Mutations in the X-linked retinitis pigmentosa 2 gene cause progressive degeneration of photoreceptor cells. The retinitis pigmentosa 2 protein (RP2) is similar in sequence to the tubulin-specific chaperone cofactor C. Together with cofactors D and E, cofactor C stimulates the GTPase activity of native tubulin, a reaction regulated by ADP-ribosylation factor-like 2 protein. Here we show that in the presence of cofactor D, RP2 protein also stimulates the GTPase activity of tubulin. We find that this function is abolished by mutation in an arginine residue that is conserved in both cofactor C and RP2. Notably, mutations that alter this arginine codon cause familial retinitis pigmentosa. Our data imply that this residue acts as an “arginine finger” to trigger the tubulin GTPase activity and suggest that loss of this function in RP2 contributes to retinal degeneration. We also show that in Saccharomyces cerevisiae, both cofactor C and RP2 partially complement the microtubule phenotype resulting from deletion of the cofactor C homolog, demonstrating their functional overlap in vivo. Finally, we find that RP2 interacts with GTP-bound ADP-ribosylation factor-like 3 protein, providing a link between RP2 and several retinal-specific proteins, mutations in which also cause retinitis pigmentosa.

Retinitis pigmentosa is a degenerative disease and the major cause of heritable blindness (1). It is caused by mutations in a dizzying variety of genes; some are structural proteins of photoreceptors, many are involved in photoreception and transduction, and a few are seemingly unrelated ubiquitously expressed proteins (see RetNet, www.sph.uth.tmc.edu/retnet/). Most (80–90%) cases of X-linked retinitis pigmentosa are caused by mutations in two genes, XR2P and XR3P (2). These were isolated by positional cloning and shown to encode, respectively, the plasma membrane by myristoylation and palmitoylation of its amino terminus (6).

RP2 has amino acid sequence similarity over half of its length to the tubulin-specific chaperone protein cofactor C (3). It has been shown that cofactor C acts in a pathway together with four other tubulin-specific chaperone proteins (cofactors A, B, D, and E) to chaperone quasi-native α- and β-tubulin subunits released from the cytosolic chaperonin CCT and assemble the αβ-tubulin heterodimer. Each step in this pathway has been deduced from in vitro reconstitution experiments using purified components (7–9). The pathway hinges on the formation of a supercomplex containing α- and β-tubulin and cofactors C, D, and E. The hydrolysis of GTP by tubulin, stimulated by cofactors C and D, is part of the heterodimer assembly reaction; the stimulated hydrolysis of GTP by β-tubulin acts as a switch for the release from the supercomplex of native, newly made tubulin heterodimers (10). Cofactor C and D in combination have also been shown to be a GTPase activator (GAP) for native tubulin; cofactor E enhances this tubulin-GAP activity (10). These biochemical data are consistent with the wealth of genetic evidence on cofactor homologs in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe (11–14). In S. cerevisiae, these genes were first identified in screens for chromosomal loss and supersensitivity to microtubule poisons. Cin1, Cin2, Cin4, and Pmc2 are the putative homologs of cofactor D, cofactor C, Arl2 (see below), and cofactor E, respectively.

The mammalian Arl (ADP ribosylation factor (ARF)-like) proteins constitute a family of Ras-related small GTP-binding proteins with at least eight members (15–18). They share 40–60% amino acid sequence identity with ARF proteins but do not act as cofactors in the cholera toxin-induced ADP-ribosylation of Gαs nor do they possess the other biochemical activities that characterize ARF proteins (19–21). Arl2 modulates the tubulin-GAP activity of cofactors C and D (22), and mutations in its putative yeast homologs affect microtubule stability (11, 12, 23). Mutations in Arl2 in Arabidopsis thaliana also result in the loss of microtubules (24, 25). Given the sequence similarity between cofactor C and RP2 and the interaction of Arl2 with tubulin-folding cofactors, we decided to compare the functional and biochemical properties of RP2 and cofactor C and to investigate the possibility that RP2 might interact with one or more Arl proteins. Our data show that RP2 acts as a GAP for tubulin in concert with cofactor D, but unlike cofactor C, it does not catalyze tubulin heterodimerization. We also show that both cofactor C and RP2 partially complement Cin2 deletion in S. cerevisiae and that RP2 binds to Arl3 in a nucleotide and myristoylation-dependent manner.
triangles

