Published in final edited form as:

Brain Res. 2012 September 20; 1474: 29–39. doi:10.1016/j.brainres.2012.07.057.

zRICH, a Protein Induced During Optic Nerve Regeneration in Zebrafish, Promotes Neuritogenesis and Interacts with Tubulin

Satya S. Pathi, Soumia Jose, Suman Govindaraju, Juan A. Conde, Hannah E. Romo, Karthik R. Chamakura, Cheryl J. Claunch, Ana Benito-Martín, Madhavi Challa-Malladi, Maribel González-García, and Rafael P. Ballesteros¹

Department of Biological and Health Sciences and Department of Chemistry, Texas A&M University-Kingsville, Kingsville, TX 78363

Abstract

Mammals do not regenerate axons in their central nervous system (CNS) spontaneously. This phenomenon is the cause of numerous medical conditions after damage to nerve fibers in the CNS of humans. The study of the mechanisms of nerve regeneration in other vertebrate animals able to spontaneously regenerate axons in their CNS is essential for understanding nerve regeneration from a scientific point of view, and for developing therapeutic approaches to enhance nerve regeneration in the CNS of humans. RICH proteins are a novel group of proteins implicated in nerve regeneration in the CNS of teleost fish, yet their mechanisms of action are not well understood. A number of mutant versions of the zebrafish RICH protein (zRICH) were generated and characterized at biochemical and cellular levels in our laboratory. With the aim of understanding the effects of RICH proteins in neuronal axon outgrowth, stable transfectants derived from the neuronal model PC12 cell line expressing zRICH Wild-Type or mutant versions of zRICH were studied. Results from differentiation experiments suggest that RICH proteins enhance neuronal plasticity by facilitating neurite branching. Biochemical co-purification results have demonstrated that zRICH binds to the cytoskeletal protein tubulin. The central domain of the protein is sufficient for tubulin binding, but a mutant version of the protein lacking the terminal domains, which cannot bind to the plasma membrane, was not able to enhance neurite branching. RICH proteins may facilitate axon regeneration by regulating the axonal cytoskeleton and facilitating the formation of new neurite branches.

Keywords

Axon Regeneration; Teleost; Cytoskeleton; Neuron Differentiation; 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase

1. Introduction

In mammals and birds (warm-blooded vertebrates), there is a significant difference in the capacity for axon regeneration when comparing the peripheral nervous system (PNS) versus the central nervous system (CNS). The PNS of these animals is able to mount a sustained

© 2012 Elsevier B.V. All rights reserved.

¹To whom correspondence should be addressed: Department of Biological and Health Sciences, Texas A&M University-Kingsville, 700 University Blvd., Kingsville, TX 78363. Tel: (361) 593-3793. Fax: (361) 593-3800. karp002@tamuk.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

regeneration response after nerve fiber injury, which is absent in their CNS (Ramon y Cajal, 1928; Bahr and Bonhoeffer, 1994; Horner and Gage, 2000; Olson, 2002). Damage to the nerves in the CNS leads to degeneration and loss of function. In humans, this lack of regeneration capacity leads to a number of permanent medical conditions after trauma or disease that results in injury to the axons of neurons in the CNS. A classic example within this category is paralysis after spinal cord injury, which seriously impacts the quality of life of the affected individuals. Learning about how to improve the regeneration response after nerve damage in humans is a relevant biomedical goal. Interestingly, cold-blooded vertebrates show nerve regeneration capacity in both their PNS and CNS (Agranoff and Ford-Holevinski, 1984; Grafstein, 1991; Bernhardt, 1999). The optic nerve of teleost fish and amphibians has served as a model system for successful axonal regeneration in the CNS. The retinal ganglion cells (RGCs) are capable of regenerating the optic nerve axons, which eventually connect with their tectal targets in the brain, resulting in functional recovery of visual function. This model of nerve regeneration offers numerous advantages for the study of this phenomenon at various levels (Agranoff and Ford-Holevinski, 1984; Bernhardt, 1999).

Cellular signals play a key role in the process of nerve regeneration. The environment of the PNS in all vertebrates, and of the CNS in cold-blooded vertebrates, provides trophic support for the process of axon regeneration (Fu and Gordon, 1997; Bernhardt, 1999; Schwab, 2004). Conversely, in the adult CNS of warm-blooded vertebrates, not only does the environment not provide the same level of trophic support; in fact, it directly inhibits and suppresses axon outgrowth after injury (Bahr and Bonhoeffer, 1994; Fawcett and Asher, 1999; Horner and Gage, 2000; Olson, 2002; Woolf and Bloechlinger, 2002; Spencer et al., 2003; Schwab, 2004). Glial cells seem to play essential roles in these phenomena (Bunge, 1993; Schwab et al., 1996; Fu and Gordon, 1997; Bernhardt, 1999; Fawcett and Asher, 1999; Spencer et al., 2003; Schwab, 2004). Using animal research model systems, alteration of these extrinsic factors that affect the neurons has already proved powerful in the enhancement of nerve regeneration in the mammalian CNS (Bray et al., 1987; Horner and Gage, 2000; Fouad et al., 2001; Olson, 2002; Schwab, 2002; Schwab, 2004; Benowitz and Yin, 2007).

Intrinsic neuronal determinants are also essential for successful axon regeneration (Chen et al., 1995; Caroni, 1997; Bernhardt, 1999; Neumann and Woolf, 1999). Mammalian neurons can show variable responses to a supportive environment, and they often lose regenerative capacity during development. Much research is still needed to understand the molecular determinants that mediate axon re-growth. Biochemical and molecular studies with systems that show successful nerve regeneration have identified numerous proteins that are induced in neurons that are in the process of regenerating their axon. These proteins are often referred to as axonal growth associated proteins (GAPs) (Skene, 1989; Caroni, 1997; Bernhardt, 1999; Plunet et al., 2002). The identification and the study of axonal GAPs are essential for gaining a complete view of nerve regeneration and for the effectiveness of therapeutic interventions in humans. The types and families of axonal GAPs identified and our knowledge of their function in nerve repair have been growing steadily. They include cell adhesion molecules (Vielmetter et al., 1991; Paschke et al., 1992; Laessing et al., 1994; Giordano et al., 1997; Becker et al., 2004; Haenisch et al., 2005), membrane receptors and transporters (Hieber et al., 1992; Hammarberg et al., 2000; Liu et al., 2002), intracellular signaling proteins (Yankner et al., 1990; Aigner et al., 1995; Strittmatter et al., 1995; Benowitz and Routtenberg, 1997; Tanabe et al., 2000; Bomze et al., 2001), cytoskeletal proteins (Heacock and Agranoff, 1976; Burrell et al., 1979; Glasgow et al., 1992; Glasgow et al., 1994; Asch et al., 1998; Hieber et al., 1998), transcription factors (Herdegen et al., 1997; Tsujino et al., 2000), etc. Despite our growing knowledge on axonal GAPs, our

current view is still limited, and much needs to be discovered in this interesting field through further studies.

