and functions in blood cell migration and apoptosis. Its binding affinity for HA might have been acquired in the vertebrate lineage.

Keywords Hyaluronan · Hyaluronan synthase (HAS) · Ci-Link · Xlink · Ascidian · Heparin

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Introduction

In vertebrates, Xlink domains (link module) are found in two types of genes (Day and Prestwich 2002). One type includes lecticans, such as aggrecan, neurocan, and versican, and their associated hyaluronan and proteoglycan-binding link proteins (HAPLNs) (Ruoslahti 1996; Spicer et al. 2003; Yamaguchi 2000). These genes encode components of the extracellular matrix (ECM) that confer distinct physical propertied to the ECM by binding a specific glycosaminoglycan (GAG) called hyaluronan, or hyaluronic acid (HA). The Xlink domain mediates the specific binding of lecticans to hyaluronan (Brissett and Perkins 1996). These lecticans and HAPLNs are characterized by their possessing two tandemly duplicated Xlink domains (Spicer et al. 2003). The other type of vertebrate gene that possesses the Xlink domain is found on the surface of lymphocytes, where the Xlink domain is found in genes such as *CD44*, *LYVE1*, *TNFAIP6 (TSG-6)*, and *stabilin* (Banerji 1999; Nottenburg et al. 1989; Politz et al. 2002; Wisniewski and Vilcek 1996). These genes encode cell surface molecules that are involved not only in lymphocyte migration, such as homing in lymphoid tissues and recruitment to sites of inflammation, and also mediate signal transduction to regulate cellular behavior (Cichy and Pure 2003; Kzhyshkowska et al. 2006; Lesley et al. 2004; Ponta et al. 2003; Prevo et al. 2001). The Xlink domain

plays a central role in the migration of lymphocytes by binding specifically to hyaluronan.

Recent advances in comparative genome analyses have shed new light on molecular evolution in metazoans. One observation is that while the Xlink domain is found in a wide variety of animals, such as *Caenorhabditis elegans*, sea urchins, ascidians, and amphioxus (Finn et al. 2006; http://pfam.sanger.ac.uk/), hyaluronan synthase (HAS), which is responsible for the biosynthesis of hyaluronan (Weigel et al. 1997), is thought to have been acquired by the ancestor of vertebrates (Salzberg et al. 2001). These observations imply that the vertebrate Xlink domain acquired its specific binding affinity for hyaluronan relatively soon after the divergence from invertebrate chordates, such as in ascidians.

To examine the molecular evolution of the Xlink domain, we examined hyaluronan and the Xlink domain of ascidians. Our previous comprehensive analyses of domain shuffling in the chordate lineage revealed that the genes for lecticans evolved via domain shuffling, which occurred in the vertebrate ancestors, and that ascidians possess genes having a single Xlink domain (Kawashima et al. 2009). One ascidian gene, *Ci-Link1*, is expressed in certain blood cells, and so its gene structure and putative function are more similar to those of the second type of vertebrate Xlink domain genes, such as *CD44*, or those expressed on the surface of lymphocytes (Kawashima et al. 2009). In this report, we present evidence that hyaluronan does not exist in ascidians and that the Xlink domain of Ci-Link1 shows binding affinity for heparin. Based on these results, we discuss the evolution of Xlink domain genes and their role during vertebrate evolution.

Materials and Methods

Molecular Phylogenetic Analyses

Phylogenetic analyses of the amino acid sequences of HAS-glycosyl transferase and the sequences of the link modules were performed using PhyML ver. 3.0 (Guindon and Gascuel 2003). The evolutionary model was selected using PROT-TEST ver. 2.0 (Abascal et al. 2005) and LG+G was applied according to the Akaike information criterion (AIC). Confidence values for each node were calculated using bootstrapping.

In Situ Hybridization

In situ hybridization of *Ciona* embryos was performed following the previous reports (Ogasawara et al. 2002; Yasuo and Satoh 1994). Probes for *Ci-Link1* and *Ci-Link2* were prepared using a clone from the Ciona Gene Collection (Cluster 02838 for *Ci-Link1* and Cluster 15981 for *Ci-Link2*; (Satou et al. 2002). Because ascidian tunic shows strong non-specific binding of RNAs, in situ hybridization for the specimens of metamorphosis stage was usually suffered from high background. We overcome this problem by increasing yeast RNA up to 1 mg/ml in hybridization buffer and by decreasing the amount of probes. By this method, almost no background staining was observed as shown in Wada (2010).

