Expression of rhizobium chitin oligosaccharide fucosyltransferase in zebrafish embryos disrupts normal development a

Semino, C.E.; Allende, M.L.; Bakkers, J.; Spaink, H.P.; Robbins, P.P.

Expression of *Rhizobium* Chitin Oligosaccharide Fucosyltransferase in Zebrafish Embryos Disrupts Normal Development[a]

CARLOS E. SEMINO,[b,d] MIGUEL L. ALLENDE,[b] JEROEN BAKKERS,[c] HERMAN P. SPAINK,[c] AND PHILLIPS P. ROBBINS[b]

[b]*Center for Cancer Research, Building E17, Room 125, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA*
[c]*Institute for Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 Al Leiden, the Netherlands*

ABSTRACT: In this report we present data about the effect of the *Rhizobium* NodZ enzyme on zebrafish development. We injected zebrafish embryos with a plasmid expressing NodZ protein, and we confirmed that the enzyme is active and has chitin oligosaccharide fucosyltransferase (NodZ) activity *in vitro*. In addition, the embryos injected with the NodZ-expressing plasmid, but not with a control plasmid, showed malformations or bends in the tail, and in some cases shunted tail structures and fused somites. These results clearly indicate that the likely substrates for this enzyme, chitin oligosaccharides and free *N*-glycans, have essential functions during early vertebrate embryogenesis.

INTRODUCTION

The symbiotic relationship between bacteria of the genera *Rhizobium* and legumes results in the formation of a nitrogen-fixing root organ, the nodule.[1] The bacteria respond to specific compounds secreted by the plant roots (flavonoids) by production and excretion of lipochitin oligosaccharides (modified chitin oligosaccharides, also known as Nod factors), signaling molecules that act as plant morphogens promoting root infection and the consequent nodulation.[2,3,6] The backbone of the Nod factors consists of an oligomer of 3 to 5 residues of *N*-acetylglucosamine, *N*-acylated on the nonreducing end. This backbone is synthesized by three enzymes, NodA, NodB, and NodC. NodC is the first enzyme in this pathway and is responsible for the synthesis of chitin oligosaccharides.[4,5,7] Additional gene products, such as NodZ, a chitin oligosaccharide fucosyltransferase, are involved in chemical modifications that have been shown to determine host specificity.[8]

Polymeric chitin has been found in the epidermal cuticle of teleost fish,[22] and more recently chitin oligosaccharides have been isolated from *Xenopus laevis* and zebrafish embryos.[10,11] In addition, genes highly homologous to NodC have been cloned from *X. laevis*, zebrafish, and mouse (DG42, ZDG42, and MDG42, respectively[9,10,13]). In *Xenopus* and zebrafish, these genes are expressed during a short window of time during de-

[a] This work was supported by Grant GM31318 (to P. P. Robbins). M. L. Allende was supported by a Public Health Service fellowship (HD 07818).
[d] Tel: (617) 258-8057; fax: (617) 258-8315; e-mail: seminoraimon@wccf.mit.edu.

velopment, between the gastrula and neurula stages.[9–11] We are interested in investigating the possible biological function of chitin oligosaccharides during vertebrate embryogenesis, in particular to learn whether chitin oligosaccharides act as signaling molecules as they do during the nodulation process in plants.

Previously, using a cell-free *in vitro* transcription/translation system, we showed that the DG42 protein is able to catalyze synthesis of chitin oligosaccharides that vary in chain length from three to six residues of *N*-acetylglucosamine, as well as polymeric chitin.[14] We have also demonstrated that when cell extracts from frog and zebrafish embryos of the appropriate developmental stage are used it is possible to obtain *in vitro* synthesis of chitin oligosaccharides.[10] In these extracts, chitin oligosaccharide synthase activity, but not hyaluronate (HA) synthase activity, can be inactivated with a specific anti-DG42 antibody.[10] Similar results have also been found in carp embryos.[15]

In this report we present data that support our previous observations on the effect of the NodZ enzyme on zebrafish development. We injected zebrafish embryos with a plasmid expressing NodZ, and we confirmed that the enzyme is active and has chitin oligosaccharide fucosyltransferase activity *in vitro*. The embryos injected with the NodZ-expressing plasmid, but not with a control plasmid, show defects similar to those seen when purified NodZ protein was injected.[15]

