

# Molecular Characterization of Calymmin, a Novel Notochord Sheath-Associated Extracellular Matrix Protein in the Zebrafish Embryo

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**ABSTRACT** During the screening of a zebrafish postsomitogenesis embryo cDNA library, we have identified a cDNA corresponding to a novel type of protein localized to the notochordal sheath-associated extracellular matrix (ECM) of the embryo. The 4.049-kb mRNA encodes a predicted polypeptide of 1,207 amino acids (122 kDa, pI 10.50) with a potential signal peptide of 20 amino acids. After the signal peptide, the mature protein consists of 1,187 amino acids (119 kDa, pI 10.46), for which the name “Calymmin” (from Greek *χαλνμμα*, to envelop, to cover) is proposed. The Calymmin mRNA is highly and transiently expressed by the notochord cells of the embryo from the 10- to 12-somite stage to the pharyngula period (13 and 24 hours postfertilization, respectively), and light and electron microscopical immunolocalization analysis revealed that the protein was specifically localized within a granular and filamentous layer of the ECM compartment surrounding the notochord. In zebrafish *no tail* mutants (*ntl*<sup>tc41</sup>), in which the notochord precursor cells are present but fail to differentiate, the Calymmin protein was not detected, confirming the notochord origin of Calymmin. These results indicate that Calymmin is a novel constitutive protein of the ECM compartment associated to the perinotochordal sheath in the zebrafish embryo, which is specifically expressed by the differentiating notochord cells. © 2002 Wiley-Liss, Inc.

**Key words:** zebrafish embryogenesis; notochord; sheath; extracellular matrix; *no tail* mutant

## INTRODUCTION

The notochord is a midline mesodermal embryonic structure common to all members of the phylum chordata, which has essential functions in patterning the paraxial mesoderm (Herrmann et al., 1990; Dietrich et al., 1993; Pourquie et al., 1993), the neuroectoderm (van Straaten et al., 1988; Bovolenta and Dodd, 1991; Yamada et al., 1993; Placzek et al., 1993), and other tissues (Wiertz-Hoessels et al., 1987; Stern et al., 1991; Danos and Yost, 1995), through notochord-derived signals. In the zebrafish (*Danio rerio*), systematic muta-

tional analysis of early development has identified several notochord-expressed genes, such as *no tail* (*ntl*) and *floating head* (*flh*) (Halpern et al., 1993; Schulte-Merker et al., 1994; Talbot et al., 1995; Odenthal et al., 1996; Stemple et al., 1996; Currie and Ingham, 1996; Fouquet et al., 1997; Blagden et al., 1997; Amacher and Kimmel, 1998), in which loss-of-function mutations cause defects in the formation and/or maintenance of the notochord and within other tissues. Nevertheless, the notochord also serves as the major skeletal element of the embryo, and shows important mechanical processes, such as elongation, straightening and stiffening, during neurogenesis and somitogenesis stages. The developing notochord is located in the midline immediately below the neural tube, and is composed of large vacuolated cells surrounded by a thin epithelial sheath. The notochord sheath is believed to resist the internal pressure building up during vacuolisation of the notochord, and with increased pressure and resistance to it, its greater stiffness may permit the notochord to elongate and straighten without being buckled by the surrounding tissues (Adams et al., 1990).

Several immunohistochemical and ultrastructural studies in both tetrapods (e.g., Pavola et al., 1980; Hay, 1984; Camón et al., 1990; Gotz et al., 1995; Ghanem, 1996) and cyclostomes (Kimura and Kamimura, 1982; Welsch et al., 1991) have demonstrated the presence of different extracellular matrix (ECM) proteins in intercellular spaces and under the basal lamina of the notochordal sheath, such as type I and II collagen fibers, sulphated glycosaminoglycans, glycoproteins (fibronectin, laminin, and tenascin), and proteoglycans (e.g., decorin). However, the complete molecular nature of the notochord sheath-associated ECM proteins, and their possible role in the modulating signaling to or differentiation of neighbouring embryonic tissues (e.g.,

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Ashkenas et al., 1996), are still poorly understood. In this work, we report the isolation of a new notochord-specific gene in the zebrafish embryo, which encodes a novel secreted protein with no homology to any of the

known proteins present in the data bases. The spatio-temporal distribution of its mRNA during embryogenesis, together with the ultrastructural localization of the protein, which we named Calymmin, indicate that this molecule is a novel component of the notochord sheath-associated ECM in the zebrafish.

## RESULTS AND DISCUSSION

### Identification of a New Gene Specifically and Transiently Expressed During the Development of the Zebrafish Notochord

During the screening of a zebrafish postsomitogenesis embryo cDNA library for orthologs of mammalian cytoskeletal components (Cerdà et al., 1998), a poly (A<sup>+</sup>)-bearing 3.273-kb clone (c29-77) with a single open reading frame of 945 amino acids was isolated. The predicted protein encoded by clone c29-77 presented no homology to other proteins in the current databases, and because of this intriguing fact, we examined the pattern of expression of the corresponding mRNA during development of wild-type zebrafish embryos by whole-mount in situ hybridization (Fig. 1A–F). The expression of the c29-77-derived mRNA became detectable in embryos approximately between the 10- and 12-somite stage (approximately 13 hours postfertilization [hpf]; not shown), and by the 15-somite stage (approximately 16 hpf) the developing notochord cells are notably labelled (Fig. 1A–C). As the embryos developed during the pharyngula period (prim-5 stage; 24h hpf), the expression developed into a gradient with a high point in the posterior part of the extended tail (Fig. 1D,E). By this stage, it was apparent that the staining was specific for the notochord cells, the signal being not detected in cells of the floor plate or the hypochord, located dorsally and ventrally, respectively, with respect to the notochord (Fig. 1E). Approximately by the prim-15 stage (34 hpf), the expression in the notochord cells located along the trunk gradually decreased to-

