Biochemical characterization of changes in gene expression that accompany optic nerve regeneration has led to the identification of proteins that may play key roles in the regeneration process. In this report, a cDNA encoding gRICH70, a novel isoform of the regeneration-induced gRICH68 protein, has been identified and characterized in goldfish. Both gRICH68 and gRICH70 show significant homology (34–36%) to mammalian 2',3'-cyclic-nucleotide 3'-phosphodiesterases (CNPases), hence the name goldfish regeneration-induced CNPase homolog (gRICH). The predicted 431-amino acid gRICH70 protein is 88% homologous to gRICH68, and the retinal mRNA for gRICH70 is coordinately induced with gRICH68 mRNA during optic nerve regeneration. Enzymatic analysis of recombinant proteins confirms that both gRICH proteins possess CNPase activity. Despite the relatively limited sequence homology, the kinetic constants obtained suggest that both gRICH proteins are at least as efficient as recombinant mouse CNP1 in catalyzing the hydrolysis of 2',3'-cyclic-nucleotide-3'-phosphate (cyclic-nucleotide 3'-phosphodiesterase (CNPase)).

The goldfish visual system has been used extensively in CNS nerve regeneration studies (2, 7). After optic nerve crush, the retinal ganglion cells undergo morphological and biochemical changes (8–10) and regrow their axons to reform connections with the tectum with a high degree of spatial specificity (11, 12), resulting in recovery of visual function (13). Studies of the molecular correlates of optic nerve regeneration have implicated several proteins in the process of regrowth, including cytokines (14–16), cell adhesion molecules (17), ion channels (18), transcription factors (19), and several proteins of as yet unknown function (15, 20–25).

p68/70 is a doublet of acidic proteins that is markedly induced in goldfish retinal ganglion cells and transported into the optic nerve during optic nerve regeneration (15). The doublet was purified from goldfish tissues, and a polyclonal antibody was generated and used to demonstrate a marked increase of expression of p68/70 in the retinal ganglion cells during regeneration (26). More recently, peptide sequences obtained from the purified p68/70 (27) were used to isolate a corresponding cDNA from a goldfish nerve-regenerating retina cDNA library (28). The encoded protein was designated RICH (regeneration-induced CNPase homolog) to underscore the homology with mammalian CNPases, enzymes highly expressed in mammalian myelin with an as yet unknown biochemical function (29). The region of homology between RICH and CNPases was localized to the COOH terminus of the proteins (28), a region that contains the catalytic activity of mammalian CNPases (30).

The RICH protein showed several characteristics that supported its relationship with the p68/70 doublet, including a similar isoelectric point (acidic) and amino acid composition. The RICH mRNA was found to be induced during regeneration as well (28).

It remained unclear which of the two peptides in the p68/70 doublet was encoded by gRICH. It had been established previously that the p68/70 protein components migrated anomalously in SDS-PAGE, probably due to effects of protein shape and low isoelectric point and of possible post-translational modifications (27). These characteristics probably account for the...
differences in apparent molecular weights of p68/70 on SDS-PAGE gels and the predicted molecular mass for gRICH68 of approximately 45 kDa (28).

In the present report, a cDNA encoding a second isoform of RICH has been isolated and sequenced. It encodes a slightly larger protein that is highly homologous to RICH. The corresponding mRNA is also induced during regeneration with a very similar time course to that for the initially characterized cDNA. The evidence reported here supports the identity of these proteins with the p68/70 doublet components, and hence, they have been renamed gRICH68 and gRICH70. Moreover, the enzymatic properties of each of the gRICH proteins have been determined, confirming that both are highly active CNPases.

MATERIALS AND METHODS

**Animals—Goldfish (Carassius auratus) 6–9 cm in body length were obtained from Grassy Forks Fisheries (Martinsville, IN) and maintained in aerated tanks at 25 °C. The intraorbital, unilateral optic nerve crush procedure has been described previously (13).**

**Library Screening—** The isolation of the clone g-RICH-1 from a λ cDNA library from goldfish optic nerve-regenerating retina has been described previously (28). It contained two unrelated cDNA inserts (28). A PCR fragment containing the full ORF of gRICH68 is described below under “Bacterial Expression and Purification of gRICH Proteins and mCNP1.” Approximately 20 ng of this fragment (template) and an antisense primer were used to synthesize a radiolabeled probe (5 × 10^6 cpm/μg) by asymmetric PCR. The probe was used to screen a goldfish genomic library (kindly provided by Dr. N. Schechter) by standard hybridization techniques (31). Clone g-RICH4 was purified by further rounds of hybridization, and its insert was mapped by restriction analysis. Southern blotting using the same probe was used to determine the restriction fragments bearing sequences of interest. The full RICH-related cDNA insert from the g-RICH-1 clone and a fragment from the g-RICH4-gene genomic insert (part of exon 1) were sequenced (both strands). DNA sequencing was performed by the dideoxynucleotide chain termination method by using a Sequenase DNA polymerase kit (U.S. Biochemical Corp./Amersham Corp.). Sequence analysis was performed with the University of Wisconsin Genetics Computer Group and DNASTAR software packages. The sequence reported here has been submitted to the GenBank database.