Functional Overlap between RP2 and Cofactor C

Plasmid Construction—DNA fragments encoding human RP2, cofactor C, BART (binder of Arl2) and carboxyl-terminally His₆-tagged Arl2, Arl3 and Arl4 were generated by PCR using reverse transcribed human testis RNA (CLONTECH, Inc., Palo Alto, CA) as template. These were inserted into the pET23b vector (Novagen, Inc., Madison, WI). From these plasmids, mutated cDNAs (encoding RP2-R118F, RP2-ΔS6, cofactor C-R262A, Arl3-T26N, and Arl3-Q70L) were generated by PCR, using the recombinant pET23b plasmids as template, with specific mismatched primers and the QuikChange site-directed mutagenesis kit (Strategene Inc., La Jolla, CA). For the expression of glutathione S-transferase-RP2 (GST-RP2), a fragment encoding human RP2 was generated and subcloned into the pGEX-4T-1 vector (Strategene Inc., La Jolla, CA) in frame with the GST-coding region. For expression in yeast, human C cofactor wild type and R262A were subcloned from pET23b into pRS413-GPD carrying the HIS3 gene and the 2 μ ori (26). CIN2 was cloned into the same vector by PCR from a yeast genomic library. All constructs were verified by DNA sequencing.

Expression and Purification of Proteins—Bovine brain tubulin (27) was obtained free from associated proteins by passage through phosphocellulose (28). The cytosolic chaperonin CCT and cofactors B, D, and E were purified as described previously (8, 9, 29). Full-length, untagged wild type and mutant cofactor C were expressed in Escherichia coli BL21 (DE3) cells using the expression plasmids described above. Harvested cells were lysed using a French press and clarified by centrifugation, and the recombinant proteins were purified through two dimensions as follows: 1) a cation exchange column of SP-Sepharose (Amersham Biosciences), developed with a linear gradient of 10–250 mM sodium phosphate buffer (pH 6.8) containing 1 mM each DTT, MgCl₂, and EGTA and 2) an anion exchange column (Q15, Amersham Biosciences), also developed using a phosphate buffer gradient (10–400 mM sodium phosphate buffer, pH 7.3). The recombinant proteins were monitored by SDS-PAGE analysis of fractions emerging from the columns. Wild type and mutant RP2 proteins were expressed from pET23b recombinant plasmids harbored in E. coli BL21(DE3). Cell lysates were cleared by centrifugation at 100,000 × g and applied to a Q15 anion exchange column developed with a linear gradient of NaCl (0.01–0.7 M NaCl in 10 mM phosphate buffer, pH 7.4, 0.5 mM MgCl₂, 1 mM EGTA, and 1 mM DTT). The purified material from this dimension was loaded on a column of hydroxyapatite (Pentax, American International Chemical Co., Inc., Natick, MA) in 0.5 mM MgCl₂, 50 μM CaCl₂, and 1 mM DTT. Elution was performed in the same buffer using a linear gradient of 0–0.25 M sodium phosphate buffer, pH 6.7. Fractions enriched in RP2 were further purified by application to a MonoQ anion exchange column (Amersham Biosciences) and eluted in 20 mM Tris-HCl, pH 8, and 1 mM DTT via a 0.0–0.5 M gradient of MgCl₂. Following each dimension of purification, aliquots of fractions emerging from the column were analyzed by Western blotting using a polyclonal anti-RP2 antibody for the detection of immunoreactive material. His₆-tagged Arl2, Arl3, Arl4, and GST-tagged RP2 were affinity-purified on cobalt resin (CLONTECH, Inc., Palo Alto, CA) and agarose-coupled glutathione (Amersham Biosciences), respectively, according to the manufacturer’s recommended protocols.

