

# Identification of Nogo-66 Receptor (NgR) and Homologous Genes in Fish

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The Nogo-66 receptor NgR has been implicated in the mediation of inhibitory effects of central nervous system (CNS) myelin on axon growth in the adult mammalian CNS. NgR binds to several myelin-associated ligands (Nogo-66, myelin associated glycoprotein, and oligodendrocyte-myelin glycoprotein), which, among other inhibitory proteins, impair axonal regeneration in the CNS of adult mammals. In contrast to mammals, severed axons readily regenerate in the fish CNS. Nevertheless, fish axons are repelled by mammalian oligodendrocytes *in vitro*. Therefore, the identification of fish NgR homologs is a crucial step towards understanding NgR functions in vertebrate systems competent of CNS regeneration. Here, we report the discovery of four zebrafish (*Danio rerio*) and five fugu (*Takifugu rubripes*) NgR homologs. Synteny between fish and human, comparable intron-exon structures, and phylogenetic analyses provide convincing evidence that the true fish orthologs were identified. The topology of the phylogenetic trees shows that the extra fish genes were produced by duplication events that occurred in ray-finned fishes before the divergence of the zebrafish and pufferfish lineages. Expression of zebrafish NgR homologs was detected relatively early in development and prominently in the adult brain, suggesting functions in axon growth, guidance, or plasticity.

## Introduction

In contrast to mammals, lesioned axons readily regenerate in the fish central nervous system (CNS) and reestablish appropriate connections with their targets (Gaze 1970). This success of axonal regeneration depends on intrinsic properties of fish neurons and on a growth-promoting environment for regenerating axons (Stuermer et al. 1992). Fish CNS myelin as well as oligodendrocytes are permissive substrates for axonal growth and do not induce growth cone collapse (Bastmeyer et al. 1991; Ankerhold et al. 1998). Therefore, homologs of mammalian inhibitory proteins such as the IN-1 antigen (Caroni and Schwab 1988), myelin associated glycoprotein (MAG) (Mukhopadhyay et al. 1994), and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al. 2002) are most probably not present in fish (Bastmeyer et al. 1991; Wanner et al. 1995). On the other hand, rat and bovine CNS myelin as well as rat oligodendrocytes are inhibitory substrates for regenerating goldfish retinal axons *in vitro*, indicating that fish axons are sensitive to mammalian neurite growth inhibitors (Bastmeyer et al. 1991; Wanner et al. 1995).

In mammals, the Nogo-66 receptor (NgR) has been proposed to play an important role in the mediation of axonal growth inhibition (Fournier, GrandPré, and Strittmatter 2001; Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002b). NgR is a glycosylphosphatidylinositol (GPI)-linked, leucine-rich repeat (LRR) protein that binds to the 66 amino acid (aa) loop between the two C-terminal hydrophobic domains of Nogo (termed Nogo-66) (GrandPré et al. 2000) and transduces Nogo-66 mediated axonal inhibition (Fournier, GrandPré, and Strittmatter 2001). The recent discovery that MAG and OMgp also require the interaction with NgR for their inhibitory effects (Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002b) is

suggestive of a common signaling pathway for these myelin inhibitors.

It is an intriguing question whether fish possess an NgR ortholog and what its function might be in this class of organisms. Because CNS axons regenerate successfully in fish, the functions of fish NgR proteins are likely to differ from the mammalian function as transducer of growth inhibition. However, NgR might be involved in the mediation of the inhibitory effect of mammalian CNS myelin on fish axons *in vitro*. In this context, we set out to identify NgR homologs in fish. Four different NgR-related genes were cloned and sequenced from zebrafish, and five genes were uncovered in the fugu genome. While our study was in progress, two additional NgR homologs were identified in human and rodents (Pignot et al. 2003), and the five fugu NgR genes were discovered (He et al. 2003). Phylogenetic relationships among all available NgRs demonstrate that this family of genes was produced by separate duplication events in the ancestors of vertebrates and early in actinopterygian evolution. In zebrafish, the mRNA distribution during development and in different adult tissues is consistent with a potential role for these proteins in the regulation of axon growth and plasticity.

## Materials and Methods

Cloning of zfNgR, zfNgRH1a, zfNgRH1b, and zfNgRH2a

By searching the NCBI database, zebrafish genomic sequences homologous to human NgR were identified and amplified from cDNA with specific primers (supplementary table 1). Sequences were completed performing 5'-RACE and 3'-RACE, respectively. In brief, we extracted mRNA from a pool of 48 h postfertilization (hpf) zebrafish embryos (FastTrack™ 2.0 kit; Invitrogen) and used 0.9 µg/reaction as template for the synthesis of either first-strand 5'-Ready cDNA using 5'-CDS and SMART II oligonucleotides or of 3'-Ready cDNA using 3'-CDS primer, according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit; BD Clontech). PCR fragments were directly

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subcloned into the pCRII cloning vector (Invitrogen), and plasmid DNA was prepared using the QIAprep<sup>®</sup> 8 Miniprep Kit (Qiagen). Both DNA strands were sequenced using the Abi Prism<sup>®</sup> BigDye<sup>™</sup> Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an AbiPrism 3100 Genetic Analyzer. Single sequences were assembled using SeqMan<sup>™</sup>II of the DnaStar software package (GATC Biotech).

#### Sequence Alignments and Phylogenetic Analyses

Zebrafish and fugu genomic sequences were obtained using blast algorithms (Altschul et al. 1997) at NCBI ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)), Ensembl ([www.ensembl.org/Danio\\_rerio/blastview](http://www.ensembl.org/Danio_rerio/blastview); [www.ensembl.org/Fugu\\_rubripes/blastview](http://www.ensembl.org/Fugu_rubripes/blastview)) and the Doe Joint Genome Institute ([aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=fugu6](http://aluminum.jgi-psf.org/prod/bin/runBlast.pl?db=fugu6)) Web pages. The fugu cDNAs were deduced from the respective genomic sequences by comparison with the zebrafish cDNAs. The human, macaque, rat, and mouse sequences were already available in GenBank (accession numbers gi20127617, gi9280024, gi21311542, gi28496903, gi27701808, gi25056697, gi27500320, and gi29244159). Exon-intron structures were examined by comparison of cDNA against genomic sequences, considering the GT-AG rule of splice donor and acceptor sites.