RESULTS AND DISCUSSION

NodZ-expressing Embryos Show Chitin Oligosaccharide Fucosyltransferase Activity

The NodZ enzyme transfers a fucose residue from the sugar donor GDP-β-L-fucose (GDP-Fuc) to the C-6 position of the GlcNAc residue at the reducing end of chitin oligosaccharides.[8] Previously, we have shown that injection of the purified NodZ enzyme into early embryos induces abnormalities visible at 24 hours of development[15] and that the effect is similar to that produced by injection of a chitinase[11] or an anti-DG42 protein antibody.[11,15] We were interested in determining whether the recombinant NodZ enzyme was able to elicit the same effect, which would confirm the specificity of the prior results.

The *NodZ* gene of *Bradyrhizobium japonicum* was cloned into the expression vector pcDNA3 downstream of the cytomegalovirus (CMV) promoter, which is able to direct high levels of RNA expression in zebrafish embryos.[21] Fertilized zebrafish embryos at the one-cell stage were injected with this construct (pcDNA3-BJ-NodZ) or with the parental plasmid pcDNA3 as a control (see legend of FIG. 1). After injection, the embryos were raised under standard conditions.[12] At 12 hours (early somite stage) NodZ enzyme activity was assayed. NodZ is specific for chitin oligosaccharides of 2–6 residues of *N*-acetylglucosamine. We incubated cell extracts from the two groups of injected embryos in the presence of GDP[^{14}C]-Fuc, Mg^{2+}, Ca^{2+}, Mn^{2+}, ATP and chitin pentaose (see legend to FIG. 1). After incubation, the water soluble and neutral material was applied to a thin-layer chromatography (TLC) plate. The embryos injected with the plasmid pcDNA3-BJ-NodZ (FIG. 1, lane A), but not the control embryos (FIG. 1, lane B), show fucosyltransferase activity. The [^{14}C]fucosylated compound has a mobility identical to fucosylated chitin pentaose by both TLC (FIG. 1, lane A), and HPLC (data not shown). This result clearly indicates that the embryos express functional NodZ enzyme, with the same properties found before with the *E. coli*–expressed protein.[15]