**RNA Protection Assays—** The protocol was used for the RNase protection assays and the total RNA isolation from goldfish retinas in a time course experiment have been described previously (28). A 183-base pair EcoRI-BamHI fragment from the gRICH70 ORF, highly divergent from the gRICH68 corresponding sequence, was selected as template and subcloned into pBluescript-KS (Stratagene), generating plasmid pbBS-gRICH70. The EcoRI-BamHI plasmid pbBS-gRICH70-EB was digested with EcoRI, and a 234-base-long riboprobe (10^6 cpm/μg) was synthesized by run-off transcription using T7 RNA polymerase (Life Technologies, Inc.) by standard protocols (31). To generate sense RNA, pbBS-gRICH70-EB was digested with BamHI, and T3 RNA polymerase (Life Technologies, Inc.) was used in the synthesis reaction. Four μg of the retina total RNAs (0, 2, 5, 9, 20, and 35 days post-optic nerve crush (PC)) were used in the sample tubes. For the sense controls, several amounts of sense RNA (0, 0.3, 1, 3, 10, and 30 pg) were mixed with 4 μg of yeast tRNA. The RNAs were hybridized to 4 × 10^6 cpm of antisense riboprobe. After the RNase protection assay procedure, the protected fragments (188 bases in sample tubes, 187 bases in sense controls) were separated in a 6% acrylamide sequencing gel. Radiolabeled DNA markers and 2 × 10^6 cpm of the antisense riboprobe were run in the gel as references. PhosphorImager (Molecular Dynamics) quantitation was performed as described previously (28).

**Bacterial Expression and Purification of gRICH Proteins and mCNP1—** All of the enzymes used were from Life Technologies unless otherwise indicated. The plasmid pK2K33–2 (Clontech) was modified for the purpose of expression of the gRICH proteins and mCNP1. The unique BamHI site was removed by digestion with BamHI, treatment with Klenow DNA polymerase, and blunt end religation. The polyclonal crush procedure has been described previously (13). A PCR fragment containing the full ORF of gRICH68 and gRICH70 flanked by BgII sites, as well as the full ORF of mCNP1 flanked by BamHI sites, was a subclone DNA insert from cloning gRICH-8 and a subclone that fuses sequences from the g-RICH4 genomic clone and the g-RICH1 cDNA clone were used as templates in the gRICH68 and gRICH70 PCR reactions. For the mCNP1 reaction, mouse brain total RNA was used as template in a reverse transcriptase-PCR reaction. The fragments were subcloned in pKKR2 generating the pKKR2-gRICH68, pKKR2-gRICH70, and pKKR2-mCNP1 constructs. In all cases, the subcloned PCR fragments were fully sequenced. No mutations were detected in the gRICH68 and gRICH70 ORF fragments, and only silent mutations were detected in the mCNP1 ORF fragment.

**Escherichia coli (XLI-Blue strain, Stratagene) were transformed with the pKKR2-gRICH68, pKKR2-gRICH70, and pKKR2-mCNP1 plasmids and grown at 37 °C in 0.5 liters of LB medium until they reached an optical density of 600 nm of 0.3. The isopropyl-m-thiogalactoside (0.4 mM) was added to the cultures to induce expression of the gRICH68, gRICH70, and mCNP1 corresponding ORF fragments.**

**Determination of Kinetic Constants—** CNPase activity was measured with an alkaline phosphatase (AP)-coupled assay method based on a previously published procedure (35). The reactions were performed in 50 mM Tris-HCl (pH 7.5), 0.2 mM MgCl2, 1 mM sodium molybdate, 0.001% Brij 35, and 0.02 mM 2-aminoethylbenzenesulphonate (AEB), as substrate (Sigma). Five ng of H7-gRICH68, 2.5 ng of H7-gRICH70, and 10 ng of H7-mCNP1 were used in the assays. The CNPase reaction was stopped by boiling for 1 min, and the coupled reaction was initiated then by the addition of 50 μl of CAP-buffer (300 mM Tris-HCl, pH 9, 21 mM MgCl2) containing 1 unit of calf intestine AP (Boehringer) and was incubated at 37 °C for 20 min. The released inorganic phosphate was detected by a sensitive chromogenic method (34). All measurements were performed in triplicate and were corrected with control values obtained in parallel in assays containing all of the components but the specific CNPase enzyme. The measured rates were used to obtain Lineweaver-Burk plots using SigmaPlot software (Jandel).