RICH proteins (Regeneration Induced CNPase Homologs) were discovered as a doublet of acidic axonal GAPs that were induced in the RGCs of the goldfish eye during the regeneration of the optic nerve (Heacock and Agranoff, 1982; Wilmot et al., 1993). Biochemical analyses showed that they can form dimers of highly elongated monomers, which seem to be able to shuttle between the plasma membrane and the cytosol (Leski and Agranoff, 1994). Cloning of cDNAs allowed the discovery of homology with mammalian CNPases (2',3'-cyclic nucleotide 3'-phosphodiesterases) (Ballesterio et al., 1995; Ballesterio et al., 1997; Ballesterio et al., 1999), enzymes that are very abundant in myelin-producing glial cells (Sprinkle, 1989). 2',3'-cyclic nucleotides were considered artificial substrates for long, but recent reports have detected them and suggested possible involvement in adenosine and Ca²⁺ signaling pathways (Jackson et al., 2009; Azarashvili et al., 2010). Mammalian CNPases have been shown to facilitate the formation of membranous extensions in glial cells, probably through the regulation of the tubulin cytoskeleton (Laezza et al., 1997; Bifulco et al., 2002; Lee et al., 2005; Rasband et al., 2005). Recombinant goldfish and zebrafish RICH proteins were shown to have phosphodiesterase activity (Ballesterio et al., 1997; Ballesterio et al., 1999). Based on the alignment with mammalian CNPases, three putative domains were predicted in RICH proteins (Ballesterio et al., 1995; Ballesterio et al., 1997). The N-terminal domain of the protein (approximately first one-third of the protein) is acidic in nature, shows limited homology to the acidic domains found in some intermediate filament proteins, and is completely divergent from the equivalent region in mammalian CNPases. The central domain (spanning almost the last two-thirds of the protein) shows significant homology to mammalian CNPases. The C-terminal domain is a small domain that bears an isoprenylation motif, which is also present in mammalian CNPases, suggesting a conserved role in membrane association. Mutagenesis studies demonstrated that the central domain is sufficient and essential for catalysis and that the C-terminal domain is necessary for membrane association (Ballesterio et al., 1999; Challa et al., 2006).

Despite the advances described above, the roles of RICH proteins as axonal GAPs in the teleost optic nerve model of nerve regeneration are not well understood. In this report, the effects of protein expression of Wild-Type (WT) or different mutant versions of zebrafish RICH (zRICH) on neuritogenesis have been analyzed in PC12 cells differentiated to sympathetic neuron phenotype with NGF, and the ability of zRICH to interact with tubulin has been explored with co-purification assays.

2. Results

2.1. Generation of stable PC12 transfectants overexpressing zRICH proteins

PC12 cells are used as a model system of neuronal differentiation, as treatment with NGF induces the formation of neurites and the expression of markers of sympathetic neurons (Greene and Tischler, 1976). In a previous study, transient transfections with the eukaryotic expression plasmids encoding WT or mutant versions of zRICH were carried out to study the effect of expressing the proteins on PC12 differentiation in response to NGF. The expression of the different zRICH protein versions did not have an effect on the percentage of PC12 cells that extended neurites in response to NGF (approximately 35% of the cells) (Challa et al., 2006), although the proteins seemed to affect neurite branching and the extent of the neurite arbor in the transfected cells. However, accurate quantitation of the effects on neurite branching could not be assessed, due to the fact that these experiments were performed with relatively high cell densities, and to the transient nature of the expression in the transfected cells. To facilitate the performance of detailed morphometric analyses of the effects of RICH protein expression on neuritogenesis in PC12 cells treated with NGF, stable

transfectant cell lines were generated by transfecting eukaryotic expression plasmids encoding zRICH-WT, zRICH(172-410), and zRICH-H334A (as well as a plasmid encoding β -galactosidase, used in negative controls) into PC12 cells. The cells that integrated the constructs into their genome were selected with the neomycin antibiotic derivative G418. The two mutant versions of zRICH utilized have significant biochemical and cellular differences from WT zRICH. zRICH(172-410) is a catalytically active 2',3'-cyclic nucleotide 3'-phosphodiesterase that cannot attach to membranes, while zRICH-H334A is catalytically inactive, but still binds cellular membranes (Challa et al., 2006). Diagrams of the three proteins are shown in Figure 1A. Immunoblotting with anti-RICH antibody was performed to confirm that the stable transfectants express the zRICH proteins (Figure 1B). The results suggest that all three zRICH proteins are expressed by the corresponding stable transfectants.

2.2. zRICH induces neuritogenesis of transfected PC12 cells

Differentiation experiments (by NGF treatment) were performed with the stable transfectants. Morphometric analyses were conducted to assess the effects of the zRICH proteins on neuritogenesis. The expression of the zRICH proteins was detected by immunocytochemistry. As expected from our previous results with transient transfections (Challa et al., 2006), expression of the various versions of zRICH protein did not cause PC12 differentiation on their own, but the cells were responsive to differentiation triggered by NGF (Figure 1C). Interestingly, expression of zRICH-WT resulted in a significant increase in the number of branching points found in neurites produced by PC12 cells in response to NGF, resulting in a higher number of secondary neurite branches and an overall higher level of neurite arborization (Figure 1D). The effect was surprisingly more pronounced by expression of the mutant zRICH-H334A. Conversely, the expression of zRICH(172-410) did not result in any increase in neurite branching. In fact, although not statistically significant for each parameter, the expression of zRICH(172-410) had a modest negative effect on the number of primary neurites and on neurite branching in PC12 cells. The results suggest that RICH proteins promote neuron plasticity by facilitating neurite branching, which could contribute to the regenerative capacity of neurons after injury.

2.3. zRICH interacts with tubulin

Mammalian CNPases have been shown to interact and regulate the tubulin cytoskeleton, providing a mechanistic basis for its effects on the formation of membranous extensions in glial cells (Bifulco et al., 2002; Lee et al., 2005). While RICH proteins have a highly divergent N-terminal domain from mammalian CNPases, they display a functional and highly homologous catalytic domain. Additionally, the effects on neurite branching are consistent with an interaction of RICH and the cytoskeleton. Pull-down co-purification assays were performed with purified proteins to focus directly on the putative interaction of zRICH with the tubulin cytoskeleton, using protocols that are based on the procedures previously described for mammalian CNPases (Lee et al., 2005). Positive control experiments confirmed the interaction of tubulin with purified recombinant mCNP1 protein (Figure 2A), validating the procedure utilized. The specificity of the interaction was shown by the absence of tubulin in pull-down experiments performed with other purified recombinant proteins used as negative controls (Gal4AD, Bcl-2). The experiments showed that bovine brain tubulin does co-purify with recombinant WT zRICH protein in pull-down assays (Figure 2B), suggesting that tubulin can interact directly with the zRICH protein. Recombinant histidine-tagged purified proteins were utilized in the pull-down assays, and SDS-PAGE analyses were used to assess the purification by affinity chromatography (Figure 2C).