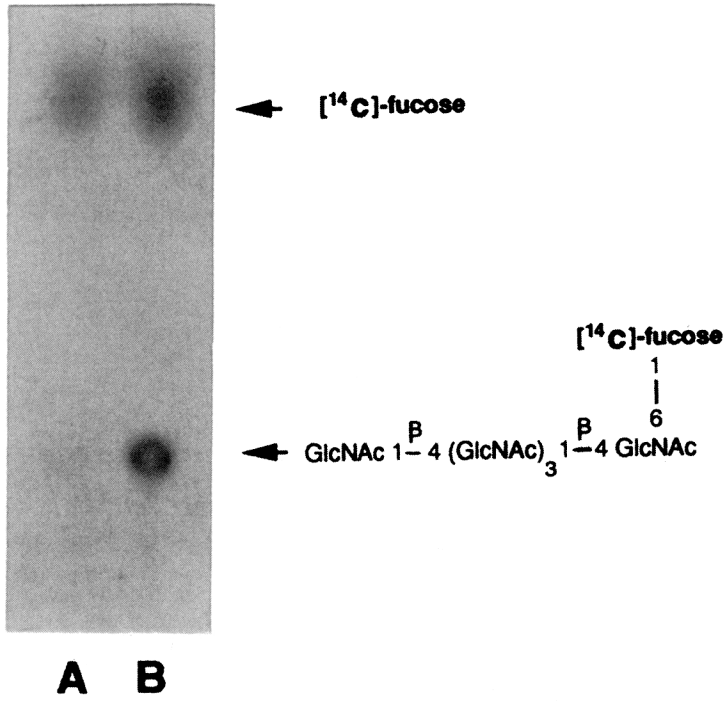

FIGURE 1. Chitin oligosaccharide fucosyltransferase activity in NodZ-expressing embryos. The plasmid pMP2452, containing the *NodZ* gene,[23] was digested with Xba I and Bam HI, and the 950 bp *NodZ* fragment was cloned into pB SK (+/–) (Stratagene). The resulting construct, pB SK(+/–)-Bj-NodZ, was digested with Sac I and Xho I, and the 1 Kb insert encoding *NodZ* was subcloned into pcDNA3 (Invitrogen). This construct, pcDNA3-Bj-NodZ, has the *NodZ* gene under the control of the human cytomegalovirus (CMV) enhancer-promoter sequence. 5 to 8 nL of the plasmids pcDNA3-Bj-NodZ or pcDNA3 (0.2 mg/mL in 0.2 M KCl) were microinjected into one cell–stage zebrafish embryos (40–50 per experiment), which were raised to 12 hours of development and used to prepare cell extracts, as described before.[10] The cell extracts (50 μL) were incubated as described[23] at room temperature for 30 minutes with 1 mM of ATP; 5 mM of Mg^{2+}, Ca^{2+} and Mn^{2+}; 50 nCi of GDP-β-[^{14}C]-L-fucose (272 mCi / mmol) (DuPont, NEN); and 1 mM chitin pentaose standard (penta-*N*-acetyl-D-chitopentaose) (Seikagaku America, FL). The reaction was stopped by adding 100 μL water and boiling for two minutes. The sample was centrifuged at 14,000 rpm for 10 minutes, and the supernatant was loaded onto a mixed ion-exchange column (400 μL of Rexyn I-300 H-OH, Fisher Scientific Co.) prepared in a Pasteur pipette. The column was washed twice with 200 μL of water. The water-soluble material eluted from the column (neutral) was concentrated and loaded on a thin-layer chromatography (TLC) plate. The TLC was performed on silica gel G plates (5 × 10 cm) (Merck) in *n*-butanol / ethanol / water, 5 : 4 : 3 (vol/vol). The external standards used were [^{14}C]fucosylated chitin oligosaccharides from 2 to 6 residues of *N*-acetylglucosamine obtained after incubation of chitin oligosaccharides in the presence of GDP [^{14}C]fucose and pure NodZ enzyme expressed in *E. coli.*

The FIGURE shows the chromatographic mobility of free [^{14}C]fucose and also the molecular structure of the fucosyl β(1–6)-chitopentaose. **A:** Embryos microinjected with pcDNA3; **B:** embryos microinjected with pcDNA3-Bj-NodZ plasmid.

Embryos Expressing the NodZ Enzyme Develop an Abnormal Phenotype

Embryos microinjected with the NodZ-expressing (pcDNA3-Bj-NodZ) plasmids and the control (pcDNA3) plasmids were raised and visually inspected at 6, 12, 24, and 48 hours postfertilization. Among the embryos injected with the control plasmid (FIG. 2A), approximately 5% showed abnormalities (n=112), which were comparable to the background defects normally found associated with the injection procedure. On the other hand, by 24 hours, embryos injected with the NodZ-expressing plasmid presented a phenotype that was similar to that seen previously when purified NodZ enzyme,[15] anti-DG42 antibody,[15] or chitinase[11] were injected. Approximately 70% (n=145) of embryos in this group were affected, although with varying severity. Moderately affected individuals showed slight malformations or bends in the tail (FIG. 2B). The more severely affected individuals had shunted or absent tail structures and often showed fused somites (FIG. 2C).

It was recently shown that the DG42 protein is involved in synthesis of HA,[16,17] and it was proposed that chitin oligosaccharides may function *in vivo* as primers for this process.[18] Preliminary data from our group suggests that the chitin oligomers produced by the DG42 protein can, in fact, act as a template for HA biosynthesis but that free