**Paper Chromatography Separation of CNPase Reaction Products—** CNPase reactions were performed with 10 mM 2′,3′-cAMP in CNPase reaction buffer for 30 min at 30 °C in a final volume of 50 μl. One μg each of H7-gRICH68, H7-gRICH70, and H7-mCNP1 were used. The products of the reactions were separated by paper chromatography as described previously (35). Five μl of each of the reactions were loaded side by side, and 5 μl each of a 10 mM solution of 2′,3′-cAMP, 2′-AMP, or 3′-AMP, employed as standards for comparison (Sigma). The developed chromatogram was photographed under short wave ultraviolet light transillumination.

**Transient Transfections into HEK-293 Cells—** The PCR fragments containing the ORFs for mCNP1, gRICH68, and gRICH70 (described above under “Bacterial Expression and Purification of gRICH Proteins and mCNP1”) were subcloned into the unique BgII site of pCMVneo.
A third clone (g-RICH-1) was obtained in the original screening of the goldfish retinae. Clone g-RICH-G4 was isolated in this screening, and partial sequence of the region immediately upstream of that of the g-RICH-1 cDNA insert was determined. The sequence obtained showed continuous homology to that of the g-RICH-8 cDNA up to the 5'-untranslated region, 25 bases upstream of the initiator ATG codon, suggesting that this sequence corresponded to a single exon. The composite sequence is represented in Fig. 1. Evidence presented below has indicated the identity of the two isoforms of RICH with the components of the p68/70 protein doublet. The first isoform (28) corresponds to the faster migrating protein of the p68/70 doublet, while the novel isoform described here corresponds to the slower migrating protein; hence, they have been renamed gRICH68 and gRICH70, respectively. The predicted gRICH70 protein is 431 amino acids in length, 20 amino acids longer than gRICH68, and has a predicted molecular mass of 46,972 Da and a calculated isoelectric point of 4.39.

An alignment of gRICH70 with gRICH68 is presented in Fig. 2. The two isoforms are highly homologous (88% amino acid identity) over their entire sequence, and both proteins contain an identical isoprenylation consensus sequence (CTIL) at the COOH terminus (37). The homology with mammalian CNPases is clearly localized to the COOH terminus (Fig. 2), corresponding to the catalytically active region of these proteins (30).

**gRICH70 mRNA Is Induced in Nerve-regenerating Retinas during Goldfish Optic Nerve Regeneration**—A significant induction of the gRICH68 mRNA during goldfish optic nerve regeneration has been previously demonstrated by a specific RNase protection assay (28). In similar studies of the gRICH70 mRNA, induction was detected in the regenerating retinas (R lanes) as early as 2 days PC and continued to increase up to 20 days PC, declining at later times (Fig. 3A). No significant induction was detected in the control retinas (C lanes). This time course of the induction resembled that seen previously for the gRICH68 mRNA, suggesting coordinate regulation of gRICH68 and gRICH70 mRNAs. Sense RNA controls were included in the assay for accurate quantitation in a PhosphorImager. The quantitation indicated a maximal induction of gRICH70 mRNA of 6-fold over control retina levels 20 days PC (Fig. 3B). It also suggested that the levels of gRICH70 mRNA are approximately 2–3-fold lower than those of gRICH68 in normal retinas. These retinal RNA samples have been previously tested by an RNase protection assay with a goldfish 18 S rRNA riboprobe and yielded a band of similar intensity in all of the lanes (28).

The induction detected with whole retinas is likely to be an underestimation of the induction in the retinal ganglion cell layer, since constitutive expression of p68/70 proteins has been demonstrated in other retinal layers (26).

**Bacterial Expression and Purification of gRICH Proteins and mCNP1**—The predicted gRICH proteins showed very significant homology to the mammalian myelin CNPases, particularly in the catalytic region of these proteins, including an isoprenylation motif at the COOH terminus (Fig. 2 and Ref. 28). CNPases hydrolyze 2',3'-cyclic-nucleotide monophosphates specifically to the 2'-nucleotide monophosphate product, but their in vivo role is as yet unclear (29).