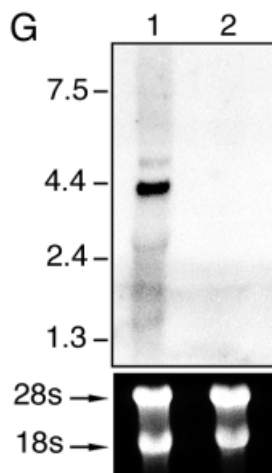
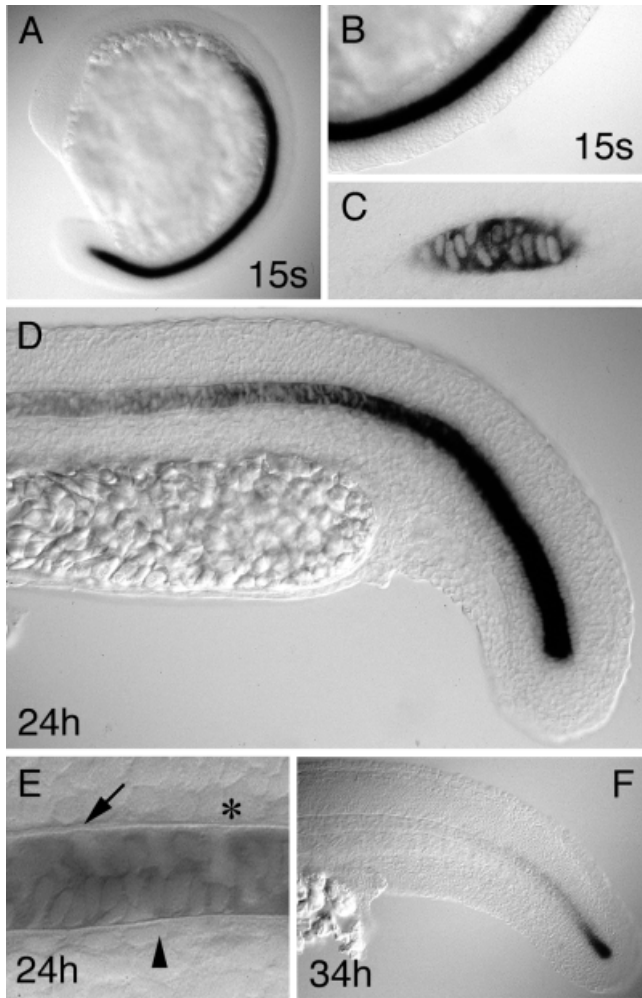


Fig. 1. Expression of Calymmin mRNA during zebrafish embryogenesis as detected by whole-mount in situ hybridization (A–F) and Northern blot (G). Lateral views of embryos with rostral to the left (A,B,D–F) and 2- to 3- $\mu$ m cross-section (C). **A–C:** Expression of the mRNA in notochord precursor cells in 15-somite stage embryos; C shows that transcripts are specifically expressed by the vacuolated notochord cells. **D,E:** Pharyngula period (24 hours postfertilization [hpf]). In D, the expression of c29-77 cDNA in the notochord shows a gradient with a higher level of transcripts toward the cells located in the tail region. E shows specific expression in the notochord cells at the level of the trunk, whereas both the adjacent floor plate (asterisk) and hypochord (arrowhead) cell layers are negative; the notochord sheath is indicated by an arrow. **F:** 34 hpf-embryos showing that expression becomes restricted to notochord cells located in the posterior region of the tail (F), where the notochord cells are differentiating. The results shown were obtained by using DIG-labelled riboprobes derived from full-length c29-77 (3.273 kb) and are identical when using riboprobes synthesized from c39a (4.049 kb). **G:** Northern blot showing the detection of a ~4.1-kb mRNA in 14- to 19-somite-stage zebrafish embryos (lane 1), whereas the message was not detected in adult fish (lane 2). Thirty micrograms of total RNA were loaded per lane. The size of molecular markers and ribosomal RNA is indicated on the left.

ward the anterior region of the embryo, and the signal exclusively remained within the cells forming the notochord primordium in the posterior tail region (Fig. 1F). At later stages, the signal completely disappeared from the embryo (not shown).

By using RNA probes derived from the full-length clone c29-77, Northern blot analysis indicated the accumulation of high amounts of the corresponding mRNA in the embryo but not in the adult fish. Figure 1G shows a clear hybridization band of approximately 4.1 kb on total RNA from 14- to 19-somite stage zebrafish embryos (lane 1), whereas no signal was observed in RNA extracted from the whole adult fish (lane 2). In agreement with these observations, *in situ* hybridization on adult frozen sections by using probes derived from clone c29-77 of different length did not show specific signals (data not shown). Taken together, the results of the expression analysis suggested that the gene represented by clone c29-77 was highly and transiently expressed in the notochord cells during formation of the notochord during the segmentation period. Thus, the expression of the c29-77 gene in the notochord cells occurs markedly later in development than the *no tail* (*ntl*) gene, the zebrafish homologue of the widely conserved *Brachyury* gene (Schulte-Merker et al., 1994) that is expressed during the late blastula and gastrula periods and in the tail bud during segmentation regulating the early formation of the notochord (Schulte-Merker et al., 1992; Odenthal et al., 1996).

### Isolation of Calymmin Full-length cDNA and Amino Acid Sequence Analysis

Judging from the molecular size of the hybridization signal observed in 14- to 19-somite stage embryos in Northern blot experiments, we assumed that the N terminus of the c29-77 encoded protein was missing. To isolate the full-length cDNA of the protein, the zebrafish postsomitogenesis and neurula cDNA libraries were screened under high stringency by using random-primed labelled probes corresponding to the most 5' sequence of clone c29-77. Three different clones were isolated, named c39a, c38b, and c20a, from which clone c39a (4.049-kb) presented a poly (A<sup>+</sup>) tail and the longest sequence at the 5' end, and was selected as the representative cDNA. The c39a clone contained a single open reading frame of 3,635 nt starting from nucleotide 1, without any stop codon at the 5' region and four putative translation initiation codons at nucleotides 14, 38, 41, and 56. The first three of these ATG codons were preceded by an adenine residue at position -3, but none of them presented a guanidine at position +4, which partially fulfilled the consensus sequence for eukaryotic translation initiation sites (Kozak, 1987); thus, the first ATG codon was taken as the translation initiation site. The open reading frame of c39a ended at nucleotide 3635 with a *taa* stop codon followed by two consensus polyadenylation sites (*aataaa*) beginning at nucleotides 3778 and 4008.