2.4. The catalytic domain of zRICH is sufficient for interaction with tubulin

A deletion mutagenesis study allowed the characterization of several biochemical and cellular roles for different domains of the protein (Challa et al., 2006). The deletion mutant protein zRICH(1-410) lacks the small C-terminal domain that bears an isoprenylation motif, and remains a catalytically active cyclic nucleotide phosphodiesterase, but cannot bind to cellular membranes; the deletion mutant protein zRICH(172-424) lacks the acidic N-terminal domain that is highly divergent from mammalian CNPases and retains both catalytic cyclic nucleotide phosphodiesterase activity and binding to membranes. The double deletion mutant zRICH(172-410), which lacks both of the terminal domains, has been described earlier (catalytically active 2',3'-cyclic nucleotide 3'-phosphodiesterase that cannot attach to membranes). A diagram representing the structure of these three mutant proteins is shown in Figure 3A. These three mutants were utilized in tubulin co-purification tests (pull-down assays) to learn about the domains of zRICH that are involved in the interaction with tubulin. Tubulin was able to bind to all three deletion mutant proteins (Figure 3B), indicating that neither the acidic N-terminal domain, nor the C-terminal small membrane binding domain, are essential for the interaction. Therefore, the central catalytic domain highly homologous to mammalian CNPases seems sufficient to pull-down tubulin in the assays. The affinity purified deletion mutant proteins used in these pull-down assays were separated by SDS-PAGE to confirm purity and specificity of binding to the resin (Figure 3C).

2.5. Catalytic activity is not required for the interaction of zRICH with tubulin

Since the catalytic domain of zRICH is sufficient for binding to tubulin, a relevant question is whether catalytic activity is required for the interaction. Tubulin co-purification tests were performed with zRICH-H334A to study if the catalytically inactive mutant protein is still capable of interaction. Figure 1A shows a diagram of this mutant. The pull-down assays showed that the zRICH-H334A mutant protein was able to bind and bring down tubulin (Figure 4A). zRICH-WT was used as positive control for the tests. Recombinant purified zRICH-H334A was analyzed by SDS-PAGE to assess the purification by affinity chromatography (Figure 4B).

2.6. The N-terminal domain of zRICH does not interact with tubulin

The catalytic domains of both zRICH and mammalian CNPase are sufficient for interaction with tubulin (see Figure 3B and (Lee et al., 2005), respectively). A mutant version of mammalian CNPase comprising the N-terminal domain was shown to be incapable of pulling down tubulin in co-purification assays. The N-terminal domain of zRICH shows no homology to the corresponding region of mammalian CNPase, and may be involved in different functions. To assess whether this domain in zRICH is capable of interacting with tubulin, a new mutant version of zRICH containing exclusively the acidic N-terminal domain, termed zRICH(1-171), was generated utilizing recombinant DNA technologies and affinity purification of recombinant protein. A diagram of the new protein is presented in Figure 5A. The zRICH(1-171) protein was tested in tubulin co-purification tests (pull-down assays) in comparison with zRICH-WT (positive control). The results showed that zRICH(1-171) does not pull-down tubulin (Figure 5B), suggesting that this region is not involved in interactions with the cytoskeletal protein. An SDS-PAGE of the purified recombinant zRICH(1-171) protein used in these assays is presented in Figure 5C. Previous experiments demonstrated that zRICH-WT protein migrates anomalously in SDS-PAGE (well above its predicted molecular weight) due to the highly acidic nature of its N-terminal domain (Challa et al., 2006). As expected from the previous observations, the mutant protein that contains only this domain did demonstrate the anomalous migration as well, approximately 20 kDa above its predicted molecular weight (Figure 5C).

3. Discussion

Zebrafish and goldfish RICH proteins are induced in RGCs after optic nerve injury (Ballesterio et al., 1997; Ballesterio et al., 1999), but their roles in axon repair are not well understood yet. These teleost proteins share significant homology with mammalian CNPases over the C-terminal two-thirds of the proteins, corresponding to their catalytic domain (which confers them 2',3'-cyclic nucleotide 2' phosphodiesterase activity) (Ballesterio et al., 1999; Challa et al., 2006). Both proteins also share other biochemical and cellular characteristics, such as the ability to form dimers (Sprinkle, 1989; Leski and Agranoff, 1994), and to interact with membranes through a C-terminal peptide sequence that bears a prenylation motif (De Angelis and Braun, 1994; Leski and Agranoff, 1994; Challa et al., 2006). Interestingly, mammalian CNPases have been shown to facilitate the formation of branched membranous extensions in glial cells (Lee et al., 2005). RICH proteins are induced during the process of nerve fiber regeneration, which involves the formation and extension of neurites, and it is transported down these extensions through slow axonal transport, accumulating in the varicosities and branching points, where they were hypothesized to participate in the regulation of the cytoskeleton (Wilmot et al., 1993; Challa et al., 2006). To study whether RICH proteins participate in the extension of neurites or the formation of new branches in neurons, stable transfectants of the pro-neuronal PC12 cell line were generated to express high levels of zRICH during the differentiation triggered by NGF treatment (Figure 1). Morphometric analyses demonstrated that the expression of zRICH resulted in an increase in the formation of neurite branches in the differentiating neurons. Other proteins induced during nerve regeneration, such as GAP-43 and CAP-23, have been shown to facilitate neurite sprouting during nerve regeneration and neuronal differentiation (Bomze et al., 2001). It is believed that by increasing the plasticity of the neurons, these proteins enhance the potential for successful nerve regeneration. Of note, the overexpression of zRICH-H344A in PC12 cells had a (statistically significant) stronger effect in the induction of neurite branching than zRICH-WT (Figure 1). It can be hypothesized that the lack of 2', 3'-cyclic nucleotide 2' phosphodiesterase catalytic activity makes the protein more active in neurite branch formation, but further information would be needed to understand the mechanisms involved. Other proteins do use catalysis to regulate their own activity. A classic example would be G-proteins (Hepler and Gilman, 1992), which turn themselves off by hydrolysis of bound GTP to GDP plus inorganic phosphate. Mutant versions of CNPase unable to bind to membranes were unable to elicit branching of membrane extensions in glial cells (Lee et al., 2005). Similarly, the mutant zRICH(172-410) was incapable of inducing neurite branching in differentiating PC12 cells (Figure 1). Future experiments should test whether the effect on neuritogenesis requires only the C-terminal membrane attachment domain, only the N-terminal acidic domain, or both of these domains, by generating and testing additional stable transfectants. Based on the requirement of membrane binding for the effect of mammalian CNPases in the formation of membranous extensions in glial cells, our current hypothesis is that the zRICH(1-410) mutant will not be able to promote neurite branching in PC12 cells. Much harder to predict, but perhaps more novel and interesting, is to determine whether the N-terminal domain is involved in enhancing neuritogenesis, and if it is, to elucidate the mechanism involved.