To explore further the significance of the homology between the gRICH proteins and mammalian CNPases, gRICH68 and gRICH70 proteins were expressed and purified from E. coli. mCNP1 was also purified using the same system. Bacterial expression constructs were designed so that the full ORFs for the three proteins were fused in frame to a sequence encoding...
a heptahistidine tag. The NH₂-terminal heptahistidine-tagged proteins (H7-gRICH68, H7-gRICH70, and H7-mCNP1) could therefore be purified from the bacteria to near homogeneity by a single column affinity purification method (described under “Materials and Methods”). Five mg of each of the purified proteins were analyzed by SDS-PAGE (Fig. 4). While the three proteins (mCNP1, gRICH68, and gRICH70) are of similar length (400, 411, and 431 amino acids long, respectively), their apparent molecular weight in SDS-PAGE is significantly different. While mCNP1 seems to migrate accordingly with the predicted molecular weight, gRICH proteins migrate significantly more slowly, slightly below the 71 kDa molecular mass standard (Fig. 4). These electrophoretic mobilities support the assignment of gRICH proteins with the p68/70 doublet.

**FIG. 1. Sequence of gRICH70 cDNA and its encoded protein.** The sequence represents a composite of the sequence derived from the g-RICH-1 cDNA clone and the sequence from the g-RICH-G4 genomic clone (exon 1). The sequence contains partial 5'- and 3'-untranslated regions and the full ORF for the 431-amino acid-long predicted gRICH70 protein, as represented below the DNA sequence with the one-letter amino acid codes. Both strands of the DNA were fully sequenced.

**FIG. 2. Homology of gRICH70 with gRICH68 and mCNP1.** The alignment of gRICH68 and gRICH70 predicted proteins was obtained with the BESTFIT program of the Genetics Computer Group software. gRICH70 is 20 amino acids longer than gRICH68 and shows 88% amino acid identity. Positions conserved are indicated with an asterisk under the alignment. An x is substituted in those positions also conserved in mCNP1. Note that all of the homology with mCNP1 is localized in the carboxyl terminus of the gRICH proteins.
anomalous migrations may be dependent on the sequence of the proteins themselves, since the relevant post-translational modifications do not occur in bacteria.

gRICH Proteins Are Novel and Efficient CNPases—The purified H7-gRICH68 and H7-gRICH70 were tested for CNPase activity. Assays with H7-mCNP1 were performed for comparison. Initial rates of reaction were determined in assays performed in triplicate at several concentrations (0.25–8 mM) of the substrate 2΄,3΄-cAMP, and the values were used to obtain Lineweaver-Burk plots. A representative experiment is presented in Fig. 5. The plots show the average ± S.D. of triplicate measurements and are representative of three independent determinations for each enzyme. Error bars not visible were contained within the symbol.

values for all three (H7-mCNP1, H7-gRICH68, and H7-gRICH70) fall well within the range determined for mammalian CNPases purified from myelin. The $V_{\text{max}}$ values are slightly lower than those reported with highly purified mammalian CNPases (approximately 800–1000 units/mg) (29). In other experiments, a different bacterial expression system (New England Biolabs), which generates proteins fused to the E. coli maltose-binding protein (MBP), was used to purify MBP-mCNP1 and MBP-gRICH68. Both enzymes were active and yielded virtually identical kinetic constants to those obtained with the corresponding heptahistidine-tagged proteins (data not shown).

The CNPase activity assay employed does not distinguish the product isomer (2΄-NMP or 3΄-NMP) generated in the reac-

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Fig. 3. gRICH70 mRNA expression during regeneration. A, RNase protection assay of gRICH70. The expression of gRICH70 mRNA during optic nerve regeneration was studied using an RNase protection assay. Total RNA was isolated from both control (C) and regenerating (R) goldfish retinas 0, 2, 5, 9, 20, and 35 days postcrush. Four μg of each were hybridized to a gRICH70-specific riboprobe (234 bases). After RNase digestion, the protected fragments (188 bases) were separated by denaturing gel electrophoresis in a 6% sequencing gel, and the gel was dried and then exposed to autoradiographic film. The position of the protected fragments is indicated with an arrow. Assays containing sense RNA standards were included for accurate quantification. The time course shows a detectable induction of gRICH70 mRNA 5 days postcrush, with continuing increases up to 20 days postcrush and declining levels afterward. B, PhosphorImager quantification of the assay. The expression of gRICH70 mRNA in both control and nerve-regenerating retinas is 2–3-fold lower than that previously determined for gRICH68, but the induction time course is very similar for both. Maximal induction (approximately 6-fold) occurs 20 days postcrush (8-fold was previously observed for gRICH68).