Based on these observations, clone c39a was assumed to encode a full-length polypeptide of 1,207 amino acids, with a molecular mass of 122 kDa and a pI of 10.50. A noteworthy feature of the primary sequence is its elevated content in glycine residues (20.2%). The first 20 amino acids in the sequence fulfill the criteria for a signal peptide, indicating that this protein may be synthesized in the endoplasmic reticulum and processed through the Golgi apparatus (Von Heijne, 1986). After removal of the signal peptide, the mature predicted protein consists of 1,187 amino acids, with a molecular mass of 119 kDa and a pI of 10.46, and two low-complexity glycine-rich regions (Pagni et al., 2001), from amino acids 2 to 587 and from 792 to 984. The protein sequence also showed several conserved motifs, such as one glycosaminoglycan attachment site, two asparagine (N)-, and six mucin type O-glycosylation sites (Hansen et al., 1998), respectively, and many potential N-myristoylation sites dispersed along the entire polypeptide.

On sequence comparisons using the BLAST-algorithm (Altschul et al., 1997), the c39a-encoded protein did not show significant homology to any of the known proteins present in the databases, nor were any putative conserved domains found. However, further analysis by recursive PSI-BLAST searches (Corpet et al., 2000) of the novel protein identified herein, which we named Calymmin, identified four consecutive prodomains in its amino acid sequence of 105 to 175 amino acids long (Fig. 2A). Three of these domains are located toward the N terminus of the peptide and are separated by short portions of amino acids of 16 and 12 residues in length, whereas the fourth domain is located 278 amino acids downstream from the third domain. The four Calymmin prodomains exhibit different degrees of homology to portions of the amino acid sequence of some polypeptides from the databases with unknown functions. These peptides included the *Drosophila melanogaster* cg6639, a serine protease-like peptide, and cg11581 proteins (Adams et al., 2000), and the *Mycobacterium tuberculosis* hypothetical 56.9-kDa protein cy10h4.20c (Cole et al., 1998), and the *M. leprae* hypothetical 49.8-kDa protein (Eiglmeier, et al., 1993), both of which also contain forkhead-associated domains (Durocher et al., 1999) at their C terminus (Fig. 2A). The sequence alignment of the zebrafish Calymmin domains with the Calymmin-like domains from these procaryotic and eucaryotic proteins indicated 32–50.7% identity and 42.6–64% homology to the zebrafish domains, calculated on the corresponding aligned amino acid sequences. The consensus amino acid sequence of this novel domain after the alignment showed four GGY(P/G)(N/X) motifs, approximately conserved among the proteins, separated by glycine and proline residues (Fig. 2B).

The amino acid sequence alignment analysis of the Calymmin clone c39a with the other clones isolated from the postsomitogenesis library, c29-77 and c38b, revealed that these cDNAs encoded slightly different



