Regulation of NF-κB/Rel by IκB is essential for ascidian notochord formation

Narudo Kawai, Hiroki Takahashi, Hiroki Nishida, Hideyoshi Yokosawa

Department of Biochemistry, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan
Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan
Department of Developmental Biology, Division of Morphogenesis, National Institute for Basic Biology, Okazaki 444-8585, Japan

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Abstract

We previously reported that two NF-κB/Rel family members are involved in notochord formation of the ascidian Halocynthia roretzi. Here, we present evidence that the NF-κB/Rel signaling pathway plays important roles in the notochord formation in another ascidian, Ciona intestinalis. We first found that two NF-κB/Rel family members of C. intestinalis, Ci-rel1 and Ci-rel2, are splice variants: Ci-rel1 is a typical member, while Ci-rel2 is a C-terminally truncated short one. Ectopic expression of GFP-fusion proteins in the C. intestinalis notochord revealed that Ci-rel1 transiently moved into the nucleus in the initial tailbud stage, when concomitant expression of Ci-IκB, a C. intestinalis IκB homologue, was observed, indicating that Ci-rel1 is transiently activated in this stage. Ci-rel1, as well as Ci-rel2, is capable of binding to the κB sequence present upstream of Ci-IκB, suggesting that Ci-IκB is a target gene of Ci-rel1. Reporter gene assay suggests that the expression of Ci-IκB in the notochord is controlled by its κB sequence. Gene silencing of Ci-IκB by injection of the corresponding antisense morpholino oligonucleotide resulted in impairment of notochord formation in C. intestinalis, particularly in a defect in intercalation of notochord cells. Taken together, the results suggest that the regulation of Ci-rel1 by Ci-IκB, whose transcription is regulated by Ci-rel1, in the tailbud stage is essential for notochord formation in C. intestinalis.

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Introduction

Ascidians, commonly known as sea squirts, are marine invertebrates that belong to the most primitive branch of the chordate phylum, the Urochordate. The swimming tadpole-type larva of an ascidian, whose tail contains an axial notochord flanked by muscle, a dorsal neural tube and a ventral endoderm strand, is thought to represent the most simplified and primitive chordate body plan. For example, the larva of Ciona intestinalis, the most cosmopolitan ascidian species, has about 2600 cells that include only 40 notochord cells (Satoh, 1994). In other words, the ascidian larva represents an ideal model of early chordate development: it possesses the basic developmental and morphologic features of vertebrates but has the cellular and genomic simplicity of invertebrates. In addition, the ascidian embryo provides a good experimental system to explore the expression and functions of developmental genes because ascidian fertilized eggs develop rather quickly into tadpole-type larvae with several distinct types of tissues and organs and because the lineage of ascidian embryonic cells is well documented (Corbo et al., 2001; Gregorio and Levine, 2002; Satoh, 2001).

With respect to factors functioning in notochord formation of ascidian tadpole-type larvae, it has been reported that Brachyury, which contains the DNA-binding T-box domain, plays a critical role in ascidian embryo-
genesis: Ascidian Brachyury genes, As-T of Halocynthia roretzi (Yasuo and Satoh, 1993, 1994) and Ci-Bra of C. intestinalis (Corbo et al., 1997), are expressed exclusively in notochord precursor cells and the timing of gene expression coincides with clonal restriction of the notochord lineage at the 64-cell stage. Over-expression of As-T induced ectopic notochord formation (Yasuo and Satoh, 1998), and misexpression of both As-T (Yasuo and Satoh, 1998) and Ci-Bra (Takahashi et al., 1999) caused transformation of endodermal and neuronal lineages into notochord cells. In addition, we reported that two splice variants of the NF-κB/Rel family, As-rel1 and As-rel2, are involved in the formation of the notochord in H. roretzi (Shimada et al., 2001): Embryos that had been injected with As-rel1 mRNA showed a short tail phenotype, that is, reduction of the number of notochord cells and a defect in tail elongation. This phenotype was rescued by coinjection of As-rel2 mRNA. Thus, NF-κB/Rel family proteins function in ascidian notochord formation. The regulatory mechanisms, however, have not yet been determined.

Transcription factors belonging to the NF-κB/Rel family contain highly conserved Rel homology domains (RHDs) required for DNA binding and dimerization, and they regulate the expression of downstream target genes involved in various phenomena such as morphogenesis, immune response, cell growth, and programmed cell death (Ghosh and Karin, 2002; Ghosh et al., 1998; Karin and Lin, 2002; May and Ghosh, 1998; Verma et al., 1995). Regulatory mechanisms underlying the functions of NF-κB/Rel family members have been extensively studied, and several modes of their regulation have been proposed (Ghosh and Karin, 2002; Karin and Lin, 2002). One proposed basic regulatory mechanism in the canonical NF-κB/Rel signaling pathway is based on the inhibitor protein IκB-mediated mechanism. Without extracellular stimuli, the NF-κB/Rel protein is sequestered in the cytoplasm through binding to IκB because IκB masks the nuclear localization signal (NLS) of NF-κB/Rel. In response to extracellular stimuli, the IκB protein is phosphorylated by the oligomeric IκB kinase IKKβ and then polyubiquitinated and degraded by the proteasome, resulting in the release of the NF-κB/Rel protein, which moves from the cytoplasm to the nucleus, where it binds to regulatory elements, κB sequences, of target genes to trigger their transcription.

