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Cloning and Characterization of zRICH, a 2′,3′-Cyclic-Nucleotide 3′-Phosphodiesterase Induced During Zebrafish Optic Nerve Regeneration

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Abstract: We previously reported cloning of cDNAs encoding both components of a protein doublet induced during goldfish optic nerve regeneration. The predicted protein sequences showed significant homology with the mammalian 2′,3′-cyclic-nucleotide 3′-phosphodiesterases (CNPases). CNPases are well-established markers of mammalian myelin; hence, the cDNAs were designated gRICH68 and gRICH70 (for goldfish Regeneration-Induced CNPase Homologues of 68 and 70 kDa). Homologous cDNAs have now been isolated from zebrafish encoding a highly related protein, which we have termed zRICH. RNase protection assays show that zRICH mRNA is induced significantly (fivefold) in optic nerve regenerating zebrafish retinas 7 days following nerve crush. Western blots show a single band in zebrafish brain and retina extracts, with immunoreactivity increasing threefold in regenerating retinas 21 days postcrush. Immunohistochemical analysis indicated that this increase in zRICH protein expression is localized to the retinal ganglion cell layer in regenerating retina. We have characterized and evaluated the relevance of a conserved β-ketoacyl synthase motif in zRICH to CNPase activity by means of site-directed mutagenesis. Two residues within the motif, H334 and T336, are critical for enzymatic activity. A cysteine residue within the motif, which corresponds to a critical residue for β-ketoacyl synthase, does not appear to participate in the phosphodiesterase activity. **Key Words:** 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase—Retinal ganglion cell—Teleost.
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The CNS of warm-blooded vertebrates does not support nerve regeneration, in sharp contrast with the regenerative potential of their PNS. This difference has obvious clinical implications, and the study of the biochemistry involved in axonal regrowth is thus of considerable biomedical interest (Bahr and Bonhoeffer, 1994). In contrast, the CNS of cold-blooded vertebrates possesses marked regenerative potential. The teleost and amphibian optic nerves have been used extensively as examples of successful regeneration in the vertebrate CNS (Agranoff et al., 1976; Agranoff and Ford-Holevinski, 1984; Grafstein, 1991). Following injury, the axons of the retinal ganglion cells regenerate and reconnect with their targets in the tectum, maintaining topological specificity (Sperry, 1948, 1963; Attardi and Sperry, 1963). Biochemical studies with these systems have led to the identification of several proteins that may play a role in axonal regeneration (Heacock and Agranoff, 1982; Benowitz and Lewis, 1983; Skene, 1989; Grafstein, 1991; Glasgow et al., 1992; Paschke et al., 1992; Herdegen et al., 1993).

A doublet of acidic proteins on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) shown to be induced in regenerating retinal ganglion cells of goldfish was designated p68/70 to reflect the apparent molecular weights of the components (Heacock and Agranoff, 1982). The protein doublet was purified from brain tissues and was shown to represent two related proteins, able to form heterodimers and homodimers and at least partially associated with the plasma membrane (Leski and Agranoff, 1994). Extensive use of a polyclonal antibody against the purified protein (Wilmot et al., 1993) confirmed the induction of p68/70 in retinal ganglion cells following optic nerve crush. The doublet was found in other tissues as well (Wilmot et al., 1993). A partial peptide sequence was used to clone cDNAs encoding a p68/70-related protein (Ballestero et al., 1995). Sequence analysis showed significant homology to 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase), a marker enzyme of mammalian myelin (Sprinkle, 1989). Subsequently, the encoded pro-

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Abbreviations used: CNPase, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase; βKAS, β-ketoacyl-acyl carrier protein synthase; βKAT, β-ketoacyl-CoA thiolase; mCNP1, mouse 2′,3′-cyclic-nucleotide 3′-phosphodiesterase I protein; ORF, open reading frame; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WT, wild-type.

tein was designated gRICH (for goldfish Regeneration-Induced CNPase Homologue). The corresponding mRNA is expressed in several tissues and is strongly induced in goldfish retinas during optic nerve regeneration (Ballestero et al., 1995). Recently, a novel cDNA encoding a protein highly homologous to gRICH was also cloned (Ballestero et al., 1997). The corresponding mRNA was similarly induced in retinas of regenerating optic nerves. Recombinant proteins were expressed in both prokaryotic and eukaryotic systems and found to possess CNPase activity. The gRICH proteins were identified as novel nonmammalian members of the CNPase family, implicating these enzymes for the first time in nerve regeneration (Ballestero et al., 1997). A highly specific polyclonal antibody was generated against recombinant gRICH peptide and was used to confirm the identity of the two cloned goldfish proteins with the components of the p68/70 doublet. The proteins were correspondingly renamed gRICH68 and gRICH70. The antibody was used in immunodepletion experiments to suggest that these gRICH proteins are responsible for almost all of the CNPase activity detected in goldfish retinas (Ballestero et al., 1997).

The present study characterizes an analogous protein induced during optic nerve regeneration in the zebrafish, a species that is emerging as a model system for classic and molecular genetic studies on neural development (Streisinger et al., 1981; Eisen, 1996; Gaiano and Hopkins, 1996). We have previously observed a single gRICH-like protein in the zebrafish nervous system (Wilmot et al., 1993), and experimental evidence for successful optic nerve regeneration in this species has been reported (Bernhardt et al., 1996). The regenerating zebrafish optic nerve thus presented the opportunity to learn whether CNPase activity is induced during optic nerve regeneration in another species and also to learn which features of the peptide sequence were most highly conserved.

MATERIALS AND METHODS

Animals

Zebrafish (*Danio rerio*) 1–3 cm in body length were obtained from local aquariums and maintained in aerated tanks at 25–28°C. Intraorbital optic nerve crushes were performed with the zebrafish under tricaine anesthesia as described previously for the goldfish (Springer and Agranoff, 1977). All the experiments were performed in accordance with the guidelines of the National Institutes of Health.