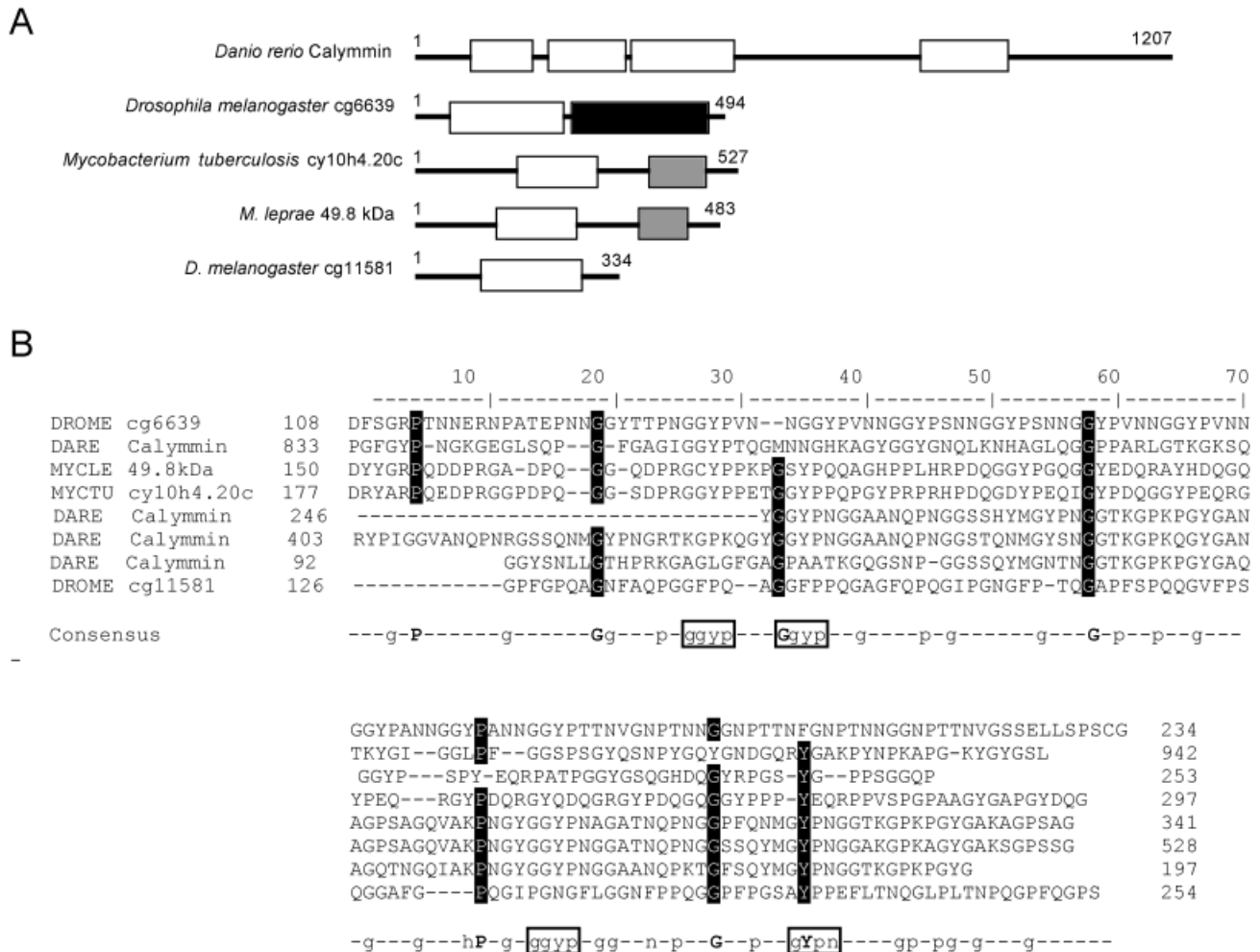


Fig. 2. Structure of the zebrafish Calymin prodomains and homologies with other proteins with similar amino acid sequences. **A:** Schematic diagram of zebrafish Calymin and other proteins containing Calymin-like prodomains. The location and length of the Calymin-like domains are indicated by open boxes, and the grey and black boxes indicate the forkhead-associated and trypsin family associated domains, respectively. **B:** Sequence alignment of the Calymin-like prodomains from the zebrafish Calymin and those from the proteins indicated in A.

after recursive PSI-BLAST analysis. The dashes are inserted to optimize the alignment, and black boxed amino acids indicate conserved residues. In the consensus sequence, upper case indicates invariant residues, lower case residues present in at least half of the domains, dashes indicate nonconserved residues, and h indicates hydrophobic residues. The boxes indicates the position of the GGY(P/G)(N/X) approximately conserved motifs.

polypeptides. Thus, insertions or deletions of one or of groups of 34-85 amino acids, with a sequence highly homologous to the Calymmin prodomains, were observed between or within the second and third prodomain defined in the c39a encoded protein. In addition, amino acid substitutions in all three first prodomains were observed among the c39a, c38b, and c29-77 predicted polypeptides. Whether these differences in the amino acid sequence of the cDNAs indicate the existence of isoforms of the Calymmin protein or duplicate gene copies, or whether they simply arise by alternative splicing of the mRNA, remains to be investigated.

## Protein Characterization and Cellular Localization of Calymmin During Embryogenesis

Synthetic peptides representing different segments of the N and C terminus of the c29-77 predicted protein were used to raise guinea pig antibodies to characterize and localize the Calymmin protein in the zebrafish embryo. The antisera obtained against the sequence QNMGYPNGGTKGPKPGYGAK resulted in positive signals in the notochord in preliminary immunocytochemical experiments on 20-hpf embryos; thus, the most reactive antiserum (Calym1-1) was further affinity purified to specifically recognize the Calymmin

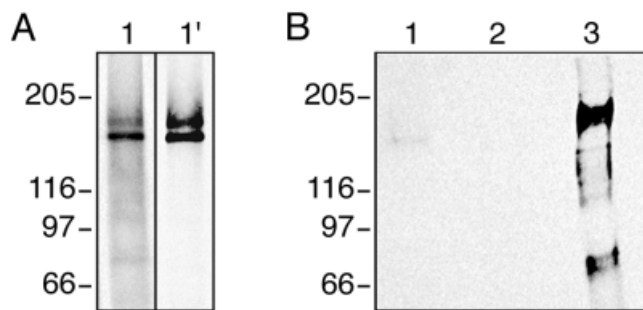


Fig. 3. Biochemical characterization of zebrafish Calymmin. **A:** Radiolabeled products obtained by in vitro transcription-translation of zebrafish clone c39a in a rabbit reticulocyte lysate (lane 1), and subsequent immunoblot detection of the products with the anti-Calymmin antibody (lane 1'). **B:** Immunoblot detection of Calymmin polypeptides from 24 hours post fertilization embryos showing the low-Triton supernatant fraction (lane 1), the high salt supernatant fraction (lane 2), and the NP-40 insoluble cytoskeletal fraction (lane 3). The relative molecular masses of the reference proteins are indicated on the left.

polypeptide in subsequent immunoblotting and immunolocalization experiments.

In vitro transcription-translation of Calymmin clone c39a by using the reticulocyte lysate system yielded two radioactively labelled products with an apparent molecular mass of approximately 175 kDa and 160 kDa (Fig. 3A, lane 1). Both products were recognized by the anti-Calymmin antibody, although the 175-kDa band appeared as a broader less intense reactive band when compared with the 160-kDa band (Fig. 3A, lane 1'). In 24-hpf zebrafish embryos, the detection of the Calymmin protein was carried out by immunoblotting after cell fractionation experiments, during which we also determined the solubility of the protein (Fig. 3B). In these experiments, the bulk of Calymmin was recovered in the nonionic detergent insoluble fraction, as indicated by the detection of a strong reactive band of an apparent molecular mass of approximately 180 kDa in the cytoskeletal pellet (Fig. 3B, lane 3), whereas the soluble fraction of the protein was very low and already released in the presence of 0.2% NP-40 (Fig. 3B, lanes 1 and 2). The detection of a broader reactive band corresponding to Calymmin, in both in vitro transcription-translation and embryo protein extracts, may suggest the existence of posttranslational modifications of the peptide both in vivo and in cell-free systems. This would agree with the various N- and mucin type O-glycosylation conserved motifs, and the high number of potential N-myristoylation sites found in the amino acid sequence of Calymmin (e.g., Deichaite et al., 1988).