Due to the biochemical similarities between RICH proteins and mammalian CNPases, it is possible that the cellular effects of these proteins on the formation of membranous extensions (in glial cells and neurons, respectively) share underlying mechanisms. The overexpression of mammalian CNPases results in cytoskeletal modifications and cell morphology changes, and these effects require the interaction of the protein with tubulin (Lee et al., 2005). Co-purification experiments with purified proteins suggested a direct interaction of tubulin with mammalian CNPases. Similar experiments were performed to analyze whether zRICH can interact with tubulin. The results suggest that tubulin does

indeed interact specifically with zRICH (Figure 2). Similarly to the case of mammalian CNPase (Lee et al., 2005), the N-terminal domain and the C-terminal isoprenylation motif are not required for the interaction with tubulin, since even the deletion mutant lacking both regions was capable of interaction (Figure 3). Interestingly, although the experiments showed that the catalytic domain is sufficient for interaction (Figure 3), the experiments with the catalytically deficient zRICH-H334A mutant suggest that enzymatic activity is not necessary for interaction (Figure 4). Although not yet tested, it could be speculated that the same will be true for mammalian CNPases. It would be interesting to learn whether catalytically inactive versions of mammalian CNPases have pronounced effects on cell morphology, as observed in PC12 cells with zRICH-H334A (Figure 1). The N-terminal domain of mammalian CNPase did not interact with tubulin (Lee et al., 2005). Since the corresponding region of the protein is very different in zRICH, a new mutant protein consisting only of this region in zRICH was generated and tested for interaction with tubulin. The experiments indicated that this region is not sufficient for interaction with tubulin (Figure 5). The function of the N-terminal domain of CNPases and RICH proteins remains unclear.

Mammalian CNPases were used in tubulin polymerization assays to demonstrate a very rapid reaction that resulted in formation of large copolymers of tubulin and CNPase, as detected by increases of absorbance at 350 nm and fluorescence microscopy observations of the copolymer (Lee et al., 2005). Tests with zRICH-WT did not show an increase in absorbance at 350 nm (Perez-Ballester, unpublished results). In mammalian CNPases, a region of the protein preceding the C-terminal prenylation motif is essential for the copolymerization effects. This region does not show high similarity with zRICH, which may explain the different effects. Although both proteins interact with tubulin, the mechanism and effects of this interaction may therefore exhibit subtle differences for these two proteins.

RICH proteins are induced during nerve regeneration in teleost fish, and their effect on the tubulin cytoskeleton may affect the plasticity of neurons by facilitating the formation of new neurite branches. The absence of this protein in regenerating mammalian CNS neurons may contribute to the diminished regeneration response to axon injury. As indicated above, forcing the expression of a combination of two proteins able to induce neuronal plasticity (GAP-43 and CAP-23) was able to enhance nerve regeneration in a model animal system (Bomze et al., 2001). Combinatorial therapies offer the best chances of success in promoting nerve regeneration in humans that have suffered nerve damage in their CNS (Benowitz and Yin, 2007). RICH proteins could become an additional pro-regeneration factor for the development of such combinatorial therapies. Future studies should target a better understanding of the mechanisms of interaction of RICH with the cytoskeleton and its effects on neuritogenesis, as well as the coordination with the functions of other pro-regeneration factors.

4. Experimental Procedures

4.1. PC12 stable transfections

PC12 cells (rat pheochromocytoma, ATCC CRL-1721) were cultured as previously described (Vancha et al., 2004). The eukaryotic expression plasmids pcDNA3-zRICH-WT, pcDNA3-zRICH(172-410), pcDNA3-zRICH-H334A, and pcDNA3-βgal were used for transfection (Challa et al., 2006). Transfections were performed by lipofection with Lipofectamine-2000 reagent (Invitrogen) using protocols recommended by the manufacturer. Forty-eight hours after transfection, the growth medium was supplemented with 500 μg/ml G-418 (Promega). Transfected cells that integrate the plasmids into their genome were selected for 14–21 days by culture in medium with G-418 and then pooled. Expression of the transgenes in the stable transfectants was analyzed by Western blotting

with rabbit anti-RICH polyclonal antibody or with rabbit anti- β -Galactosidase antibody (Chemicon), following protocols described previously (Challa et al., 2006). The antibodies were used at a 1:1,000 dilution and incubations were performed at 4 °C for at least 4 h on a rocking platform (VWRbrand).

4.2. PC12 differentiation assays and analyses

For the differentiation assays, PC12 stable transfectants were seeded in multiwell-12 plates with approximately 5,000 cells per well (3 wells per stable transfectant, and 3 experiments were conducted in total, so that enough well separated cells could be used for the analyses described below). The cells were treated with 100 ng/ml of NGF (Sigma) in RPMI medium with low serum content (1% HS and 0.5% FBS). The medium was replaced every 2 days. After 5 days of treatment, the cells were fixed with 4% paraformaldehyde and later processed for immunocytochemistry as previously described (Challa et al., 2006). Briefly, the cells were permeabilized with phosphate buffered saline (PBS) containing 0.2% Triton-X100 and probed with rabbit anti-RICH (Ballesterio et al., 1999) at 1:1,000 dilution, incubating the plates on a rocking platform (VWRbrand) for at least 4 h at 4 °C. Goat-anti-rabbit antibody coupled to alkaline phosphatase (Invitrogen) was used as secondary antibody (1:1,000 dilution, incubated for at least 4 h at 4 °C), and detection was achieved by utilizing chromogenic substrate (NBT-BCIP, Promega; 15 min at room temperature). Cells were then observed under brightfield/phase contrast microscopy (Olympus CK40), and photographs were obtained with a Pixera Penguin digital camera. Morphometric analysis was performed on differentiated cells expressing high levels of the proteins encoded by the transgenes (cells were considered differentiated if they showed at least 1 neurite that was 3 times the length of the cell body). Numbers of primary neurites arising from the cell body, number of secondary neurites (arising from another neurite), and the number of branching points were determined. Statistical analyses consisted of one-way ANOVA conducted with a Microsoft Excel worksheet designed for this purpose. The worksheet calculates the F-factor by comparing the data sets, and estimates the probability (p) of obtaining that result assuming that the samples came from the same population (null hypothesis: all means are equal; alternate hypothesis: not all means are equal), based on the F-distribution. After rejection of the null hypothesis ($p < 0.05$), Tukey-HSD post-hoc analysis was utilized for assessing significance in comparison of the individual samples, specifically to compare the results obtained with cells expressing a zRICH protein variant with control cells, estimating the probability (p) of obtaining the result observed assuming that the two samples came from the same population. The averages of any two samples were considered statistically different whenever p was lower than 0.05. A summary of the ANOVA + Tukey-HSD post-hoc analysis results is provided as electronic supplementary materials.

