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Original Article

Chitin oligosaccharides as candidate patterning agents in zebrafish embryogenesis

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ABSTRACT In this work we investigate the possible function of *N*-acetyl-chitooligosaccharides (NACOs) produced during zebrafish (*Danio rerio*) development. First, we show that NACOs are synthesized *in vivo* during early embryogenesis in the zebrafish. Second, we demonstrate that injection of a pure bacterial chitinase into one-cell stage embryos elicits developmental defects in which the posterior trunk and tail of developing embryo are severely affected. In addition, an endogenous chitinase activity detected both intra- and extracellularly is described, suggesting that cells may secrete it into the extracellular space. Moreover, this compartmentalization appears to be functionally relevant as inhibition of the extracellular, but not the intracellular, endogenous chitinase activity causes morphological defects similar to those seen in embryos injected with chitinase 63. Finally, analysis of the expression of the zebrafish *ZDG42* gene, which has been suggested to be involved in synthesis of NACOs, is described. Transcripts are detected from late blastula stage, during gastrulation, and move as an anterior-posterior wave of expression in adaxial mesoderm during somitogenesis.

KEY WORDS: *N-acetyl-chitooligosaccharides (NACOs), zebrafish embryo patterning, chitin oligosaccharide synthesis, zebrafish chitinase inhibition, ZDG42 gene*

Introduction

Chitin oligosaccharides or *N*-acetyl-chitooligosaccharides (NACOs), short linear chains of *N*-acetylglucosamine (β-1,4-linked GlcNAc), were initially described in bacteria as part of the Nod factors of *Rhizobium* (Long, 1989; Lerouge *et al.,* 1990; Dénarié and Collimore, 1993; Kamst *et al.,* 1997). Chitin polymer (100 or more residues of GlcNAc) is a cell wall component in fungi (Cabib *et al.,* 1982) and is the major exoskeleton component in arthropods (Richards, 1951; Hunt, 1970). More recently, chitin polymer has been found also in the epidermal cuticle of the Teleost fish (Wagner *et al.,* 1993), suggesting that vertebrates posses the enzymatic machinery necessary for chitin synthesis. Ten years ago it was reported that *N*-acetylchitooligosaccharides elicited significant chemotactic responses in human neutrophils (Tokoro *et al.,* 1988) and in mouse leukocytes *in vitro* (Susuki *et al.,* 1986). The authors concluded that the results suggest the presence of specific receptors to NACOs in the surface of these cell types (Tokoro *et al.,* 1988). This was the first demonstration of the biological activity of NACOs in a vertebrate scenario. At the same time, in a screen for genes which are expressed at high levels in early *Xenopus* embryos, Rosa *et al.* (1988) isolated a cDNA named *DG42*. It was later shown (Bulawa and Wasco, 1991; Bulawa, 1992) that the protein encoded by *DG42* had sequence similarity at the amino acid level to a number of proteins such as the *Rhizodium* NodC protein, which has been shown to synthesize NACOs (Geremía *et al.,* 1994; Spaink *et al.,* 1994), to the *Streptococcus* HasA protein, which has been reported to be a hyaluronan (HA) synthase (Dougherty and van de Rijn, 1992) and to fungal chitin synthases (Bulawa and Wasco, 1991). More recently, cDNAs homologous to *DG42* were isolated from zebrafish and mouse, and were named *ZDG42* and *MDG42*, respectively (Semino *et al.,* 1996). In addition, several mammalian proteins belonging to this family were subsequently described: HAS and HAS2 -or MDG42- in mouse, and HuHAS1 and HAS2 (human) which are involved in the synthesis of hyaluronic acid (HA) (Itano and Kimata, 1996; Shyjan *et al.,* 1996; Spicer *et al.,* 1996; Watanabe and Yamaguchi, 1996). The *Xenopus* DG42 gene is

Abbreviations used in this paper: NACOs, *N*-acetyl-chitooligosaccharides; GlcNAc, *N*-acetylglucosamine; HA, hyaluronic acid or hyaluronate; hpf, hours post fertilization; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography.

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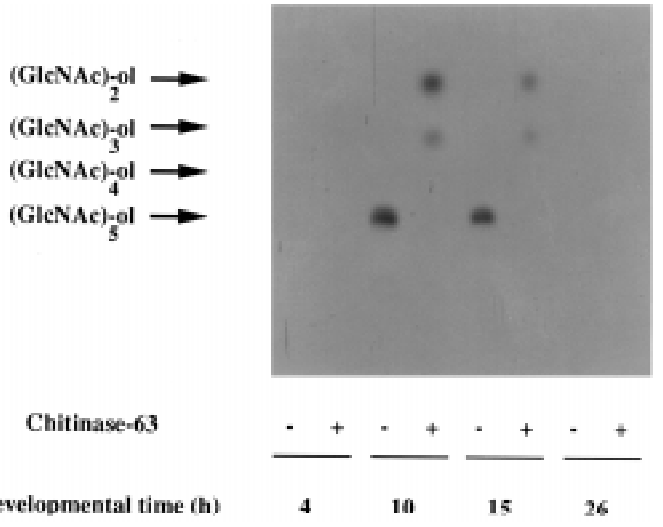

Fig. 1. *In vivo* production of *N*-acetyl-chitooligosaccharides, NACOs, by late gastrula stage embryos. *Embryos from four developmental stages (500 each) (4 h, late blastula; 10 h, late gastrula; 15 h, somitogenesis; 26 h, postsomitogenesis) were boiled and extracted with mild alkali solution (10 mM NaOH). The alkali extract was neutralized, centrifuged and loaded on a mixed ion exchange column (see Materials and Methods). The neutral water-soluble material was concentrated and injected into an HPLC column, and the volume of solvent corresponding to the retention time of the penta-N-acetyl-chitopentaose was collected, concentrated and labeled by reduction with NaB-[^{3}H]H$_4$ (see Materials and Methods). The [^{3}H]labeled material from the four developmental times were loaded again in the same HPLC column after purification with a mixed ion exchange column (see Materials and Methods). The peak of radioactivity eluted in the position of penta-N-acetyl-chitopentaositol was collected and analyzed by TLC after incubation with (+) or without (-) chitinase 63 (see Materials and Methods). The following standards were used: (GlcNAc)$_2$-ol, di-N-acetyl-chitobiositol; (GlcNAc)$_3$-ol, tri-N-acetyl-chitotriositol; (GlcNAc)$_4$-ol, tetra-N-acetyl-chitotetraositol; and (GlcNAc)$_5$-ol, penta-N-acetyl-chitopentaositol.*

expressed during early embryogenesis, between the gastrula and neurula stages (Rosa *et al.,* 1988). Northern analysis showed that the zebrafish *DG42* homolog is expressed within a similar developmental timeframe (Semino *et al.,* 1996). In this work, we analyze the expression of the zebrafish *ZDG42* gene by *in situ* hybridization analysis. *ZDG42* transcripts are detected beginning at the late blastula stage in the future germ ring, in the embryonic shield during gastrulation, and move as an anterior-posterior wave of expression in the adaxial mesoderm during somitogenesis. It was recently postulated, by studies of sequence analysis among the vertebrate *HAS* gene family, that the *Xenopus DG42* gene belongs to the *HAS 1* group and has been proposed to be named *XHAS 1*, whereas *MDG42* and *ZDG42* (Semino *et al.,* 1996) belong to the *HAS 2* group and were named *MHAS 2* and *ZHAS 2*, respectively (Spicer and McDonald 1998). However, here we follow the original nomenclature of *ZDG42* instead of *ZHAS 2.*

We showed in previous work that recombinant *Xenopus DG42* protein expressed in an *in vitro* transcription/translation system is able to catalyze both the synthesis of NACOs, which vary in chain length from three to six residues of *N*-acetylglucosamine, and of chitin polymer (Semino and Robbins, 1995). Cell extracts prepared from either frog or zebrafish embryos of the appropriate stage (gastrula or neurula) are also able to synthesize NACOs *in vitro* (Semino *et al.,* 1996). The same result was later described in carp embryos (Bakkers *et al.,* 1997). We now show that, concurrent with the expression of the *ZDG42* gene, it is possible to detect, at the expected developmental stages, NACOs synthesized in zebrafish embryos *in vivo*.