Library screenings

A PCR-generated *Bgl*II fragment containing the open reading frame (ORF) of gRICH68 (Ballestero et al., 1997) was used to prepare a radiolabeled probe (5×10^9 cpm/μg) with [α-^{32}P]dATP (ICN) by standard random priming protocols (Sambrook et al., 1989). A zebrafish 16–20-h embryo λgt10 cDNA library (kindly provided by Dr. John Kuwada) was screened ($\sim 10^6$ clones) with the probe. Hybridization and washing procedures were performed as previously described (Ballestero et al., 1997). A single clone, zRICH-C1, was identified and isolated by two further rounds of plate purification. The clone insert was subcloned into pGEM3Zf(+) (Promega) and analyzed by restriction digestion, and fragments of it were subcloned into pSP73 (Promega) and pBluescript-KS (Stratagene) and sequenced by the dideoxynucleotides method using a Sequenase T7 DNA polymerase kit (USB-Amersham). A *Pst*I–*Sst*I fragment ($\sim$0.6 kb) from the zRICH-C1 cDNA was used to generate a radiolabeled probe as described above. The probe was used to screen ($\sim 1.75 \times 10^6$ clones) a zebrafish 24-h embryo λ-ZAP cDNA library (Stratagene), and at least 40 additional positive clones were identified. Four such clones, zRICH-C3, zRICH-C5, zRICH-C6, and zRICH-C7, were isolated by two further rounds of plate purification and excised as plasmids (Stratagene protocols), and their inserts were preliminarily characterized by end-sequencing and restriction analysis. Clone zRICH-C3 was further sequenced by the use of specific oligonucleotides designed to the effect (oligonucleotides synthesized at the University of Michigan DNA core facility). Sequence analysis was performed with the University of Wisconsin-GCG and DNASTAR software packages.

RNase protection assays

The procedures used for the RNase protection assays and the total RNA isolation have been described previously (Ballestero et al., 1995). Total RNA was purified from 70 control retinas (left eye) and from 70 nerve-regenerating retinas (right eye) 7 days after the right optic nerve was crushed. An *Sst*I–*Bam*HI ($\sim$0.4-kb) fragment from the zRICH-C1 clone was selected as template and subcloned into pSP73 (Promega), generating plasmid pSP-zRICH1-SB0.4, which was digested with *Eco*RI, and a 481-base-long riboprobe (10^9 cpm/μg) was synthesized by run-off transcription using T7 RNA polymerase (GibcoBRL) by standard protocols (Sambrook et al., 1989). Four micrograms of the retinal RNA was used in the sample tubes. For the control, the tube contained 4 μg of yeast tRNA. The RNAs were hybridized to 4×10^5 cpm of antisense riboprobe. After the RNase protection assay procedure, the protected fragments (427 bases) were separated in a 6% polyacrylamide sequencing gel. Radiolabeled DNA markers and 2×10^3 cpm of the antisense riboprobe were electrophoresed in the gel as references. The gel was exposed to a PhosphorImager screen (Molecular Dynamics) and quantitated using the manufacturer's software.

Protein extract preparation and western blot analysis

Protein extracts from goldfish brain, zebrafish brain, and zebrafish retinas (nerve-regenerating and control, 21 days post-crush) were prepared by a protocol similar to that described for goldfish retinas (Ballestero et al., 1997). Twenty zebrafish retinas were used in each sample. Protein concentrations were determined (Bio-Rad reagent) on a Beckman Biomek-1000 automated workstation. One hundred micrograms of total protein from the extracts was separated by 10% SDS-PAGE (Bio-Rad miniapparatus), transferred to nitrocellulose, and immunoblotted with anti-gRICH antiserum (Ballestero et al., 1997). The western blot procedure has been described in detail elsewhere (Ballestero et al., 1997). The blots were photographed with a Kodak DC40 digital camera for quantitation with Kodak 1D image analysis software. The intensities of the bands were normalized to positive controls (50 ng of purified H7-gRICH68).

CNPase tests with protein extracts

All CNPase assays were performed by the alkaline phosphatase method as previously described (Ballestero et al., 1997) using 4 m*M* 2′,3′-cyclic AMP as substrate. Four micrograms of extracted protein was used as the enzyme source. Assays were performed in triplicate. Control values were obtained from assays with all of the components except the extract, and these were subtracted from the values obtained with the extracts. Immunodepletion analyses of the extracts were performed essentially as previously described (Ballestero et al., 1997). Twenty-five microliters of a 2.5 μg/μl dilution of the extracts was used. In all cases, control immunodepletions were performed with the preimmune serum obtained from the same rabbit. All the immunodepletion procedures were performed in triplicate. The specific activities determined were then normalized to the results obtained with the preimmune controls for the same extract.

Immunohistochemical analysis of retinal sections

Whole eyes were removed 7 days postcrush and fixed in 4% paraformaldehyde for 1 h at room temperature. The eyes were rinsed in 0.1 *M* sodium phosphate (pH 7.0). Eyes were cryoprotected sequentially in 5, 10, 15, and 20% sucrose followed by 30 min in 20% sucrose/OCT (2:1 vol/vol) and then frozen in liquid nitrogen that had been cooled in isopentane. Sectioning was performed at −20°C, and 5-μm sections were mounted on polylysine-coated slides.

Sections were allowed to dry and warm to room temperature before blocking for 1 h at room temperature with 20% goat serum in diluting buffer (0.1 *M* sodium phosphate, 0.15 *M* NaCl, 0.1% sodium azide, and 0.5% Triton X-100). The gRICH polyclonal antiserum was diluted 1:500 with 1% goat serum in diluting buffer. Sections were washed for 30 min with diluting buffer and then incubated with fluorescein isothiocyanate-labeled goat anti-rabbit antibody (Sigma) diluted 1:1,000 in dilution buffer. The sections were washed before mounting in 60% glycerol, 0.1 *M* sodium carbonate (pH 9.0), and 0.4 mg/ml phenylenediamine.

Bacterial expression constructs for zRICH wild-type (WT) and mutant proteins

PCR was used to generate a *Bgl*II-flanked DNA fragment containing the full ORF of zRICH by a strategy similar to that used for gRICH proteins (Ballestero et al., 1997). The fragment was subcloned into the *Bam*HI site of the vector pKKR2 (Ballestero et al., 1997), generating the vector pKKR2-zRICH-WT. For the generation of the site-directed mutants, the pKKR2-zRICH-WT vector was used as template, and standard PCR mutagenesis protocols were used (Innis et al., 1990) with specific oligonucleotides designed to the effect. An *Sst*I–*Kpn*I (~0.3-kb) fragment of the PCR products was substituted for the original one in pKKR2-zRICH-WT, generating the bacterial expression plasmids for each mutant.