4.3. Purification and analysis of recombinant proteins

The prokaryotic expression plasmids pKKR2-zRICH-WT, pKKR2-zRICH-H334A, pKKR2-zRICH(1-410), pKKR2-zRICH(172-424), and pKKR2-zRICH(172-410) have been described elsewhere (Ballesterio et al., 1999; Challa et al., 2006). A DNA fragment encoding zRICH(1-171), a deletion mutant derived from zRICH-WT, was generated by PCR mutagenesis essentially as previously described (Innis et al., 1990). Briefly, the PCR reactions were performed using the plasmid pKKR2-zRICH-WT (Ballesterio et al., 1999) as template. The oligonucleotide primers zR1F (GGT GAA CAG GGA TCC ACC ATG GAA GCT GAA CAG) and zR513R (CGC TTT CTC AAG CTT TCA TTG AAC TCC ATC AGC CT) were used for the amplification reactions (oligonucleotides were obtained from Sigma-Genosys). After digestion with *Bam*HI and *Hind*III, the fragment was subcloned into pBluescript-KS plasmid (Stratagene), and its sequence was confirmed (sequencing reactions were performed with the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit from PE Biosystems; DNA sequence results were obtained at the Texas A&M

University-College Station core facility). The fragment was subcloned from pBluescript-KS to pKKR2 to generate the construct pKKR2-zRICH(1-171). Similar procedures were used to generate the constructs pKKR2-Gal4AD and pKKR2-hBcl2, except that the plasmid pSP73 (Promega) was used for intermediate cloning and sequencing steps. The templates used for the PCR reactions were pGAD10 (Clontech) and pSFFVneo-hBcl2 (Boise et al., 1993), respectively. The oligonucleotide primers GAL4ADNNF (GGT GAA CAG GGA TCC ACC ATG AAT TTT AAT CAA AGT GGG) with GAL4ADR (CGC TTT CTC GAA TTC AAG CTT CTA CTC TTT TTT TGG GTT TGG), and HBCL2NF1 (GGT GAA CAG AGA TCT TCC ACC ATG GCG CAC GCT GGG AG) with HBCL2BR1 (CGC TTT CTC AAG CTT TCA CTT GTG GCC CAG ATA G) (obtained from Sigma-Genosys) were used for the amplification reactions. *Escherichia coli* (XL10-Gold strain, Stratagene) were transformed with the pKKR2 plasmid constructs and used for the expression and affinity purification of the heptahistidine-tagged recombinant proteins as previously described (Ballesterio et al., 1997). For the electrophoretic analyses, approximately 5 µg of each of the purified recombinant proteins were run on a 12% SDS-PAGE gel and the proteins were detected by staining with Coomassie Brilliant Blue (Sambrook et al., 1989). Molecular weight markers were run for size comparisons.

4.4. Tubulin co-affinity purification assays (pull-down)

Five µg of purified brain tubulin (Cytoskeleton) was mixed with 10 µg of the purified recombinant proteins tested in a total volume of 100 µl of 2X-STT-PIC buffer (0.2 M NaCl, 0.02 M Tris pH 7.5, 0.5% Triton-X100, and a protease inhibitor cocktail that contains 1 mM PMSF, 1 µg/ml Aprotinin, 1 µg/ml Leupeptin and 1 µg/ml Pepstatin). The protein mixture was incubated at 4 °C for 2–3 h on a rocking shaker (Nutator). For the pull-down procedure, Ni-NTA (Quiagen) beads were added and incubated with the protein mixture in the presence of 20 mM imidazole for 4–16 h at 4 °C. The heptahistidine-tagged recombinant proteins and any complexed tubulin were collected by centrifugation at 2,000 g for 10 min at 4 °C in Biofuge Fresco (Heraeus). The beads were washed 3 times with 2X-ST buffer with 10 mM imidazole. Protein complexes were released in 2X-ST buffer with 250 mM imidazole. Eluted co-purified tubulin was analyzed by Western blotting with mouse anti-α-tubulin monoclonal antibody (Sigma) at 1:2,000 dilution (incubated for at least 4 h at 4 °C on a rocking platform). Goat-anti-mouse IgG coupled to Horseradish Peroxidase (Calbiochem) was used as secondary antibody (at 1:5,000 dilution; incubated as above). Positive controls for the Western blot procedures were performed with approximately 500 ng (Figure 2) or 400 ng (Figures 3, 4 and 5) of purified tubulin. The blots were developed with enhanced chemiluminescent substrate (Amersham) and detected with a Kodak 440 Imager Station. Each figure showing the result of a co-affinity purification assay is representative of three independent experiments (15 assays were performed in total).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by NIH-MBRS-SCORE grant S06 GM08107 to M.G.G. and R.P.B., by Welch Foundation Grant AC-0006 to the Department of Chemistry at Texas A&M University-Kingsville, and by Texas A&M University-Kingsville research award 280811. The content of this research article is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl phosphate
CNPase	2',3'-cyclic nucleotide 3'-phosphodiesterase
GAP	growth associated protein
NBT	nitroblue tetrazolium
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
RGC	retinal ganglion cell
RICH	Regeneration Induced CNPase Homolog
SDS	sodium dodecyl sulfate
WT	Wild-Type

References

- Agranoff, BW.; Ford-Holevinski, TS. Biochemical aspects of the regenerating goldfish visual system. In: Elam, JS.; Cancalon, P., editors. Axonal transport in neuronal growth and regeneration. Plenum Press; New York: 1984. p. 69-86.
- Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, Botteri F, Brenner HR, Caroni P. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell*. 1995; 83:269–278. [PubMed: 7585944]
- Asch WS, Leake D, Canger AK, Passini MA, Argenton F, Schechter N. Cloning of zebrafish neurofilament cDNAs for plasticin and gefiltin: increased mRNA expression in ganglion cells after optic nerve injury. *J Neurochem*. 1998; 71:20–32. [PubMed: 9648847]
- Azarashvili T, Stricker R, Reiser G. The mitochondria permeability transition pore complex in the brain with interacting proteins - promising targets for protection in neurodegenerative diseases. *Biol Chem*. 2010; 391:619–629. [PubMed: 20370325]
- Bahr M, Bonhoeffer F. Perspectives on axonal regeneration in the mammalian CNS. *Trends Neurosci*. 1994; 17:473–479. [PubMed: 7531889]
- Ballesterio RP, Dybowski JA, Levy G, Agranoff BW, Uhler MD. Cloning and characterization of zRICH, a 2',3'-cyclic-nucleotide 3'-phosphodiesterase induced during zebrafish optic nerve regeneration. *J Neurochem*. 1999; 72:1362–1371. [PubMed: 10098837]
- Ballesterio RP, Wilmot GR, Agranoff BW, Uhler MD. gRICH68 and gRICH70 are 2',3'-cyclic-nucleotide 3'-phosphodiesterases induced during goldfish optic nerve regeneration. *J Biol Chem*. 1997; 272:11479–11486. [PubMed: 9111061]
- Ballesterio RP, Wilmot GR, Leski ML, Uhler MD, Agranoff BW. Isolation of cDNA clones encoding RICH: a protein induced during goldfish optic nerve regeneration with homology to mammalian 2', 3'-cyclic-nucleotide 3'-phosphodiesterases. *Proc Natl Acad Sci U S A*. 1995; 92:8621–8625. [PubMed: 7567986]
- Becker CG, Lieberoth BC, Morellini F, Feldner J, Becker T, Schachner M. L1.1 is involved in spinal cord regeneration in adult zebrafish. *J Neurosci*. 2004; 24:7837–7842. [PubMed: 15356195]
- Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci*. 1997; 20:84–91. [PubMed: 9023877]
- Benowitz LI, Yin Y. Combinatorial treatments for promoting axon regeneration in the CNS: strategies for overcoming inhibitory signals and activating neurons' intrinsic growth state. *Dev Neurobiol*. 2007; 67:1148–1165. [PubMed: 17514713]
- Bernhardt RR. Cellular and molecular bases of axonal regeneration in the fish central nervous system. *Exp Neurol*. 1999; 157:223–240. [PubMed: 10364435]