To study the localization of Calymmin during embryonic development, whole-mount immunocytochemistry on wild-type zebrafish embryos was carried out (Fig. 4). Under the conditions used, Calymmin protein was first detected in embryos at approximately the 15-somite stage (16.5 hpf), where the positive signals appeared restricted to the anterior developing notochord in the tail (not shown). By the 18-somite stage (17.5 hpf), the

Calymmin staining appeared distributed within the external sheath surrounding the notochord cells along the embryo (Fig. 4A,C) but much less intense signals were observed in the cells located in the most posterior region of the notochord (Fig. 4B). The examination of cross-sections of embryos at the pharyngula period (24 hpf) labelled with anti-Calymmin antibody confirmed the perinotochordal localization of the Calymmin products (Fig. 4D), and this observation was corroborated by using newly raised antisera against amino acid sequences of Calymmin different than those selected for the Calym1-1 antisera (data not shown). However, in the whole-mount preparations, slightly visible reactive deposition products were also seen close to what likely appeared to be the notochord cell nucleus (Fig. 4C), which was further investigated by immunofluorescence microscopy on cryostat sections of the embryo labelled with the Hoechst fluorescent dye to visualize the cell nucleus. As shown in Figure 4E, the anti-Calymmin reactive products were exclusively found in a juxtanuclear position within the notochord cells situated in the most posterior region of the notochord, whereas the more anterior cells, possibly in advanced differentiating stages, became strongly labelled in the notochord sheath. This characteristic pattern of intracellular localization of Calymmin strongly suggests its presence within the Golgi complex of the notochord cells (Camón et al., 1990) at the initial stages of differentiation, which would be consistent with the presence of a signal peptide detected at the N terminus of Calymmin. At later stages of differentiation, Calymmin appears to be secreted to the ECM compartment of the notochord to form a component of the notochordal sheath.

To further explore the origin of Calymmin in the zebrafish embryo, we examined the occurrence of the protein in *no tail* (*ntl*) mutant embryos by whole-mount immunocytochemistry and immunoblotting. In *ntl* embryos, the notochord precursor cells are present in the trunk but fail to differentiate, and consequently the notochord is completely missing from the posterior part of the embryo, resulting in fusion of the somites (Halpern et al., 1993; Schulte-Merker et al., 1994). In addition, in the *ntl* mutants, the tail does not form, and this phenotype is stronger in the *ntl*<sup>tc41</sup> allele, which shows a very short postanal region (Odenthal et al., 1996; Fig. 5B). Therefore, the *ntl* mutant represented a suitable model to investigate our previous hypothesis on the notochord origin of perinotochordal Calymmin immunoreactivity. The results of these experiments showed that, unlike in the wild-type embryos (Fig. 5A), in the *ntl* mutants the Calymmin protein was not immunologically detected, neither in whole-mount preparations (Fig. 5B) nor in cytoskeletal protein extracts (Fig. 5C) from 22-hpf embryos. Thus, these findings confirm that the zebrafish Calymmin polypeptide is specifically synthesized and secreted into the extracellular space by the differentiating notochord cells.

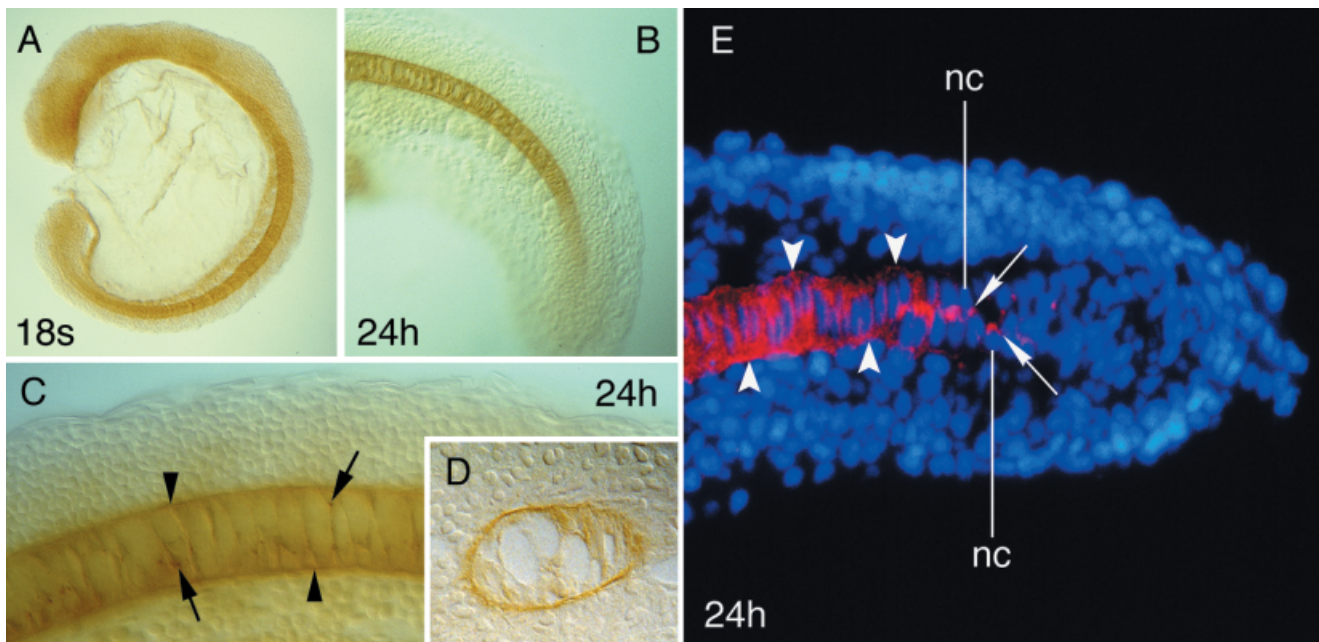


Fig. 4. Localization of Calymmin in wild-type zebrafish embryos by whole-mount immunocytochemistry (A–D) and immunofluorescence microscopy (E). Views of embryos with rostral to the left (A–C), cross-section of labelled embryos (D), and cryostat section (E). **A**: 18-somite stage embryo showing staining within the developing notochord. **B,C**: Embryos at the pharyngula period (24 hours postfertilization [hpf]) showing Calymmin staining at the notochord sheath (C), which is less intense at the level of the tail (B). The arrowheads point to the notochord sheath, whereas the arrows indicate subcellular staining deposits close to the nucleus of the notochord cells. **D**: Cross-section of labelled embryos