Fig. 4. Expression and purification of recombinant CNPases in E. coli. gRICH68, gRICH70, and mCNP1 were expressed in E. coli as heptahistidine-tagged proteins. The recombinant proteins were purified to near homogeneity by nickel-nitrilotriacetic acid affinity chromatography. Five μg of each protein were run in a 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Molecular weight marker sizes are indicated on the left. Note the abnormal migration of the gRICH proteins, approximately 20–25 kDa above their predicted molecular mass.

Fig. 5. Enzymatic characterization of gRICH proteins. The purified proteins were used in CNPase assays with 2΄,3΄-cAMP as substrate as described under “Materials and Methods.” The initial velocities determined at several substrate concentrations were used to generate Lineweaver-Burk plots. The plots show the average ± S.D. of triplicate measurements and are representative of three independent determinations for each enzyme. Error bars not visible were contained within the symbol.
gRICH Proteins Are Equivalent to the p68/70 Doublet Components—To compare directly the gRICH68 and gRICH70 proteins encoded in the cDNAs with the p68/70 doublet from goldfish, a polyclonal antibody recognizing specifically gRICH proteins was generated. Purified H7-gRICH68 was used as the antigen for rabbit immunizations (see “Materials and Methods”), and the antibody was characterized using the purified proteins. The anti-gRICH antibody recognized both H7-gRICH68 and H7-gRICH70 and showed very weak cross-reactivity with H7-mCNP1.

Constructs for the eukaryotic expression of the encoded gRICH68 and gRICH70 proteins were generated and transfected transiently into HEK-293 cells, a system that can produce isoprenylated proteins. Control transfections were performed with the parental plasmid (pCMVneo) and with a construct expressing mCNP1 as well. Protein extracts were obtained from the transfected HEK-293 cells (HEK-neo, HEK-mCNP1, HEK-gRICH68, and HEK-gRICH70 extracts) as well as from goldfish nerve-regenerating and control retinas 35 days PC (gf-retina-R35 and gf-retina-C35 extracts).

The protein extracts were analyzed by Western blot (Fig. 7). The anti-gRICH antibody detected a doublet of proteins in the 68–70-kDa area in the goldfish extracts. Additionally, the doublet was induced in the optic nerve-regenerating retinas and comigrated perfectly with the major protein products detected in the HEK-gRICH68 and HEK-gRICH70 extracts. All these data indicate that gRICH68 and gRICH70 proteins are identical to the p68/70 doublet components. Quantification of the Western blot signal demonstrated an induction of the gRICH68/gRICH70 proteins detected in the regenerating retinas of 4.17 ± 1.40 fold, a result correlating with the induction detected previously for the p68/70 doublet itself (26). No specific signal was detected in the HEK-neo or HEK-mCNP1 extracts (Fig. 7).

Analysis of the Enzymatic Activity of Recombinant and Native gRICH Proteins—The protein extracts used for the Western blot analysis were also tested for CNPase activity by the previously described AP-coupled method. A representative experiment is presented in Fig. 8. As expected, the HEK-mCNP1, HEK-gRICH68, and HEK-gRICH70 extracts showed high specific activity compared with the control HEK-neo extract (Fig. 8A). The activity detected in the HEK-mCNP1 extract confirmed the presence of the enzyme, indicating that the absence of a band in the Western blot (Fig. 7) was not due to lack of expression but to the specificity of the anti-gRICH antibody. An estimation of the $V_{\text{max}}$ of the eukaryotic recombinant gRICH68 and gRICH70 proteins can be calculated based on the levels quantitated in the Western blot (Fig. 7; assuming that anti-gRICH recognizes equally the bacterial and eukaryotic proteins) and the specific activity levels of the extracts. This calculation yields values in the range of 500–1000 $\mu$mol min$^{-1}$ mg$^{-1}$, correlating fairly well with the values obtained with the purified bacterial proteins, suggesting that the post-translational modifications in eukaryotic cells do not significantly affect the catalytic properties of the enzymes.

The CNPase enzyme activity of the recombinant and native gRICH proteins was measured using the hydrolysis of $2',3'$-cAMP as substrate as described under “Materials and Methods.” Reactions were driven to near completion by an excess of enzyme. Standards were run for comparison. The chromatogram was air-dried and photographed under short wave ultraviolet light. All three enzymes (gRICH68, gRICH70, and mCNP1) generated $2'$-AMP specifically. The origin and direction of the chromatogram are indicated with an arrow.

![Under short wave ultraviolet light](FIG.6)

**FIG. 6.** Analysis of reaction products by paper chromatography. Paper chromatography was performed to separate the products of the reactions with $2',3'$-cAMP as substrate as described under “Materials and Methods.” Reactions were driven to near completion by an excess of enzyme. Standards were run for comparison. The chromatogram was air-dried and photographed under short wave ultraviolet light. All three enzymes (gRICH68, gRICH70, and mCNP1) generated $2'$-AMP specifically. The origin and direction of the chromatogram are indicated with an arrow.