In this study, we investigated the roles of the NF-κB/Rel signaling pathway in notochord formation in C. intestinalis. All of the genomic sequences in this species have been determined (Dehal et al., 2002). Moreover, an EST database for this species has been constructed (Satou et al., 2002), and Ci-IκB, a C. intestinalis IκB homologue, has been isolated (Kawai et al., 2002). We first isolated two splice variants of the NF-κB/Rel family in C. intestinalis, Ci-rel1 and Ci-rel2. In situ hybridization experiments revealed specific expressions of Ci-rel1, Ci-rel2 and Ci-IκB genes during C. intestinalis embryo-genesis. Specific over-expression of GFP fusion proteins in the C. intestinalis notochord revealed that Ci-rel1 transiently moved into the nucleus at the beginning of notochord formation, when concomitant expression of Ci-IκB gene was observed. Moreover, it was demonstrated that both Ci-rel1 and Ci-rel2 are capable of binding to the 5′ upstream sequence of Ci-IκB, suggesting that Ci-IκB is a direct target gene of Ci-rel1. Reporter gene assay suggests that the expression of Ci-IκB in the notochord is controlled by its κB sequence. Gene silencing of Ci-IκB by injection of the corresponding antisense morpholino oligonucleotide resulted in impairment of notochord formation in C. intestinalis, particularly in a defect in intercalation of notochord cells in the middle tailbud stage. Thus, Ci-rel1 regulates the Ci-IκB gene at the level of transcription, and Ci-IκB regulates Ci-rel1 probably at the level of nuclear transport, which creates an autoregulatory feedback loop. We propose that the regulation of Ci-rel1 by Ci-IκB through this autoregulatory feedback loop in the tailbud stage is essential for notochord intercalation in C. intestinalis.

Materials and methods

Animals and embryos

Adult C. intestinalis was collected from several locations in Japan. Adults were maintained in a natural seawater aquarium at 18°C under constant light to prevent the spawning of eggs. To fertilize eggs, gametes from two or more individuals were mixed for 1.5–2 min and excess sperm was removed. Embryos were cultured at 18°C and tadpole-type larvae began to hatch about 18 h after fertilization (Whittaker, 1973).

Genome and cDNA data resources

A genome database of C. intestinalis has been constructed by the Department of Energy Joint Genome Institute (Walnut Creek, CA, USA) (http://genome.jgi-psf.org/ciona4/ciona4.home.html). Gene products predicted from the genome sequence of C. intestinalis are designated by gene product ID numbers (Dehal et al., 2002). The release version 1.0 of the genome and the predicted product sequence data were used in this study.

A cDNA database of C. intestinalis has been constructed by Kyoto University (Sakyo-ku, Kyoto, Japan) (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). The C. intestinalis Gene Collection Release version 1, which contains cDNA clones for 13,464 genes, covering nearly 85% of mRNA species, EST sequences from both the 5′ and 3′ ends from approximately 90,000 cDNA clones and temporal and spatial expression profiles (Satou et al., 2002), was used in this study.
Isolation and sequence determination of Ci-rel1 and Ci-rel2

We found cDNAs for Ci-rel2 in clones of the C. intestinalis EST database described above. Its cluster ID is 10502; one clone (cieg22h11) from the egg cDNA library containing the entire coding sequence. cDNA clones for Ci-rel1 were screened from a λZAP library from tailbud-stage embryos using as probe a DIG-labeled PCR fragment (nucleotides 2479–2755) that had been obtained from the EST database described above and labeled by a DIG detection kit (Roche). Nucleotide sequences of cDNAs for both genes were determined for both strands using a Big-Dye terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 377 DNA sequencer (Perkin Elmer).

In situ hybridization

RNA antisense probes for in situ hybridization were prepared using DIG RNA labeling Mix (Boehringer-Mannheim) according to the manufacturer’s protocol. Ci-rel1 (768–1374 bp), Ci-rel2 (1697–2243 bp) and Ci-IxB (919–1525 bp) cDNAs were linearized at the 5’ end and transcribed with T7 RNA polymerase (Roche). Whole-mount specimens were hybridized in situ using DIG-labeled antisense and sense RNA probes essentially according to the method described previously (Corbo et al., 1997).