- Bifulco M, Laezza C, Stingo S, Wolff J. 2',3'-Cyclic nucleotide 3'-phosphodiesterase: a membrane-bound, microtubule-associated protein and membrane anchor for tubulin. *Proc Natl Acad Sci U S A*. 2002; 99:1807–1812. [PubMed: 11842207]
- Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell*. 1993; 74:597–608. [PubMed: 8358789]
- Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JH. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. *Nat Neurosci*. 2001; 4:38–43. [PubMed: 11135643]
- Bray GM, Villegas-Perez MP, Vidal-Sanz M, Aguayo AJ. The use of peripheral nerve grafts to enhance neuronal survival, promote growth and permit terminal reconnections in the central nervous system of adult rats. *J Exp Biol*. 1987; 132:5–19. [PubMed: 3323406]
- Bunge RP. Expanding roles for the Schwann cell: ensheathment, myelination, trophism and regeneration. *Curr Op Neurobiol*. 1993; 3:805–809. [PubMed: 8260833]
- Burrell HR, Heacock AM, Water RD, Agranoff BW. Increased tubulin mRNA in the goldfish retina during optic nerve regeneration. *Brain Res*. 1979; 168:628–632. [PubMed: 435987]
- Caroni P. Intrinsic neuronal determinants that promote axonal sprouting and elongation. *Bioessays*. 1997; 19:767–775. [PubMed: 9297967]
- Challa M, Chapa GR, Govindaraju S, Gonzalez-Garcia M, Ballesterio RP. Characterization of the domains of zRICH, a protein induced during optic nerve regeneration in zebrafish. *Brain Res*. 2006; 1100:42–54. [PubMed: 16765331]
- Chen DF, Jhaveri S, Schneider GE. Intrinsic changes in developing retinal neurons result in regenerative failure of their axons. *Proc Natl Acad Sci U S A*. 1995; 92:7287–7291. [PubMed: 7638182]
- De Angelis DA, Braun PE. Isoprenylation of brain 2',3'-cyclic nucleotide 3'-phosphodiesterase modulates cell morphology. *J Neurosci Res*. 1994; 39:386–397. [PubMed: 7884818]
- Fawcett JW, Asher RA. The glial scar and central nervous system repair. *Brain Res Bull*. 1999; 49:377–391. [PubMed: 10483914]
- Fouad K, Dietz V, Schwab ME. Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. *Brain Res Brain Res Rev*. 2001; 36:204–212. [PubMed: 11690617]
- Fu SY, Gordon T. The cellular and molecular basis of peripheral nerve regeneration. *Mol Neurobiol*. 1997; 14:67–116. [PubMed: 9170101]
- Giordano S, Laessing U, Ankerhold R, Lottspeich F, Stuermer CA. Molecular characterization of E587 antigen: an axonal recognition molecule expressed in the goldfish central nervous system. *J Comp Neurol*. 1997; 377:286–297. [PubMed: 8986886]
- Glasgow E, Druger RK, Fuchs C, Lane WS, Schechter N. Molecular cloning of gefiltin (ON1): serial expression of two new neurofilament mRNAs during optic nerve regeneration. *EMBO J*. 1994; 13:297–305. [PubMed: 8313874]
- Glasgow E, Druger RK, Levine EM, Fuchs C, Schechter N. Plasticin, a novel type III neurofilament protein from goldfish retina: increased expression during optic nerve regeneration. *Neuron*. 1992; 9:373–381. [PubMed: 1379821]
- Grafstein, B. The goldfish visual system as a model for the study of regeneration in the central nervous system. In: Cronly-Dillon, JR., editor. *Development and plasticity of the visual system*. Macmillan; London: 1991. p. 190-205.
- Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A*. 1976; 73:2424–2428. [PubMed: 1065897]
- Haenisch C, Diekmann H, Klinger M, Gennarini G, Kuwada JY, Stuermer CA. The neuronal growth and regeneration associated Cntn1 (F3/F11/Contactin) gene is duplicated in fish: expression during development and retinal axon regeneration. *Mol Cell Neurosci*. 2005; 28:361–374. [PubMed: 15691716]