Generation of Antiserum to Cofactor C, RP2, and Arl3—Purified recombinant proteins were used as antigens for the production of antisera in rabbits (Arl3, cofactor C) or guinea pigs (RP2) (Cocalico Biologicals Inc., Reamstown, PA). For this purpose, Arl3 was first coupled to keyhole limpet hemocyanin. The resulting sera were tested for their titer and specificity by Western blotting against both their respective antigens and whole cell lysates.

In Vitro Tubulin Folding Reactions—In vitro tubulin refolding assays (10 μl) were performed in folding buffer (20 mM Mes, pH 6.8, 20 mM KCl, and 1 mM each DTT, MgCl₂, and EGTA), containing 10 μM [α-32P]GTP (2.5 μCi), purified bovine brain tubulin (3 μM), the cytosolic chaperonin CCT (1.5 μM) and various combinations of cofactors B, D, E (6 μg/ml each), and C (6 μg/ml) or RP2 (6–300 μg/ml). Reactions were incubated at 30 °C for 1.25 h and analyzed by native gel electrophoresis (30) followed by autoradiography to assess the incorporation of non-exchangeable radiolabeled GTP into refolded tubulin dimers.

Tubulin-GAP Assays—Purified bovine brain tubulin (1 μg) was incubated at 37 °C in the presence of 25 μM γ-[32P]GTP (10 μCi) in folding buffer in the presence of various combinations of tubulin-folding cofactors and RP2 (see Fig. 1 legend). Release of radiolabeled inorganic phosphate was monitored by 1 min intervals as described above.

Complementation Assay in S. cerevisiae—A CIN2 deletion strain (413.4B:MATα ade2 his3 leu2 lys2 ura3 cin2::LEU2) (11) was transformed according to the lithium acetate method with either pRS413 alone or pRS413 carrying the wild type or mutant versions of RP2 or cofactor C. CIN2-pRS413 was used as a positive control. Transformants were selected on synthetic medium. Single colonies were picked and...
**RESULTS**

**Functional Comparison of RP2 and Cofactor C**—Human RP2 and human cofactor C share 53% similarity and 29% identity over a domain of ~200 amino acids. The untagged, bacterially expressed proteins were purified to homogeneity through several dimensions of ion exchange chromatography. Using these purified recombinant proteins, together with the cytosolic chaperonin CCT and the other tubulin-specific chaperone protein RP2 (9), we performed in vitro α-tubulin refolding reactions in the presence of radiolabeled GTP. These reactions, tubulin is cycled through the folding (CCT-driven) and heterodimerization (tubulin-folding cofactors) machinery, in the presence of exchangeable GTP bound to α-β-tubulin in the αβ heterodimer (10, 31). The products were analyzed by non-denaturing gel electrophoresis (Fig. 1A). We found that despite its sequence similarity, RP2 was unable to substitute for cofactor C in these in vitro folding reactions and hence cannot participate in the heterodimerization of tubulin under these experimental conditions. Increasing the concentration of RP2 in these reactions had no effect (data not shown). However, reactions containing RP2, cofactors D, and E, and native tubulin showed tubulin-GAP activity (Fig. 1B). No GTP hydrolysis was observed in control reactions containing either RP2 alone or RP2 plus tubulin. We conclude that RP2, like cofactor C, acts in concert with cofactor D as a tubulin-GAP. As is the case with cofactor C, cofactor E enhances this tubulin-GAP activity but is not essential to it (Fig. 1B). A higher concentration of RP2 relative to cofactor C is needed to elicit comparable tubulin-GAP activity; possible reasons for this difference are discussed below.