Construction of expression plasmids and electroporation

The plasmid pBraGFP was constructed from pEGFP-C1 (Clontech): pEGFP-C1 was cut with NheI and AseI to remove the CMV promoter, end-filled with the Klenow fragment of DNA polymerase (Takara), and then cut with HindIII. This blunt-ended fragment was then ligated to an 890-bp Ci-Bra genomic upstream fragment to give pBraGFP. To generate pCI-neo-Flag-Ci-rel1, pBraGFP-Ci-rel1, pBraGFP-Ci-rel2, and pBraGFP-Ci-IxB, used in electroporation experiments, full-length Ci-rel1, Ci-rel2, and Ci-IxB cDNAs were inserted into the KpnI–SmaI, PstI–SmaI, and PstI–BamHI sites of pBraGFP, respectively, after subcloning of full-length Ci-rel1 containing KpnI and SmaI sites by PCR using a Ci-rel KpnI forward primer, 5’-GGTACCATGGATAGAATT-3’, and a Ci-rel1 SmaI reverse primer, 5’-CCCCGGCTCTCGACAGTT-3’, subcloning of full-length Ci-rel2 containing PstI and SmaI sites by PCR using a Ci-rel PstI forward primer, 5’-CTGCAGTCATGGAACACGAT-3’, and a Ci-rel2 SmaI reverse primer, 5’-CCCCGGCCCTATCGCATGAA-3’, and subcloning of full-length Ci-IxB containing PstI and BamHI sites by PCR using a Ci-IxB PstI forward primer, 5’-CTGCAGTCATGCTAA-3’, and a Ci-IxB BamHI reverse primer, 5’-GGATCCCTGGTTCTCTTCT-3’. The PCR products were subcloned in the pGEM-T-vector (Promega). To generate pBraCi-actin 5C-GFP, full-length Ci-actin 5C cDNA was inserted into the PstI–SmaI sites of pBraGFP-N1, which had been prepared from pEGFP-N1 (Clontech) by cutting with HindIII to remove the CMV promoter, end-filled with the Klenow fragment of DNA polymerase, and then ligated to the 890-bp Ci-Bra genomic upstream fragment.

Electroporation was carried out using a BioRad gene pulser with a capacitance extender according to the method described previously (Corbo et al., 1997). The electroporated embryos were allowed to develop at 18°C in agar-coated dishes with Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate until the desired time point. A typical round of electroporation yielded hundreds of embryos expressing the transgene. Multiple rounds of electroporation were carried out with the respective transgenes.

Construction of antisense morpholino oligonucleotides (M-oligos) and microinjection

For gene silencing for Ci-rel and Ci-IxB, 25-mer antisense morpholino oligonucleotides (M-oligos) corresponding to the Ci-rel sequence (5’-CTATCCCATATTGAATGATTTGAGCC-3’) and Ci-IxB sequence (5’-TGTAGCGTTGGTGCTTATTAGCAT-3’) were constructed by Gene Tools (Oregon, USA). The vitelline coats of unfertilized C. intestinalis eggs were removed by treatment with 1% sodium thioglycollate and 0.05% protease E as previously described (Mita-Miyazawa et al., 1985), and the thus-formed naked (dechorionated) eggs were thoroughly washed in MFSW and were then microinjected with 2.5 pmol of antisense M-oligos together with the pBraCi-actin 5C-GFP plasmid (as a control) in a volume of 30 pl using a PA-S1 micromanipulator (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Injected eggs were inseminated and reared at 18°C in MFSW containing 50 μg/ml streptomycin sulfate. The embryos were then further cultured until the control embryos had reached the desired stage.

Mammalian cell culture, transfection and electrophoretic mobility shift assay (EMSA)

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C under a 5% CO2 atmosphere. Transfection was performed using Metafectene transfection reagent (Biontex) according to the manufacturer’s protocol. Ci-rel1 and Ci-rel2 expression plasmids, pCI-neo-Flag-Ci-rel1 and pCI-neo-Flag-Ci-rel2, used in transfection experiments were constructed according to the method reported previously (Kawai et al., 2003).

HEK293T cells that had been transfected with the Flag-Ci-rel1 or Flag-Ci-rel2 expression plasmid were washed with phosphate-buffered saline and collected by centrifugation at 15,000 × g for 20 s at 4°C. The resultant pellet was suspended in 320 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, complete protease inhibitor cocktail (Roche)
and 10 μM MG132 (Peptide Institute) and incubated for 15 min on ice. Twenty microliters of buffer A containing 10% Nonidet P-40 were added to the cells, and the cell suspension was then agitated with a vortex mixer for 10 s. The cell lysate was subjected to centrifugation at 15,000 × g for 1 min at 4°C, and the resulting pellet was suspended in 40 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, complete protease inhibitor cocktail and 10 μM MG132) and incubated for 15 min at 4°C. The nuclear lysate was then subjected to centrifugation at 15,000 × g for 5 min at 4°C to give the nuclear extract. Protein concentration of the nuclear extract was determined by using a BCA protein assay reagent kit (Pierce).

The sequence 5′-GGGAATCCCCA-3′ (−893 to −884 bp upstream) was used to make a probe for the 5′-upstream sequence of Ci-IxB. The sense and antisense oligonucleotides used as probes were end-labeled with [γ-32P]ATP (NEB) by using T4 polynucleotide kinase (Roche), annealed, and then purified by phenol extraction and ethanol precipitation. EMSA was performed essentially as described previously (Garner and Revzin, 1981). The nuclear extract (10 μg) was incubated with [γ-32P]ATP-labeled probes (15,000 cpm) in 20 μl of binding solution (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 50 ng/μl poly(dI-dC) (Roche), 2.5% glycerol and 0.05% Nonidet P-40), and the mixture was incubated for 30 min at room temperature. For competition assays, the nuclear extract was incubated with a 200-fold molar excess of the unlabeled oligonucleotide for 5 min at room temperature prior to incubation with the end-labeled oligonucleotide probe. For supershift assays, the nuclear extract was incubated with 2.5 μg of anti-Flag antibody (Sigma) for 1 h at 4°C prior to incubation with the end-labeled oligonucleotide probe. Resulting DNA–protein complexes were electrophoresed on a 4% polyacrylamide gel in Tris–glycine buffer (50 mM Tris, 0.38 M glycine and 2 mM EDTA, pH 8.5), vacuum-dried, and visualized by autoradiography.