Rapid screening of mutant RICH constructs for enzyme activity

Escherichia coli (XLI-Blue strain) bacteria transformed with the bacterial expression plasmids were grown in a miniculture (3 ml) overnight. Two hundred microliters of the culture was centrifuged in a microfuge at top speed for 1 min, the supernatant was removed, and the pellet was resuspended by brief vortex-mixing in 100 μl of STE-0.1% T buffer [10 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 1 m*M* EDTA, and 0.1% Triton X-100]. The suspension was centrifuged as above, and the supernatant was tested for CNPase activity by the alkaline phosphatase method. Two microliters of each supernatant was added as the source of enzyme in a 100-μl reaction containing 1 m*M* 2′,3′-cyclic AMP and incubated for 10 min at 22°C. Control reactions were performed with the WT bacteria and with bacteria harboring the plasmid without any insert. The presence of blue product generated by the alkaline phosphatase method was assessed visually.

Protein purification and determination of kinetic constants

The zRICH-WT and the R332A, H334A, and C339A mutants were expressed in bacteria as heptahistidine-tagged proteins and were purified essentially as previously described (Ballestero et al., 1997). All CNPase assays were performed as above, and minus enzyme control values were subtracted. Enzyme specific activities were determined with 4 m*M* 2′,3′-cyclic AMP in 20-min assays at 30°C. Five nanograms of H7-zRICH-WT and H7-zRICH-C339A, 100 ng of H7-zRICH-R332A, and 6 μg of H7-zRICH-H334A purified proteins were used in the assays, which were performed in triplicate. For the determination of the catalytic constants, the assays were performed essentially as described for the goldfish proteins (Ballestero et al., 1997). Five nanograms of H7-zRICH-WT and H7-zRICH-C339A and 100 ng of H7-zRICH-R332A purified proteins were used. The assays were performed in triplicate, and results were used to generate Lineweaver–Burk plots with Sigmaplot software (Jandel). All enzyme activity determinations were made between 5 and 20% substrate consumption and were linear with respect to enzyme concentration and time of incubation.

RESULTS

Cloning of zRICH cDNAs

A radiolabeled probe generated from the ORF of gRICH68 was used to screen a zebrafish embryo cDNA library. One positive clone, designated zRICH-C1, was isolated. The cDNA insert was subcloned and analyzed by sequencing. The results indicated that it contained a partial ORF highly related to that of the gRICH68 and gRICH70. A fragment from the zRICH-C1 cDNA was used to screen a second zebrafish embryo cDNA library. Four clones were isolated and characterized by restriction analysis and end-sequencing. All the clones contained an ~3-kb insert. The results suggested that the clones were unique but represented the same mRNA transcript. The clone zRICH-C3 was selected for further characterization. A full ORF could be completed from combined sequencing of clones zRICH-C1 and zRICH-C3. The predicted ORF encoded a 424-amino acid protein, designated as zRICH (Fig. 1).

zRICH is highly homologous to gRICH68 and gRICH70

Alignment of the zRICH protein sequence with those of gRICH68, gRICH70, and mouse CNPase I protein (mCNP1) showed high homology (Fig. 2) to gRICH68 and gRICH70 along their entire sequence (75 and 78% amino acid identity, respectively; 30% with mCNP1). The N-terminal third showed the highest divergence among RICH proteins, and the alignment presented several gaps. This region does not show significant homol-

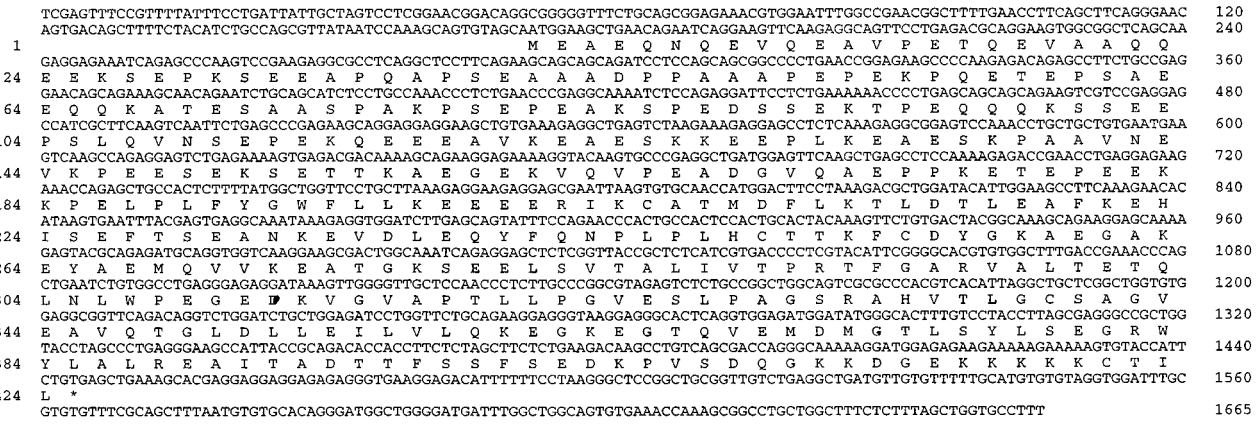

FIG. 1. cDNA and protein sequence of zRICH. The zebrafish cDNA clone zRICH-C1 was purified by means of probes derived from the gRICH68 cDNA and low-stringency hybridization conditions. A second screening with probes derived from zRICH-C1 resulted in the isolation of the zRICH-C3 cDNA clone. The partial sequence shown is a composite derived from the zRICH-C1 and zRICH-C3 cDNA clones. The complete ORF codes for a 424-amino acid protein as indicated by the single letter abbreviation code.

ogy with mCNP1. The putative catalytic domain (approximately the C-terminal two-thirds; from amino acid 187 in zRICH) shows high conservation with both goldfish RICH proteins (~88% amino acid identity) and mCNP1 (46% amino acid identity), including the presence of a prenylation CaaX box at the C terminus (Clarke, 1992). This sequence has been shown to be required for prenylation in CNPases and for membrane attachment (Braun et al., 1991; De Angelis and Braun, 1994).