In previous experiments we reported that zebrafish embryos injected with an anti-DG42 antibody develop abnormally; the posterior trunk and tail of embryos are severely affected (Bakkers *et al.,* 1997). Injection of this antibody into embryos at the one cell stage inhibits NACO synthase activity *in vitro* as well as *in vivo* (C. Semino, unpublished results). Consistent with these results, developmental defects similar to those observed after injection of anti-DG42 antibody were observed in two related experiments. First, such defects were seen in zebrafish embryos injected with pure NodZ protein (Bakkers *et al.,* 1997), a specific NACO modifying enzyme which transfers a fucose residue from GDP-fucose to the position C-6 of the reducing end of NACOs (Quinto *et al.,* 1997). And second, a similar phenotype was elicited by injection of a plasmid encoding NodZ under the control of the CMV promoter in zebrafish embryos (Semino *et al.,* 1998). These observations suggest that *in vivo* modification of the NACO structure, in this case by adding a new fucose residue, may functionally inactivate the molecule leading to the observed developmental defect (Bakkers *et al.,* 1997; Semino *et al.,* 1998). In this work, we have continued these studies by injecting a pure preparation of a bacterial chitinase, chitinase 63, used previously to inhibit synthesis of HA and NACOs *in vitro* (Semino *et al.,* 1996). Embryos injected with chitinase 63 show a significant reduction in the *in vitro* and *in vivo* synthesis of NACOs and HA and present developmental defects that closely mimic those seen with injection of anti-DG42 antibody or of NodZ protein (Bakkers *et al.,* 1997; Semino *et al.,* 1998). In addition, an endogenous chitinase activity in zebrafish embryos is described. The chitinase activity is detected both intra- and extracellularly, suggesting that it may be secreted by the cells into the extracellular space. Moreover, this compartmentalization appears to be functionally relevant as inhibition of the extracellular, but not the intracellular, endogenous chitinase, causes morphological defects similar to those seen in embryos injected cytoplasmically with chitinase 63. These results suggest that NACO synthesis and degradation may have an essential role during zebrafish embryonic development.

Results

Chitin oligosaccharides (NACOs) are produced in vivo by zebrafish embryos

Previously, we obtained *in vitro* synthesis of NACOs with frog and zebrafish enzyme preparations from gastrula and neurula stage embryos (Semino *et al.,* 1996). In this work, our major interest was to know whether or not the embryo naturally produces NACOs and, if so, whether they have an essential function during embryogenesis. As the cytoplasmic levels of UDPGlcNAc are relatively high in eukaryotic cells, we believed it would be possible to purify and detect NACOs from zebrafish embryos. Harvested embryos from four developmental stages (early blastula until postsomitogenesis, 500 embryos of each stage) were boiled and extracted with mild alkali solution (10 mM NaOH) (see Materials and Methods). NACOs were then purified from the

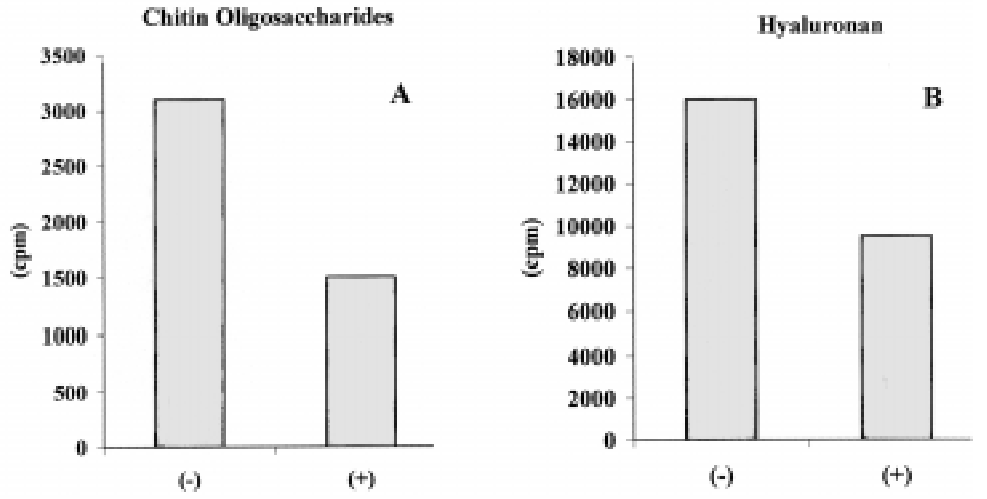

Fig. 2. Inhibition in the synthesis of NACOs and HA in embryos injected with chitinase 63. *Zebrafish embryos at one cell stage were microinjected with approximately 5 nl of BSA (1 mg/ml) (n=110) (-) or chitinase 63 (0.01-0.02 mg/ml) (n=120) (+) and were cultured for 6-8 h. Fifty (50) embryos (of each group) were removed and used to prepare cell extracts and incubated in vitro in presence of UDP-[^{14}C]GlcNAc as described in Materials and Methods. The incubations were stopped by boiling and extracted with water. The material present in the soluble fraction was analyzed by HPLC (for detection of chitin oligosaccharides) and FPLC (for detection of HA) (see Materials and Methods).* **(A)** *[^{14}C]GlcNAc-chitin oligosaccharides (NACOs) and* **(B)** *[^{14}C]GlcNAc-hyaluronan (HA) produced in vitro with two different extracts: embryos injected with BSA (control) (-), or chitinase 63 (+).*

extract and radiolabeled by reduction with NaB-[^{3}H]H$_4$. The [^{3}H]labeled material was fractionated by HPLC and the peaks of labeled reduced chitopentaose (penta-*N*-acetyl-[^{3}H]chitopentaositol) detected by radioactivity were collected and analyzed by TLC (Fig. 1). Late gastrula and neurula stage samples have a compound with the chromatographic characteristics of penta-*N*-acetyl-chitopentaositol (Fig. 1, 10 h and 15 h respectively). After treatment with chitinase 63 the samples showed the expected degradation products: di-*N*-acetyl-chitobiositol and tri-*N*-acetyl-chitotriositol (Fig. 1, 10 h+ and 15 h+). The sample from postsomitogenesis stage embryos showed detectable amounts of penta-*N*-acetyl-chitopentaositol by HPLC, but cannot be detected in the TLC result (Fig. 1, 26 h).

Microinjection of chitinase 63 induces developmental defects

Since injection of anti-DG42 antiserum causes abnormalities in the development of the trunk and tail of zebrafish embryos (Bakkers *et al.,* 1997), we sought to confirm that these effects are due to the specific inactivation in the synthesis of NACOs and/or HA. Previously, we observed that chitinase 63 inhibits the biosynthesis of HA and causes degradation of NACOs *in vitro* (Semino *et al.,* 1996). Therefore, we injected chitinase 63 (0.01-0.02 mg/ml, see below) into one cell stage embryos, and analyzed NACO and HA synthase activity *in vitro* at 10-12 hpf (late gastrula). Chitinase 63 injected embryos show a reduction in the synthesis of both NACOs (Fig. 2A) and HA (Fig. 2B) and develop morphological defects which become visible at 22-24 hpf (see below, Fig. 3A). In addition, a reduction of 46% (Standard deviation, S.D.= 8.1 %) in the amount of extractable penta-*N*-acetyl-chitopentaose labeled with NaB-[^{3}H]H$_4$ was obtained with embryos injected with the bacterial chitinase 63, compared with control embryos injected with BSA in PBS (see Materials and Methods) (not shown). In a parallel experiment we also analyzed the amount of HA produced in embryos, of the same developmental age, injected with chitinase 63 *versus* controls injected with BSA in PBS. The water soluble material extracted from both groups of embryos was radiolabeled by reduction with NaB-[^{3}H]H$_4$, analyzed chromatographically and by its sensitivity to hyaluronate lyase and quantitated (see Materials and Methods). A reduction of 27% (S.D.= 5.2%) in the amount of [^{3}H]HA was obtained in the group of embryos injected with chitinase 63 (not shown).