Construction of reporter genes and reporter gene assay

The genomic PCR was carried out to release a 1.0-kb fragment from the 5′ flanking region of Ci-IxB. This 1.0-kb fragment, which contains 1.0 kb upstream of the initiation codon, was fused to the lacZ reporter gene in the vector pPD1.27 (Fire et al., 1990) to generate pPD1.27-Ci-IxB 1.0 kb. A mutation construct, pPD1.27-Ci-IxB1.0 kb mut, was obtained by introducing one-base substitution using mutated PCR primer. The mutated kxB sequence is 5′-GGCAATCCCC-3′, in which the mutated nucleotide is underlined. A deletion construct, pPD1.27-Ci-IxB 1.0 kb ΔkxB, in which the kxB sequence 5′-GGGAATCCCC-3′ had been deleted, was obtained by PCR amplification. All regions derived from PCR amplification were verified by sequencing.

Electroporation of the constructs into fertilized eggs was carried out as described above. Embryos were fixed 8 h after insemination and subjected to in situ hybridization with a lacZ probe (1291–2116 bp) as described above.

Results

Ci-rel1 and Ci-rel2, C. intestinalis NF-κB/Rel family members, are splice variants

We first searched the C. intestinalis EST database for NF-κB/Rel family homologues, and we found an EST clone, cieg22h11, which is predicted to include the full length of H. roretzi As-rel2-like cDNA, in this database and determined its nucleotide sequence. This cDNA clone, designated Ci-rel2 (GenBank accession number AK114901), consists of 1741 nucleotides with a poly(A) tail, and its single open reading frame encodes 290 amino acids containing an RHD. Since Ci-rel2 lacks a C-terminal region as does As-rel2 (Shimada et al., 2001), this protein seems to have no transcriptional activity (Kawai et al., 2003). Next, to isolate a C. intestinalis NF-κB/Rel family homologue having a C-terminal region, we carried out screening of a tailbud-embryo cDNA library using a C. intestinalis C-terminal region found in the EST database as a probe. We isolated three clones and determined their nucleotide sequences. It was found that three clones have one identical sequence. This cDNA clone, designated Ci-rel1 (GenBank accession number AY692136), consists of 3121 nucleotides with a poly(A) tail, and its single open reading frame encodes 707 amino acids, containing an RHD identical to that of Ci-rel2 and a C-terminal region. The homology searches among the RHDs revealed that the Ci-rel1 protein has the highest homology (63%) with H. roretzi As-rel1 and also has the highest homology (48%) with zebrafish c-rel among vertebrate NF-κB/Rel family members. (The amino acid sequence alignment and the phylogenetic tree are shown in Figs. S1 and S2, respectively, in Supplementary material.)

The fact that Ci-rel1 and Ci-rel2 contain identical RHDs indicates the possibility that both are splice variants. To understand how Ci-rel1 and Ci-rel2 mRNAs are produced, we searched the genome database and found the Ci-rel genomic sequence. Comparison of genomic and cDNA sequences revealed that the genome has one sequence encoding the RHD (4272 nucleotides), that the Ci-rel1-specific sequence is located 1402 bp downstream from the RHD sequence, and that the intron (1402 nucleotides) for Ci-rel1 contains a stop codon and a polyadenylation signal sequence, which are located 311 and 757 bp downstream, respectively, from the RHD sequence (Fig. 1). These results suggested that the Ci-rel1 mRNA is generated from pre-mRNA by splicing, while a short mRNA of Ci-rel2 is generated because the intron for Ci-rel1, which contains a stop codon 311 bp downstream from its 5′-end, is not excised.
Fig. 1. Ci-rel1 and Ci-rel2 are splice variants. Schematic representation of the structures of the Ci-rel gene and mRNAs of Ci-rel1 and Ci-rel2. The exons are indicated by filled boxes and numbered from 1 to 9. The stop codon in the alternative exon for Ci-rel2 is indicated by an open arrowhead.

(Fig. 1), strongly suggesting that Ci-rel1 and Ci-rel2 are splice variants as are As-rel1 and As-rel2 (Kawai et al., 2003).

In situ localization of Ci-rel1, Ci-rel2 and Ci-IκB transcripts during C. intestinalis embryogenesis

To identify the spatial and temporal patterns of expressions of Ci-rel1, Ci-rel2, and Ci-IκB during C. intestinalis embryogenesis, we carried out in situ hybridization experiments with whole-mount preparations of embryos in the respective developmental stages using DIG-labeled Ci-rel1, Ci-rel2, and Ci-IκB antisense RNA probes (Fig. 2): Strong hybridization signals specific for Ci-rel1 and Ci-rel2 were detected from fertilized egg to middle gastrula and weak ones were detected in larva. However, these specific signals were not detected in embryos from neural plate stage to middle tailbud stage (Figs. 2A, B). In the case of Ci-rel2 expression, a weak signal was detected also in neurula-stage embryos (Fig. 2B). The presence of both Ci-rel1 and Ci-rel2 transcripts in unfertilized egg (data not shown) suggested that both proteins exist maternally. In the case of Ci-IκB expression, strong specific signals were detected from unfertilized egg to 32-cell stage embryo and weak ones peaked at neurula and initial tailbud stages (Fig. 2C). It should be noted that Ci-IκB-specific signals were detected in