Nucleotide sequences were translated using BioEdit (Hall 1999) and aligned as amino acids using ClustalW (Thompson, Higgins, and Gibson 1994). The alignment was edited by hand and then converted back into nucleotides to produce a codon alignment. The multispecies NgR amino acid alignment was also used in a model-based HMMER 2.3 (Sean Eddy) search of the NCBI nonredundant (protein), ENSEMBL, and Doe Joint Genome Institute databases for invertebrate (*Drosophila melanogaster*, *Anopheles gambiae*) and ascidian (*Ciona intestinalis*) orthologs. Phylogenies of NgR-related sequences were reconstructed using maximum-parsimony (MP) and genetic distance-based methods with PAUP\* version 4.0 (Swofford 2002) and using a maximum-likelihood (ML) approach with MrBayes (Huelsenbeck and Ronquist 2001). The transition transversion ratio (1:1.41) was estimated using PAUP and included in the MP analyses. One MP analysis considered characters from the complete alignment, and the second excluded third codon positions. Two methods for estimating genetic distance were used, HKY85 (Hasegawa, Kishino, and Yano 1985) and LogDet (Lockhart et al. 1994). Neighbor-joining (Saitou and Nei 1987) trees were reconstructed from genetic distances. Support for nodes in the MP and NJ trees was assessed using 1,000 bootstrap reiterations (Felsenstein 1985). ML trees were reconstructed from the nucleotide alignment with  $nst = 6$  (Tavare 1986) and rates =  $inv\gamma$  (Yang 1993) and from the amino acid alignment using the blossom model (Henikoff and Henikoff 1992). In the amino acid-based analysis, rate variation among positions was assumed to follow a continuous gamma distribution approximated from eight discrete rate categories. The parallel version of MrBayes (Huelsenbeck and Ronquist 2001) was used on four processors, one for each of four Markov Chains.

Mega (Kumar, Tamura, and Nei 1994) was used to identify amino acid residues unique to one subfamily of NgR genes. We compared evolutionary rates between the human NgR genes using a test developed by Tajima (Tajima 1993) as implemented in Mega (Kumar, Tamura, and Nei 1994). Signal peptides were predicted using SignalP version 1.1 at <http://www.cbs.dtu.dk/services/SignalP/> (Nielsen et al. 1997) and GPI-anchor sites using big-PI Predictor at [http://mendel.imp.univie.ac.at/sat/gpi/gpi\\_server.html](http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html) (Eisenhaber, Bork, and Eisenhaber 1999).

#### Radiation Hybrid Mapping and Synteny Analyses

Zebrafish NgRH1a, NgRH1b, and NgRH2a were mapped on the LN54 radiation hybrid panel using standard conditions (Hukriede et al. 1999) and the respective web interface (<http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi>). Because no unequivocal result was obtained for NgR on this panel, this receptor was mapped on the Goodfellow T51 radiation hybrid panel (Research Genetics, Inc.) by instant mapping at <http://134.174.23.167/zonrhmapper/instantMapping.htm>.

For synteny analysis (Woods et al. 2000), other zebrafish genes and ESTs already mapped on the LN54 and T51 radiation hybrid and HS maps ([http://zfin.org/cgi-bin/mapper\\_select.cgi](http://zfin.org/cgi-bin/mapper_select.cgi)) were assigned to putative human orthologs by BlastX searches (Altschul et al. 1997) against the NCBI human nonredundant (nr) protein sequence database (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). For EST clones that have been sequenced at the 5' and 3' ends, both sequences were used for BlastX searches. If the results of these searches had expect scores ( $E$  values) of  $-5$  or less, the putative orthologs were further tested with reciprocal searches against the zebrafish subset of nr sequences and dbEST databases. A human ortholog was confirmed if the original zebrafish gene or EST (or a gene or EST that showed highly significant overlap with the original sequence) was in the top 15 matches of the reciprocal search by TblastN. Fugu synteny data were retrieved with MartView ([http://www.ensembl.org/Fugu\\_rubripes/martview](http://www.ensembl.org/Fugu_rubripes/martview)).

#### Southern Blotting

Genomic DNA was prepared from one individual *honkong* inbred adult zebrafish and digested with *Bcl I*, *Bgl II*, *Hind III*, and *Pst I*, respectively. After electrophoretic separation, genomic DNA fragments were transferred to a Hybond N<sup>+</sup> nitrocellulose membrane (Amersham Biosciences) under alkaline conditions (Chomczynski and Qasba 1984). The DNA probe was labeled using the AlkPhos Direct labeling kit (Amersham Biosciences) and applied according to the manufacturer's protocol, with longer prehybridization and washing times and increased washing volumes. Blots were developed with CDP-Star detection reagent (Amersham Biosciences).

To determine the expected number and sizes of bands to be detected, restriction maps of the subcloned and sequenced southern probe and of the relevant genomic region were established using MapDraw<sup>™</sup> of the DnaStar software package (GATC Biotech). Because of

polymorphisms in the genomic sequences, the size of the expected fragments remains an estimation.

## RT-PCR

One hundred zebrafish embryos (~100 mg) for each stage or 50 mg of various adult tissues were used for preparation of total RNA with the RNeasy Mini Prep Kit (Qiagen) following manufacturer's instructions. Muscle tissue was additionally subjected to proteinase K digestion (200 µg/30 mg tissue). First-strand cDNA was synthesized under standard conditions with the Superscript First-Strand Synthesis System (Invitrogen) using oligo(dT)<sub>12-18</sub> primer. Zero-transcriptions (without Superscript II in the reaction) were performed in parallel, to control for genomic DNA contaminations in subsequent PCRs. Amount and quality of the different cDNA samples were evaluated comparing the expression level of the housekeeping gene actin. Primer informations are listed in supplementary table 1.