To analyze potential morphological disruptions, we injected chitinase 63 into one-cell stage embryos and raised them to two days post fertilization (see Materials and Methods). High concentrations of chitinase 63 (0.1 mg/ml) were lethal shortly after injection (embryos did not progress past the blastula stage), whereas highly diluted chitinase (0.001 mg/ml) produced no detectable effect. Since we were interested in determining whether chitinase 63 has an effect during the period in which NACO synthesis is observed, we arrived at a working concentration of about 0.01-0.02 mg/ml in which approximately 50% of the embryos showed developmental defects visible at 24 hpf, although the severity of the defect (described below) was not uniform (Fig. 3A). The variability observed within a batch of chitinase 63 injected embryos could be due to differences in the injection volume, unequal distribution of the injected material, or uneven susceptibility among the animals to the enzyme. In a series of injection experiments we quantitated the number of affected individuals and compared it to control injected embryos (Fig. 3B). A small number of embryos in each experiment (under 5%) develop non-specific abnormalities due to the injection procedure. These abnormalities were observed in control experiments where embryos were injected with BSA (in PBS), or with PBS alone, and can usually be distinguished from the specific effect of the chitinase 63 (Fig. 3B). However, to avoid any possible confusion between "background" vs. specific embryonic malformations, we have simply quantitated "affected" *vs.* normal for each experiment. The quantitative analysis for these experiments is shown in Figure 3B.

Since the concentration of chitinase 63 which causes developmental abnormalities in about half of the injected embryos was achieved empirically, it was crucial to include a control which can rule out a non-specific effect of the chitinase. We injected a

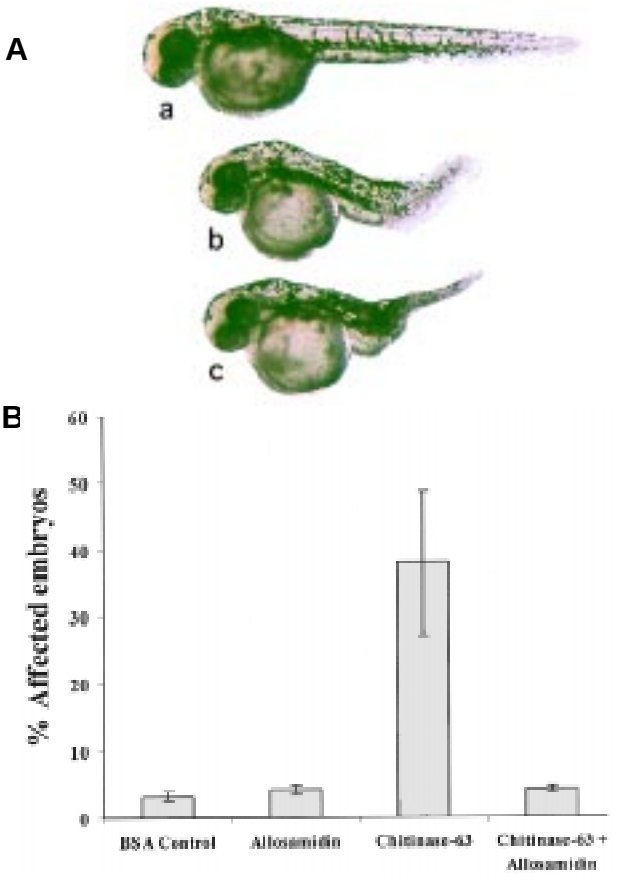

Fig. 3. Microinjection of chitinase 63 into one cell stage embryos leads to abnormal development of the trunk and tail. *Embryos at the one cell stage were microinjected with approximately 5 nl of BSA (1 mg/ml), chitinase 63 (0.01-0.02 mg/ml), chitinase 63 preincubated with allosamidin (0.2 mg/ml) and allosamidin (0.2 mg/ml). After injection, the embryos were cultured under standard conditions and viewed at 24 hpf (see text). The concentration of chitinase 63 used in this case produces visible developmental defects in approximately 50% of the embryos.* **(A)** *Top (a), control embryo microinjected with chitinase 63 + allosamidin. Center (b) and bottom (c), two affected individuals microinjected with chitinase 63 showing moderate and severe defects, respectively.* **(B)** *For each group of microinjected embryos the number of embryos that survived to gastrulation were counted (n), and the percentage of affected embryos of each experiment in the group at 24 hpf was calculated (% aff.). The number of experiments is indicated by N. The parameters N and % aff. were used in each case to calculate the standard deviation (S.D.). The graph shows the average % of affected embryos (% affected) by group and its standard deviation (S.D.) for the four different groups. BSA control: n=79, N=2, % affected=3.3, S.D.=0.75; allosamidin : n=94, N=2, % affected=3.95, S.D.=0.75; chitinase 63: n=181, N=4, % affected=38.8, S.D.=10.9; chitinase 63 + allosamidin: n=155, N=3, % affected=4.0, S.D.=0.48.*

mixture of chitinase 63 with the specific inhibitor allosamidin (chitinase 63 enzyme preincubated with 0.2 mg/ml of allosamidin, see Materials and Methods) that causes a reduction of approximately 75-80% in the enzymatic activity (not shown). Injection of this mixture did not elicit the developmental defects seen with the enzyme alone (Fig. 3B), and quantitation of abnormal appearing embryos in these experiments showed numbers that are comparable to the other two control batches, namely the injections with PBS or BSA (Fig. 3B). Likewise, injection of allosamidin alone into one cell stage embryos had no effect (Fig. 3B). In addition, the strong deleterious effect that results from injecting a high concentration of chitinase 63 (0.1 mg/ml), could be overcome by preincubation with allosamidin (0.2 mg/ml): developmental defects could be observed in approximately 60% of the embryos at 24 hpf. Therefore, allosamidin has a specific protective effect against chitinase 63. In embryos injected with chitinase 63, the observed morphological abnormalities after 24 and 48 h of development recall those seen after injection of anti-DG42 antiserum (Bakkers *et al.,* 1997) or after expression of the NodZ enzyme in early embryos (Semino *et al.,* 1998). The most severely affected individuals lacked tail structures or the tails were seriously malformed (Fig. 3A). Less affected animals showed bends or kinks in the tail, and often had irregularly shaped somites and bent notochords (Fig. 3A). Between the time of injection (0 hpf) and 12 h of development, chitinase injected embryos were indistinguishable from injected controls under the dissecting microscope (see below). In order to characterize in more detail the phenotype of the embryos injected with chitinase 63 we performed *in situ* hybridization with multiple probes representative of various axial positions and tissue types. *In situ* hybridization with *goosecoid*, a probe that labels early axial mesoderm (Schulte-Merker *et al.,* 1994a); *no-tail* (*ntl*), a probe that labels notochord (Schulte-Merker *et al.,* 1994b); and *oxt2*, a probe that levels the anterior central nervous system (Li *et al.,* 1994), did not reveal any noticeable developmental defects at these early stages (not shown). However, we did observe an effect of chitinase injection in embryos hybridized with a probe for the *Myo D* gene, which labels somitic mesoderm (Weinberg *et al.,* 1996) (Fig. 4). The two stripes of stained paraxial mesoderm normally seen at 12-13 hpf (Fig. 4A) were usually present in chitinase-injected embryos, though they were often kinked or interrupted (Fig. 4B). *MyoD* expressing cells in the somitic mesoderm, which normally form compact series of bilateral stripes, were often disorganized and sometimes absent (Fig. 4B-C). At a later timepoint (18 hpf), these defects become more pronounced (Fig. 4E-F). We have also observed occasional ectopic expression of *MyoD* within axial tissue or extending laterally beyond the normal width of somitic mesoderm (Fig. 4B and C). At 24 h, the abnormalities are overtly manifest in live embryos and become more pronounced at 48 h (Fig. 3A). Affected embryos do not develop past the third or fourth day of life. In addition, we performed control experiments to test the effect of chitinase 63 on cell viability. Biotin-dextran or chitinase 63 + biotin-dextran was microinjected into one cell stage embryos. The embryos were allowed to develop until the blastula stage and were used as donors for cell transplantation into non-injected host embryos of the same age. Donor and host embryos were allowed to develop and were observed at 24 hpf and analyzed for visible developmental defects. Defects were observed in 46% of donor embryos injected with biotin-dextran + chitinase 63 while only 4% of control embryos (injected with biotin-dextran alone) were affected as expected from our previous results with chitinase injected embryos. In the case of host embryos, also evaluated at 24 hpf, defects were observed in 16% of the embryos, both for those receiving control cells and chitinase injected cells. These defects, therefore, are due exclusively to the effect of the transplantation on normal development. Furthermore, this result suggests that the effect of the injected chitinase is cell-autonomous,

as host embryos are not affected by the presence of chitinase-bearing cells. The host embryos were then fixed and were processed for detection of transplanted cells using peroxidase-coupled antibodies to biotin (see Materials and Methods). In all cases, the transplanted cells were able to differentiate into various cell types, including neurons, muscle and notochord cells (Fig. 5). This indicates that chitinase 63 is not inherently toxic to cell viability.