![Western blot analysis of recombinant gRICH proteins expressed in eukaryotic cells: relationship with the p68/70 doublet from goldfish retina](FIG.7)

**FIG. 7.** Western blot analysis of recombinant gRICH proteins expressed in eukaryotic cells: relationship with the p68/70 doublet from goldfish retina. The purified recombinant heptahistidine-tagged proteins were used as controls (50 ng of each). Total extracts (2 $\mu$g of protein) from HEK-293 cells transfected with control plasmid (HEK-neo) or plasmids expressing mCNP1, gRICH68, or gRICH70 were loaded side by side with goldfish retina extracts (40 $\mu$g of protein) in a 10% SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed with rabbit polyclonal anti-gRICH antibody. The left three lanes show the specific recognition of the purified recombinant heptahistidine-tagged gRICH proteins. Anti-gRICH detected gRICH68 and gRICH70 in the transfected HEK-293 cells that comigrated with a doublet of ~68–70 kDa in the retina extracts. The positions of the specific doublet are indicated with arrows. Note the induction of the doublet in regenerating retinas over the control retinas, which further indicates that the recognized proteins correspond with the previously described p68/70 proteins. The blot is representative of four independent experiments.

![Western blot analysis of recombinant gRICH proteins expressed in eukaryotic cells: relationship with the p68/70 doublet from goldfish retina](FIG.7)

**FIG. 7.** Western blot analysis of recombinant gRICH proteins expressed in eukaryotic cells: relationship with the p68/70 doublet from goldfish retina. The purified recombinant heptahistidine-tagged proteins were used as controls (50 ng of each). Total extracts (2 $\mu$g of protein) from HEK-293 cells transfected with control plasmid (HEK-neo) or plasmids expressing mCNP1, gRICH68, or gRICH70 were loaded side by side with goldfish retina extracts (40 $\mu$g of protein) in a 10% SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed with rabbit polyclonal anti-gRICH antibody. The left three lanes show the specific recognition of the purified recombinant heptahistidine-tagged gRICH proteins. Anti-gRICH detected gRICH68 and gRICH70 in the transfected HEK-293 cells that comigrated with a doublet of ~68–70 kDa in the retina extracts. The positions of the specific doublet are indicated with arrows. Note the induction of the doublet in regenerating retinas over the control retinas, which further indicates that the recognized proteins correspond with the previously described p68/70 proteins. The blot is representative of four independent experiments.

### Table I

| Enzyme       | $K_m$ (mM) | $V_{\text{max}}$ (µmol min$^{-1}$ mg$^{-1}$) |
|--------------|-----------|------------------------------------------|
| HEK-mCNP1    | 1.03 ± 0.13 | 86.3 ± 9.9 |
| H7-gRICH68   | 1.23 ± 0.05 | 328.1 ± 47.2 |
| H7-gRICH70   | 0.34 ± 0.06 | 355.3 ± 68.8 |
The activity levels in goldfish retina were much lower than those in the overexpressing extracts (Fig. 8A), correlating well with the lower levels of gRICH proteins detected in the Western blot (Fig. 7). However, an induction of CNPase activity could be clearly detected in the optic nerve-regenerating retinas over the levels of control retinas (Fig. 8B). The average induction of three independent experiments was 3.54 ± 0.84-fold. The induction of the CNPase activity correlates well with the induction of the gRICH doublet detected by Western blot (Fig. 7). This result further supports the identity of gRICH68 and gRICH70 with the p68/70 doublet components. Moreover, it suggested that the gRICH proteins are contributing significantly to the total CNPase activity of the retina extracts.

The activity in nerve-regenerating retinas is due to gRICH proteins—From the analysis of the retina extracts, it could be inferred that a significant fraction of the CNPase activity detected was due to the gRICH proteins. To directly explore the proportion of the CNPase activity attributable to gRICH proteins, immunodepletion experiments with the anti-gRICH antiserum were performed. The gRICH proteins present in the extracts were immunoprecipitated by precipitation with protein A-Sepharose beads, and the immunodepleted supernatants were tested for CNPase activity. As expected, the CNPase activity was severely reduced in the HEK-gRICH68 and HEK-gRICH70 extracts by the immunodepletion procedure, while CNPase activity was not significantly affected in the HEK-mCNP1 extract (Fig. 9). Further controls indicated that the preimmune serum did not have a significant effect on the activity of any extract. In addition, the anti-gRICH antibody per se (without the protein A-Sepharose precipitation step) was able to block the activity of the gRICH enzymes only by approximately 30–40% (data not shown). The immunodepletion protocol removed most of the CNPase activity from the g-retina-R35 extract (>98%; Fig. 9). This result was confirmed by three independent experiments and provides direct evidence indicating that the majority of the CNPase activity in nerve-regenerating retinas is accounted for by the gRICH68 and gRICH70 proteins.