## Results

### Identification and Phylogenetic Analyses of Fish NgRs

Database searches uncovered four different zebrafish (*Danio rerio*) genomic sequences with significant homology to human NgR. Full-length cDNAs were identified by RT-PCR, 5'-RACE, and 3'-RACE. Analysis of fugu (*Fugu rubripes*) genomic sequences revealed the presence of five NgR-related genes. The zebrafish (zf) genes were named zfNgR (GenBank accession number AY25718), zfNgRH1a (GenBank accession number AY263332), zfNgRH1b (GenBank accession number AY263334), and zfNgRH2a (GenBank accession number AY263333), and the fugu genes received analogous names, reflecting the results of the phylogenetic analyses (see below).

Using ClustalW and BioEdit, we produced an unambiguous alignment of 17 NgR-related sequences that was 819 nucleotides long (see fig. 1 in the Supplementary Material online). The *Drosophila* Slit gene was the invertebrate sequence with the best match to the HMMER model, which was based upon an alignment of vertebrate NgR proteins. However, there are vertebrate Slit proteins that are more closely related to *Drosophila* Slit than NgR. There were no matches to the HMMER model based upon all vertebrate NgRs in the peptides annotated in the mosquito (*Anopheles gambiae*) and ciona (*Ciona intestinalis*) genome databases. Therefore, it appears that no true orthologs of NgR are present in invertebrates and ascidians. Phylogenetic trees reconstructed using maximum-parsimony (MP), maximum-likelihood (ML), and genetic distance-based methods had the same topology and produced trees comprising distinct NgR, NgRH1, and NgRH2 clades (fig. 1). This pattern was observed when all nucleotide positions were included in the analyses and also in the MP analysis, excluding third codon positions. The three NgR clades were well supported (fig. 1). Each clade included a human, mouse, zebrafish, and at least one fugu sequence, indicating that the gene duplication events producing NgR, NgRH1, and NgRH2 occurred before the divergence of ray-finned fish (actinopterygians) and tetrapods (sarcopterygians). The NgR clade also included orthologs for macaque and rat. The NgRH1 clade contained two fugu and two zebrafish genes,

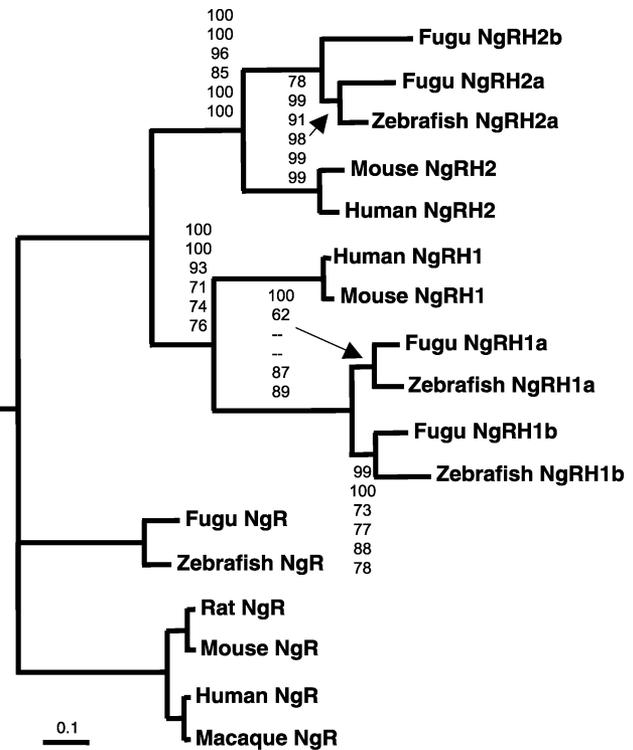


FIG. 1.—Evolutionary relationships among vertebrate NgR and NgR-related genes. Topology shown is a consensus tree based upon maximum-likelihood (ML) analysis of an amino acid (aa) alignment (273 positions [supplementary figure 1]). Placement of root reflects the results of an analysis that included the *Drosophila* Slit gene (252 aa positions [supplementary figure 2]). The same topology was reconstructed from a nucleotide alignment using maximum parsimony (MP), genetic distance—estimates, and ML. Support for nodes is 100% (all ML trees or all bootstrap trees) except where indicated. Percent bootstrap support (1,000 reiterations) is shown from top to bottom: ML analysis of amino acid alignment, ML analysis of nucleotides, MP analysis of first and second codon positions, MP analysis of all positions, HKY genetic distance, and LogDet genetic distance. Scale represents 10% protein sequence divergence. Fugu = *Fugu rubripes*; human = *Homo sapiens*; macaque = *Macaca fascicularis*; mouse = *Mus musculus*; rat = *Rattus norvegicus*; zebrafish = *Danio rerio*.

and the NgRH2 clade included two fugu genes and only one zebrafish gene (fig. 1). In all phylogenetic analyses, the relationships among the fish sequences were consistent with the hypothesis that the NgRH1 and NgRH2 duplicates were produced before the ancestors of zebrafish and fugu diverged.

The zebrafish transcripts encode proteins of 479 aa (NgR), 458 aa (NgRH1a), 457 aa (NgRH1b), and 478 aa (NgRH2a) (supplementary fig. 3). The sequences of the fugu proteins were deduced from genomic data by comparison with the zebrafish sequences, but their expression was not validated. They are 467 aa (NgR), 454 aa (NgRH1a), and 451 aa (NgRH2a) long (supplementary fig. 4). For fugu NgRH1b and NgRH2b, the first exon could not be identified, and the size of the respective proteins can therefore not be calculated. As in human, all proteins contain predicted signal sequences followed by eight leucine-rich repeat (LRR) domains that are framed by LRR amino-terminal and carboxy-terminal (LRRNT, LRRCT) domains. At the C-terminus, a region with only