Endogenous chitinase activity in early embryos

In view of the results obtained above with injections into the cytoplasm of one cell stage embryos, we tested whether delivery of chitinase 63 into the extracellular space of blastula stage embryos (3 hpf), would elicit an effect (see Materials and Methods). Blastula stage embryos were injected with identical concentrations of reagents as those used for one-cell stage cytoplasmic injections. We found that in this case the injection of chitinase 63 was far less effective compared to when it was delivered into the cytoplasm (Fig. 6B, compare to Fig. 3B). Surprisingly, however, injection of allosamidin alone was very effective, promoting trunk and tail defects and often resulting in additional malformations in the head region (Fig. 6A). As before, malformations in embryos injected extracellularly at the blastula stage were not visible by inspection under the dissecting microscope between 12-13 hpf. However, abnormalities could be visualized by *in situ* hybridization with the *MyoD* probe (Fig. 7). We observed interruption in the paraxial mesoderm expression pattern in chitinase injected embryos (Fig. 7B), similar to those seen before when the enzyme was delivered into the cytoplasm (Fig. 4B and C). Embryos injected with allosamidin, usually showed strong disruptions in the distribution of *Myo D* expressing cells. The bilateral stripes were, in most cases, highly disorganized and interrupted (Fig. 7C). The defects produced by the allosamidin suggest that the inhibitor may be interfering with an endogenous chitinase activity present during early development. To confirm this idea, we injected tetra-*N*-[^{14}C]acetyl-chitotetraose into the cytoplasm of one cell stage embryos and followed its pattern of degradation during the first 28 h of embryogenesis. The tetra-*N*-acetyl-chitotetraose was completing degraded to di-*N*-acetyl-chitobiose, the expected product of a chitinase activity, during the first 10-15 hpf (Fig. 8). In a parallel experiment, cytoplasmic injection of penta-*N*-[^{14}C]acetyl-chitopentaose, also produced the expected product of a chitinase activity: di-*N*-acetyl-chitobiose and tri-*N*-acetyl-chitotriose (not shown). To examine whether this chitinase activity can be detected extracellularly, we injected penta-*N*-[^{14}C]acetyl-chitopentaose at the blastula stage. The material was also degraded in the expected way, producing di-*N*-acetyl-chitobiose and tri-*N*-acetyl-chitotriose (Fig. 9). However, in this case a fraction of the chitopentaose still remained intact after more that 24 h (Fig. 9, lane 3). Finally, the endogenous chitinase activity and its inhibition by allosamidin were assayed *in vitro*. Chitinase activity can be assayed *in vitro* either from cells (cellular lysates) or from extracellular embryonic material (see Materials and Methods) using penta-*N*-[^{14}C]acetyl-chitopentaose as a substrate. Again, with both preparations the expected degradation products were observed: di-*N*-acetyl-chitobiose and tri-*N*-acetyl-chitotriose (Fig. 10). Interestingly, allosamidin inhibits the zebrafish chitinase activity *in vitro* by almost 100%, under the conditions tested (Fig. 10). These results strongly suggest a link between the inhibition of the endogenous chitinase activity present in the extracellular milieu and the developmental disruptions observed after injection of allosamidin at the blastula stage (Fig. 6).

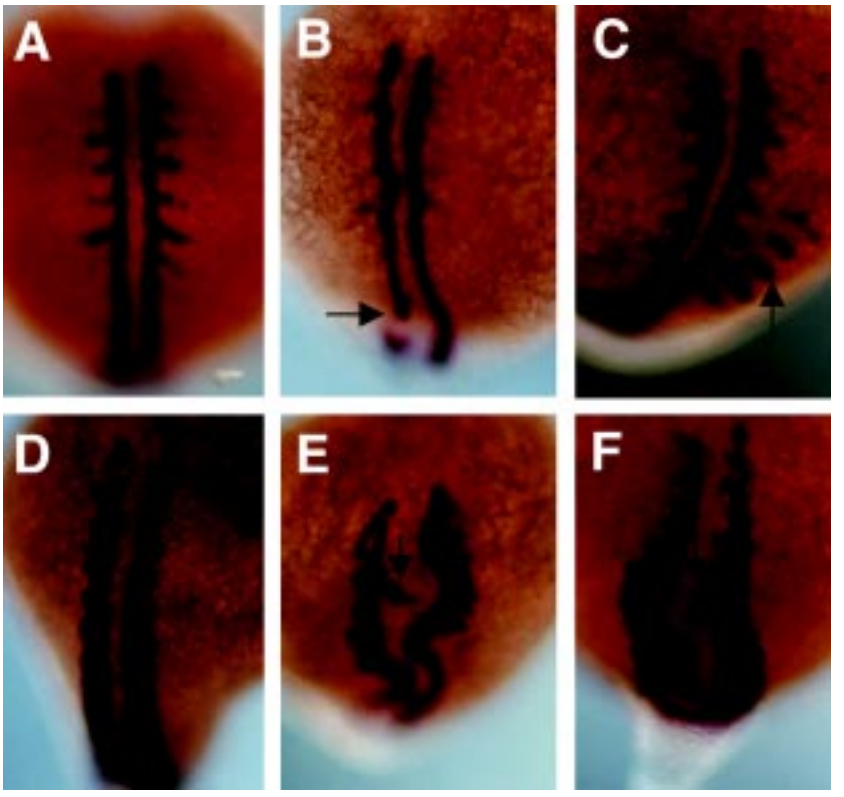

Fig. 4. ***Myo D* staining shows early mesoderm patterning defects in embryos injected at the one cell stage with chitinase 63.** *Embryos were microinjected at the one cell stage with chitinase 63 or chitinase 63 preincubated with allosamidin, as described in Figure 3, were fixed in paraformaldehyde at two developmental timepoints (12-13 h and 16-18 h) and were used to perform in situ hybridization using Myo D as a probe (see Material and Methods).* **(A)** *12-13 h control, chitinase 63 + allosamidin;* **(B** *and* **C)** *12-13 h, chitinase 63;* **(D)** *16-18 h control, chitinase 63 + allosamidin;* **(E** *and* **F)** *16-18 h, chitinase 63. The arrows indicate abnormal MyoD expression sites in chitinase 63 injected embryos (see text).*

Expression pattern of the ZDG42 gene

A 450bp *ZDG42* gene fragment was previously obtained by PCR using degenerate oligonucleotides based on the *Xenopus* DG42 sequence (Semino *et al.,* 1996). Northern blot analysis had shown that expression of *ZDG42* message in zebrafish was detectable at late blastula stage (4 hpf), reached a peak at neurula stage, decreased thereafter, and was undetectable after 24 hpf (Semino *et al.,* 1996). Similar expression profiles were reported for the *Xenopus* DG42 homolog (Rosa *et al.,* 1988). To ascertain the distribution of ZDG42 transcripts during embryogenesis, we performed *in situ* hybridization on zebrafish embryos with the 450bp *ZDG42* gene fragment as a probe (see Materials and