Induction of zRICH mRNA and protein during optic nerve regeneration in zebrafish

Levels of zRICH mRNA and protein during regeneration were established by RNase protection and western blot analysis, respectively. For basic characterization, single time points were designated based on the time courses previously determined for the goldfish (Ballestero et al., 1995, 1997). RNase protection assays (Fig. 3A) with zebrafish control and nerve-regenerating retinas 7 days postcrush showed significant induction (5.2-fold) of the zRICH mRNA (Fig. 3B). The magnitude of the induction was very close to that observed for the gRICH mRNAs at similar time points during goldfish optic nerve regeneration (Ballestero et al., 1995, 1997). Anti-gRICH antibody was used to follow zRICH protein levels by immunoblot (Fig. 3C). Immunoreactivity with a 49-kDa protein was observed in the zebrafish tissues. Control and nerve-regenerating retinas 21 days postcrush

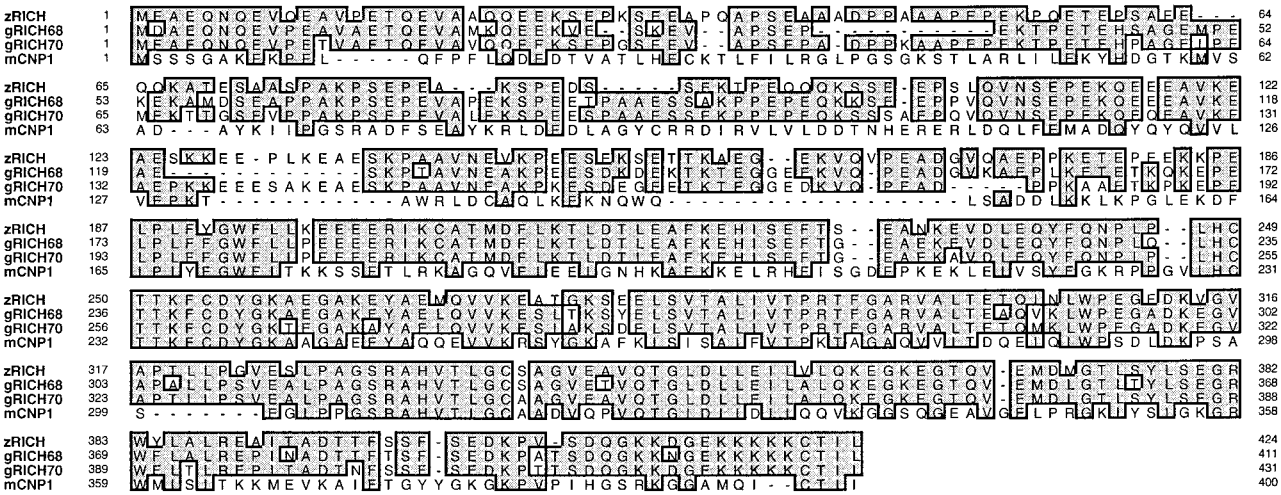

FIG. 2. Alignment of zRICH gRICH68, gRICH70, and mCNP1. The sequences of the zRICH protein (top) was aligned with those of gRICH68, gRICH70, and mCNP1 by the Clustal method. Note the high degree of amino acid identity with both gRICH68 and gRICH70. The highest divergence is localized to the N-terminal third of the molecule, the region of lowest homology with mammalian CNPases. The putative catalytic domain (C-terminal two-thirds) is highly conserved among all of the RICH proteins and shows high homology to mCNP1, including an isoprenylation motif at the C terminus.