DISCUSSION

In this report, previous biochemical studies of optic nerve regeneration in goldfish (7, 26–28) have been extended with the cloning of a cDNA encoding a novel isoform of RICH proteins. The available cDNAs have allowed the expression of the encoded proteins both in prokaryotic and eukaryotic recombinant systems, as well as the generation of a specific polyclonal antibody that recognizes the gRICH proteins efficiently. The experimental evidence obtained confirmed the identity of gRICH68 and gRICH70 with the p68/70 doublet components, two induced proteins previously identified in the goldfish optic nerve regeneration model system.

The studies performed here with the purified recombinant proteins have identified gRICH68 and gRICH70 as efficient CNPases. These novel CNPases are the first cloned nonmammalian members of this family of enzymes. Since they are nevertheless related to the mammalian CNPases, they should be helpful in delineating the regions of the protein relevant for the enzymatic activity. These highly conserved motifs will probably correspond to those relevant for the in vivo function of these proteins. Of note, those conserved sequences do not include several sequence motifs that were previously thought to be involved in catalysis (28, 29).

The gRICH68 and gRICH70 proteins showed very similar migrations in SDS-PAGE and enzymatic activity when expressed in E. coli or HEK-293 cells, suggesting that post-translational modification is not the cause of the anomalous migration of gRICH proteins, nor is it necessary for enzymatic
activity. Alternatively, the acid pl of both gRICH68 and gRICH70 relative to mCNP1 may be responsible for the anomalous migration in SDS-PAGE. The overexpression systems described here should be useful for the studies of post-translational modifications of gRICH proteins and to discern their role on the gRICH proteins function. The isoprenylation of mammalian CNPases have been shown to be important for their membrane localization and for the phenotypic effects of their overexpression (38, 39).

The two known isofoms of mammalian CNPases, CNP1 and CNP2, differ only in the amino terminus. They arise by alternative splicing and promoter usage (40, 41). The gRICH68 and gRICH70 cDNAs showed differences all along the sequence, suggesting that they are derived from two distinct genes. Both the gRICH68 and gRICH70 mRNAs showed similar time courses of induction during optic nerve regeneration (Ref. 28 and Fig. 3). The mechanisms of transcriptional induction of specific genes following nerve injury are not well understood. The time course of induction of gRICH mRNAs closely resembles that of the α-tubulin (18), with a progressive increase in messenge levels during the period of axonal growth. The levels of a nicotin acetylcholine receptor subunit mRNA follow a different time course, being induced late in the regeneration process (18), corresponding to the period of synapse reforma-

The expression systems utilized in these studies demonstrated that gRICH68 and gRICH70 are novel members of the CNPase family of enzymes. The best studied members of this family are the mammalian CNPases (29), although enzymes with CNPase activities have been detected in organisms as diverse as crustaceans (42) and even plants (43). The mammalian CNPases are very highly expressed in oligodendrocytes and Schwann cells, and they have been widely used as a marker of these glial cells (29). Additionally, CNPases are strongly associated with myelin membranes both by a hydrophobic isoprenyl group in their COOH terminus (38, 39) and by interaction with the cytoskeleton (44, 45). Overexpression exper-