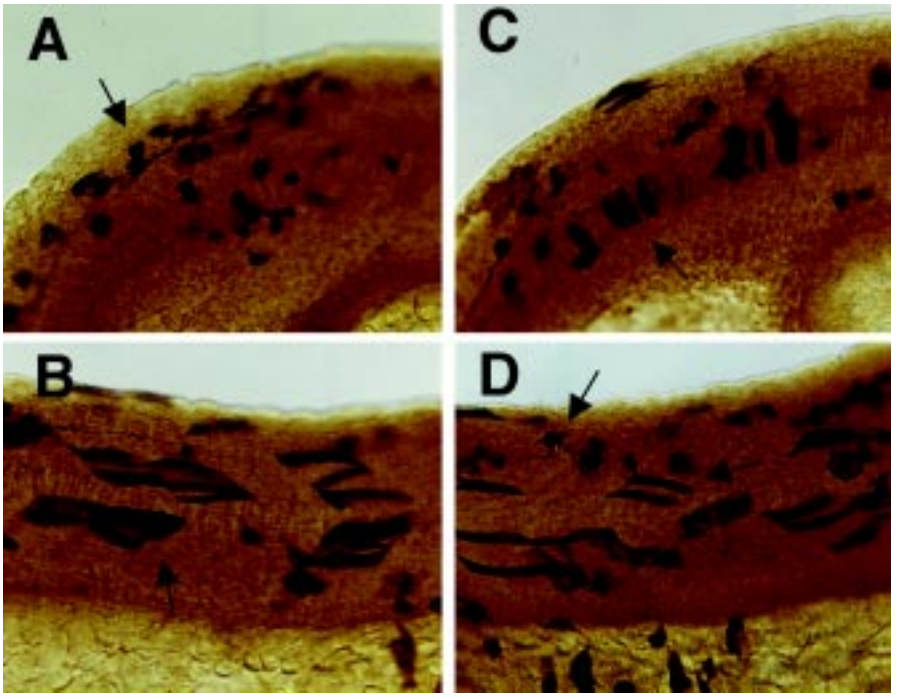

Fig. 5. Embryos transplanted with embryonic cells carrying chitinase 63. *Embryos were microinjected at the one cell stage with biotin-dextran or a mixture of biotin-dextran and chitinase 63 (0.01-0.02 mg/ml). After injection, the embryos were cultured under standard conditions to 3-4 hpf and were used for transplantation experiments. About 40-80 cells of each microinjected donor embryo were transplanted into non-injected host embryos of the same age. The host embryos were processed for detection of cells carrying biotin-dextran (derived from the transplanted cells) using peroxidase-coupled antibodies to biotin (see Materials and Methods).* **(A** *and* **B)** *Cells carrying biotin-dextran.* **(C** *and* **D)** *Cells carrying biotin-dextran and chitinase 63. Arrows indicate different cell types derived from the transplanted cells*: **(A)** *neurons*; **(B)** *muscle*; **(C)** *notochord cells*; **(D)** *neuron.*

Methods). *ZDG42* RNA can be seen starting at late blastula stage as a weak ring of staining at the blastula margin (not shown). During gastrulation, weak expression is observed around the germ ring with a slightly higher concentration at the embryonic shield (Fig. 11A). At early neurulation, weak expression is evident in presumptive mesoderm lateral to the notochord (adaxial cells) at the position of the most anterior somites (Fig. 11B and C, black arrowheads). As somitic tissues become defined in later stages, the expression of *ZDG42* decreases and is not visible by the time the somites have condensed. Therefore, *ZDG42* RNA can be detected in somitic adaxial cells during a short developmental window and follows an anterior to posterior wave of expression. At 22 h of development, *ZDG42* message can only be seen in the distal portion of the tail where somites are still in the process of being formed (Fig. 11D, black arrowhead, E and F). An additional site of *ZDG42* expression becomes visible during late somitogenesis: staining can be seen in presumptive endodermal tissue in two parallel lines lateral to the midline (Fig. 11B and C, white arrowheads). These cells continue to express ZDG42 until 24 h and they are located in a position that suggests they are part of the developing gut (Fig. 11D, white arrowhead). The elucidation of the exact fate of these expressing cells awaits further analysis.

Discussion

Previously, we have demonstrated that zebrafish embryo extracts can synthesize chitin oligosaccharides (NACOs) *in vitro*. Similar results have recently been found in carp embryos (Bakkers *et al.,* 1997). In addition, we and others have shown that the DG42 protein is able to direct the synthesis of hyaluronan (HA) *in vitro* (DeAngelis and Achyutan, 1996; Meyer and Kreil, 1996; C. Semino, unpublished results). However, NACO synthase activity, but not HA synthase activity, can be inactivated with a specific anti-DG42 antibody (Semino *et al.,* 1996). The HA synthase activity detected *in vitro* is highly dependent on the incubation conditions (Mian, 1986; Triscott and van de Rijn, 1986; Ng and Schwartz, 1989), is associated with the membrane fraction and, in addition, is strongly inhibited by chitinase 63 when cell extracts from zebrafish or frog are used as enzyme preparation, suggesting that NACOs or derivative molecules may act as templates in the synthesis of HA (Semino *et al.,* 1996; Varki, 1996). Zebrafish embryos show NACO production and accumulation *in vivo*, mainly between the gastrula and neurula stages (Fig. 1). We have attempted to disrupt the possible activities of NACOs and their potential derivatives by introducing an exogenous bacterial chitinase (chitinase 63) into developing zebrafish embryos. Embryos injected with the chitinase show a remarkable reduction in the synthesis and accumulation of NACOs and *HA in vitro* and *in vivo* (Fig. 2). Since cytoplasmatic NACOs are accessible to the enzyme during the first hours of development we therefore assume that the specific effects observed are in part due to the absence or reduction of the chitin oligosaccharides and/or HA. The injected embryos fail to develop a normal tail and have irregular or misshapen somites (Fig. 4). A similar effect was seen after injection of both the anti-DG42 antibody (Bakkers *et al.,* 1997) and a chitin oligosaccharide modifying enzyme, the NodZ fucosyltransferase (Bakkers *et al.,* 1997; Semino *et al.,* 1998), suggesting that these agents may affect the same general process. The specificity of the bacterial chitinase 63 effect is reinforced by the result of preincubating the chitinase with allosamidin, a chitinase inhibitor, which causes a significant reduction in its activity (75-80%), and in the number of defective embryos obtained when compared to injection of chitinase alone (Fig. 3B).

We also found that injection of chitinase 63 into the extracellular space of blastula stage embryos had less of an effect than cytoplasmic injection at the one cell stage (Fig. 6B). This could indicate that the biologically active substrate for the chitinase is

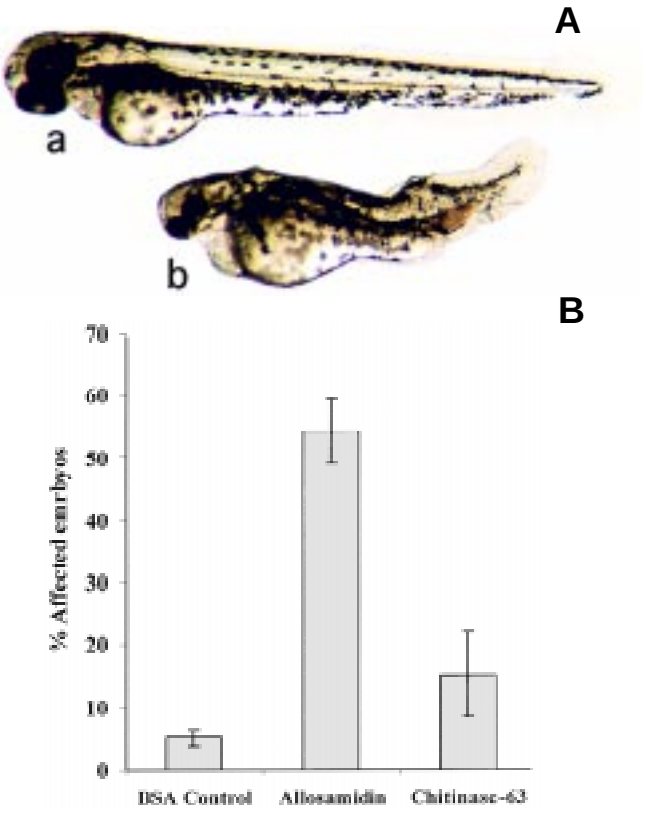

Fig. 6. **Microinjection of allosamidin into the extracellular space of blastula stage embryos leads to abnormal development.** *Embryos at blastula stage (3-4 hpf) were microinjected with approximately 5 nl of BSA 1 mg/ml), chitinase 63 (0.01-0.02 mg/ml) or allosamidin (0.2 mg/ml) in the extracellular space between the blastomeres. After injection, the embryos were cultured under standard conditions and viewed at 24 hpf (see text). The concentration of allosamidin used in this case produces visible developmental defects in approximately 50% of the embryos.* **(A)** *Top (a), control embryo injected with BSA in PBS; bottom (b), embryo injected with allosamidin (in PBS), showing severe defects in the head, trunk and tail.* **(B)** *Each group of injected embryos that survived to gastrulation was used to calculate the same parameters present in Figure 4B. BSA control: n=82, N=2, % affected=5.0, S.D.=1.5; allosamidin: n=102, N=3, % affected=54.2, S.D.=5.2; chitinase 63: n=93, N=2, % affected=15.1, S.D.=6.8.*