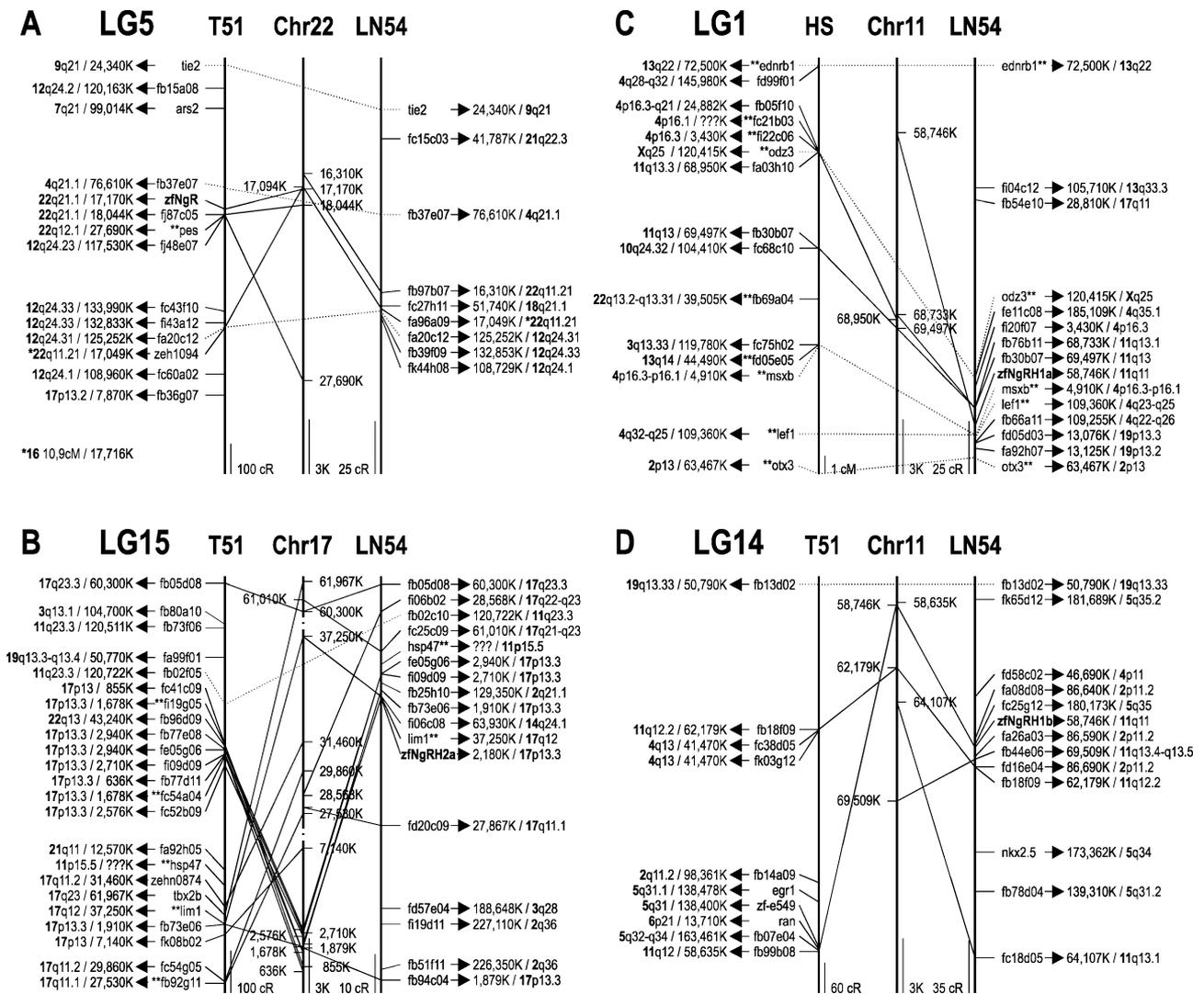


FIG. 2.—Analysis of zebrafish and human syntenic relationships. Map locations of ESTs and genes in the radiation hybrid panels (T51 and LN54) and the heatshock (HS) panel were obtained from ZFIN. The relative chromosomal locations of the human orthologs were deduced from data in LocusLink. Markers present on more than one zebrafish panel are connected by dotted lines (colored version is available as supplementary figure 5). (A) Conserved synteny of zebrafish linkage group (LG) 5 and human chromosome (Chr) 22 defined by zfNgR (bold). Asterisk (\*) indicates chromosomal position of the human ortholog of EST fa96a09 could not be identified unambiguously. (B) Conserved synteny of zebrafish LG15 and human Chr 17 defined by zfNgRH2a (bold). (C) Conserved synteny of zebrafish LG1 and human Chr 11 defined by zfNgRH1a (bold). (D) Conserved synteny of zebrafish LG14 and human Chr 11 defined by zfNgRH1b (bold). CR = centiray; cM = centimorgan; K = kilobasepairs. Double asterisks (\*\*\*) indicates that these EST have been published by Woods et al. (2000).

weak similarity among these related proteins is followed by a GPI anchorage site.

Mega (Kumar, Tamura, and Nei 1994) was used to identify amino acid residues unique to one subfamily of NgR genes. The alignment used for this survey included *Drosophila* Slit to distinguish ancestral and derived subfamily-specific residues. This alignment was shorter (252 aa [supplementary fig. 2]) than the NgR-only alignment (273 aa [supplementary fig. 1]). Eighty-three amino acids were conserved (monomorphic) in all NgRs and 57 aa were shared between the vertebrate NgRs and *Drosophila* Slit, meaning that the three NgR subfamilies differ from *Drosophila* Slit at 26 shared and monomorphic residues. The NgR subfamily is defined by six unique amino acid substitutions, four of which are derived.