- Hammarberg H, Piehl F, Risling M, Cullheim S. Differential regulation of trophic factor receptor mRNAs in spinal motoneurons after sciatic nerve transection and ventral root avulsion in the rat. *J Comp Neurol.* 2000; 426:587–601. [PubMed: 11027401]
- Heacock AM, Agranoff BW. Enhanced labeling of retinal protein during regeneration of optic nerve in goldfish. *Proc Natl Acad Sci USA.* 1976; 73:828–832. [PubMed: 1062795]
- Heacock AM, Agranoff BW. Protein synthesis and transport in the regenerating goldfish visual system. *Neurochem Res.* 1982; 7:771–788. [PubMed: 6181424]
- Hepler JR, Gilman AG. G proteins. *Trends Biochem Sci.* 1992; 17:383–387. [PubMed: 1455506]
- Herdegen T, Skene P, Bahr M. The c-Jun transcription factor--bipotent mediator of neuronal death, survival and regeneration. *Trends Neurosci.* 1997; 20:227–231. [PubMed: 9141200]
- Hieber V, Agranoff BW, Goldman D. Target-dependent regulation of retinal nicotinic acetylcholine receptor and tubulin RNAs during optic nerve regeneration in goldfish. *J Neurochem.* 1992; 58:1009–1015. [PubMed: 1371143]
- Hieber V, Dai X, Foreman M, Goldman D. Induction of alpha1-tubulin gene expression during development and regeneration of the fish central nervous system. *J Neurobiol.* 1998; 37:429–440. [PubMed: 9828048]
- Horner PJ, Gage FH. Regenerating the damaged central nervous system. *Nature.* 2000; 407:963–970. [PubMed: 11069169]
- Innis, MA.; Gelfand, DH.; Sninsky, JJ.; White, TJ. A guide to methods and applications. Academic Press; San Diego: 1990. PCR protocols.
- Jackson EK, Ren J, Mi Z. Extracellular 2',3'-cAMP is a source of adenosine. *J Biol Chem.* 2009; 284:33097–33106. [PubMed: 19801686]
- Laessing U, Giordano S, Stecher B, Lottspeich F, Stuermer CA. Molecular characterization of fish neurolin: a growth-associated cell surface protein and member of the immunoglobulin superfamily in the fish retinotectal system with similarities to chick protein DM- GRASP/SC-1/BEN. *Differentiation.* 1994; 56:21–29. [PubMed: 8026643]
- Laezza C, Wolff J, Bifulco M. Identification of a 48-kDa prenylated protein that associates with microtubules as 2',3'-cyclic nucleotide 3'-phosphodiesterase in FRTL-5 cells. *FEBS Lett.* 1997; 413:260–264. [PubMed: 9280293]
- Lee J, Gravel M, Zhang R, Thibault P, Braun PE. Process outgrowth in oligodendrocytes is mediated by CNP, a novel microtubule assembly myelin protein. *J Cell Biol.* 2005; 170:661–673. [PubMed: 16103231]
- Leski ML, Agranoff BW. Purification and characterization of p68/70, regeneration-associated proteins from goldfish brain. *J Neurochem.* 1994; 62:1182–1191. [PubMed: 8113803]
- Liu ZW, Matsukawa T, Arai K, Devadas M, Nakashima H, Tanaka M, Mawatari K, Kato S. Na,K-ATPase alpha3 subunit in the goldfish retina during optic nerve regeneration. *J Neurochem.* 2002; 80:763–770. [PubMed: 11948239]
- Neumann S, Woolf CJ. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron.* 1999; 23:83–91. [PubMed: 10402195]
- Olson L. Medicine: clearing a path for nerve growth. *Nature.* 2002; 416:589–590. [PubMed: 11948332]
- Paschke KA, Lottspeich F, Stuermer CA. Neurolin, a cell surface glycoprotein on growing retinal axons in the goldfish visual system, is reexpressed during retinal axonal regeneration. *J Cell Biol.* 1992; 117:863–875. [PubMed: 1577862]
- Plunet W, Kwon BK, Tetzlaff W. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. *J Neurosci Res.* 2002; 68:1–6. [PubMed: 11933043]
- Ramon y Cajal, S. Degeneration & regeneration of the nervous system. Oxford University Press; London: 1928.
- Rasband MN, Tayler J, Kaga Y, Yang Y, Lappe-Siefke C, Nave KA, Bansal R. CNP is required for maintenance of axon-glia interactions at nodes of Ranvier in the CNS. *Glia.* 2005; 50:86–90. [PubMed: 15657937]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. A laboratory manual. 2. Cold Spring Harbor Laboratory Press; New York: 1989. Molecular cloning.

- Schwab ME. Increasing plasticity and functional recovery of the lesioned spinal cord. *Prog Brain Res.* 2002; 137:351–359. [PubMed: 12440377]
- Schwab ME. Nogo and axon regeneration. *Curr Opin Neurobiol.* 2004; 14:118–124. [PubMed: 15018947]
- Schwab JM, Gu MF, Stuermer C, Bastmeyer M, Hu GF, Boulis N, Irwin N, Benowitz LI. Optic nerve glia secrete a low-molecular-weight factor that stimulates retinal ganglion cells to regenerate axons in goldfish. *Neuroscience.* 1996; 72:901–910. [PubMed: 8735218]
- Skene JH. Axonal growth-associated proteins. *Annu Rev Neurosci.* 1989; 12:127–156. [PubMed: 2648946]
- Spencer T, Domeniconi M, Cao Z, Filbin MT. New roles for old proteins in adult CNS axonal regeneration. *Curr Opin Neurobiol.* 2003; 13:133–139. [PubMed: 12593992]
- Sprinkle TJ. 2',3'-cyclic nucleotide 3'-phosphodiesterase, an oligodendrocyte-Schwann cell and myelin-associated enzyme of the nervous system. *Crit Rev Neurobiol.* 1989; 4:235–301. [PubMed: 2537684]
- Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. *Cell.* 1995; 80:445–452. [PubMed: 7859286]
- Tanabe K, Tachibana T, Yamashita T, Che YH, Yoneda Y, Ochi T, Tohyama M, Yoshikawa H, Kiyama H. The small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats. *J Neurosci.* 2000; 20:4138–4144. [PubMed: 10818149]
- Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K. Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. *Mol Cell Neurosci.* 2000; 15:170–182. [PubMed: 10673325]
- Vancha AR, Govindaraju S, Parsa KV, Jasti M, Gonzalez-Garcia M, Ballesteros RP. Use of polyethyleneimine polymer in cell culture as attachment factor and lipofection enhancer. *BMC Biotechnol.* 2004; 4:23. [PubMed: 15485583]
- Vielmetter J, Lottspeich F, Stuermer CA. The monoclonal antibody E 587 recognizes growing (new and regenerating) retinal axons in the goldfish retinotectal pathway. *J Neurosci.* 1991; 11:3581–3593. [PubMed: 1941096]
- Wilmot GR, Raymond PA, Agranoff BW. The expression of the protein p68/70 within the goldfish visual system suggests a role in both regeneration and neurogenesis. *J Neurosci.* 1993; 13:387–401. [PubMed: 8423482]
- Woolf CJ, Bloechlinger S. Neuroscience. It takes more than two to Nogo. *Science.* 2002; 297:1132–1134. [PubMed: 12183616]
- Yankner BA, Benowitz LI, Villa-Komaroff L, Neve RL. Transfection of PC12 cells with the human GAP-43 gene: effects on neurite outgrowth and regeneration. *Brain Res Mol Brain Res.* 1990; 7:39–44. [PubMed: 2153893]

Highlights

- zRICH promotes neurite branching in neuronal differentiation model cell line.
- Effect on structural plasticity enhanced for catalytically inactive zRICH mutant.
- zRICH interacts with the cytoskeletal protein Tubulin.
- Interaction mediated by central domain, but independent of phosphodiesterase.

Figure 1.