Biochemical Analysis of Ascidian Hyaluronan

Hyaluronan was examined in two ascidian species: *Ciona intestinalis* and *Halocynthia roretzi*. Adult *C. intestinalis* were incubated overnight in artificial seawater containing 185 kBq/ml [^{3}H]-glucosamine (specific activity 1.48 TBq/mmol). The acidic polysaccharide fraction was collected from the specimens by actinase digestion, acetylpyridinium chloride precipitation, and 80% ethanol precipitation. The fraction was digested with *Streptomyces* hyaluronidase (Seikagaku, Tokyo, Japan; (Ohya and Kaneko 1970). The radioactivity of the 80% ethanol-soluble fraction of the hyaluronidase digests was measured using a liquid scintillation counter.

In order to detect hyaluronan from *H. roretzi*, the acidic polysaccharide fraction was analyzed by electrophoresis on a cellulose acetate membrane. Chondroitin 6-sulfate from shark cartilage (Seikagaku), dermatan sulfate from pig skin (Seikagaku), and hyaluronan from *Streptococcus zooepidimicus* (Sigma, St. Louis, MO) were used as standard control. The acidic polysaccharides were detected by staining with toluidine blue (0.05% in 75% ethanol) followed by Alcian blue (0.05% in 75% ethanol).

Biochemical Analysis of the Ascidian Xlink Domain

Protein extracts from two adult *C. intestinalis* were density-fractionated under associative conditions at a density of 1.37 g/ml with the addition of CsCl (Tang et al. 1979). Each fraction was assayed for uronic acid (Bitter and Muir 1962), the ethanol-precipitated proteins were subjected to SDS-PAGE, and the gel was subsequently silver-stained (Bio-Rad) or transferred to a Hybond^{TM}-C Extra membrane (Amersham) for the heparin-binding assay. The affinities to heparin and hyaluronan were assayed by applying biotinylated heparin or biotinylated hyaluronan, respectively.

Twelve adult *C. intestinalis* were minced and extracted with 6 M guanidine HCl solution [6 M guanidine HCl containing 50 mM Tris–HCl (pH 8.0), 0.15 M NaCl, and protease inhibitors]. After dialysis against 50 mM Tris–HCl (pH 7.4) and 0.15 M NaCl, the solution was filtered through a membrane (pore size: 0.45 μm). The filtered solution was applied to HiTrap^{TM} Heparin (Amersham). The sample was eluted from the heparin column with 2 M

NaCl/50 mM Tris–HCl (pH 7.4) and subjected to two-dimensional gel electrophoresis (Mini-Protean II 2-D system, Bio-Rad) after ethanol precipitation. The electro-transferred protein was visualized with Coomassie Brilliant Blue R-250 or used for the heparin-binding assay. The amino acid sequences of the separated proteins were analyzed with the Model Procise® 494 cLC protein sequencing system (Applied Biosystems).

Results

Absence of Hyaluronan in Ascidians

HA is a repeat of *N*-acetylglucosamine and glucuronic acid, and its biosynthesis requires a specific enzyme: HAS. Vertebrates possess three types of HAS (HAS1-3; (Weigel et al. 1997), and HAS is postulated to have been acquired by horizontal gene transfer in the vertebrate ancestors (Salzberg et al. 2001). Indeed, a BLAST search of the genome sequences of *Drosophila melanogaster* and *C. elegans* retrieved chitin synthase genes (Fig. 1), while BLAST searches of the genome sequences of the ascidian *C. intestinalis* (Dehal et al. 2002) and sea urchin *Strongylocentrotus purpuratus* did not recover any similar sequences (Sea urchin genome sequencing consortium 2006). Nevertheless, we found nine HAS homologs in the amphioxus genome (Putnum et al. 2008). Unexpectedly, we found that a mosquito (*Anopheles gambiae*) also possesses genes with sequence similarity to HAS (Holt et al. 2002), although no related sequences were retrieved from the genomes of other mosquitos: *Aedes aegygypti* or *Culex quinquefasciatus* (http://metazoa.ensembl.org/index.html). Molecular phylogenetic analyses indicate that while the