![Fig. 2. Expression of Ci-rel1, Ci-rel2, and Ci-IκB during C. intestinalis embryogenesis. Whole-mount preparations of C. intestinalis embryos in various developmental stages were hybridized with DIG-labeled Ci-rel1 (A), Ci-rel2 (B), and Ci-IκB (C) antisense probes and were stained with alkaline phosphatase-conjugated anti-DIG antibody. (a) Fertilized egg (fertilized), (b) 64-cell stage embryo (64-cell), (c) early gastrula (early G), (d) middle gastrula (middle G), (e) neurula, (f) initial tailbud (initial T), (g) middle tailbud (middle T). The strongest level of expression is indicated by ++, and no signal is indicated by --. The localization of the transcript in the neurula or initial tailbud stage is indicated by an arrow, and the notochord in the initial or middle tailbud stage is indicated by an arrowhead. The scale bar is 50 μm.](image-url)
the center of embryos, where notochord cells are localized, implying that Ci-IκB functions during notochord formation.

Localization of GFP-Ci-rel1, GFP-Ci-rel2 and GFP-Ci-IκB fusion proteins in C. intestinalis embryos

The fact that NF-κB/Rel family proteins are activated and then transported into the nucleus, where they promote the transcription of target genes, implies that the intracellular localization of Ci-rel proteins reflects their function. To determine when Ci-rel proteins are activated during C. intestinalis embryogenesis and whether Ci-IκB protein affects embryogenesis, we carried out electro-poration of the respective expression plasmids, pBraGFP-Ci-rel1, pBraGFP-Ci-rel2 and pBraGFP-Ci-IκB, into fertilized eggs to over-express them specifically in the notochord using a notochord-specific Ci-Bra promoter. The electroporated eggs were inseminated and developed. In the neurula, initial tailbud and middle tailbud stages when notochord cells are aligned, embryos were fixed and fluorescence due to GFP was observed (Fig. 3): The embryos moderately overexpressing GFP-Ci-rel1, GFP-Ci-rel2, and GFP-Ci-IκB under the conditions used did not show any abnormal phenotypes in all fixed stages. (It should be noted that the embryos highly overexpressing GFP-Ci-rel1 had shortened tails, data not shown.) GFP-Ci-rel2 and GFP-Ci-IκB were detected in both the cytoplasm and nucleus in the period from neurula stage to middle tailbud stage (Figs. 3B, C). Since Ci-rel2 lacks an NLS as does H. roretzi As-rel2 (Kawai et al., 2003), it can be inferred that Ci-rel2 in the cytoplasm is associated with Ci-IκB or with itself, while Ci-rel2 is associated with Ci-rel1 and the thus formed heterodimer then moves into the nucleus. On the other hand, GFP-Ci-rel1 was detected in the cytoplasm in the neurula stage (Fig. 3Aa’), in the nucleus in the initial tailbud stage (Fig. 3Ab’) and again in the cytoplasm in the middle tailbud stage (Fig. 3Ac’).

Close investigation of GFP-Ci-rel1 localization in the period from the neurula stage to the middle tailbud stage revealed that GFP-Ci-rel1 was localized only in the cytoplasm 7.5 h after insemination, that is, in the neurula stage (Fig. 4A). After 30 min from the neurula stage, that is, in the initial tailbud stage, GFP-Ci-rel1 was found to have moved into the nucleus, where it remained for about 2 h (Figs. 4B, C). In the early and middle tailbud stages, however, GFP-Ci-rel1 was again localized in the cytoplasm (Figs. 4D, E). This transient nuclear transport of Ci-rel1 suggests that Ci-rel1 is transiently activated in response to an unknown upstream developmental signal and moves into the nucleus, promoting the transcription of target genes, in the initial tailbud stage.

Ci-rel1-induced gene expression during C. intestinalis embryogenesis

It has been reported that IκBα, one of the inhibitors of the NF-κB/Rel family, is a target gene of the NF-κB/Rel
family (Scott et al., 1993; Verma et al., 1995). We previously showed that Ci-I\textsubscript{n}B, an I\textsubscript{n}B homologue of \textit{C. intestinalis}, has the highest homology with human I\textsubscript{n}B\textsubscript{a} (Kawai et al., 2003). Thus, it can be inferred that Ci-I\textsubscript{n}B is a target gene of Ci-rel1.

To determine whether GFP-Ci-rel1 is actually activated and promotes the target gene transcription in the initial tailbud stage of \textit{C. intestinalis}, we carried out in situ hybridization with a Ci-I\textsubscript{n}B probe using embryos over-expressing GFP-Ci-rel1 or GFP-Cirel2 (Fig. 5). In control embryos, weak expression of endogenous Ci-I\textsubscript{n}B was detected in the initial tailbud stage (Fig. 5Aa). When GFP-Ci-rel1 was over-expressed, the expression of Ci-I\textsubscript{n}B was remarkably enhanced in notochord cells only in the

![Image of Figure 4](image1)

**Fig. 4.** Intracellular localization of GFP-Ci-rel1 during \textit{C. intestinalis} embryogenesis. GFP-Ci-rel1 protein was expressed in the notochord as in Fig. 3. The embryos were fixed 7.5 h (A, a), 8 h (B, b), 8.5 h (C, c), 10 h (D, d) and 11.5 h (E, e) after insemination and observed as in Fig. 3. The photographs (a–e) show merges of bright fields and fluorescence images, while the fluorescence images (A–E) show magnified versions of the boxed areas in (a–c). The nuclei are indicated by white arrows. The scale bar is 25 \textmu m.