REFERENCES

1. Sperry, R. W. (1943) J. Comp. Neurol. 79, 33–55
2. Grafstein, B. (1991) in Development and Plasticity of the Visual System (Cronly-Dillon, J. R., ed) Vol. 11, pp. 190–205, Macmillan, London
3. Ramon y Cajal, S. (1928) Degeneration and Regeneration of the Nervous System (May, R. M., ed.) Oxford University Press, London
4. Bahr, M., and Bonhoeffer, F. (1994) Trends Neurosci. 17, 473–479
5. Schwab, M. E. (1990) Trends Neurosci. 13, 452–456
6. Chen, D. F., Shaveri, S., and Schneider, G. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7287–7291
7. Agranoff, B. W., and Ford-Holevisnski, T. S. (1984) in Axonal Transport in Neuronal Growth and Regeneration (Eliam, J. S., and Canicalon, F., eds) pp. 69–86, Plenum Press, New York
8. Murray, M., and Grafstein, B. (1969) Exp. Neurol. 23, 544–560
9. Murray, M. (1973) Exp. Neurol. 39, 489–497
10. Burrell, R. H., Dokas, L. A., and Agranoff, B. W. (1978) J. Neurochem. 31, 269–298
11. Sperry, R. W. (1963) Proc. Natl. Acad. Sci. U. S. A. 50, 703–710
12. Jacobson, M., and Gaze, R. M. (1965) Exp. Neurol. 13, 418–430
13. Sprininger, W. D., and Agranoff, B. W. (1977) Brain Res. 128, 405–415
14. Heacock, A. M., and Agranoff, B. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 828–832
15. Heacock, A. M., and Agranoff, B. W. (1982) Neuron. Res. 7, 771–788
16. Glassow, E., Druger, R. K., Levine, E. M., Fuchs, E., and Schechena, N. (1990) Neuro 9, 373–381
17. Paschke, K. A., Lottspeich, F., and Stuermer, C. A. O. (1992) J. Cell Biol. 117, 863–875
18. Hieber, V., Agranoff, B. W., and Goldman, D. (1992) J. Neurochem. 58, 1009–1015
19. Herdegen, T., Bastmeyer, M., Bahr, M., Stuermer, C., Bravo, R., and Zimmermann, M. (1990) J. Neurobiol. 21, 528–543
20. Skene, J. H. P., and Willard, M. (1981) J. Cell Biol. 89, 86–95
21. Benowitz, L. I., Shashoua, V. E., and Yoon, M. G. (1981) J. Neurosci. 1, 300–307
22. Benowitz, L. I., and Lewis, E. R. (1983) J. Neurosci. 3, 2153–2163
23. Perry, G. W., Burmeister, D. W., and Grafstein, B. (1985) J. Neurosci. 44, 1142–1151
24. Skene, J. H. (1989) Annu. Rev. Neurosci. 12, 127–156
25. Schwab, J. M., Boulis, N. M., Gu, M. F., Winickoff, J., Jackson, P. S., Irwin, N., and Benowitz, L. I. (1995) J. Neurosci. 15, 5514–5525
26. Wilmot, G. R., Raymond, P. A., and Agranoff, B. W. (1993) J. Neurosci. 13, 387–401
27. Leski, M. L., and Agranoff, B. W. (1994) J. Neurosci. 62, 1182–1191
28. Ballesteros, R. P., Wilmot, G. R., Leski, M. L., Uhler, M. D., and Agranoff, B. W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8621–8625
29. Sprinkle, T. J. (1989) CRC Crit. Rev. Neurobiol. 4, 235–401
30. Kurihara, T., Folkew, A. V., and Takahashi, Y. (1987) J. Biol. Chem. 272, 2526–2561
31. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory, New York
32. Imai, Y., Matsuaihima, Y., Sugimura, T., and Terada, M. (1991) Nucleic Acids Res. 19, 2785
33. Olafson, R. W., Drummond, G. I., and Lee, J. F. (1969) Can. J. Biochem. 47, 961–966
34. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756–1758
35. Markham, R., and Smith, J. D. (1952) Biochem. J. 52, 552–557
36. Huggenovik, J. I., Collard, M. W., Stofko, R. E., Seashilz, A. F., and Uhler, M. D. (1991) Mol. Endo. Res. 5, 921–930
37. Clarke, S. (1992) Annu. Rev. Biochem. 61, 355–386
38. Brau, P. E., DeAngelis, D., Stibely, W. B., and Bernard, L. (1991) J. Neurosci. Res. 30, 540–544
39. DeAngelis, D., and Brau, P. E. (1994) J. Neurosci. Res. 39, 386–397
40. Kurihara, T., Moroh, K., Sakimura, K., and Takahashi, Y. (1990) Biochem. Biophys. Res. Commun. 170, 1074–1081
41. Scherer, S. S., Braun, P. E., Grispan, J., Collarini, E., Wang, D. Y., and Komolh, J. (1994) Neuro 12, 1363–1375
42. Diaz, A. R., and Heredia, C. F. (1996) Biochim. Biophys. Acta 1290, 135–140
43. Tyc, K., Kellenberger, C., and Filipowicz, W. (1987) J. Biol. Chem. 262, 12994–13006
44. DeAngelis, D., and Brau, P. E. (1996) J. Neurochem. 66, 2523–2531
45. DeAngelis, D., and Brau, P. E. (1996) J. Neurochem. 67, 943–951
46. Gravel, M., Peterson, J., Yong, V. W., Kottis, V., Tropp, B., and Braun, P. E. (1996) Mol. Cell. Neurosci. 7, 453–466
47. Muller, H. W., Clapshaw, P. A., and Seifert, W. (1981) J. Neurosci. 37, 947–955