**Mutation of the Arginine Conserved in RP2 and Cofactor C**—It has been proposed that GTPase activators share a common active site consisting of an arginine finger (32). Arg-118 in RP2 and Arg-262 in cofactor C constitute the only pair of conserved arginines found in these two proteins (Fig. 2A). Therefore, using site-directed mutagenesis, we engineered three mutant proteins: RP2-R118H, RP2-R262A, and RP2-R118H/262A. In vitro transcription/translation reactions were performed as in Fig. 1 and contained tubulin, cofactors D and E, and either wild type cofactor C (open diamonds) or cofactor C-R262A (open squares). C, mutation of Arg-118 (but not Ser-6) in RP2 abolishes its tubulin-GAP activity. Three tubulin-GAP assays were performed as described in the legend to Fig. 1; they contained tubulin and cofactors D and E and either RP2 (closed diamonds) or RP2-ΔS6 (open triangles) or RP2-ΔR118H (asterisks). Control reactions were performed with RP2 (crosses) or RP2 plus tubulin (open squares).

**Translation in Vitro and Binding Assays**—cDNAs encoding RP2, cofactor C, and BART were transcribed and translated in vitro using TnT rabbit reticulocyte lysate (Promega, Inc., Madison, WI) in the presence of [35S]methionine (0.8 mM/ml) at 30 °C for 1 h. Samples were cleared of ribosomes by centrifugation at 200,000 g for 1 h. Samples were spotted on toph cell suspensions onto yeast extract/peptone/dextrose plates containing 0, 1, or 5 μg/ml of benomyl.

**FIG. 2. Mutation of a conserved arginine in RP2 and cofactor C abolishes tubulin-GAP activity.** A, sequence comparison between the conserved domains in human RP2 and human cofactor C with a consensus sequence shown below. The sole conserved arginine is boxed. B, mutation of Arg-262 in cofactor C abolishes its tubulin-GAP activity. Tubulin-GAP reactions were performed as in Fig. 1 and contained tubulin, cofactors D and E, and either wild type cofactor C (open diamonds) or cofactor C-R262A (open squares). C, mutation of Arg-118 (but not Ser-6) in RP2 abolishes its tubulin-GAP activity. Three tubulin-GAP assays were performed as described in the legend to Fig. 1; they contained tubulin and cofactors D and E and either RP2 (closed diamonds) or RP2-ΔS6 (open triangles) or RP2-ΔR118H (asterisks). Control reactions were performed with RP2 (crosses) or RP2 plus tubulin (open squares).
arginine is critical for tubulin-GAP activity and suggests that it is indeed part of an arginine finger.

**Complementation Assay in S. cerevisiae—**Cofactor C and RP2 are distantly related in sequence to the yeast protein CIN2 (11, 12, 40). CIN1, CIN2, CIN4, and PAC2 (putative homologs of cofactor D, cofactor C, Arl2, and cofactor E, respectively) act together in a pathway affecting microtubule stability. Yeast harboring mutations in these genes show supersensitivity to cold and to benomyl, both of which destabilize microtubules. To test whether human cofactor C or RP2 can compensate for the loss of CIN2 in yeast, we transformed plasmids expressing wild type and mutant versions of these proteins into a CIN2 deletion strain. We assayed the resulting strains for their benomyl resistance. Incubation was at 26 °C to enhance benomyl sensitivity. Cc, cofactor C.

**FIG. 3.** Cofactor C and RP2, but neither cofactor C-R262A or RP2-R118H, complement a CIN2 deletion phenotype in yeast. CIN2, cofactor C (wild type and R262A), RP2 (wild type and R118H), and vector alone were each transformed into a CIN2 deletion strain. Individual transformants were grown in selective medium overnight and spotted in serial dilutions onto yeast extract/peptone/dextrose plates containing 0, 1.0, or 5.0 μg/ml benomyl to assess growth under these conditions. Incubation was at 26 °C to enhance benomyl sensitivity. Cc, cofactor C.