not available to the enzyme in the extracellular space, that the enzyme may be inactivated or inhibited by some component present in the extracellular space, or that it is degraded. An alternative explanation of this effect could be that the chitinase 63 is not able to compete for the substrate with its endogenous counterpart (see below). An unexpected finding from this work was the discovery of an endogenous chitinase activity in zebrafish embryos. Vertebrate chitinases have been described previously (Lundblad *et al.,* 1979; Overdijk and Steinj, 1994; Boot *et al*., 1995). In our case, the endogenous zebrafish chitinase activity appears to be localized both intra- and extracelullarly (Figs. 8 and 9, respectively). When penta-*N*-[^{14}C]acetyl-chitopentaose was injected into the extracellular space, some material remained intact after more that 24 hpf, suggesting the following possible explanations: I) The chitinase is less active in this compartment. II) It is secreted only by some areas of the embryo, or III) the NACOs are protected against the chitinase attack by interacting with a protein. Although both the intra- and extracellular chitinase activities can be strongly inhibited by allosamidin (Fig. 10), only the inhibition of the extracellular activity causes dramatic developmental abnormalities in the whole embryo (Fig. 6). This result may be explained by postulating that the intracellular chitinase is inaccessible to the inhibitor, for example, if it was localized in the endoplasmic reticulum. Alternatively, only the NACOs present in the extracellular space may be essential for proper embryonic development, suggesting that allosamidin may be competing with NACOs for a cellular receptor. If this is the case, NACOs as such, or a modified form of these molecules, could be secreted into the extracellular environment where they could have a signaling function. An interaction of NACOs with a specific cell surface receptor has been suggested previously by Tokoro *et al.* (1988) for the chemotactic response of human neutrophils to hexa-*N*-acetyl-chitohexaose *in vitro*.

In addition, we have shown that the *ZDG42* gene is expressed in a temporal and spatially regulated manner during the early embryonic stages of zebrafish development in a way that recalls the expression pattern of *DG42* gene in *Xenopus laevis* (Fig. 11). *ZDG42* gene is specifically expressed in the developing trunk and tail mesoderm, prior to the time when defects are observed in embryos injected with chitinase-63 (this work) or with anti-DG42 antiserum (Bakkers *et al.,* 1997). Expression of *ZDG42* is also detected in tissue of endodermal origin but we have not analyzed these tissues in injected animals. Interestingly, independent results obtained in *Xenopus laevis* by injection of Chitinase-63 into embryos (C. Wagner, personal communication), show similar developmental defects to those seen in zebrafish injected with chitinase-63. Defects seem to be severe, but limited to trunk and

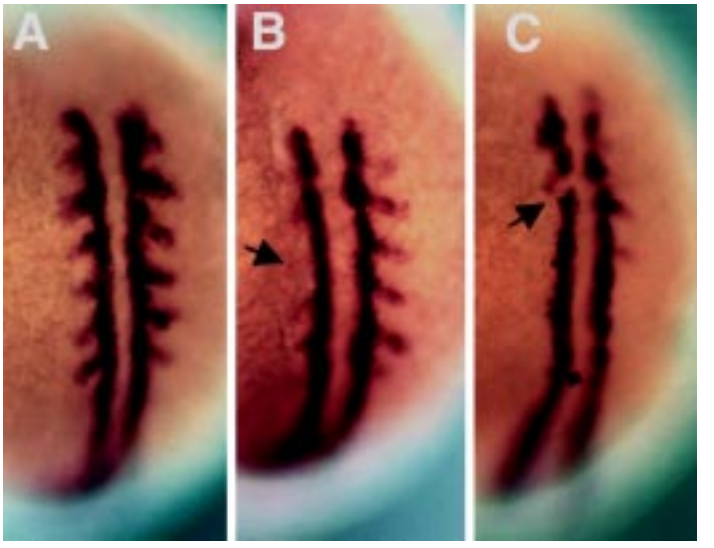

Fig. 7. *Myo D* staining shows early mesoderm patterning defects in embryos injected at blastula stage with chitinase 63 and allosamidin. *Embryos were microinjected at blastula stage with BSA, chitinase 63 or allosamidin, as described in Figure 3, and were allowed to develop until 12-13 hpf. They were fixed with paraformaldehyde and used to perform in situ hybridization using MyoD as a probe (see materials and Methods).* **(A)** *BSA control injected embryo.* **(B)** *Chitinase 63 injected embryo.* **(C)** *Allosamidin injected embryo. The arrows indicate abnormal MyoD expression sites (see text).*

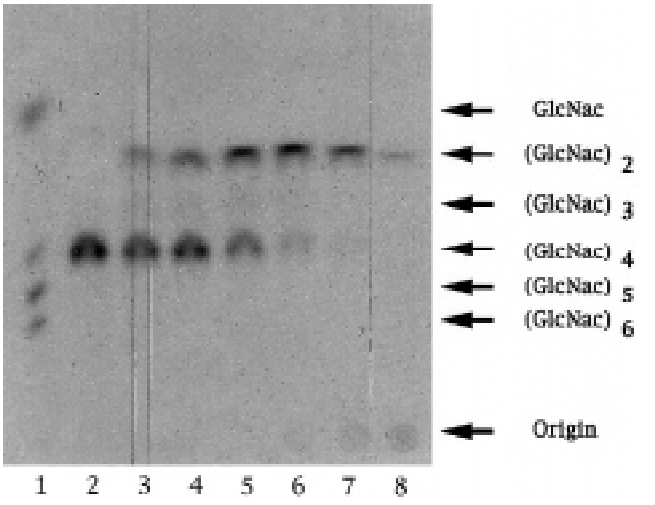

Fig. 8. **Endogenous intracellular chitinase activity in early embryos.** *Embryos at the one cell stage were microinjected with penta-N-[^{14}C]acetyl-chitopentaose (48000 cpm/μl). At different developmental times, ten embryos were boiled in 50 μl of water for two min and lysates were centrifuged. The supernatant was analyzed by TLC (see Materials and Methods).* **(1)** *Standards: GlcNAc, N-acetylglucosamine; $(GlcNAc)_2$, di-N-acetyl-chitobiose; $(GlcNAc)_3$, tri-N-acetyl-chitotriose; $(GlcNAc)_4$, tetra-N-acetyl-chitotetraose; $(GlcNAc)_5$, penta-N-acetyl-chitopentaose; $(GlcNAc)_6$, hexa-N-acetyl-chitohexaose. From lanes 2 to 8, samples of injected embryos from different developmental timepoints (in hpf):* **(2)** *0.0;* **(3)** *2.0;* **(4)** *4.5;* **(5)** *7.0;* **(6)** *10.5;* **(7)** *15;* **(8)** *28.*

tail structures leaving head development almost unperturbed.

The elucidation of the cellular function of the chitin synthesizing machinery in vertebrate embryos, that we and others have described, is an entirely new area of research. It remains to be seen whether NACOs have essential functions in animal physiology on their own or whether they are simply intermediate products which lead to the synthesis of HA. HA itself is a widely distributed glycosaminoglycan of the extracellular matrix which has been implicated in multiple developmental processes such as neurulation (Schoenwolf and Fisher, 1983; Copp and Bernfield, 1988), heart morphogenesis (Manasek *et al.,* 1973; Markwald *et al.,* 1979), and muscle differentiation (Orkin *et al.,* 1985; Yoshimura, 1985). Alternatively, chitin oligosaccharides as such, or a modified form of these molecules, could be secreted into the extracellular environment where they could act as signaling agents.