NgRH1 proteins have seven diagnostic and derived residues, whereas NgRH2 proteins share eight diagnostic residues, and of these, seven are derived. Residues at position 134 (with respect to human NgR [supplementary fig. 2]) are diagnostic for each of the three subfamilies. Note that all of these observations must be considered in light of limited species sampling (ingroup and outgroup), meaning that the number of diagnostic residues might decrease when NgR sequences of more species are available. Most of the identified diagnostic residues have either polar or charged side chains. Mapping on the published NgR structure (Barton et al. 2003; He et al. 2003) revealed that 18 out of the 21 diagnostic amino acids are surface-exposed and locate to the top and bottom part of the NgR ectodomain and not to the central, concave face

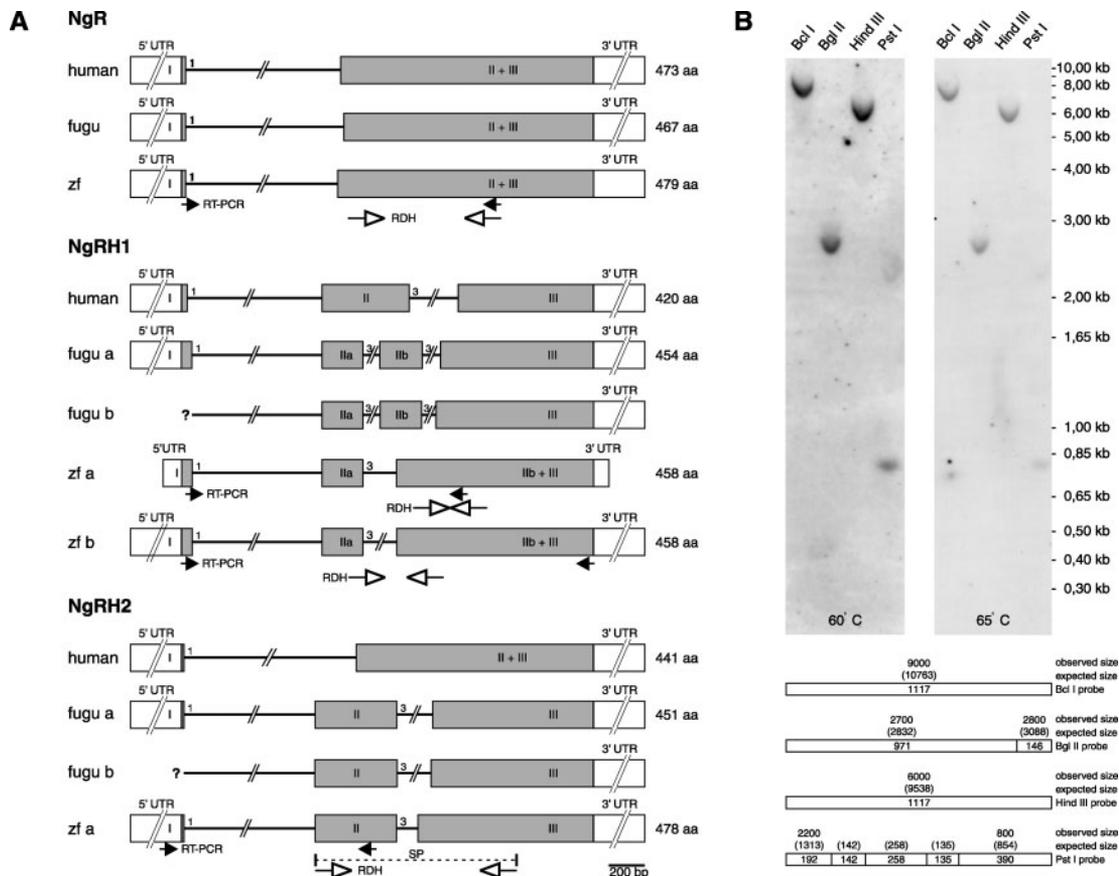


FIG. 3.—Analysis of NgR, NgRH1, and NgRH2 genes. (A) Exon-intron arrangements of NgR, NgRH1, and NgRH2 genes are schematically shown for human, fugu, and zebrafish (zf). Exons are drawn as Roman-numbered boxes, with the coding regions shaded in gray. Intron phases are specified with Arabic numbers. For fugu NgRH1b and fugu NgRH2b, the short N-terminal exons could not be identified within the genomic sequences (marked with ?). Localizations of primers for the analysis of the zebrafish genes/transcripts are indicated by closed (RT-PCR) and open (radiation hybrid mapping [RDH]) arrows, respectively. The range of the zfNgRH2a Southern probe (SP) is outlined with a dotted line. (B) Southern blot analysis of zfNgRH2a. Genomic DNA was digested with *Bcl I*, *Bgl II*, *Hind III*, and *Pst I*, respectively (top of each lane). The same DNA was used as PCR template for the generation of the NgRH2a-specific probe to avoid sequence polymorphisms. Blots were hybridized and washed at either 60°C (left) or 65°C (right). Molecular weights in kilobases (kb) are indicated on the right. The schematic drawing depicts the size of the DNA bands observed on the Southern blots, the sizes of DNA bands predicted from virtual digestion of the relevant genomic region (in brackets), and a restriction map of the Southern probe for each restriction enzyme (boxes).

of the curved  $\beta$ -sheet (data not shown). This specificity for one subclass and their predominant surface exposure probably renders these amino acids essential for the discrimination between different ligands for NgR, NgRH1, and NgRH2.

We compared evolutionary rates between the human NgR genes using a test developed by Tajima (Tajima 1993) as implemented in Mega (Kumar, Tamura, and Nei 1994). This rate test counts the number of unique substitutions in pairs of sequences relative to an outgroup (here *Drosophila* Slit). NgRH1 and NgRH2 are more similar to one another than either is to NgR (mean number of amino acid substitutions: NgR to NgRH1 = 114.9; NgR to NgRH2 = 111.8; and NgRH1 to NgRH2 = 105.2). The respective value between the whole NgR family and *Drosophila* Slit (159.9) is significantly higher. We also used this test to compare substitution rates between pairs of duplicated fish genes (fugu NgRH1a and fugu NgRH1b, zebrafish NgRH1a and zebrafish NgRH1b, and fugu NgRH2a and fugu NgRH2b) using the single-copy human ortholog as the

outgroup. In none of the comparisons did we discover a significant difference ( $P < 0.05$ ) in substitution rates between pairs of NgR genes relative to the outgroup. Of note was the large, but not significant, difference in unique substitutions between fugu NgRH1a (nine) and fugu NgRH1b (18) relative to the outgroup (human NgRH1).