![Image of Figure 5](image2)

**Fig. 5.** Ci-rel1 induced expression of Ci-I\textsubscript{n}B in the initial tailbud stage during \textit{C. intestinalis} embryogenesis. In situ hybridization with a Ci-I\textsubscript{n}B probe was carried out using embryos over-expressing GFP-Ci-rel1 (B) or GFP-Cirel2 (C) or control embryos (A) in the neurula (a), initial tailbud (b) and middle tailbud stages (c). The scale bar is 50 \textmu m.
initial tailbud stage (Fig. 5Bb), whereas when GFP-Ci-rel2 was over-expressed, expression of Ci-IkB was undetectable in the neurula, initial tailbud, and middle tailbud stages (Fig. 5C). Thus, over-expression of Ci-rel1 induced the expression of Ci-IkB, strongly suggesting that Ci-IkB is a target gene of Ci-rel1. This Ci-rel1-induced expression of Ci-IkB was observed at the same stage when the nuclear import of C-rel1 proceeds, suggesting that in the initial tailbud stage Ci-rel1 is activated and promotes the transcription of target genes, including Ci-IkB. With respect to the role of Ci-rel2, it should be noted that over-expression of Ci-rel2 suppressed the expression of Ci-IkB (compare Figs. 5Ab and Cb): This result can be explained by the speculation that Ci-rel2 inhibits the activity of Ci-rel1, leading to the suppression of Ci-rel1-induced expression of Ci-IkB. This situation appears similar to the case in which As-rel2 negatively regulates the activity of As-rel1 in H. roretzi (Kawai et al., 2003).

### Binding of Ci-rel1 to κB-like sequence of Ci-IkB

To obtain definitive evidence that Ci-IkB is a direct target gene of Ci-rel1, we searched for a κB-like consensus sequence 5’-GGGRNNYYCC-3’ (where R indicates A or G, Y indicates C or T, and N indicates any base) (Chytíl and Verdine, 1996; Parry and Mackman, 1994) in the genomic 5’ upstream sequence of Ci-IkB and found that the sequence 5’-GGGAATCCCC-3’ is located about 800 bp upstream from the 5’-end of Ci-IkB as the κB consensus sequence. Next, to determine whether Ci-rel1 and Ci-rel2 proteins can bind to this sequence directly, we carried out EMSA using the nuclear extract of HEK293T cells transiently expressing Flag-Ci-rel1 (Fig. 6A) or Flag-Ci-rel2 (Fig. 6B). EMSA revealed that Ci-rel1 and Ci-rel2 are capable of binding to this sequence (see rel extract in Figs. 6A, B). A supershift was observed in either case by the addition of anti-Flag antibody (see supershift in Figs. 6A, B), indicating that these DNA-binding activities are specific for both Ci-rel1 and Ci-rel2. In a self-competition assay, the above DNA-binding activities were decreased by the addition of an unlabeled DNA oligomer of this κB consensus sequence (see competitor in Figs. 6A, B), indicating that these DNA binding activities are specific for the κB consensus sequence. Thus, these results suggest that Ci-IkB is a direct target gene of Ci-rel1.

### κB sequence-dependent gene expression in the notochord

To investigate whether the κB sequence of Ci-IkB controls its expression in the notochord, we carried out reporter gene assay using the 1.0 kb upstream sequence of Ci-IkB and lacZ in the C. intestinalis embryos (Fig. 7A). Because Ci-rel1 moves into nucleus and induces the expression of Ci-IkB 8 h after insemination, that is, in the initial tailbud stage (Figs. 4 and 5Bb), it is possible that the κB sequence functions in this stage. A strong signal was detected in the notochord in the case of the wild type sequence (5’-GGGAATCCCC-3’) (Fig. 7B). On the other hand, weak signals were detected in the notochord and ectopic signals in the mesenchyme and epidermis in both cases of one point mutation (5’-GGCAATCCCC-3’) (Fig. 7C) and the deletion of the κB sequence (Fig. 7D). In addition, the middle tailbud stage embryos, which had been injected with lacZ gene fused with the wild type κB sequence, had no lacZ signals (data not shown). The fact that the endogenous Ci-IkB gene expression was scarcely detectable in the notochord in the middle tailbud stage (Fig. 2Cg) supports this result that the lacZ gene expression was not promoted in this stage. Thus, these results suggest that the expression of Ci-IkB in the notochord is controlled by its κB sequence.