Effects of zRICH proteins on neuronal plasticity of transfected PC12 cells. **A:** Structure of the proteins expressed in PC12 cells. There are three domains in Wild-Type zRICH protein, an amino-terminal acidic domain, a central CNPase homology domain, and a small carboxyl-terminal membrane localization domain (which contains an isoprenylation motif). The mutant protein zRICH-H334A has a highly conserved histidine amino acid residue (H334), which is essential for catalysis, substituted by an alanine. Both the acidic domain and the membrane localization domain are missing in the zRICH(172-410) mutant version of the protein. Numbers indicated are amino acid positions related to the Wild-Type protein. (AD: acidic domain, shown in red with black stripes; CD: CNPase homology domain, shown in green; MD: membrane localization domain containing isoprenylation-motif, shown in black). **B:** Western blot analysis of PC12 stable transfectants. The proteins in lysates from cells transfected with eukaryotic expression plasmids were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Immunodetection was performed with polyclonal anti-RICH antibody (left membrane) or anti- β -Galactosidase antibody (right membrane). All the three different versions of zRICH and the β -Galactosidase proteins were expressed in the corresponding stable transfectants, as shown by the presence of specific immunoreactive bands. Lysates from untransfected parental PC12 cells were used as negative controls. **C:** Differentiation characteristics of PC12 cells expressing zRICH proteins. Stable transfectant PC12 cells expressing zRICH-WT, zRICH-H334A, or zRICH(172-410) versions of zRICH protein were detected by immunocytochemistry with anti-RICH antibody. Expression of high levels of zRICH-WT or mutant versions of the protein in PC12 cells did not result in neurite extension in the absence of NGF (panels c, e, g). NGF treatment triggered neurite branching in the PC12 stable transfectants expressing the zRICH proteins (panels d, f, h). Cells expressing zRICH-WT or catalytically inactive

mutant zRICH-H334A often displayed more extensive branching than cells expressing the cytosolic mutant zRICH(172-410). The insets in panels d and f show a magnified view of neurites with arrowheads marking the location of branching points. PC12- β gal stable transfectants expressing β -galactosidase were used as controls (panels a, b). **D:** Morphometric analysis of neurites in differentiated stable transfectants. From left to right, the graphs represent the number of primary neurites, the number of secondary neurite segments, and the number of branching points per cell, respectively. Compared to the control PC12 expressing β -galactosidase, cells expressing zRICH-WT showed significant increases in branching points and formation of secondary neurites, but not in the number of primary neurites. The neurite branching effect of the catalytically deficient zRICH-H334A mutant was even more pronounced. Conversely, the cytosolic mutant zRICH(172-410) was not able to enhance neurite branching, and in fact the cells displayed slight decreases in neurite formation. The bars show the average $\pm$ SEM; n = 49 PC12- β gal, 70 PC12-zRICH-WT, 57 PC12-zRICH-H334A, and 75 PC12-zRICH(172-410) cells. Statistical analysis (Tukey-HSD): * p < 0.05, ** p < 0.01, groups were compared with PC12- β gal control; additionally, PC12-zRICH-H334A cells were compared with PC12-zRICH-WT cells (# p < 0.05, ## p < 0.01).

Figure 2.

Interaction of tubulin with zRICH-WT. **A:** Co-affinity purification (pull-down) of tubulin with mCNPase. Purified brain tubulin incubated with the indicated purified recombinant proteins were subjected to an affinity pull-down procedure. Immunodetection was performed with anti- α -tubulin antibody. Tubulin co-purified specifically with mCNPase, but was not pulled-down by the control recombinant proteins Gal4-AD or Bcl-2. Results are representative of three independent experiments. **B:** Co-affinity purification (pull-down) of tubulin with zRICH-WT. Purified brain tubulin incubated with the indicated purified recombinant proteins were subjected to an affinity pull-down procedure. Tubulin co-purified specifically with zRICH-WT, but not with negative control proteins, suggesting direct interaction between these two proteins. Results are representative of three independent experiments. **C:** Electrophoretic analysis of purified recombinant proteins used in pull-down assays. The position and size of the molecular weight standards is indicated. (Please note that zRICH-WT migrates anomalously approximately 20 kDa above the similarly sized mCNPase due to its highly acidic N-terminal domain).

Figure 3.

Interaction of tubulin with the catalytic domain of zRICH. **A:** Structure of the zRICH domain deletion mutants. The zRICH(1-410) deletion mutant is missing the small carboxyl-terminal membrane localization domain. The zRICH(172-424) deletion mutant is lacking the amino-terminal acidic domain. The zRICH(172-410) is missing both terminal domains, retaining only the central CNPase homology domain. Numbers indicated are amino acid positions related to the Wild-Type protein. (AD: acidic domain, shown in red with black stripes; CD: CNPase homology domain, shown in green; MD: membrane localization domain containing isoprenylation-motif, shown in black). **B:** Co-affinity purification (pull-down) of tubulin with zRICH deletion mutants. Purified brain tubulin and purified recombinant proteins were subjected to affinity pull-down assays. Tubulin co-purified specifically with zRICH-WT, and with all three deletion mutants zRICH(1-410), zRICH(172-424), and zRICH(172-410), indicating that the central catalytic domain is sufficient for interaction with tubulin. Specificity of the pull-down assay was confirmed with a negative control performed with the recombinant protein Gal4-AD. Results are representative of three independent experiments. **C:** Electrophoretic analysis of purified recombinant zRICH(172-424), zRICH(1-410) and zRICH(172-410) proteins. The position and size of the molecular weight standards is indicated.

Figure 4.

Interaction of tubulin with zRICH is independent of phosphodiesterase enzymatic activity.

A: Co-affinity purification (pull-down) of tubulin with zRICH-H334A. Purified brain tubulin and purified recombinant proteins were subjected to affinity pull-down assays. Tubulin co-purified specifically with zRICH-WT and with the catalytically inactive zRICH-H334A mutant version of the protein. The results indicate that catalytic activity is not required for interaction of tubulin with the central domain of zRICH. A negative control for the affinity pull-down assay was performed with the recombinant protein Gal4-AD. Results are representative of three independent experiments. **B:** Electrophoretic analysis of purified recombinant zRICH-H334A. The position and size of the molecular weight standards is indicated.

Figure 5.

The acidic domain of zRICH is not sufficient for co-purification of tubulin. **A:** Structure of the zRICH(1-171) mutant. A new deletion mutant of zRICH comprising exclusively the amino-terminal acidic domain was generated. The zRICH(1-171) deletion mutant is missing both the central catalytic domain and the small carboxyl-terminal membrane localization domain. Numbers indicated are amino acid positions related to the Wild-Type protein. (AD: acidic domain, shown in red with black stripes; CD: CNPase homology domain, shown in green; MD: membrane localization domain containing isoprenylation-motif, shown in black). **B:** Tubulin does not co-purify with zRICH(1-171) in pull-down assays. Purified brain tubulin and purified recombinant proteins were subjected to affinity pull-down assays. Tubulin co-purified specifically with zRICH-WT, but not with the deletion mutant zRICH(1-171), indicating that the amino-terminal acidic domain of zRICH is not a major determinant of the interaction of zRICH with tubulin. Specificity of the pull-down assay was confirmed with a negative control performed with the recombinant protein Gal4-AD. Results are representative of three independent experiments. **C:** Electrophoretic analysis of purified recombinant zRICH(1-171). The position and size of the molecular weight standards is indicated.