Taken together, these results show that NgR homologs with similar structural motifs as human NgR exist in actinopterygians and that this gene family has arisen through several separate duplication events. The presence of three distinct subclasses was supported by phylogenetic analyses as well as by the identification of diagnostic residues.

#### Conserved Syntenies of Fish and Human NgR, NgRH1, and NgRH2

The zebrafish genes zfNgR, zfNgRH1a, zfNgRH1b, and zfNgRH2a were mapped on LG5, LG1, LG14, and LG15 (LOD scores of 12.0, 15.7, 20.5, and 17.2),

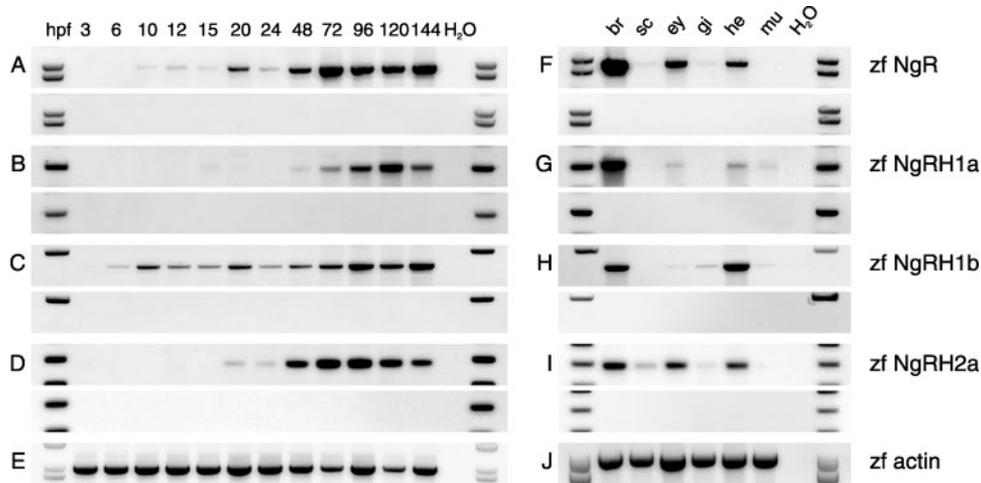


FIG. 4.—RT-PCR analysis of *zfNgR*, *zfNgRH1a*, *zfNgRH1b*, and *zfNgRH2a* mRNA expression. Expression of *zfNgR* and its homologs was examined during development (A–D, upper rows) and in various adult tissues (F–I, upper rows). A zero reverse transcriptase negative control (without SuperscriptII enzyme) was conducted with each primer pair to show that no bands are amplified because of genomic DNA contamination of the templates (lower row of A–D and F–I). RT-PCR with actin-specific primers was used as positive control to ensure that equal amounts of cDNA template were put into each reaction (E and J). hpf = h postfertilization; br = brain; sc = spinal cord; ey = eye; gi = gill; he = heart; mu = muscle; H<sub>2</sub>O = no template control.

respectively, and compared with the location of the human orthologs (fig. 2, supplementary fig. 5, and supplementary tables 2a–d). Conservation of synteny is found between zebrafish LG5 and human chromosome 22, establishing a new syntenic group containing *NgR*, the previously mapped gene *pes* (Woods et al. 2000), and four ESTs (fig. 2A). An additional, so far unrecognized synteny is formed by eight other zebrafish ESTs on LG5 and their orthologs on human chromosome 12. *ZfNgRH1a*, together with three neighboring ESTs on LG1, and *zfNgRH1b*, with four ESTs on LG14, both define new syntenies with the same region (11q12–11q13) of human chromosome 11 (fig. 2C and D), indicating that at least this part of the human genome underwent a fish-specific duplication. Mapping of *zfNgRH2a* and 14 additional ESTs extends the existing large syntenic region between LG15 in zebrafish and chromosome 17 in human (fig. 2B), including *crk* (ESTs fi19g05, fc54a04), DKFZP564A122 (EST fb92g11), and *lim1* (Woods et al. 2000).

The five *NgR*-related fugu genes were identified within the genomic sequences of scaffolds 67, 1244, 1581, 154, and 1111 (Ensembl release 11.2.1). Comparison of other predicted fugu genes in the vicinity of the *NgR*s with the chromosomal localization of the respective mammalian orthologs again revealed conserved syntenies (supplementary table 3). Orthologs of genes from scaffolds 1244 and 1581 (fugu *NgRH1a* and fugu *NgRH1b*, respectively) mapped to human chromosome 11, whereas genes of scaffolds 154 and 1111 (fugu *NgRH2a* and fugu *NgRH2b*, respectively) have orthologs on human chromosome 17, indicating a fish-specific duplication of these chromosomal segments.

These comparative mapping results support the phylogenetic analysis with the assignment of orthologous groups in the *NgR* receptor family and with fish-specific duplications of *NgRH1* and *NgRH2*.

#### Genomic Analyses of *NgR*, *NgRH1*, and *NgRH2*

Exon-intron structures and intron phasing of *NgR*, *NgRH1*, and *NgRH2* were examined for the respective human, fugu, and zebrafish genes by comparison of cDNA against genomic sequences (fig. 3A and supplementary table 4). *NgR* genes from human, fugu, and zebrafish comprise two coding exons (separated by a phase-1 intron), indicating that the true fish orthologs of human *NgR* have been identified. Human *NgRH2* also comprise two coding exons, whereas the orthologous fish genes each have an additional phase-3 intron at the same position, indicating an intron insertion in the gene of the common ancestor of zebrafish and fugu. The coding part of the first exon is identical in length for the homologous *NgRH2* (13 bp) and *NgR* (22 bp) genes. For *NgRH1*, the exon-intron structure is less conserved (fig. 3A). The human *NgRH1* gene consists of three coding exons. The respective fugu genes have an additional phase-3 intron dividing exon II. At exactly the same position, an intron is also located in the zebrafish *NgRH1a* and *NgRH1b* genes (supplementary table 4). However, the second phase-3 intron is missing in zebrafish. Compared with the orthologous human gene, the second exon in zebrafish *NgRH1a* and *NgRH1b* (IIa) is less than half in size, and the third zebrafish exon is consequently longer. Intron phasing is strictly conserved for all *NgR*-related genes, with a phase-1 intron followed by one or two consecutive phase-3 introns (fig. 3A and supplementary table 4).