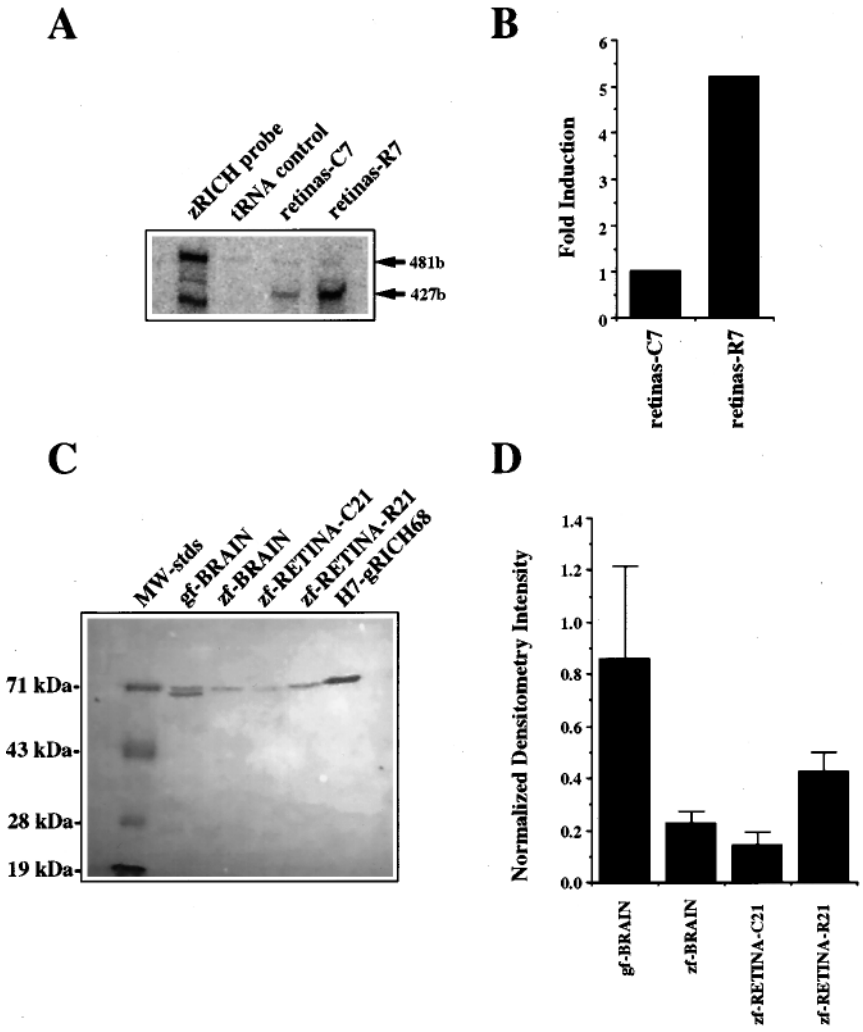

FIG. 3. zRICH mRNA and protein are induced in zebrafish retina during optic nerve regeneration. **A:** zRICH mRNA analysis by RNase protection assay. Four micrograms of zebrafish retinal RNAs (control and nerve-regenerating, 7 days postcrush) was used. A negative control (4 μg of yeast tRNA) was also included in the assay. The positions of the full-length riboprobe and the protected 427-base fragment are indicated by arrows. Notice the induction of zRICH mRNA levels in the regenerating retinas (retinas-R7 lane) compared with the control retinas (retinas-C7 lane). **B:** PhosphorImager quantitation of zRICH mRNA induction. The regenerating retinas showed 5.2-fold higher zRICH mRNA levels than the control retinas. This induction is very similar to that previously observed for both gRICH68 and gRICH70. **C:** Western blot analysis of zRICH expression in zebrafish brain and retinas. Extracts from goldfish brain (gf-BRAIN), zebrafish brain (zf-BRAIN), and zebrafish control and regenerating retinas 21 days postcrush (zf-RETINA-C21 and zf-RETINA-R21, respectively) were analyzed (100 μg of protein) by immunoblotting with anti-gRICH antibody. The gRICH68 and gRICH70 proteins are clearly identified in the gf-BRAIN lane. Cross-reactivity with a 69-kDa protein in zebrafish tissues was detected, and the zRICH band is seen to be induced in the regenerating retina lane. Fifty nanograms of purified H7-gRICH68 served as a positive control. MW-stds, molecular weight standards. The figure is representative of two independent western blots. **D:** Densitometric quantitation of relative zRICH protein levels during regeneration. The intensities of the digitized bands were normalized to the positive control lane and averaged. zRICH is induced 3.0-fold in the regenerating retinas 21 days postcrush, in accordance with previous observations for the gRICH proteins.

showed a threefold induction in zRICH levels in the regenerating retinas (Fig. 3D). The magnitude of the induction is similar to that shown for gRICH proteins at the corresponding time during regeneration (Wilmot et al., 1993; Ballestero et al., 1997).

zRICH protein is induced specifically in retinal ganglion cells of regenerating retina

To define the cellular sites of induction, sections were made of control and regenerating zebrafish retina 7 days following optic nerve crush. As demonstrated in Fig. 4, the retinal ganglion cells are stained intensely with the anti-gRICH68 antibody in the regenerating retina but not in control retina. These findings are consistent with previous studies in the goldfish (Wilmot et al., 1993).

zRICH is the major CNPase in zebrafish nervous tissues

Assays with protein extracts and immunodepletion demonstrated that gRICH proteins were the major source of CNPase activity in goldfish retina (Ballestero et al., 1997). Similar approaches were used to learn whether this was also true for zRICH. Activity assays with zebrafish retinal protein extracts showed induction of activity in the nerve-regenerating retinas versus the controls (Fig. 5A). The magnitude of the induction in activity (3.7-fold) was similar to the induction seen in protein levels by immunoblot (Fig. 3D). Immunodepletion studies of the extracts with anti-gRICH antibody confirmed that the CNPase activity in the regenerating zebrafish retinas was due primarily to zRICH (Fig. 5B). This observation was extended to complete brain extracts from both zebrafish and goldfish (Fig. 5B), suggesting that if a closer homologue of mammalian CNPases exists in teleosts, it is not abundant.

A highly conserved motif between RICH and CNPase proteins shows homology to the β-ketoacyl synthase and thiolase active site motifs

The sequence homology within the C-terminal two-thirds of RICH proteins and mammalian CNPases (Fig.

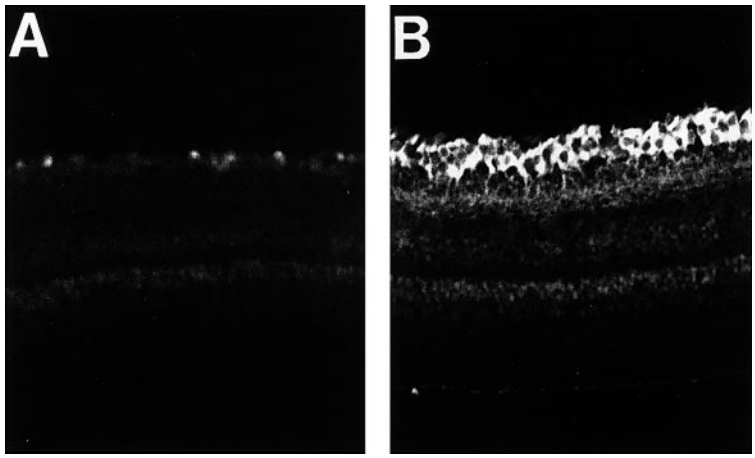

FIG. 4. Immunohistochemical analysis demonstrates induction of zRICH in retinal ganglion cells. Five-micrometer sections of (**A**) control and (**B**) postcrush zebrafish retina were incubated with a polyclonal antiserum raised against the gRICH68 protein followed by incubation with a fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody. Control sections incubated in the absence of gRICH68 antibody and exposed for the same time showed no specific staining.