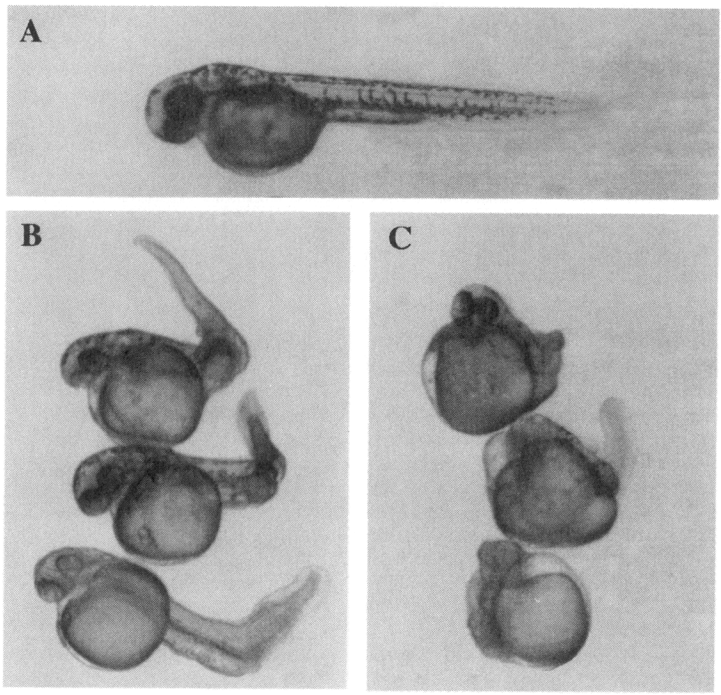

FIGURE 2. Phenotype of embryos expressing *Rhizobium* NodZ enzyme. Zebrafish embryos microinjected with pcDNA3 and pcDNA3-Bj-NodZ (see legend to FIG. 1) were cultured under standard conditions and raised to 48 hours postfertilization.[12] Photographs show embryos injected with (**A**) pcDNA3 and (**B**) pcDNA3-Bj-NodZ, showing moderate phenotype; **C,** same as **B,** presents a more severe phenotype.

chitin oligosaccharides are also generated.[19] These free oligomers could have an additional biochemical function.[2,3,6] Injection of anti-DG42 antibody into zebrafish embryos inhibits chitin synthase activity *in vitro* as well as *in vivo* and causes abnormal embryonic development: the formation of the posterior trunk and tail of the embryo are severely affected.[11,15] Consistent with this, a similar effect was obtained after injecting embryos with purified NodZ, a chitin oligosaccharide-modifying enzyme that transfers a fucose residue from GDP-β-L-fucose to the C-6 position of the reducing end of chitin oligosaccharides.[15] Chitinase-63 from *Streptomyces plicatus,* which has high affinity for chitin oligosaccharides, also produces this phenotype.[11] This suggests that *in vivo* modification of the chitin oligosaccharide structure (by adding a new fucose residue or by degradation) may functionally inactivate the molecule and generate a visible effect on embryonic development.[11,15]

The NodZ enzyme has a high affinity for chitin oligosaccharides, their natural substrates, with a K_m of 0.12 mM.[23] *N*-glycans carrying an *N,N'*-chitobiosyl structure at their reducing termini are also substrates for the enzyme, but with higher K_m.[23] *N*-glycanase activity has been reported in the early embryos of *Oryzias latipes* (Medaka fish),[20] suggesting that free *N*-glycan chains with the chitobiose at the reducing end are present in Medaka fish embryos cells; this may also be the case in zebra fish embryos. Fucosylation by NodZ may modify these oligosaccharides to an extent that causes an inhibition of their cellular function in early embryos, which leads to a visible phenotype. The glycans and/or their fucosylated derivatives should, therefore, be evaluated as alternative signaling molecules. However, it appears clear that in teleosts and amphibians, chitin oligosaccharides and free *N*-glycans have essential functions during embryogenesis.

ACKNOWLEDGMENT

We thank Nancy Hopkins for the use of laboratory facilities and zebrafish.

REFERENCES

1. LONG, S.R. 1989. *Rhizobium*-legume nodulation: Life together in the underground. Cell **56:** 203–214.
2. DENARIE, J., F. DEBELLE & J.C. PROME. 1996. *Rhizobium* lipo chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. **65:** 503–535.
3. LEROUGE, P., P. ROUCHE, C. FAUCHER, F. MAILLET, G. TRUCHET, J.C. PROME & J. DENARIE. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature (Lond.) **344:** 781–784.
4. GEREMIA, R.A., P. MERGAET, D. GEELAN, M. VAN MONTAGU & M. HOLSTERS. 1994. The NodC protein of *Azorhizobium caulinodans* is an *N*-acetyl glucosaminyltransferase. Proc. Natl. Acad. Sci. USA **91:** 2669–2673.
5. SEMINO, C.E. & M.A. DANKERT. 1994. The *in vitro* biosynthesis of functional nodulation factors (Nod Rm) produced by *Rhizobium meliloti* 1021. Cell. Mol. Biol. **40:** 1029–1037.
6. SPAINK, H. 1995. The molecular basis of infection and nodulation by rhizobia: The ins and outs of sympathogenesis. Annu. Rev. Phytopatol. **33:** 345–368.
7. SPAINK, H.P., A.H.M. WIJFJES, G.M. VAN DER DRIFT, J. HAVERKAMP, J.E. THOMAS-OATES & B.J.J. LUGTENBERG. 1994. Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum.* Mol. Microbiol. **13:** 821–831.
8. LOPEZ-LARA, I.M., L. BLOKTIP, C. QUINTO, M.L. GARCIA, G. STACEY, G.V. BLOEMBERG, G.E.M. LAMERS, B.J.J. LUGTENBERG, J.E. THOMASOATES & H. P. SPAINK. 1996. NodZ of *Bradyrhizobium* extends the nodulation host range of *Rhizobium* by adding a fucosyl residue to nodulation signals. Mol. Microbiol. **21** (2): 397–408.