### Effects of antisense M-oligos on C. intestinalis embryogenesis

Antisense M-oligo has complete resistance to nuclease digestion and heat treatment, and it binds with mRNA of the target gene to form a stable double-strand that blocks translation by ribosome. As a result, the protein of the target gene is knocked down. It has been reported that certain antisense M-oligos specifically blocked the functions of the corresponding endogenous genes to affect C. intestinalis

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Fig. 6. Ci-rel1 and Ci-rel2 bind to a κB-like sequence located upstream of Ci-IkB. (A) EMSA analysis of nuclear extracts isolated from HEK293T cells transfected with Flag-Ci-rel1 using a κB-like DNA fragment as a probe. Control extract, labeled κB fragment with control nuclear extract from non-transfected HEK293 cells; cold probe, unlabeled κB fragment with 10 μg of nuclear extract from transfected cells; rel extract, labeled κB fragment with 10 μg of nuclear extract from transfected cells; supershift, labeled κB fragment with 10 μg of nuclear extract from transfected cells and anti-Flag tag antibody (αFlag); competitor, labeled κB fragment with 10 μg of nuclear extract from transfected cells and 200-fold molar excess of unlabeled κB fragment. (B) EMSA analysis of nuclear extracts isolated from HEK293T cells transfected with Flag-Ci-rel2 using a κB-like DNA fragment as a probe. Samples were prepared as in (A) except for the nuclear extract.
embryogenesis (Satou et al., 2001). We first injected antisense M-oligo against Ci-rel to unfertilized egg together with pBraCi-actin 5C-GFP (control) that was used to identify notochord cells through fluorescence due to GFP because the presence of the Ci-Bra promoter leads to promotion of notochord-specific expression (Corbo et al., 1997). After injection, the eggs were inseminated and developed. We thought that antisense M-oligo against Ci-rel would affect Ci-rel1 and Ci-rel2 because antisense M-oligo was designed on the basis of the common RHD sequence, but no abnormality in embryogenesis or notochord formation was detected (data not shown). Next, we injected antisense M-oligo against Ci-IkB to unfertilized eggs together with pBraCi-actin 5C-GFP, and the injected eggs were inseminated and developed. In the middle tailbud stage, the embryos injected with antisense M-oligo against Ci-IkB showed an abnormal phenotype with a reduction of tail, so-called chongmague (Nakatani et al., 1999) (Figs. 8B, b). Over 90% of the injected embryos showed such an abnormality. Moreover, antisense M-oligo-injected notochord cells were ill-arranged in two rows (Fig. 8b), in contrast to control notochord cells, which were arranged in a line (Fig. 8a). It should be noted that some antisense M-oligo-injected embryos had a defect in the neural tube closure caused by abnormal notochord formation, resulting in the elongation of the notochord toward the dorsal side. These results strongly suggest that Ci-IkB plays an important role in notochord formation, especially in intercalation of notochord cells.

Since the embryos injected with antisense M-oligo against Ci-IkB showed a severe phenotype, we checked differentiation of tissues such as the tail, nerve cord and notochord in injected embryos by in situ hybridization with the following marker genes: 00124, which is specifically expressed in the nerve cord (referred to from the C. intestinalis EST database), and Noto-1, Noto-4, and Ci-fibrn, all of which are specifically expressed in the notochord in the late tailbud stage (Hotta et al., 2000, Imai et al., 2002) (Fig. 9). In the middle tailbud stage, the injected embryos showed signals for 00124 and Noto-1 (Figs. 9Ab, Bb), and in the late tailbud stage, they showed signals for 00124, Noto-1, Noto-4, and Ci-fibrn (Figs. 9Cb, Db, Eb, Fb). These results
and under a fluorescence microscope as in Fig. 3(a, b). The arrow indicates the middle tailbud stage. Embryos were observed in a bright field (A, B) or with pBraCi-actin 5C-GFP only (A, a), inseminated, and developed up to Ci-I against Ci-I is not affected by injection with antisense M-oligo against notochord formation in C. intestinalis (Shimada et al., 2001). We previously showed that the short variant As-rel2, which lacks NLS and a C-terminal putative transactivation domain, regulates the localization and transcriptional activity of the typical member As-rel1 (Kawai et al., 2003). Since in situ hybridization experiments in C. intestinalis revealed weak expression of Ci-rel2 in the neurula stage, when the initial alignment of notochord cells occurs (Fig. 2B), it is possible that Ci-rel2 regulates the activation of Ci-rel1 as does As-rel2. In fact, over-expression of Ci-rel2 suppressed the expression of Ci-IkB, a target of Ci-rel1, in the initial tailbud stage (Fig. 5Cb), possibly through its suppression of Ci-rel1.

Ci-IkB is the target gene of Ci-rel1 as is mammalian IkBx. The 5’ upstream genomic sequence of Ci-IkB has two κB-like sequences at about -800 bp and -150 bp upstream from the 5’-end of Ci-IkB. One is 5’-GGGAATCCCC-3’ at about -800 bp: It completely matches with the consensus κB sequence, and both Ci-rel1 and Ci-rel2 proteins can bind to this sequence (Fig. 6). Another is 5’-GTTACTTTCC-3’, which includes one miss-match base at about -150 bp, but neither Ci-rel1 nor Ci-rel2 proteins are able to bind to this sequence (data not shown). In any case, it can be concluded that Ci-rel proteins bind to the consensus κB sequence of Ci-IkB. In addition, the reporter gene assay showed that the wild type κB sequence of Ci-IkB induced strong gene expression in the notochord, consistent with endogenous expression of Ci-IkB in the notochord (Figs. 2C and 7B), while the mutation and the deletion of the κB sequence suppressed gene expression in the notochord and inversely induced gene expression in the mesenchyme and epidermis (Figs. 7C, D). These results suggest that the κB sequence functions in gene expression, including Ci-IkB gene, in the notochord, in which Ci-rel1 protein moves into the nucleus (Fig. 4).