2) and the conservation of the CNPase activity suggest that regions highly conserved between the teleost and mammalian enzymes may play a role in the observed activity. Also, a very highly conserved motif between mammalian CNPases and RICH proteins matches the consensus sequence of the β-ketoacyl-acyl carrier protein synthase (βKAS) active site motif {G-x(4)-[LIVMFAP]-x(2)-[AGC]-C-[STA](2)-[STAG]-x(3)-[LIVMF]}. A highly related sequence is also present in the β-ketoacyl-CoA thiolase (βKAT) active site {[LIVM]-[NST]-x(2)-C-[SAGLI]-[ST]-[SAG]-[LIVMFYNS]-x-[STAG]-[LIVM]-x(6)-[LIVM]}. The indicated motifs are critical for the enzymatic activity of these proteins related to fatty acid metabolism (Kasama-Yoshida et al., 1997). An alignment of the RICH/CNPase conserved motif and the βKAS/βKAT active site motifs is presented in Fig. 6. The absolute amino acid conservation within these motifs is not very high (boxed in Fig. 6); however, most of the requirements to match the consensus sites are present in both RICH proteins and CNPases. It is noteworthy that a critical cysteine absolutely required in the βKAS and βKAT motifs is also conserved in RICH proteins and CNPases. As shown in the alignment, the motif has been highly conserved during evolution of these enzymes.

Mutagenesis analysis of the β-ketoacyl synthase homologous motif in zRICH

The conservation of the βKAS domain between RICH proteins and mammalian CNPases suggested that it might participate in CNPase activity (hereby designated as RCD1 for RICH and CNPase Domain 1). The cysteine critical for βKAS and βKAT catalysis has been shown to act as a nucleophile that shows high reactivity toward cysteine-modifying reagents and forms a covalent intermediate in each of the respective reactions (Stoops et al., 1983; Davis et al., 1987; Thompson et al., 1989). To test the hypothesis that some of the amino acids of the RCD1 domain might be important for CNPase activity in zRICH, a site-directed mutagenesis approach was used. Five residues within the RCD1 of zRICH were selected

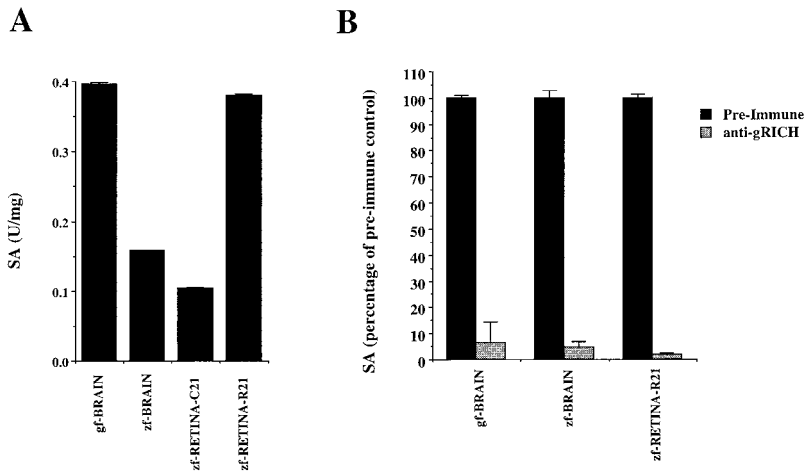

FIG. 5. zRICH is the major CNPase in zebrafish retina and brain. **A:** CNPase activity in zebrafish brain and retina extracts. Activity assays were performed by an alkaline phosphatase-coupled method with 4 m*M* 2′,3′-cyclic AMP as substrate. The regenerating retina extract 21 days postcrush (zf-RETINA-R21) showed 3.7-fold higher CNPase activity than did the control retina extract (zf-RETINA-C21), indicating the induction of the zRICH protein (gf-BRAIN, goldfish brain; zf-BRAIN, zebrafish brain). Data are average ± SD (bars) values from triplicate determinations. **B:** Immunodepletion analysis of brain and retina extracts. The gf-BRAIN, zf-BRAIN, and zebrafish regenerating retina protein extracts were immunodepleted of RICH proteins with the anti-gRICH antibody, and CNPase activity was measured in the supernatants. Results were normalized to immunodepletion with control preimmune serum. The results demonstrate that, as was seen in the goldfish, RICH proteins are the major source of CNPase activity in the regenerating retina of the zebrafish. This is also true of brain extracts from both goldfish and zebrafish. Data are average ± SD (bars) value from triplicate determinations. SA, specific activity.

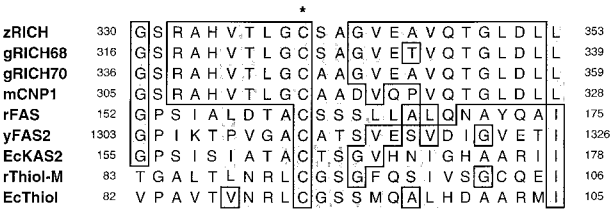

FIG. 6. Alignment of a highly conserved motif between RICH and CNPase with the β-ketoacyl synthase and thiolase active site motifs. Sequence identity is indicated with the boxes. There is high amino acid identity among the RICH proteins and mCNP1 and very limited identity with the βKAS and βKAT sequences. Amino acids that conform to the βKAS and βKAT active site motifs {PS00606, G-x(4)-[LIVMFAP]-x(2)-[AGC]-C-[STA](2)-[STAG]-x(3)-[LIVMF]; PS00098, [LIVM]-[NST]-x(2)-C-[SAGLI]-[ST]-[SAG]-[LIVMFYNS]-x-[STAG]-[LIVM]-x(6)-[LIVM], respectively} are shaded. The sequence from the RICH and CNPase proteins is generally in agreement with the βKAS and βKAT active site motifs. The critical cysteine for catalysis for βKAS and βKAT, present also in the RICH proteins and mCNP1, is indicated by an asterisk. rFAS, rat fatty acid synthase; yFAS, *Saccharomyces cerevisiae* fatty acid synthase; EcKAS2, *Escherichia coli* ketoacyl synthase 2; rThiol-M, rat thiolase-M; EcThiol, *Escherichia coli* thiolase.

based on the presumed potential of the side chain for participation in catalysis: S331, R332, H334, T336, and C339. All five residues were initially mutated to alanine, generating the recombinant protein H7-zRICH-5A. Analysis of bacterial extracts (described in Materials and Methods) indicated that the CNPase activity of H7-zRICH-5A was severely reduced compared with that of the WT protein H7-zRICH-WT (Fig. 7). This result suggested that the domain could indeed participate in catalysis or else be in a critical position for structural stability of the proteins. Each of the residues was individually mutated to alanine. Analysis of extracts indicated that H7-zRICH-S331A had activity levels similar to the WT protein, and, surprisingly, this was also true for the H7-zRICH-C339A mutant (Fig. 8). H7-zRICH-R332A had a significantly lower but detectable level of activity in the test, whereas basically no activity was detected for H7-zRICH-H334A and H7-zRICH-T336A (Fig. 8). Gel electrophoresis analysis of the extracts