To determine whether a second *NgRH2* gene exists in zebrafish (as in fugu), Southern blots were hybridized with a *zfNgRH2a*-specific probe. From virtual digestion of genomic sequences, a single band of 10.7 kb (*Bcl I*) and 9.5 kb (*Hind III*), respectively, or multiple bands (3.1 kb and 2.8 kb for *Bgl II* and 1.3 kb, 0.85 kb, 0.26 kb, 0.14 kb, and 0.13 kb for *Pst I*) were expected for a single-copy gene

(fig. 3B). As predicted, one (*Bcl I* and *Hind III* digest) or two bands (*Bgl II*) were detected on Southern blots under high-stringency (65°C) as well as low-stringency (60°C) conditions (fig. 3B). The 3.1-kb band of the *Bgl II* digest is weakly labeled because only a minor part of the probe (146 bp) overlaps with it. For *Pst I*, two bands (0.85 kb and a weakly ~2.2 kb) were observed. The other predicted bands (0.13 kb, 0.14 kb, and 0.26 kb) were too small for detection. Differences between predicted and observed band sizes in all four digests can be explained by restriction-length polymorphisms of the blotted genomic DNA compared with the database sequences used for fragment prediction. Because no additional bands appeared in the Southern blot hybridization, we found no indication for the presence of a second NgRH2 gene (*zfNgRH2b*) in zebrafish.

#### RT-PCR Analyses of *zfNgR*, *zfNgRH1a*, and *zfNgRH2a* mRNA Expression

Transcription of NgR, NgRH1a, NgRH1b, and NgRH2a during zebrafish development and in different adult tissues was analysed by RT-PCR (fig. 4A–D and F–I; upper rows). In zebrafish embryos, NgR mRNA expression started at about 10 h postfertilization (hpf) and increased thereafter (fig. 4A). The absence of an NgR PCR product at 3 hpf and 6 hpf is not the result of degraded RNA or bad template cDNA, because a strong signal was detectable in the actin-positive control in these early stages (fig. 4E). Considerable NgR mRNA levels were only detected after 20 hpf, paralleling the development of the nervous system. Onset of transcription of the homologous receptors NgRH1a and NgRH2a was time-delayed with respect to NgR. Low mRNA levels were revealed for NgRH2a at 20 hpf (fig. 4D) and even later for NgRH1a at 48 hpf (fig. 4B), whereas significant expression was detectable only at 48 hpf (NgRH2a) and 96 hpf (NgRH1a). NgRH1b had the broadest expression pattern of the zebrafish NgR paralogs. Transcription was observed at all stages after 3 hpf, with higher mRNA levels at older stages of development (fig. 4C).

In adult zebrafish, expression of NgR was predominantly observed in brain but also in eye and heart (fig. 4F). Faint bands indicating very low expression levels were visible in spinal cord and gill. NgRH2a transcripts were detected in a comparable pattern, except for a lower level in brain (fig. 4I). Neither NgR nor NgRH2a were found in muscle. NgRH1a was almost exclusively expressed in brain, with marginal levels detectable in eye, heart, and muscle (fig. 4G). Expression of NgRH1b mRNA was highest in brain and heart, and low levels were observed in eye, gill, and muscle (fig. 4H).

These results show that the expression of zebrafish NgR family members is developmentally regulated and occurs most prominently in brain.

#### Discussion

Here, we report the discovery of NgR in zebrafish and fugu. We also uncovered fish genes that are orthologs of two recently described human NgR-related genes, NgRH1 and NgRH2 (Barton et al. 2003; Pignot et al. 2003). The

fugu genes were recently identified independently by another group (He et al. 2003). Our phylogenetic analyses show that duplication events before the divergence of tetrapods (sarcopterygians) and ray-finned fish (actinopterygians) and in actinopterygia before the divergence of the zebrafish and fugu ancestors have led to the current diversity of NgR in vertebrates. NgR is considered to be an important receptor for the restrictive effects of CNS myelin on axon growth in the adult mammalian CNS because it interacts with several myelin inhibitors. It binds with high affinity to an extracellular segment of Nogo (Nogo-66) as well as to MAG and OMgp (Fournier, GrandPré, and Strittmatter 2001; Domeniconi et al. 2002; Liu et al. 2002; Wang et al. 2002b), suggesting a convergence of signaling pathways. The identity of the NgRH1 and NgRH2 ligands is yet unknown, because they do not bind to either Nogo-66, MAG, or OMgp (Barton et al. 2003; Pignot et al. 2003).

Although fish axons are capable of regeneration, they are nevertheless repelled by mammalian CNS myelin and oligodendrocytes (Bastmeyer et al. 1991). In the case that Nogo-66, MAG and/or OMgp, which are expressed in mammalian oligodendrocytes, induce this repulsion, an interaction with an akin NgR receptor might be involved in the mediation of the cross-species inhibitory effect. Moreover, the question arises as to what functions homologous NgRs and ligands might have in fish. With the identification of fish NgR genes, these issues can now be addressed. Four members of the zebrafish NgR receptor family and five NgR-related fugu genes were discovered. Syntenic chromosomal positions and phylogenetic relationships provide convincing evidence that we have identified the true zebrafish and fugu orthologs of human NgR, NgRH1, and NgRH2, respectively. Analysis of the genomic structures and the determination of diagnostic amino acid residues also confirmed the assignment of orthologous groups within the NgR receptor family. Their phylogeny demonstrates clearly that the duplication events that produced three clades of vertebrate NgR genes occurred before the divergence of ray-finned fishes (actinopterygians) and tetrapods (sarcopterygians). However, no invertebrate or ascidian NgR gene could be identified.

The duplication of fugu NgRH2 and of fugu and zebrafish NgRH1 and the tree topology shown in figure 1 are consistent with the predictions of the ancient fish-specific genome duplication hypothesis (Amores et al. 1998). In this case, the zebrafish NgRH2b gene has yet to be discovered, or a specific loss has to be assumed.