at the level of the trunk showing positive and specific immunolocalization of Calymmin in the notochord sheath. Note the high vacuolization of the notochord cells that are surrounded by the sheath. **E**: Immunofluorescence localization of Calymmin (red color) in the notochord cells located in the tail and anterior trunk of 24-hpf embryos. The nucleus of the notochord cells can be visualized by the Hoechst fluorescent dye (blue color). The arrows indicate the juxtannuclear localization of Calymmin within the notochord cells, likely in the Golgi apparatus, whereas the arrowheads point to the appearance of Calymmin at the notochordal sheath. nc, nucleus of the notochord cells.

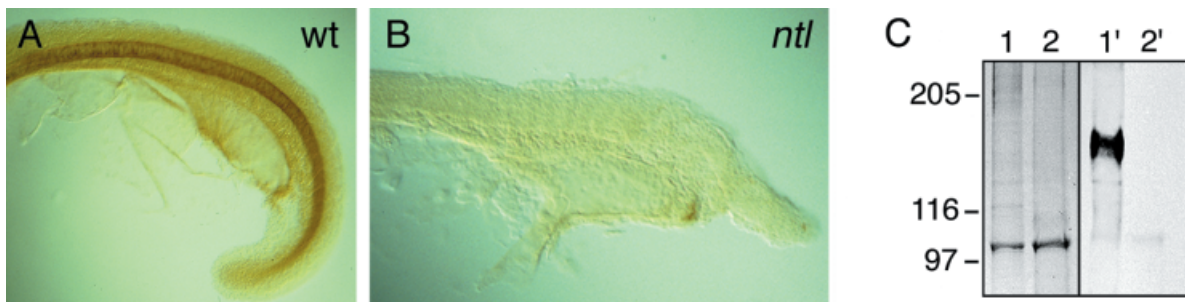


Fig. 5. Detection of Calymmin in wild-type and *ntl* mutant zebrafish at 22 hours postfertilization embryos. **A,B**: General views of wild-type (A) and *ntl* mutant (B) embryos (rostral to the left) after whole-mount immunocytochemistry. Note the absence of the notochord and tail in the *ntl* mutants, in which Calymmin was not detected. **C**: Coomassie brilliant blue-stained sodium dodecyl sulphate-polyacrylamide gel electrophore-

sis of cytoskeletal fractions from wild-type (lane 1) and *ntl* embryos (lane 2), and the corresponding immunoblot (lanes 1' and 2'), showing the absence of the anti-Calymmin reactive band in the mutant embryos. The relative molecular masses of the reference proteins are indicated on the left.

By using immunogold labelling protocols with the “silver enhancement” technique, after the application of the anti-Calymmin antibody in standard whole-mount procedures, we were able to specifically localize Calymmin within the notochordal sheath of the embryo (Fig. 6). The ultrastructural morphology of the zebrafish notochordal sheath resembles that found in chicks and amphibians (e.g., Bruns and Gross, 1970;

Kayahara, 1982; Jurand and Malacinski, 1983; Camón et al., 1990), and it appears formed by a multilayered structure composed by three main compartments, a proximal (to the notochord cells) thin basal lamina, a wider perinotochordal region of clearly striated fibrils, and an outer layer of similar size of loosely organized matrix associated with electron-dense granules (Fig. 6A,B,D,E). Immunoelectron microscopy showed immu-