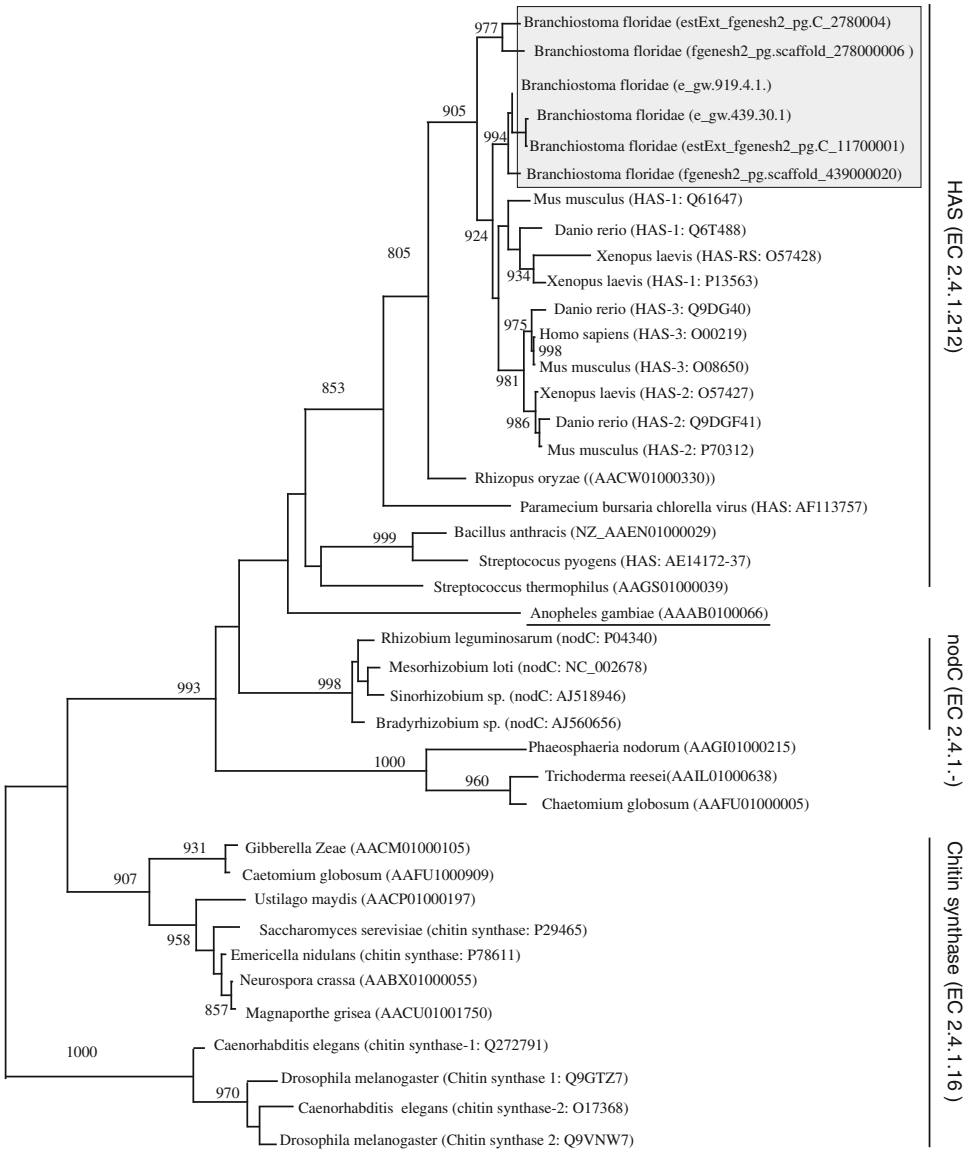

Fig. 1 Molecular phylogenetic tree of hyaluronic acid synthase (HAS) and chitin synthase. The tree was constructed using 183 amino acid sites. The numbers on the nodes are the bootstrapping values calculated using maximum likelihood analysis. Although we recovered nine gene models from amphioxus genomes, three pairs of them had sequences quite similar to the residues utilized in the phylogenetic analysis. Consequently, only six representative sequences were used in the analysis (hatched in *gray*). The mosquito (*Anopheles gambiae*) sequence is underlined