Fish NgR proteins show the same overall structure as human NgR, and the LRRNT, LRR and LRRCT domains are strikingly similar (>60%) at the amino acid-sequence level (supplementary figs. 3 and 4). In mammals, this region has been shown to be responsible for ligand binding (Fournier, GrandPré, and Strittmatter 2001; Domeniconi et al. 2002; Wang et al. 2002b). Its three-dimensional crystal structure has a curved topology of prominently repeating  $\beta$ -strands (Barton et al. 2003; He et al. 2003). He et al. (2003) proposed that a functional, ligand-binding site of NgR is located on the concave face of this  $\beta$ -sheet because it contains predominantly amino acid residues conserved between all three NgR subclasses. However, our analyses,

which included more NgR-related genes and *Drosophila* Slit as a reference for an ancestral LRR-containing protein revealed that most of these residues are also conserved in Slit and are most probably structure determining (supplementary fig. 2). Residues responsible for defining ligand specificity are expected to be conserved in only one of the three NgR subclasses. Because of the inclusion of fish sequences, we were able to substantiate the analysis of Barton et al. (2003) and to identify distinct residues that are diagnostic for each of the NgR subclasses in fish and mammals. Most of these amino acids are surface-exposed, polar, or charged residues (supplementary fig. 2) and might well be involved in specific receptor-ligand interactions. These predictions have to be confirmed by future biochemical or mutagenic data.

The high sequence conservation of the LRRNT/LRR/LRRCT domain between fish and mammals combined with the presence of diagnostic amino acids suggests that zebrafish NgR may be able to bind to and mediate the repellent signal of these mammalian CNS myelin inhibitors in cross-species *in vitro* assays. Future studies will have to show whether molecular interactions between fish NgR and mammalian Nogo-66, MAG, or OMgp are indeed possible. Another attractive hypothesis would be a function of the NgR family members in axonal growth and guidance, particularly because they exhibit a striking sequence similarity to the Slit proteins that have already been shown to confer a similar task (Brose et al. 1999).

An important unanswered question is the identity of the natural ligand(s) of zebrafish NgR and its function. Fish oligodendrocytes probably lack growth-inhibiting molecules because they support axonal elongation *in vitro* (Bastmeyer et al. 1991; Stuermer et al. 1992). At present, neither MAG nor OMgp have been identified in fish. Although an orthologous Nogo-66 is present in actinopterygians (Klinger, unpublished data; Oertle et al. 2003), it is not clear whether it is expressed in fish myelin, displays binding properties to NgR, and mediates any inhibitory activity. Additional ligands conferring a yet unidentified function that is not related to axonal growth inhibition are expected. It is now possible to determine whether zebrafish NgR or additional receptors recognizing other myelin inhibitors (such as the Nogo-A-specific region or semaphorins) mediate growth cone collapse upon contact with mammalian CNS myelin *in vitro*. In addition, it has to be clarified whether zebrafish also possess a homologous p75<sup>NTR</sup> receptor. In mammals, p75<sup>NTR</sup> has been described as a coreceptor of the GPI-linked NgR receptor that transduces the inhibitory activity of myelin-associated inhibitors to the neuron (Wang et al. 2002a). Two zebrafish EST clones with adequate sequence similarity have been identified (Oertle, unpublished data), but future studies must elucidate the potential involvement of p75<sup>NTR</sup> homologous protein(s) in the signaling pathway of fish NgR.

The mRNA distribution for the zebrafish NgRs is consistent with a function in the development of CNS structures and in the regulation of axon growth and plasticity. The expression of NgR parallels the development of the nervous system, whereas NgRH1a and

NgRH2a are detectable in embryos with a more mature CNS (fig. 4). Future immunostaining experiments will show whether the NgR protein is actually present on growing axons, as would be expected. In the adult zebrafish, the expression pattern of NgR corresponds to that in mouse, where it is also predominantly detected in brain (Fournier, GrandPré, and Strittmatter 2001). Northern analysis revealed low mRNA levels also in heart and kidney but not in other peripheral tissues. In zebrafish, we also observed NgR expression in heart. In addition, mRNA transcripts were detected in the eye, spinal cord, and gill. As in human and rat (Pignot et al. 2003), expression of the homologs zfNgRH1a and zfNgRH2a is predominantly observed in brain, although the NgRH2a mRNA level is lower than for NgR. Low transcription of NgRH1 in muscle was observed in zebrafish as well as in rat (this study; Pignot et al. 2003). In the spinal cord of both organisms, only low levels are detected for all three transcripts. This might result from a downregulation of the respective genes, as it was recently shown for NgR (Josephson et al. 2002, 2003). Therefore, members of the zebrafish NgR family are expressed in a pattern similar to that in mammals and probably convey evolutionarily conserved functions.

Several mammalian myelin proteins have been shown to exert axonal growth inhibition via a common receptor complex composing NgR. Because fish are capable of CNS axon regeneration but nevertheless express NgR homologs, additional, yet unidentified, ligands and new functions of these receptors are expected. With the present identification of homologous NgR genes, the zebrafish is a potent model organism for *in vivo* analyses of these putative functions.

### Supplementary Material

The following figures and tables are available as Supplementary Material online. Supplementary figure 1: Unambiguous alignment of 17 NgR-related sequences; supplementary figure 2: Vertebrate NgR proteins aligned with *Drosophila* Slit; supplementary figure 3: Amino acid sequence alignment of zebrafish NgRs with human NgR; supplementary figure 4: Amino acid sequence alignment of fugu NgRs with the human NgR; supplementary figure 5: Colored version of figure 2; supplementary table 1: Overview of primers and their sequences; supplementary table 2: Mapping data for the analysis of conserved synteny between NgRs in zebrafish and human; supplementary table 3: Mapping data for the analysis of conserved synteny between NgRs in fugu and mammals; and supplementary table 4: Exon and intron sizes of NgR, NgRH1, and NgRH2.

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