9. ROSA, F., T.D. SARGENT, M.L. REBBERT, G.S. MICHAELS, M. JAMRICH, H. CRUZ, E. JONAS, J.A. WINKLES & I.B. DAWID. 1988. Accumulation and decay of DG42 gene products follow a gradient pattern during *Xenopus* embryogenesis. Dev. Biol. **129:** 114–123.
10. SEMINO, C.E., C.A. SPECHT, A. RAIMONDI & P.W. ROBBINS. 1996. Homologs of the *Xenopus* developmental gene *DG42* are present in zebrafish and mouse and are involved in the synthesis of Nod-like chitin oligosaccharides during early embryogenesis. Proc. Natl. Acad. Sci. USA **93:** 4548–4553.
11. SEMINO, C.E., M.L. ALLENDE & P.W. ROBBINS. 1998. Temporal synthesis and degradation of chitin oligosaccharides during embryonic zebrafish development. Submitted.
12. WESTERFIELD, M. 1994. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish *(Brachydario rerio)*. University of Oregon Press.
13. BULAWA, C.E. & W. WASCO. 1991. Chitin and nodulation. Nature (Lond.) **353:** 710.
14. SEMINO, C.E. & P.W. ROBBINS. 1995. Synthesis of "Nod"-like chitin oligosaccharides by the *Xenopus* developmental protein DG42. Proc. Natl. Acad. Sci. USA **92:** 3498–3501.
15. BAKKERS, J., C.E. SEMINO, H. STROBAND, P.W. ROBBINS, J.W. KIJNE & H.P. SPAINK. 1997. An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish. Proc. Natl. Acad. Sci. USA **94:** 7982–7986.
16. MEYER, M.F. & G. KREIL. 1996. Cells expressing the DG42 gene from early *Xenopus* embryos synthesize hyaluronan. Proc. Natl. Acad. Sci. USA **93:** 4543–4547.
17. DEANGELIS, P.L. & A.M. ACHYUTHAN. 1996. Yeast-derived recombinant DG42 protein of Xenopus can synthesize hyaluronan *in vitro.* J. Biol. Chem. **271:** 23657–23660.
18. VARKI, A. 1996. Does DG42 synthesize hyaluronan or chitin? A controversy about oligosaccharides in vertebrate development. Proc. Natl. Acad. Sci. USA **93:** 4523–4525.
19. SEMINO, C.E. & P.W. ROBBINS. 1997. *Xenopus* developmental protein DG42 expressed in yeast synthesize variable ratios of hyaluronate and chitin oligosaccharides *in vitro* depending on incubation conditions. Submitted.
20. SUZUKI, T., A. SEKO, K. KITAJIMA, Y. INOUE & S. INOUE. 1994. Purification and enzymatic properties of peptide:*N*-glycanase from C3H mouse-derived L-929 fibroblast cells. J. Biol. Chem. **269:** 17611–17618.
21. GIBBS, P.D.L., A. PEEK & G. THOROGAAD. 1994. An *in vivo* screen for the luciferase transgene in zebrafish. Mol. Mar. Biol. Biotech. **3:** 307–316.
22. WAGNER, G.P., J. LO, R. LAINE & ALMEDER. 1993. Chitin in the epidermal cuticle of a vertebrate (*Paralipophrys triglodites,* Blenniidae, Teleostei). Experientia **49:** 317–319.
23. QUINTO, C., A.H.M. WIJFJES, G.V. BLOEMBERG, L. BLOK-TIP, I.M. LOPEZ-LARA, B.J.J. LUGTENBERG, J.E. THOMAS-OATES & H.P. SPAINK. 1997. Proc Natl. Acad. Sci. USA **94:** 4336–4341.