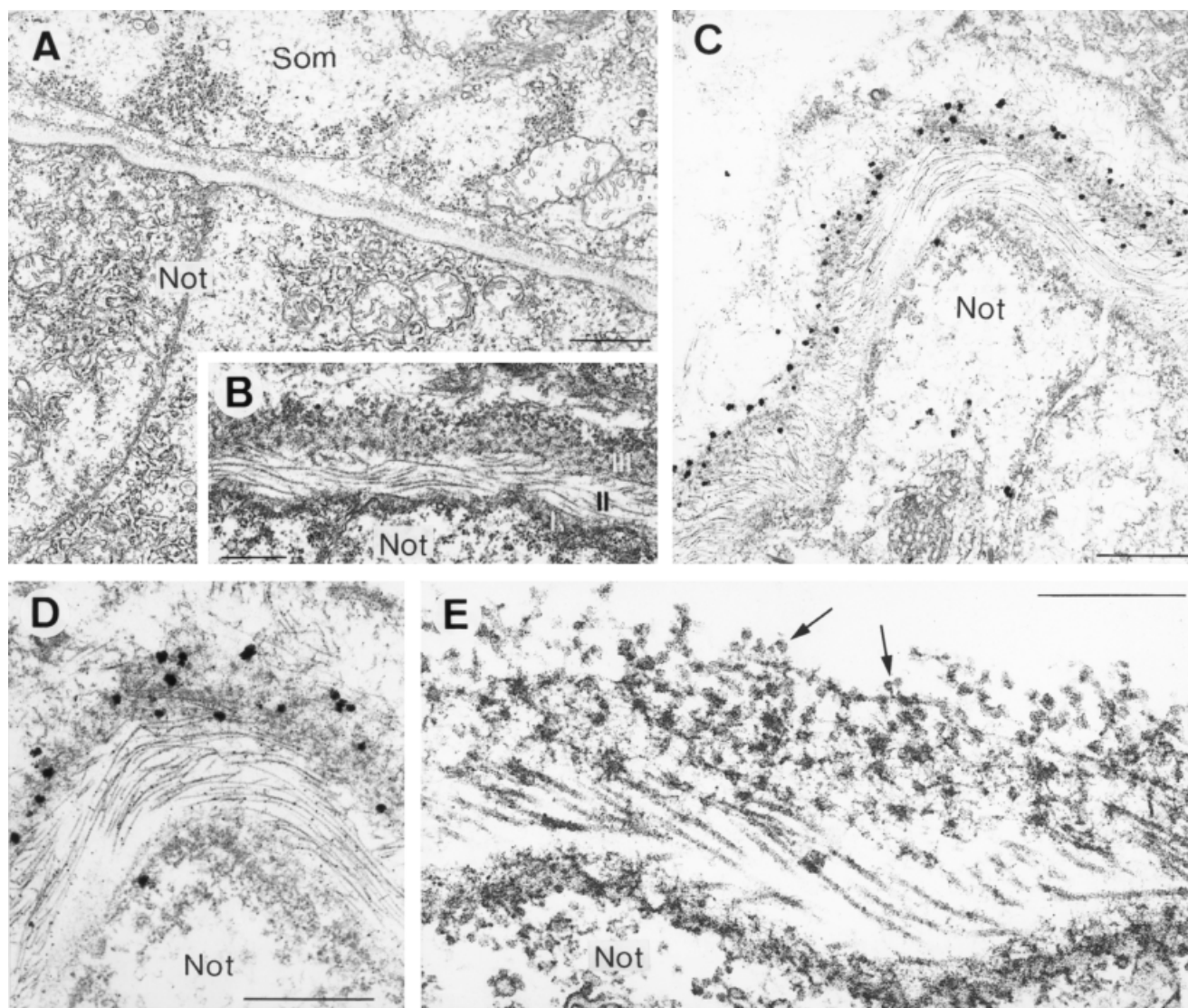


Fig. 6. Ultrastructure of the notochordal sheath in 24-hours postfertilization zebrafish embryos (A,B,E), and ultrastructural localization of Calymmin (C,D). **A:** General view of two adjacent notochord cells surrounded by the notochordal sheath, separating the notochord from the developing somites (Som). Note the presence of vacuoles and secretory vesicles within the notochord cells. **B:** Electron photomicrograph of the notochordal sheath, showing a proximal (to the notochord cells) basal lamina (I), a perinotochordal region of clearly striated fibrils (II), and an outer loosely organized and granular matrix (III). **C,D:** Immunogold localization of Calymmin at the outer granular layer of the notochordal sheath,

whereas the rest of the sheath compartments appears unlabelled by the antibody. **E:** Ultrastructure of the notochordal sheath after conventional electron microscopy procedures in the presence of high concentration of detergents to unravel its organization. The granular-attached fibrillar nature of the Calymmin-labelled sheath compartment is indicated by arrows. Below it lies the microfibrillar layer, showing a characteristic collagen-like cross-banding pattern, and the basal lamina of the notochord cells. Not, notochord cells. Scale bars = 2.5  $\mu\text{m}$  in A, 0.25  $\mu\text{m}$  in B, 1  $\mu\text{m}$  in C, 0.5  $\mu\text{m}$  in D, 0.3  $\mu\text{m}$  in E.

nolabel particle enrichment, corresponding to Calymmin specifically at the outer granular layer of the notochordal sheath, whereas the proximal layer with the microfibrils showing a collagen-like cross-banding pattern, and the basal lamina of both the notochord cells and somites were not labelled by the antibody (Fig. 6C,D). Further ultrastructural analysis, by using detergents at relatively high concentrations to unravel the notochordal sheath structure, also revealed that the Calymmin-containing matrix was in fact composed

of fibrils of similar size to those observed in the collagen-like region, that here appeared coupled to electron dense granules (Fig. 6E). The filamentous and granular structure of the notochordal sheath compartment containing Calymmin is suggestive of the presence of proteoglycans, as was previously suggested (Kenney and Carlson, 1978; Camón et al., 1990). Considering that the Calymmin amino acid sequence shows a conserved glycosaminoglycan attachment motif, this finding suggests that Calymmin may represent itself a

novel type of proteoglycan. However, in the absence of more conclusive biochemical evidence, and because Calymmin shows no consistent homology to other known proteoglycans core proteins, such as decorin, biglycan, fibromodulin, aggrecan, or versican, the biochemical nature of Calymmin remains to be determined.

### CONCLUSIONS

The findings presented in this study have identified a novel ECM protein in the zebrafish, named Calymmin, to indicate its localization within the notochordal sheath of the embryo. Calymmin presents a unique amino acid sequence with no homology to other known proteins in the databases, although conserved domains to those found in the Calymmin polypeptide have been identified in some procaryotic and eucaryotic proteins of unknown functions. The pattern of expression of Calymmin mRNA in the notochord cells and the likely localization of the protein within the Golgi apparatus of wild-type notochord cells, but not in those from *ntl* mutant embryos, suggest that Calymmin is specifically expressed and secreted by the differentiating notochord cells during the formation of the notochord and the initial stages of segmentation. The Calymmin protein, therefore, appears to be a specific product secreted by the zebrafish embryonic notochord, which is known to be the source of several important signalling molecules (e.g., Sonic hedgehog) essential for neural patterning and muscle differentiation (Odenthal et al., 1996; Blagden et al., 1997). Thus, although Calymmin seems to be a constitutive protein of the notochordal sheath of the embryo, with likely structural functions, it could well play additional roles during notochord and somite differentiation that remain to be investigated.

Although in the present work Calymmin mRNAs have not been detected in the adult fish, Calymmin or Calymmin-like polypeptides have been observed in some adult tissues in preliminary immunofluorescence microscopy experiments (data not shown). Similar to what we reported here for the embryo, in these experiments the anti-Calymmin antibodies notably labelled filamentous structures around the muscle tissue, that also appear to form a separate compartment from the basal lamina of these cells. Therefore, Calymmin, or Calymmin-related proteins, may well have additional functions also within adult tissues.