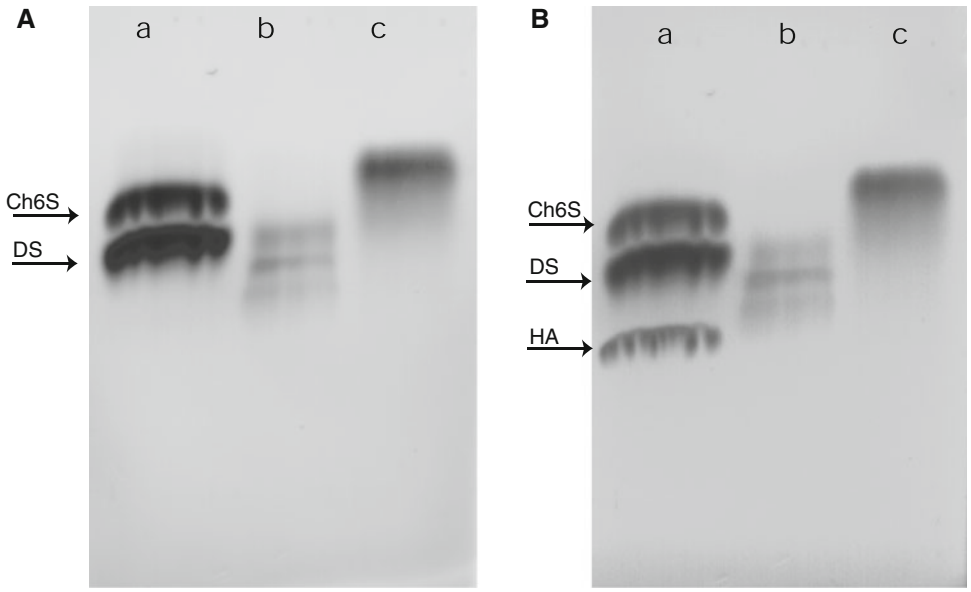

Fig. 2 Detection of hyaluronan from *Halocynthia roretzi*. The acidic polysaccharide fraction collected from adult *H. roretzi* was electrophoresed on a cellulose acetate membrane. Although some acidic polysaccharides are detected with toluidine blue stain (**a**), no hyaluronan is detected with Alcian blue (**b**). *Lane a*: standard control [chondroitin 6-sulfate (Ch6S) and dermatan sulfate (DS) were detected using toluidine blue, and subsequent staining with Alcian blue detected hyaluronan]; *lane b*: the acidic polysaccharide fraction from the body, excluding the tunic; *lane c*: the acidic polysaccharide fraction from the tunic

amphioxus HASs cluster with the vertebrate homologs, the mosquito HAS diverged from the basal node of HAS (Fig. 1). This suggests that the mosquito and amphioxus-plus-vertebrates acquired HAS independently via distinct horizontal gene transfers.

In order to confirm the absence of hyaluronan from the ascidian body, we conducted biochemical analyses in two species of ascidian: *C. intestinalis* and *H. roretzi*. The [^{3}H]-acidic polysaccharide fraction was collected from an adult *C. intestinalis* that was incubated with [^{3}H]-glucosamine and this was digested with *Streptomyces* hyaluronidase. No apparent increase in [^{3}H]-radioactivity was observed in the 80% ethanol-soluble fraction after hyaluronidase digestion (Ohya and Kaneko 1970; data not shown). In addition, from the acidic polysaccharide fraction collected from adult *H. roretzi*, we did not detect a band that stained with Alcian blue at the position corresponding to hyaluronan after electrophoresis on a cellulose acetate membrane (Fig. 2). Therefore, ascidians definitely lack hyaluronan in their bodies.

Xlink Domains in Ascidians

Although no genes homologous to HAS, or hyaluronan itself, exist in ascidians, we found two genes that possess Xlink domains in the genome sequence (Kawashima et al. 2009), which are also found in *C. savignyi* genome (Small et al. 2007). The alignment of the amino acid sequence of the Xlink domain is shown in Fig. 3a. Cysteine residues that are required for the three-dimensional structure were conserved in the Xlink domain of ascidian genes (Fig. 3a; Sandy et al. 1990). *Ci-Link1* also possesses an F5_F8_type_C functional domain (Fig. 3b; Kawashima et al. 2009). This motif is involved in binding with phospholipids and phosphatidylserine (Foster et al. 1990; Ortel et al. 1994) and the gene product is likely expressed on the cell surface.

We have already shown that *Ci-Link1* is expressed in certain blood cells in juveniles (Kawashima et al. 2009). In larvae, expression is detected in endodermal tissue (Fig. 4a). During metamorphosis, in addition to the endodermal expression, it is expressed in the cell mass of the absorbed tail, where extensive apoptosis is observed (Chambon et al. 2002; Fig. 4b). The expression of *Ci-Link2* has been detected in the neural complex of juveniles (Fig. 4c). The expression is restricted to the neural gland which is suggested to have function in regulation of blood volume (Ruppert 1990; Burighel and Cloney 1997). The epithelium of the neural gland is reported to consist of phagocytic cells (Burighel and Cloney 1997). We do not detect expression of *Ci-Link2* in early embryogenesis or larval stage. According to the EST analysis, *Ci-Link2* also shows expression in the adult heart as well as the neural gland (Satou et al. 2005).