**FIG. 4.** RP2 binds to GTP-Arl3 (ADP-ribosylation factor-like protein 3). A, His8-tagged Arl3 (lane A2) or Arl3 (lane A1) bound to cobalt affinity beads or beads alone (lane C) were incubated in the presence of [35S]-radiolabeled BART, RP2, or cofactor C either in the presence or absence of GTP-γ-S. Eluted products were analyzed by SDS-PAGE. I, input translated BART, RP2, or Cofactor C corresponding to 40% of the amount used in the other lanes. B, interaction between RP2 and either wild type or mutant forms of His8-tagged Arl3. Wild type and His8-tagged mutant (Q71L and T30N) forms of purified recombinant Arl3 were incubated with [35S]-radiolabeled RP2 and analyzed as described in A. C, GST-RP2 binds to Arl3 in a whole tissue extract. Immobilized GST-RP2 or GST alone was incubated with a total soluble protein extract from bovine testis. Bound and eluted material was analyzed by Western blotting with either an anti-Arl3 or anti-β-actin antibody. Left-hand panel, Western blot of input bovine testis soluble extract (I) corresponding to the amount used in the lanes shown in the center panel. Center panel, Western blot of material binding to beads (lane C) or to GST alone. −L and +L, material binding to GST-RP2 in the absence or presence, respectively, of bovine testis lysate. Right-hand panel, Western blot of (I) tissue extract corresponding to the amount used in the adjacent binding experiments; GTP, GDP, GTP-γ-S, +L, material bound to GST-RP2 in the presence of GTP, GDP, GTP-γ-S (all at 1 μM) or no nucleotide.

Arl3 binds most strongly to RP2, reinforcing our conclusion that it is GTP-Arl3 that binds to RP2 (Fig. 4B). The interaction between RP2 and Arl3 was also found using purified tagged proteins (data not shown), demonstrating that the interaction is direct and not mediated by some third protein.

To confirm the binding of RP2 to GTP-Arl3 in tissue extracts, we expressed RP2 as a GST fusion protein, immobilized it on agarose-bound glutathione beads, and examined the ability of these beads to specifically bind proteins contained in an unfraccionated tissue extract. These experiments were done in the presence of GTP, GDP, GTP-γ-S, or no nucleotide. In each case, the bound proteins were eluted, resolved by SDS-PAGE and detected by Western blotting with an anti-Arl3 antiserum. These experiments showed that binding of Arl3 is strongest in the presence of GTP-γ-S (Fig. 4C), supporting a specific interaction between RP2 and GTP-Arl3 in the context of whole cells.

We have shown that Arl2 inhibits the tubulin-GAP activity of the tubulin-specific chaperones known as cofactors C and D (22). However, we found that neither wild type nor mutant...
His$_6$-tagged Arl3 modulated the tubulin-GAP activity of RP2 and cofactor D, nor did RP2 behave as a GTPase activator of Arl3 (data not shown). However, these negative results could result from using bacterially synthesized Arl3 protein that is both tagged and unmodified, as well as recombinant RP2, which is not acylated.

Because our data established that RP2 binds to GTP-Arl3, we decided to see whether there was differential binding among the RP2 mutant proteins that we used in the GAP experiment shown in Fig. 2. We found that in vitro translated RP2-R118H binds to the GTPase-defective form of Arl3 more weakly than wild type RP2, whereas RP2-S6 bound much more strongly to this form of Arl3 and to wild type Arl3 (Fig. 5A). Since deletion of serine 6 in RP2 is known to prevent its myristoylation (6), we tested whether the enhanced binding of RP2-S6 might be a result of its inability to acquire this post-translational modification. To do this, we included an inhibitor of myristoylation (3β,6α-hydroxymyristic acid) (38) in in vitro translation reactions and showed that a greater proportion of wild type RP2 is bound to Arl3 when myristoylation is inhibited (Fig. 5B). We conclude that myristoylation of RP2 indeed weakens its binding to Arl3 while increasing the nucleotide dependence of that interaction.