### EXPERIMENTAL PROCEDURES

#### Fish and Embryo Culture

Breeding zebrafish were maintained as described by Westerfield (1995). Wild-type and null allele carrying embryos for *no tail* (*ntl*<sup>tc41</sup>; Odenthal et al., 1996) were cultured in E2 culture medium (Westerfield, 1995) containing 0.2 mM 1-phenyl-2-thiourea (PTU; Sigma) to prevent pigment formation. Embryos were staged according to Kimmel et al. (1995).

#### cDNA Library Screening and Expression Analysis

Approximately  $1 \times 10^6$  plaques of a postsomitogenesis zebrafish embryo (generously provided by Drs. R. Riggelman, K. Helde, and D. Grunwald, Department of Human Genetics, Eccles Institute, University of Utah, Salt Lake City, UT) and neurula (a kind gift of Prof. José Campos-Ortega, Institute for Developmental Biology, University of Cologne, Germany)  $\lambda$ -ZAP II cDNA libraries were screened under high stringency by using a 458-bp fragment corresponding to the 5'-end of clone c29-77. The positive, poly (A<sup>+</sup>)-bearing cDNA clones were excised (ExAssist Helper Phage, Stratagene, La Jolla, CA), and sequenced on both strands by using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA). The complete nucleotide sequence of zebrafish Calymmin (clone c39a) is available from GenBank, EMBL, and DDBJ under accession code AF102865. Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany), Pharmacia (Freiburg, Germany), and New England Biolabs (Schwalbach, Germany).

Isolation of total RNA from zebrafish embryos and whole adult fish and Northern blot and in situ hybridization on frozen adult tissue sections were performed as already described by Cerdà et al. (1999). Whole-mount hybridization in situ was carried out as described (Reifers et al., 1998), by using DIG-labelled riboprobes generated from the complete c29-77 and c39a cDNAs. Pictures were taken on manually dissected embryos cleared and mounted in 70% glycerol and on 3- $\mu$ m plastic sections of labelled embryos with a Zeiss Axioskop (Zeiss, Oberkochen, Germany).

#### Antibody Production, Immunoblotting, and Whole-Mount Immunocytochemistry

Guinea pig antibodies specific for Calymmin were obtained by immunization with synthesized peptides (Schnölder et al., 1992), representing various parts of the amino acid sequence as deduced from cDNA sequencing. The sequence of these peptides represented putative antigenic sites (Jameson and Wolf, 1988) of Calymmin and were controlled for low homology with other proteins by sequence database searches. The antibodies were affinity-purified on the iodoacetyl-immobilized peptide (UltraLink Iodoacetyl; Pierce, Rock-Ford, IL) as previously described (Cordes et al., 1997). All results shown in this study were obtained with antibodies against the amino acid sequence QN-MGYPNGGTKGPKPGYGAK of the Calymmin encoding clone c39a.

The cytoskeletal protein fractions of embryos were prepared following the protocol by Herrmann and Wiche (1983), or alternatively, embryos were homogenized in high Triton lysis buffer (Herrmann and Wiche, 1983) without EGTA and DNase I, mixed with  $3 \times$  Laemmli sodium dodecyl sulphate (SDS) sample buffer and boiled for 3–5 min at 95°C. Procedures for SDS-



polyacrylamide gel electrophoresis (SDS-PAGE) in 7–8% acrylamide gels and immunoblotting were as described (Cerdà et al., 1999); blots were incubated overnight at 4°C with the anti-Calymmin antibody Calym1-1 (1:200). Clone c39a was transcribed and translated in vitro in the presence of [<sup>35</sup>S]methionine in a rabbit reticulocyte lysate system by using T3 RNA polymerase according to the manufacturer's instructions (Promega, Heidelberg, Germany), and the products were subjected to SDS-PAGE and immunoblotting as described above.

Whole-mount immunocytochemistry was performed on embryos fixed with freshly prepared 4% paraformaldehyde for 30–90 min at 4°C, and subsequently treated essentially as described by Westerfield (1995) but by using 0.1% saponin in the reaction and washing buffers. The Calym1-1 antibody was used at a dilution of 1:50 overnight at 4°C, and secondary horseradish peroxidase-coupled antibodies at a dilution of 1:4,000 for 4 hr at 4°C. Pictures were taken as described for the whole-mount in situ hybridization. For immunofluorescence microscopy on cryostat sections, 24-hpf embryos were fixed and processed as described elsewhere (Cerdà et al., 1998). The ~5-μm-thick cryostat sections were incubated with Calym1-1 (1:50) for 1 hr at room temperature, in the presence or absence of the Hoechst fluorescent dye (10 μg/ml) to stain the cell nucleus. The incubation with secondary antibodies coupled to Texas Red (BioTrend, Köln, Germany) was performed for 30 min at room temperature. Pictures were taken with a Zeiss Axiophot by using TMY films (Eastman Kodak Co., Rochester, NY).

### Immunoelectron Microscopy

For conventional thin-section studies, embryos were processed as described (Rose et al., 1995). For immunoelectron microscopy on 24-hpf embryos, the specimens were treated as for whole-mount immunocytochemistry, but after the incubation with the anti-Calymmin antibodies, the embryos were incubated with secondary antibodies coupled to 1.4-nm gold particles (1:50 dilution) overnight at room temperature with shaking. After three washing steps for 20 min each with PBS, embryos were fixed in 2.5% (vol:vol) glutaraldehyde in cacodylate buffer pH 7.4 for 30 min, washed with cacodylate buffer for 15–30 min, and washed again with 1.2 M sucrose in 50 mM Hepes buffer pH 5.8. Silver enhancement was carried out for 8 min at room temperature in the dark by using the HQ Enhancement kit (BioTrend), followed by two 15-min washes, first in Hepes buffer 1.25 M sodium thiosulfate and then in water. Samples were post-fixed in 1.5% OsO<sub>4</sub> for 30 min, washed with double-distilled water for 10 min, dehydrated, and embedded in Epon. Electron photomicrographs were taken by using a Zeiss electron microscope EM 910 (LEO Electron Microscope Optics, Oberkochen, Germany).

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