Materials and Methods

Animals, microinjection and cell transplantation

Adult zebrafish, *Danio rerio*, were maintained under standard conditions (Westerfield, 1994). Embryos were obtained by natural spawning at 28ºC, and embryos were dechorionated (Westerfield, 1994). Times of development refer to hours post fertilization (hpf) and were staged according to Kimmel *et al.* (1995). Chitinase 63 (Robbins *et al.,* 1988) was injected at 0.01-0.02 mg/ml protein in PBS (Phosphate Buffered Saline). As a control for the chitinase injections, the enzyme was mixed with the chitinase inhibitor allosamidin (Koga *et al.,* 1987) with a final concentration of 0.2 mg/ml and incubated for 1 h at 0ºC before use. In addition, allosamidin alone (at the same concentration), tetra-*N*-[^{14}C]acetyl-chitotetraose (48000 cpm/μl) or penta-*N*-[^{14}C]acetyl-chitopentaose (67000 cpm/μl) were injected. About 5nl of the appropriate solution was delivered into the cytoplasm of one-cell stage embryos (intracellular) or into a blastula of 3 hpf (extracellular). One cell stage injected embryos, were allowed to develop, and after 5-6 h, unfertilized or non-dividing embryos were removed. The number of such embryos was usually constant between experimental, control and uninjected batches under the conditions tested. Transplants were carried out as described previously (Ho and Kimmel, 1993) and were performed to study the potential cytotoxic effect of the chitinase on cell viability. Donor embryos were microinjected at the one cell stage with 10% Phenol red and 4% biotin-dextran (molecular probes) or with Phenol red, biotin-dextran and chitinase 63 (0.01-0.02 mg/ml). Donor embryos were allowed to develop until the blastula stage (3-4 hpf) and were used to transfer cells (40-80) into non-injected host embryos of the same developmental age. Donors and host embryos were phenotyped at 24 hpf and fixed with 4% paraformaldahyde. Different cell types derived from the transplanted cells carrying biotin-dextran were identified in host embryos after detection with anti-biotin peroxidase-coupled antibodies.

In situ hybridization

The full length *MyoD* (Weinberg *et al.,* 1996), *goosecoid* (Schulte-Merker *et al.,* 1994a), *no-tail (ntl)* (Schulte-Merker *et al.,* 1994b), otx2 (Li *et al.*, 1994), 450bp *ZDG42* (Semino *et al.,* 1996) cDNAs were used as templates for synthesis of digoxigenin-labeled RNA probes according to the supplier (Boehringer Mannheim Biochemicals). *In situ* hybridization was carried out as described previously (Jowett and Lettice, 1992).

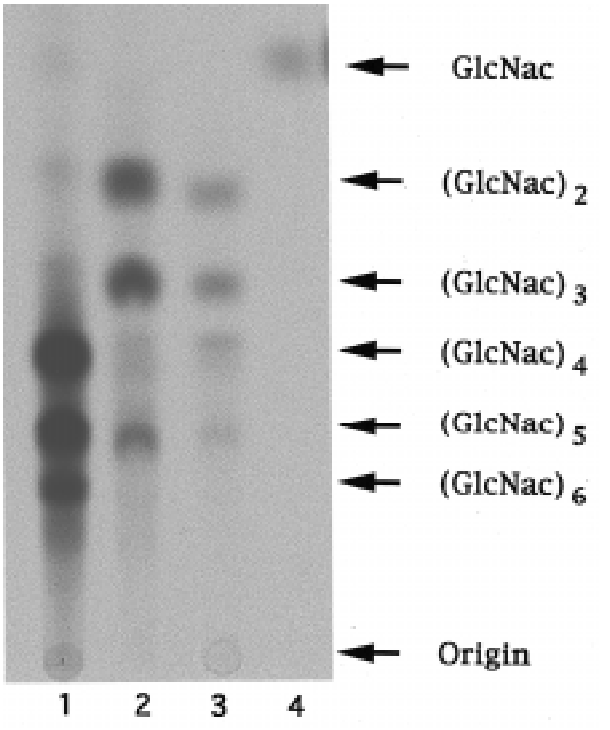

Fig. 9. **Endogenous extracellular chitinase activity in early embryos.** *Embryos were microinjected extracellularly at the blastula stage with penta-N-[^{14}C]acetyl-chitopentaose (67000 cpm/μl). At 10 and 24 hpf, a sample of ten injected embryos were processed and analyzed as described in Figure 8.* **(1)** *Standards of tetra-N-acetyl-chitotetraose, penta-N-acetyl-chitopentaose and hexa-N-acetyl-chitohexaose.* **(2)** *Sample of 10 hpf injected embryos.* **(3)** *Sample of 24 hpf injected embryos.* **(4)** *Standard of N-acetylglucosamine, GlcNAc. $(GlcNAc)_2$, di-N-acetyl-chitobiose and $(GlcNAc)_3$, tri-N-acetyl-chitotriose.*

In vitro incubations

Dechorionated zebrafish embryos (50) were microinjected at the one cell stage with BSA (Bovine Serum Albumin) or bacterial chitinase 63 in PBS (see below), raised to midgastrula or late gastrula stage (6-8 or 10-12 hpf) and were dissociated using Ca^{2+} and Mg^{2+} free solution (Westerfield, 1994). Cells were washed twice with the same solution, were resuspended and homogenized in 100 µl of lysis buffer. Cell extracts were prepared as described (Semino *et al.,* 1996). Standard incubations were carried out with 100 µl of cell extract in 12 mM $MgCl_2$, 5 mM *N*-acetylglucosamine, 20 µM Lognac (a competitive inhibitor of *N*-acetyl-β-D-glucosaminidases (CarboGen), 25 mM Tris/HCl (pH 7.4), and either 0.2 µCi (7.4 KBq) UDP-*N*-acetyl-D-[^{14}C]Glucosamine (UDP-[^{14}C]GlcNAc) (307 mCi/mmol) (DuPont NEN), or 0.2 µCi (7.37 KBq) UDP-[^{14}C]Glucuronic acid (UDP-[^{14}C]GlcA) (320.2 mCi/mmol) (DuPont NEN). Where indicated, UDP-glucuronic acid (UDP-GlcA) (100 µM) or UDP-*N*-acetylglucosamine (UDP-GlcNAc) (50 µM) were also added. The final volume was adjusted to 150 µl with water. Incubations were carried out at room temperature for 60 min and were stopped, after the addition of 200 µl water, by boiling for 2 min. The tubes were centrifuged and the precipitates washed twice with 100 µl of water. The combined supernatants were: 1) filtered through a DEAE-Sephacel (Sigma) column equilibrated in water, and the products in the effluent were fractionated by HPLC (High Performance Liquid Chromatography) for analysis of chitin oligosaccharides (NACOs) or 2) filtered through a Superose 12 HR 10/30 column by FPLC (Fast Protein Liquid Chromatography, Pharmacia Biotech Inc.) for analysis of hyaluronic acid (HA).

Zebrafish endogenous chitinase activity assay

Dechorionated zebrafish embryos (50) from 8-10 hpf, were resuspended in PBS (with 10 mM Ca^{2+} and Mg^{2+}) and gently pipetted up and down to dissociate the embryos from the yolk sac. The embryos were washed twice in the same buffer and then were resuspended in 100 µl of Ca^{2+} and Mg^{2+} free solution to dissociate the cells (Westerfield, 1994). The cells were centrifuged at 500 rpm for 5 min obtaining a supernatant and a pellet (cells). The supernatant (extracellular components) were placed into a tube and kept at -70ºC. The cell pellet was resuspended in 100 µl of lysis buffer and used to prepare cell extracts as described (Semino *et al.,* 1996). The chitinase activity was assayed by incubating penta-*N*-[^{14}C]acetyl-chitopentaose (10000 cpm) with 5 µl of either extracellular fraction or cell extracts for 60 or 180 min at 30ºC. Where indicated, allosamidin (0.2 mg/ml) was added to the incubation mix. The incubation was stopped, after adding 20 µl of water, by boiling for 2 min. The tubes were centrifuged, and the supernatant was concentrated and loaded onto a TLC (Thin Layer Chromatography) plate for analysis of the products (see Chromatographic techniques).