In C. intestinalis, notochord formation consists of two phases after gastrulation. The first phase is driven by mediolaterally direct intercalation of notochord cells, and the second phase involves the vacuolation and extension of individual cells (Miyamato and Crowther, 1985; Munro and Odell, 2002). We discovered that GFP-Ci-rel1 protein moves into the nucleus at the first phase of notochord formation (Figs. 3A and 4), at the stage at which it promotes transcription of the Ci-IkB gene (Fig. 5B). These results indicate that there is a relationship between activation of the NF-κB/Rel signaling pathway and notochord formation. In connection with this, it should be noted that both C. intestinalis Pellino (Hotta et al., 2000), which functions as a scaffold protein in the NF-κB/Rel signaling pathway (Jensen and Whitehead, 2003; Jiang et al., 2003; Strelow et al., 2003), and Ci-pelle (K. Hotta et al., unpublished), which is a C. intestinalis homologue of interleukin-1 receptor-associated kinase (IRAK) functioning in the NF-κB/Rel signaling pathway, are specifically induced in the notochord by Ci-Bra, a key factor of C. intestinalis notochord formation (Hotta et al., 2000). In addition, it has recently been found that Ci-Ube2, a C. intestinalis homologue of ubiquitin-conjugating enzyme E2, is also specifically induced in the notochord by Ci-Bra (Hotta et al.,

Discussion

In this study, we isolated Ci-rel1 and Ci-rel2, C. intestinalis NF-κB/Rel family homologues, and determined that they are splice variants. Analyses of the intracellular localization of GFP-fusion proteins and in situ hybridization revealed that Ci-rel1 is transiently activated in the initial tailbud stage. We also demonstrated that Ci-IkB is a direct target gene of Ci-rel1. Moreover, we found that knockdown of Ci-IkB induces abnormal lining of notochord cells but has little effect on differentiation of the nerve cord and notochord. These results provide evidence that the regulation of Ci-rel1 by Ci-IkB is essential for notochord formation in C. intestinalis.

The ascidian C. intestinalis has two typical and short splice variants of the NF-κB/Rel family, Ci-rel1 and Ci-rel2, as does the ascidian H. roretzi (Shimada et al., 2001). We previously showed that the short variant As-rel2, which
unpublished). This suggests that there is a relationship between ubiquitin-dependent proteolysis and notochord formation. Thus, it is possible that degradation of substrates, including Ci-\(\text{I}_n\)B, by the ubiquitin-dependent proteolytic system is induced through the upstream signaling pathway in notochord cells, which allows Ci-rel1 to move into the nucleus, leading to promotion of the transcription of target genes, including Ci-\(\text{I}_n\)B.

In gene-knockdown experiments, we first used antisense M-oligo against Ci-rel but found that it had little effect on embryogenesis (data not shown). This result can be explained by the assumption that Ci-rel1 and Ci-rel2 exist as maternally expressed proteins, since their mRNAs are strongly expressed in unfertilized eggs (data not shown). NF-\(\kappa\)B/Rel family proteins are stable in other animals; for example, the half life of human c-Rel is about 12 h (Chen et al., 1998). Since antisense M-oligo does not affect proteins, Ci-rel1 and Ci-rel2 proteins may function normally in Ci-rel-knockdown embryos. Thus, Ci-rel1 and Ci-rel2 proteins that have accumulated in unfertilized eggs may function during embryogenesis up to the middle tailbud stage. In contrast, the knockdown of Ci-\(\text{I}_n\)B induced the short tail phenotype and the abnormal alignment of notochord cells (Fig. 8). Since the Ci-\(\text{I}_n\)B knockdown embryos showed two rows of notochord cells (Fig. 8) and the expression of Ci-\(\text{I}_n\)B occurs in the neurula and initial tailbud stages, the first phase of notochord formation (Fig. 2C), it can be concluded that Ci-\(\text{I}_n\)B plays a key role in the intercalation of notochord cells. In contrast, the knockdown of Ci-\(\text{I}_n\)B did not affect differentiation of the nerve cord and notochord in the middle and late tailbud stages (Fig. 9). In M-oligo-injected embryos, in which Ci-\(\text{I}_n\)B is thought not to function, Ci-rel1 may be continuously activated in the early and middle tailbud stages, resulting in abnormal formation of notochord. Thus, it can be inferred that a key factor of the NF-\(\kappa\)B/Rel pathway is Ci-\(\text{I}_n\)B in the intercalation of notochord cells and that the inhibition of Ci-rel1 activity by Ci-\(\text{I}_n\)B at the later phase of the tailbud stage is important for the intercalation of notochord cells.

In conclusion, we propose that the NF-\(\kappa\)B/Rel signaling pathway functions in notochord formation in C. intestinulis. Ci-rel1 is transiently activated and promotes transcription of target genes, including Ci-\(\text{I}_n\)B, in the tailbud stage.
stage, which, in turn, leads to the repression of Ci-rel1 by Ci-IrB. This Ci-IrB-mediated regulation of Ci-rel1 through an autoregulatory feedback loop is essential for notochord formation in C. intestinalis.

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Appendix A. Supplementary data


References