PROTEIN	MOTIF SEQUENCE	ACTIVITY
H7-zRICH-WT	330 G S R A H V T L G C S A G V 343	+++
H7-zRICH-5A	A A A A A	-
H7-zRICH-S331A	A	+++
H7-zRICH-R332A	A	+
H7-zRICH-H334A	A	-
H7-zRICH-T336A	A	-
H7-zRICH-C339A	A	+++

FIG. 7. Analysis of zRICH mutant proteins in bacterial extracts. Bacteria expressing several zRICH mutant proteins in the RCD1 domain were lysed partially by detergent treatment, and the supernatant was tested for CNPase activity. The amount of the blue product was estimated visually and rated − to +++. The H7-zRICH-C339A protein showed WT levels of activity, whereas the H7-zRICH-H334A and H7-zRICH-T336A showed no detectable activity.

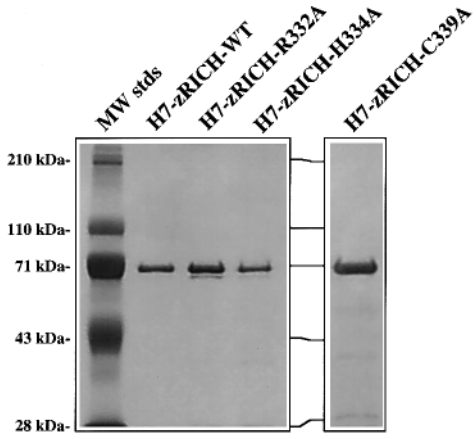

FIG. 8. Purification of H7-zRICH-WT, H7-zRICH-R332A, H7-zRICH-H334A, and H7-zRICH-C339A recombinant proteins. The recombinant proteins were purified from the bacterial lysates by a single nickel affinity chromatography step. The purified proteins were analyzed by SDS-PAGE (10% acrylamide). Five micrograms of H7-zRICH-WT, H7-zRICH-R332A, and H7-zRICH-H334A and 10 μg of H7-zRICH-C339A were loaded in the gel. Both the WT and the mutant proteins migrated at ~69 kDa. MW stds, molecular weight standards.

indicated similar levels of all the mutants in the extracts (data not shown). The H7-zRICH-WT and mutant proteins exhibited anomalously slow migration on SDS-PAGE relative to the predicted mass of the proteins. This anomalous migration has also been observed for gRICH68 and gRICH70, where it was attributed to the highly acidic amino acid composition of these proteins (Ballestero et al., 1997).

Purification and kinetic analysis of RCD1 mutants of zRICH

The results with the extracts suggested that although the RCD1 domain in zRICH is critical for catalysis, there are significant differences with the function of the βKAS motif. To analyze these results in detail, some of the proteins were purified for kinetic analysis. Figure 8 shows 5 μg each of purified H7-zRICH-WT, H7-zRICH-R332A, and H7-zRICH-H334A and 10 μg of purified H7-zRICH-C339A resolved by SDS-PAGE. The specific activity of the mutant proteins was determined with a fixed concentration of substrate (4 m*M*). The H7-

TABLE 1. *Apparent specific activities of the recombinant purified enzymes for 2′,3′-cyclic AMP*

Enzyme	Specific activity (μmol/min/mg)	Fold reduction
H7-zRICH-WT	130.58 ± 6.92	—
H7-zRICH-R332A	17.15 ± 0.61	7.6
H7-zRICH-H334A	<0.001	>130,000
H7-zRICH-C339A	76.98 ± 6.40	1.7

Data are average ± SD values from triplicate determinations. Fold reduction was calculated by comparison with the WT activity.

zRICH-WT enzyme showed a specific activity of 130.58 ± 6.92 μmol/min/mg (Table 1). The specific activity of the H7-zRICH-C339A mutant was only reduced 1.7-fold. Conversely, the H7-zRICH-R332A mutant had 7.6-fold lower specific activity than the WT enzyme (Table 1). The specific activity of the H7-zRICH-H334A mutant protein was reduced at least by 5 orders of magnitude, rendering the protein basically devoid of CNPase activity (Table 1).

The three purified enzymes with detectable activity were further analyzed for their kinetic properties. Initial rates were determined with increasing substrate concentrations, and the results were plotted as Lineweaver–Burk plots. A representative example is presented in Fig. 9. Kinetic constants were derived from the plots and averaged (Table 2). The data again indicate that only a relatively minor effect in the kinetic properties is derived by the mutation C339A, with a 1.2-fold increase in K_m and 1.3-fold reduction of V_{max}. The H7-zRICH-R332A protein, however, clearly has a more pronounced effect on both K_m (7.8-fold increase) and V_{max} (5.6-fold decrease), suggesting that residues in this domain participate both in substrate binding and in catalysis. As demonstrated previously for the gRICH proteins (Ballestero et al., 1997), the product of the reaction for the H7-zRICH-WT enzyme was demonstrated by paper chromatography to be the 2′-adenosine monophosphate exclusively (data not shown).

DISCUSSION

The present study reports the cloning of cDNAs that encode a single protein (zRICH) that is highly homologous with two previously described goldfish regeneration-associated proteins, gRICH68 and gRICH70. zRICH shows all of the characteristics previously described for the gRICH protein doublet (Ballestero et al., 1995): an N-terminal variable domain not conserved in mammalian CNPases, a putative catalytic domain highly conserved within the RICH family and highly homologous with mammalian CNPases, and a C-terminal isoprenylation box preceded by a polybasic motif (Fig. 2). Characterization of the regenerating zebrafish visual system shows that both zebrafish mRNA and protein are significantly induced during regeneration (Fig. 3) and are correlated with an increase in retinal CNPase activity. Furthermore, zRICH accounts for almost all of the CNPase activity present in zebrafish nervous tissues (Fig. 5), confirming the conclusion that there is only one RICH protein and also that the possible presence of a myelin CNPase in this tissue is therefore minimal.