**DISCUSSION**

Retinitis pigmentosa 2 protein, RP2, is similar in sequence over half its length to the tubulin-specific chaperone cofactor C. We investigated the functional similarities and differences between the two proteins. We demonstrate here that the two proteins have overlapping but not identical functions. Both stimulate the GTPase activity of native tubulin, in both cases only with the cooperation of cofactor D. However, only cofactor C participates in the heterodimerization of newly folded tubulin subunits. Whereas the ADP-ribosylation factor-like 2 protein (Arl2) regulates the tubulin-GAP activity of cofactors C and D (22), the related protein Arl3, to which RP2 binds specifically (see below), does not affect the tubulin-GAP activity of RP2 and cofactor D. The fact that RP2 and cofactor C are not functionally identical is not surprising given that they only share one of two putative domains and that the former is largely a membrane-associated protein, whereas the latter is cytosolic.

Remarkably, we found that mutation of the only conserved arginine residue present in both RP2 and cofactor C (R118H in RP2 and R262A in cofactor C) causes a total loss of tubulin-GAP activity in each protein. The mutation R118H in RP2 is one that has been shown to cause familial retinitis pigmentosa (3). This mutation does not, however, lead to the mislocalization or destabilization of the RP2 protein (2, 6). When expressed in cultured cells (either tagged or untagged), RP2-R118H is expressed at high levels and is targeted to the plasma membrane in a manner indistinguishable from wild type RP2. Combining these genetic studies with the biochemical data presented here, we conclude that the loss of tubulin-GAP activity itself may cause retinitis pigmentosa in families harboring this mutation.

The active site of many different GAPs is hypothesized to contain an arginine finger (32). It is likely that the arginine residue we have mutated plays this role in cofactor C and RP2 for two reasons. First, it is the only arginine that is invariant in all sequenced RP2 and cofactor C proteins (2). Second, mutation of this arginine leads to a total loss of GAP activity, while leaving the stability and chromatographic properties of both proteins unchanged. From the latter fact we conclude that mutation of the conserved arginine does not lead to a global rearrangement or misfolding of the proteins. If structural analysis confirms our inference that cofactor C and RP2 act as
tubulin-GAPs through an arginine finger, this would be very striking given the fact that tubulin has a very different GTP binding pocket than the small GTP-binding proteins (39).

CIN2, the putative homolog of cofactor C in yeast, acts together with CIN1, CIN4, and PAC2 (the putative homologs of cofactor D, Arl2, and cofactor E, respectively) in a pathway affecting microtubule stability (11, 12, 40). We have shown here that cofactor C and RP2 can partially complement a deletion in CIN2. However, when mutated at the conserved arginine residue, neither protein can restore benomyl resistance to the yeast strain affecting microtubule stability (11, 12, 40). We have shown here that RP2-R118H shows a weak affinity to Arl3, RP2-ΔS6 binds more strongly to it than its wild type counterpart as does unmyristoylated RP2. Our data imply that the absence of myristoylation causes the stronger binding of RP2-ΔS6. It is possible that in vivo Arl3 binds to unmodified RP2 and participates in its targeting.

The interaction of RP2 and Arl3 is particularly interesting since Arl3 has recently been shown to bind to PDE δ, the β subunit of rod-specific cyclic GMP phosphodiesterase (43), a molecule involved in phototransduction. PDE δ also interacts with the retinitis pigmentosa GTPase regulator (44); mutations in the gene (XR3P) encoding this protein are the major cause of X-linked retinitis pigmentosa. Thus, it is tempting to speculate that Arl3 links RP2 with RGPR and PDE δ in a common pathway necessary for the maintenance of rod photoreceptor cells.

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