Ascidians possess genes that contain the Xlink domain, but they lack hyaluronan. Therefore, we sought to identify the ligand to which the Xlink domains of *Ci-Link* genes bind. We tested whether either of the *Ci-Link* genes could

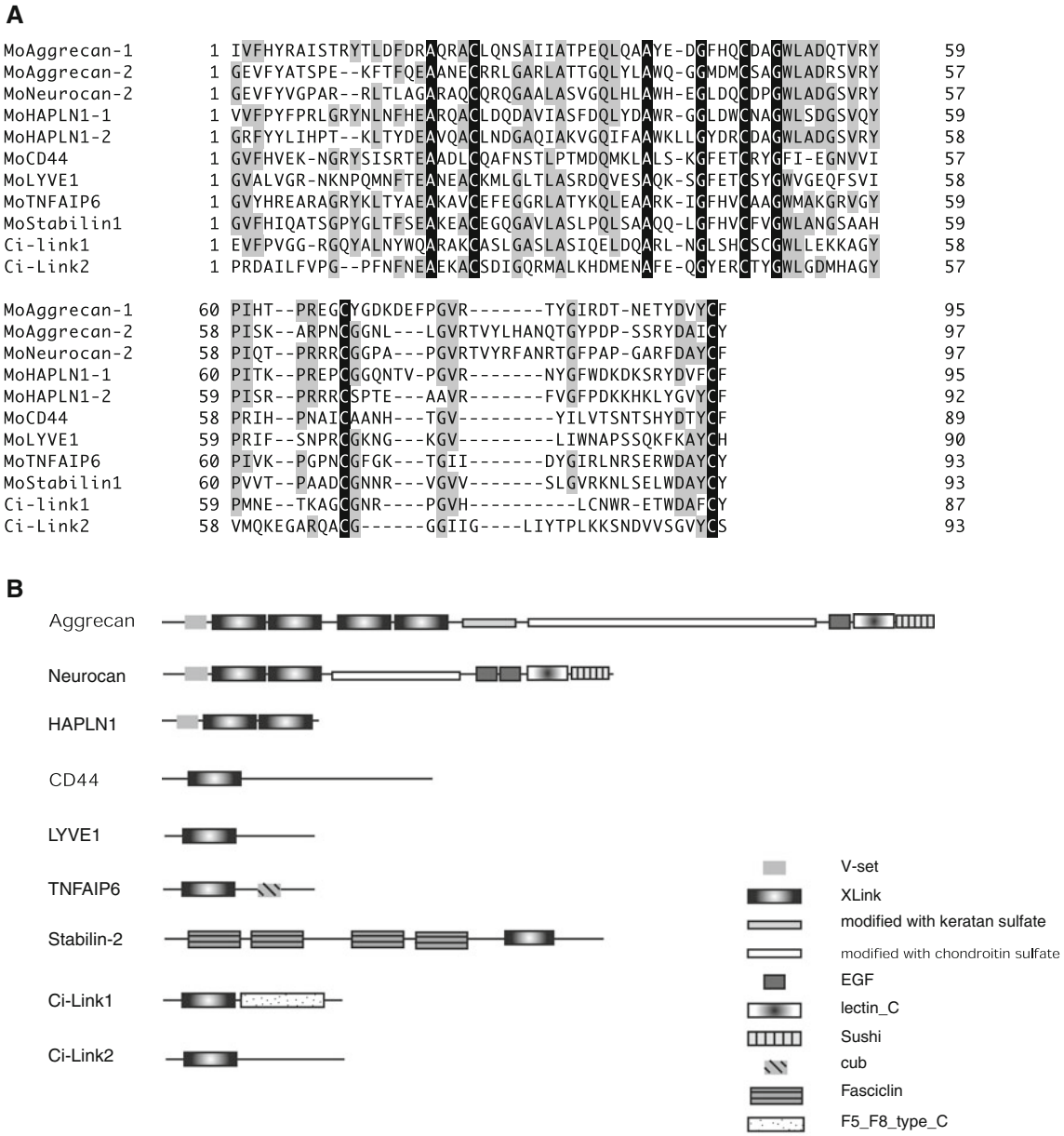

Fig. 3 Alignment of the amino acid sequences of Xlink domains and the domain architecture of Xlink-positive genes. **a** Alignment of the amino acid sequences of Xlink domains constructed using Clustal X (Thompson et al. 1997) and corrected by eye. Residues with identical amino acid are shown in dark, and residues shared by more than half of the sequences are light. **b** Domain architecture of genes with the Xlink domain