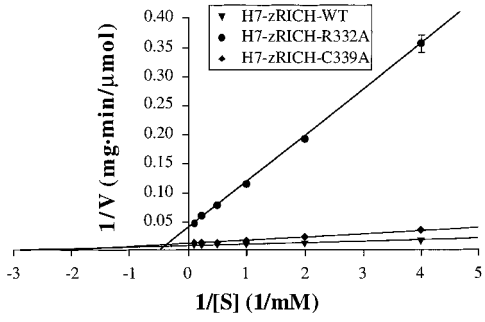

FIG. 9. Enzyme kinetics analysis of H7-zRICH-WT, H7-zRICH-R332A, and H7-zRICH-C339A recombinant proteins. The recombinant proteins (5 ng of H7-zRICH-WT and of H7-zRICH-C339A and 50 ng of H7-zRICH-R332A) were used in CNPase assays with several concentrations of 2′,3′-cyclic AMP (Lineweaver–Burk plots). Assays were performed in triplicate, and data are average ± SD (bars) values. A representative example is shown. Small errors are contained within the symbol. Kinetic constants were determined from axis intercepts. The C339A mutation did not affect dramatically the kinetic properties of the enzyme, whereas both the K_m and V_{max} are significantly affected in the R332A mutant.

TABLE 2. *Apparent kinetic constants of the recombinant purified enzymes for 2′,3′-cyclic AMP*

Enzyme	K_m (m*M*)	V_{max} (μmol/min/mg)
H7-zRICH-WT	0.32 ± 0.04	150.2 ± 8.4
H7-zRICH-R332A	2.50 ± 0.60	26.7 ± 2.7
H7-zRICH-C339A	0.39 ± 0.07	117.2 ± 26.1

Data are average ± SD values from at least three independent experiments performed in triplicate. Constants were determined from the axis intercepts on Lineweaver–Burk plots.

Analysis of the sequences of the goldfish and zebrafish RICH proteins indicates homology to the βKAS active site motif. This motif, together with a closely related one in the active site of enzymes that catalyze a complementary reaction (βKAT), was also recently reported in a CNPase protein cloned cloned from chickens (Kasama-Yoshida et al., 1997). The sequence alignments indicate that the βKAS motif lies in one of the domains most conserved between RICH proteins and CNPases (Fig. 6), a result that led to the suggestion that this motif is a component of the active site of the enzymes (Kasama-Yoshida et al., 1997). This hypothesis was tested in the present report by the generation of several site-directed mutants in which amino acids of the domain were mutated to alanine. The cysteine critical for βKAS and βKAT activities does not in fact appear to participate in the CNPase activity of zRICH (Fig. 6 and Tables 1 and 2), indicating differences in the catalytic mechanisms of βKAS and βKAT from that of CNPase. On the other hand, an arginine and a histidine within the conserved domain do seem critical for CNPase activity. The H334A mutation resulted in a reduction in specific activity of >5 orders of magnitude (Table 1). It is notable that the corresponding residues are not considered relevant to the activity of βKAS or of βKAT, and in some isoforms, alanine is the natural amino acid present at those positions (Fig. 6). These results, however, do not rule out a role for cysteine in the cellular function of these enzymes.

Despite the widespread use of CNPase as a myelin and oligodendrocyte marker protein, the 2′,3′-cyclic nucleotide substrates used for its assay are not considered to be natural substrates, and the true cellular role of the mam-

malian CNPases thus remains unknown (Sprinkle, 1989). Expression of CNPase in naive cells results in morphological alterations, including the presence of membranous extensions (De Angelis and Braun, 1994). Overexpression in the oligodendrocytes of transgenic mice resulted in extra myelin structures that did not ensheathe the axons in a normal fashion (Gravel et al., 1996). The CNPase activity of CNPases and RICH proteins is considered not to reflect their physiological function (Sprinkle, 1989), but the conservation of this catalytic property between these two families of proteins suggests that it might nevertheless reflect a common relevant aspect of their cellular roles. The mutant forms of zRICH devoid of CNPase activity reported here and corresponding mutants in the homologous amino acids in mammalian CNPases may prove useful in the eventual elucidation of the true substrates of both mammalian CNPases and the RICH proteins.

Earlier studies on RICH proteins in the goldfish (Heacock and Agranoff, 1982) indicated that the gRICH proteins migrate down the newly regenerated optic nerve at a slow rate of axonal transport, characteristic for cytoskeletal elements and their associated proteins. In this regard, a relationship between mammalian CNPase and tubulin (Dyer and Benjamin, 1989; Laezza et al., 1997) as well as with actin (De Angelis and Braun, 1996) has been proposed. These findings and the well-known association of mammalian CNPase with oligodendroglia and myelin might be reconciled with the neuronal source of gRICH and zRICH proteins in fishes by considering a common theme: In each instance, i.e., neurite outgrowth from retinal ganglion cells in optic nerve regeneration and elaboration of myelin by oligodendroglia, a cell is elaborating relatively immense amounts of both cytoskeletal elements and plasma membrane, manyfold in excess of what is already present in the cell body. The hypothesis then follows that RICH proteins play a role in a cytoskeleton/membrane-coordinated synthetic process, the nature of which remains for the present unknown, as does the biological substrate for the superfamily of CNPases, of which mammalian CNPase and the RICH proteins are members.

Nerve regeneration in the zebrafish has been studied previously by several investigators, and several specific proteins have been shown to participate in optic nerve regeneration. Plasticin and gefiltin are two neurofilament proteins first characterized in goldfish and later shown to be induced in the zebrafish retinal ganglion cells following optic nerve crush (Asch et al., 1998; Canger et al., 1998). GAP-43 and α-tubulin are induced following optic nerve crush. It is likely that more detailed genetic analysis of the signals controlling these genes as well as zRICH will provide insights into the regulation of nerve regeneration (Bormann et al., 1998). Furthermore, the advantages of the zebrafish as an experimental system and the zRICH mutant proteins described here may prove useful in elucidating the functional role of zRICH and other members of the CNPase superfamily in nerve regeneration.

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