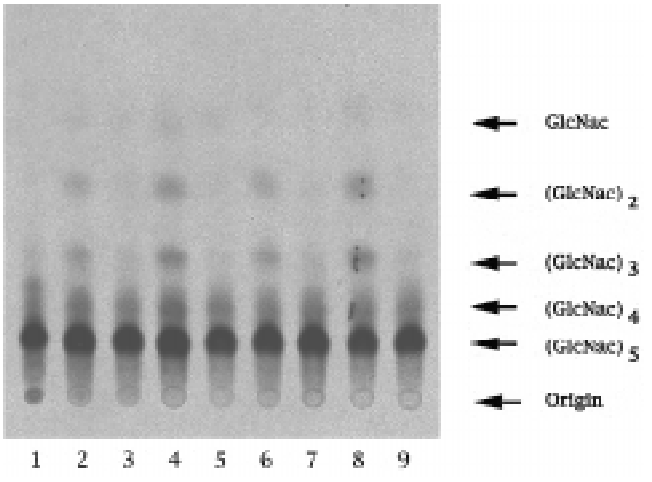

Fig. 10. ***In vitro* inhibition of the embryonic chitinase by allosamidin.** *Embryonic extracellular components (soluble extracellular matrix) and intracellular components (cell extracts) were used to assay the inhibition by allosamidin of the endogenous chitinase activity in vitro, using penta-N-[^{14}C]acetyl-chitopentaose (67000 cpm/µl) as a substrate (see Materials and Methods).* **(1)** *Standard of penta-N-acetyl-chitopentaose;* **(2)** *extracellular components incubated for 60 min;* **(3)** *same as 2 + allosamidin;* **(4)** *same as 2 but incubated for 180 min;* **(5)** *same as 4 + allosamidin;* **(6)** *intracellular components incubated for 60 min;* **(7)** *same as 6 + allosamidin;* **(8)** *same as 6 but incubated for 180 min;* **(9)** *same as 8 + allosamidin. Standards: same as Figure 8.*

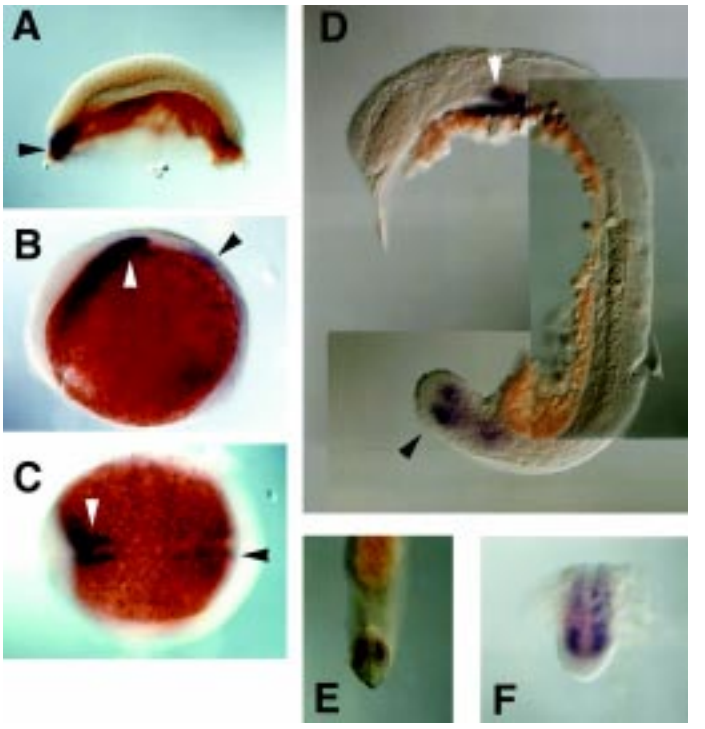

Fig. 11. Expression of ZDG42 mRNA during the first 22 h of development. *Fertilized zebrafish embryos from different developmental times (6-22 hpf) were fixed in 4% paraformaldehyde and used to perform in situ hybridization with the ZDG42 probe (see Materials and Methods).* **(A)** *Six hours lateral view (early gastrulation) staining the germ ring (black arrow).* **(B)** *Twelve hpf lateral view (dorsal is up and anterior is left) and* **(C)** *twelve hpf (early neurulation) dorsal view, anterior is up. Expression is found in mesoderm lateral to the notochord (black arrows), and also in presumptive endodermal tissue (white arrows).* **(D)** *Twenty-two hpf, lateral view, anterior is up and dorsal is right. Note expression in distal tail mesoderm (black arrow) and in the area of the developing gut (white arrow).* **(E** *and* **F)** *close up views of a tail expression at 22 hpf. Expression is seen in the tail mesoderm flanking the notochord.*

Chromatographic techniques

HPLC was carried out with an analytical Microsorb 5-µm (NH2) column (46x250 mm, Rainin Instruments) under isocratic conditions (water/acetonitrile, 30:70, vol/vol), run at 40ºC at a flow rate of 2 ml/min. One ml fractions were collected. Pure chitin oligosaccharides (Seikagaku America, St. Petersburg, FL) of 2 to 5 units of β-1,4-linked *N*-acetylglucosamine were used as internal standards and detected by UV absorption at 210 nm. FPLC was carried out with a Superose 12 HR 13/30 column (Pharmacia Biotech Inc.), equilibrated in PBS, 100 mM NaCl, 0.1% Tween 20, at a flow

rate of 0.5 ml/min with fractions of 0.5 ml. Blue dextran and $CoCl_2$ were used as indicators of total exclusion and inclusion volumes, respectively. Thin layer chromatography (TLC) was performed on silica gel G plates (10x20 cm, Merck) in n-butanol/ethanol/water, 5:4:3 (vol/vol) (Semino and Robbins, 1995). Radioactivity was measured in Ecoline scintillation solution in a liquid scintillation counter (Beckman LS 3801). TLC plates were exposed to Kodak x-ray film (X-Omat AR).

Enzymes

For analysis of labeled material, *Streptomyces plicatus* chitinase 63 (Robbins *et al.,* 1988) was used in 25 mM phosphate buffer (pH 6.5) and incubated at 37ºC for two hours at a final concentration of 10 µg/ml. Incubations with hyaluronate lyase (10 units) from *Streptomyces hyaluroliticus* (Sigma) were carried out for 4 h in 25 mM sodium acetate buffer (pH 5.0).

In vivo extraction of chitin oligosaccharides (NACOs) and hyaluronic acid (HA)

For the extraction of NACOs, dechorionated zebrafish embryos (500) from four different developmental stages (blastula, 4 hpf; late gastrula, 10 hpf; neurulation, 15 hpf; and postsomitogenesis, 26 hpf), were dechorionated and dissociated using Ca^{2+} and Mg^{2+} free solution (Westerfield, 1994), and the cells were washed twice with the same solution. They were resuspended in 2 ml of 10 mM NaOH and boiled at 100ºC for 10 min. The alkali supernatants (2 ml) were neutralized with HCl, clarified by centrifugation at 14000 rpm for 20 min at 4ºC, and loaded on a mixed ion exchange column (Rexyn I-300, H-OH) (10x40 mm) (Fisher Scientific Co.). The column was washed once with 2 ml water and the combined material eluted from the column (4 ml) was concentrated and injected into an HPLC column (see Chromatographic techniques). The fractions corresponding to the elution volume of penta-*N*-acetylchitopentaose were collected in each case, concentrated, and labeled by reduction with NaB-$[^3H]H_4$. For isolation of HA, 50 embryos (10 hpf), injected or not with chitinase 63, were resuspended in 0.4 ml of PBS (with Ca^{2+} and Mg^{2+}) and dissociated from the yolk sac by gently pipetting up and down. The cells were then washed twice with the same buffer. The embryos were resuspended in 0.4 ml water and boiled for two min. The tubes were centrifuged at 10000 rpm for 10 min. The supernatants were treated with alkaline phosphatase (Calf, Boehringer Mannhein Biochemicals) and then labeled by reduction with NaB-$[^3H]H_4$ as described above. The labeled material was filtered through a size exclusion Superose 12 HR 13/30 column, and the high molecular weight radiolabeled material (>10^5 Da) was characterized by its change in size after treatment with hyaluronate lyase from *Streptomyces hyaluroliticus* (Sigma, see Enzymes).

Labeling of oligosaccharides and hyaluronic acid by reduction with NaB-$[^3H]H_4$

The material to be labeled was resuspended in 50 µl of 10 mM NaOH and incubated for 16 h at room temperature with 1 mCi NaB-$[^3H]H_4$ (100 mCi/mmol). In the case of neutral oligosaccharides, the reaction was stopped by filtering the material through a mixed ion exchange column (Rexyn I-300, H-OH) (10x20 mm) (Fisher Scientific Co.) equilibrated in water. The column was washed twice with 2 ml water, and the combined labeled and neutral material eluted from the column (4 ml) was concentrated and frozen at -20ºC. For charged polysaccharides, like HA, the reaction was stopped by adding 40 mM HCl and the material was filtered through a FPLC Superose 12 HR 13/30 column (Pharmacia Biotech, Inc.) (see Chromatographic techniques).

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