be isolated as a heparin-binding protein, another glycosaminoglycan that is present in ascidians (Cavalcante et al. 2000). First, we tested whether any proteins in *Ciona* exist that can bind hyaluronan or heparin. In the density-fractionated proteins, we detected a 45-kDa heparin-binding protein (Fig. 5a). However, the 45-kDa protein did not show affinity for hyaluronan, and no other hyaluronan-binding protein was detected (data not shown). Then, we examined whether *Ci-Link1* or *Ci-Link2* encodes the 45-kDa heparin-binding protein. The 45-kDa protein was isolated as a spot from a two-dimensional electrophoresis gel (Fig. 5b), and its amino acid sequence was analyzed. The resultant amino acid sequences were SLXEVF and SSXQEV, which match *Ci-Link1* completely (Cluster 02838; Satou et al. 2002). No other gene models of *C. intestinalis* possess the stretch of amino acid sequence, including Ci-Link2. Therefore, we concluded that *Ci-Link1* encodes the 45-kD heparin-binding protein and that it does not show binding affinity for hyaluronan (Fig. 6).

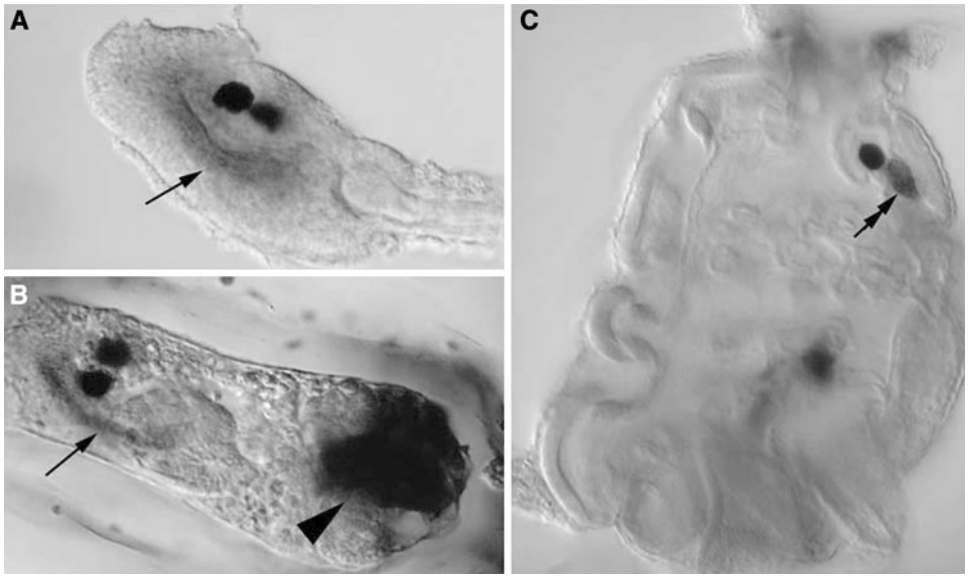

Fig. 4 Expression pattern of the ascidian Xlink domain genes *Ci-Link1* (**a**, **b**) and *Ci-Link2* (**c**). **a** The expression of *Ci-Link1* in larval endoderm (*arrow*). **b** In a metamorphosing juvenile, *Ci-Link1* is expressed in the cell mass of the absorbed tail (*arrowhead*). Endodermal expression is still detected (*arrow*). **c** The expression of *Ci-Link2* in the neural complex of a juvenile (*double arrow*)

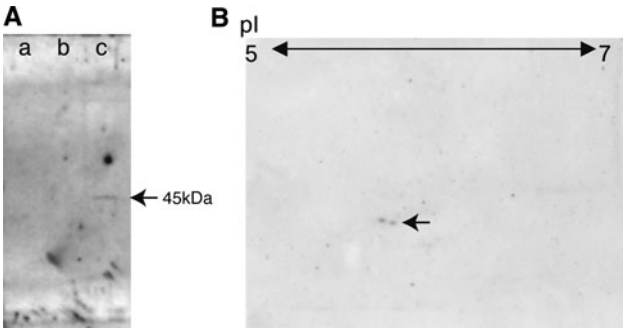

Fig. 5 The heparin-binding protein of *Ciona intestinalis*. **a** Density-fractionated proteins were assayed for affinity with heparin using biotinylated heparin. The densities of fractions a through c are 1.371, 1.412, and 1.455, respectively. A 45-kDa protein shows affinity for biotinylated heparin (*arrow*). **b** In a two-dimensional electrophoresis gel of heparin-affinity purified protein, two spots are detected using biotinylated heparin (*arrows*). These two spots turned out to have identical N-terminal amino acid sequences, which are also encoded by *CiLink*

Discussion

The Xlink domain and hyaluronan have two distinct roles in vertebrates. First, through binding with hyaluronan via the Xlink domain, lecticans confer distinct physical properties to the ECM (Ruoslahti 1996; Yamaguchi 2000). The other role of Xlink domain genes is in lymphocyte migration. In some cases, lymphocytes recognize their target site through hyaluronan, and this recognition is mediated by Xlink-positive cell surface molecules, such as CD44 and LYVE-1 (Banerji 1999; Cichy and Pure 2003; Ponta et al. 2003; Prevo et al. 2001). Vertebrate Xlink domains perform their role through specific binding to hyaluronan (Brissett and Perkins 1996).

From an evolutionary perspective, our previous analyses indicated that the Xlink domain was originally involved in blood cell migration because one of the ascidian Xlink-domain genes had a gene structure similar to cell surface Xlink genes in terms of a single Xlink domain for each gene. In addition, *Ci-Link1* is expressed in certain blood cells (Kawashima et al. 2009). *Ci-Link1* is also expressed in the tail cell mass, which undergoes apoptosis during metamorphosis (Fig. 4b). Therefore, *Ci-Link1* might also be involved in apoptosis. Notably, Ci-Link1 possesses the F5_F8_type_C domain (Kawashima et al. 2009), which shows binding affinity to phosphatidylserine (PS) (Foster et al. 1990; Ortel et al. 1994). The exposure of PS on the cell surface is used as a recognition signal for phagocytosis. PS is also used as a recognition signal in ascidian apoptosis (Cima et al. 2003). In vertebrates, this process is mediated by stabilins, which also possess the Xlink domain (Fig. 3b; Park et al. 2008). From this aspect, the expression of *Ci-Link2* in the neural gland is interesting, because the neural gland contain phagocytic cells (Burighel and Cloney 1997). Although it is mere speculation based only on the expression, *Ci-Link2* may also be involved in the phagocytosis.

Another notable evolutionary aspect of the Xlink–hyaluronan complex is that although the Xlink domain is found in a relatively wide range of animals, including *C. elegans*, sea urchins, amphioxus and ascidians, hyaluronan is found only in vertebrates. Indeed HAS was reported to be a candidate vertebrate-specific gene acquired through horizontal gene transfer from prokaryotes (Salzberg et al. 2001). Recent advances in genome analyses provide further evidence that no HAS is found in *C. elegans*, sea urchins, or ascidians. However, several HAS genes were detected in the amphioxus genome. Therefore, HASs are probably not

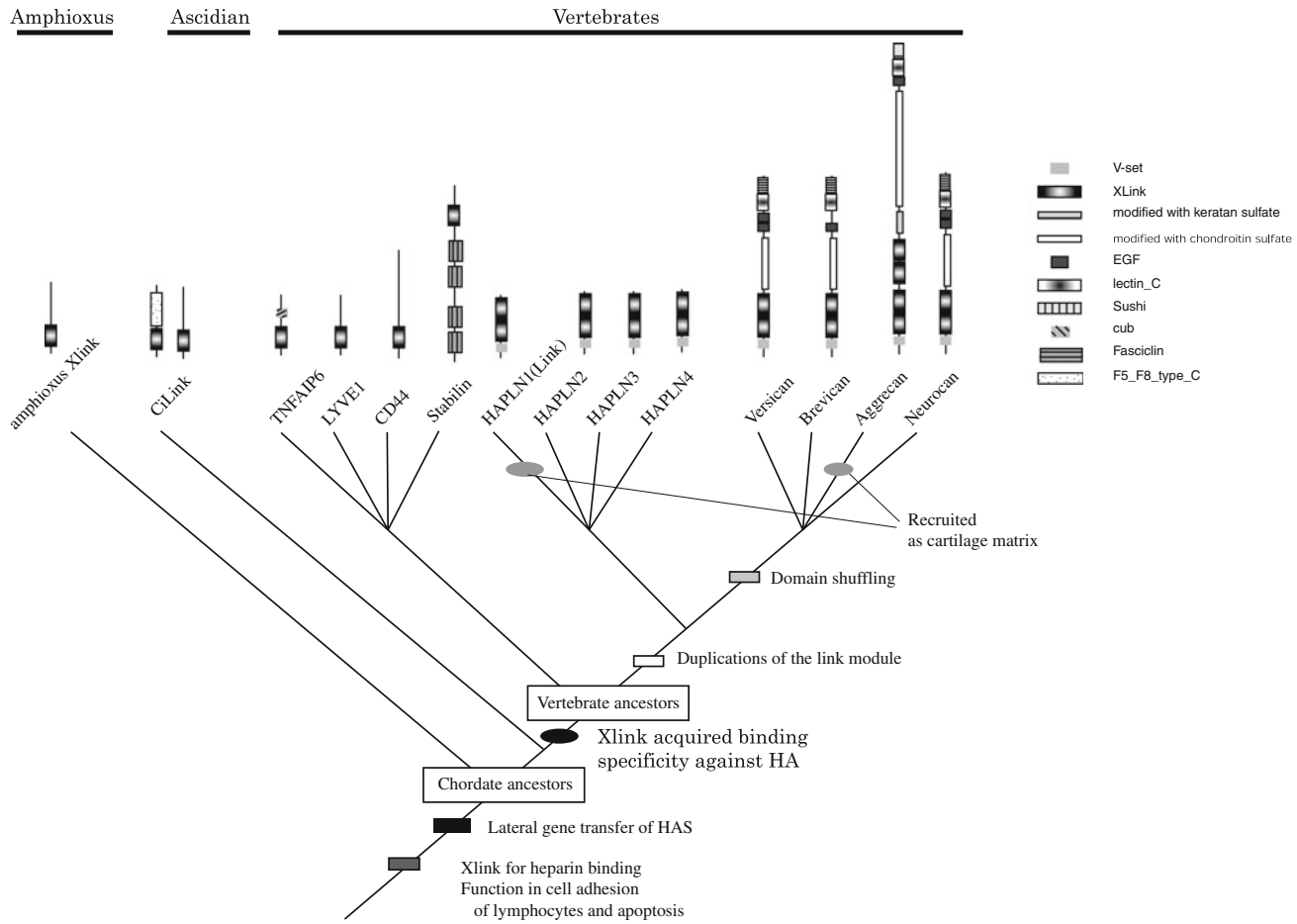

Fig. 6 Evolutionary scheme of the Xlink domain in chordates

vertebrate-specific genes, but were acquired in the common ancestors of vertebrates and amphioxus. Referring to the phylogenetic trees based on genome analyses, which support the sister grouping of vertebrates and ascidians with amphioxus as the outgroup (Putnum et al. 2008), HAS was likely acquired in the chordate ancestors. Ascidians are likely to have lost the HAS genes secondarily.

Although ascidians lost HAS secondarily, they have not lost the Xlink domain, perhaps because the ascidian Xlink domain has functions that are not related to hyaluronan. Supporting this idea, we present evidence that the ascidian Xlink domain binds another type of GAG: heparin. This is consistent with the fact that ascidians possess heparin (Cavalcante et al. 2000), but lack hyaluronan (present study). *Ci-Link1* may be involved in regulating blood cell migration by binding heparin. Since most invertebrates, except amphioxus, possess Xlink domains but lack HAS genes, the Xlink domain of invertebrates may function to mediate binding with heparin. It should be noted that we cannot exclude the possibility that Ci-Link1 binds heparin not via its Xlink domain, but via distinct stretches of aminoacids. However, we think it is quite likely that Xlink domain is involved because SPACR binds heparin through via hyaluronan binding motif (HABM) (Zhao et al. 2008).

Based on our results, when the Xlink domain obtained its specific binding affinity for hyaluronan is not certain. When the chordate ancestors acquired HAS, the Xlink domain might have developed a binding affinity for hyaluronan in addition to heparin. In that case, one might imagine that the ascidian Xlink domain would show affinity to hyaluronan. However, we found evidence that *C-Link1* does not show affinity for hyaluronan. Therefore, we prefer the idea that the Xlink domain functioned as a heparin-binding molecule in protochordates and acquired hyaluronan specificity in the vertebrate ancestors. Further analyses of the binding affinity of the Xlink domains in amphioxus will help improve our understanding on the evolution of the Xlink domain. Perhaps the acquisition of hyaluronan strongly influenced the subsequent evolution of vertebrates. For example, for vertebrate cartilage to function as a shock absorber, the molecular complex of aggrecan and hyaluronan is essential. Without hyaluronan,

vertebrates might not have joints between their bones. Therefore, hyaluronan might be essential for the evolution of the highly motile body of vertebrates. Modification of the binding affinity of the Xlink domain may also have played a critical role in vertebrate evolution.

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